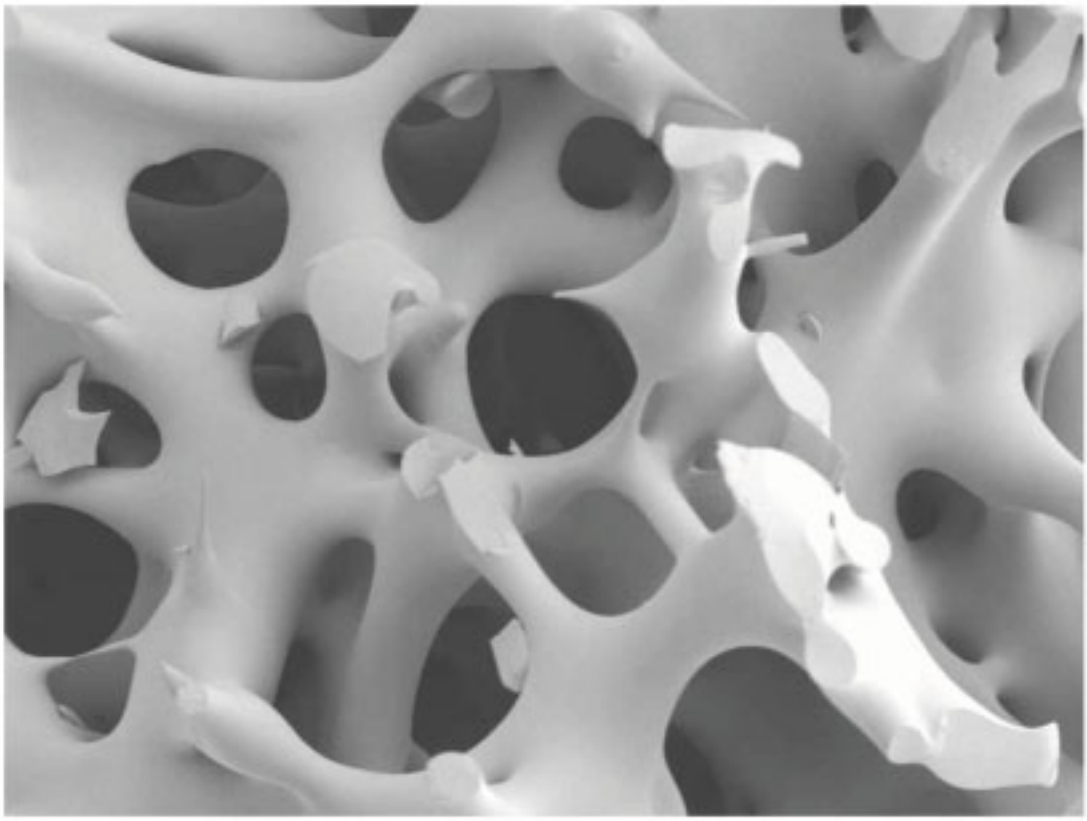
Julia Elisabeth Boni

Improvements to biorelevant dissolution testing: lyophilized media, buffer alternatives and miniaturized apparatus



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Improvements to biorelevant dissolution testing: lyophilized media, buffer alternatives and miniaturized apparatus

Dissertation

zur Erlangung des Doktorgrades

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der Johann Wolfgang Goethe - Universität

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von

Julia Elisabeth Boni

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Love all, trust a few. Do wrong to none.

William Shakespeare, *All's Well That Ends Well*

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ABBREVIATIONS

ANOVA	analysis of variations
API	active pharmaceutical ingredient
AUC	area under the curve
BA	bioavailability
BCS	biopharmaceutical classification system
BE	bioequivalence
BI	Boehringer Ingelheim Pharma GmbH & Co.KG
BI 20	NCE of BI
BIBF	NCE of BI
BIMT	NCE of BI
BIXX	NCE of BI
BP	British Pharmacopoeia
DLS	dynamic light scattering (s. PCS)
DMSO	dimethlene sulfoxide
DSC	differential scanning calorimetry
E-PC	egg phosphatidylcholine
Fab	FaSSIF blank buffer (without bile salts / lecithin)
Feb	FeSSIF blank buffer (without bile salts / lecithin)
FaSSIF	Fasted State Simulating Intestinal Fluid
FaSSIF / FeSSIF CM	conventional method FaSSIF / FeSSIF
FaSSIF / FeSSIF FD	freeze-dried FaSSIF / FeSSIF
FDA	Food and Drug Administration
FeSSIF	Fed State Simulating Intestinal Fluid
FIP	International Pharmaceutical Federation
GIT	gastrointestinal tract
GMO	glycerol monooleate
HIF	Human Intestinal Fluid
ICH	international conference on harmonisation
IVIVC	*in vivo in vitro* correlation
JP	Japanese Pharmacopoeia
KFT	Karl Fisher Titration
LPC	lysophosphatidylcholine
MeOH	methanol
MM	mixed micelles
NaAc	sodium actetate
NaCl	sodium chloride
NaGC	sodium glycocholate

NaO	sodium oleate
NaTC	sodium taurocholate
NBE	new biological entity
NCE	new chemical entity
NNLS	non-negative least square method
PCS	photon correlation spectroscopy (s. DLS)
PCS	phosphatidylcholine
PdI	polydispersity index
PE	polyethylene
P-gp	P-glycoprotein
PhEur	European Pharmacopoeia
QC	quality control
R&D	research and development
RH	relative humidity
SEIF	Simulated Endogenous Intestinal Fluid
SEM	scanning electron microscopy
SIF™	Simulated Intestinal Fluid
S-PC	soy phosphatidylcholine
SUPAC	scale-up and post-approval changes
tBA	tert-butyl alcohol
T_c	collapse temperature
T_{eut}	eutectic temperature
T_g'	glass transition temperature
USP	United States Pharmacopeia

I Overview - General Introduction

Problem identification

Prerequisite for absorption of drugs after oral intake is the dissolution of the active principle in the gastrointestinal tract (GIT). For poorly soluble drugs this is often the most critical factor. Poor dissolution of the active pharmaceutical ingredient (API) in the intestine leads to low absorption, which, in turn, can result in lack of efficacy. The Biopharmaceutical Classification System (BCS) developed by Amidon et al.[1] is a useful tool to estimate absorption problems. If absorption problems are likely, it may be necessary to develop sophisticated formulations, employing excipients which can enhance dissolution and maintain supersaturated solutions in the GI tract. In order to facilitate rational drug development, *in vitro* test systems which are predictive for absorption in man are necessary, e.g. those using biorelevant media.

Another important aspect in early formulation work is the limited availability of drug substance. Therefore formulation development as well as *in vitro* testing should be done with low consumption of drug substance. As many experiments are necessary to formulate difficult drugs, high throughput methods for formulation work as well as for *in vitro* tests are highly desirable to minimize the amount of drug needed per experiment.

The present thesis covers:

- A method for manufacturing "instant" dissolution media
- Development of appropriate *in vitro* tests
- Miniaturisation of *in vitro* tests
- Increase in throughput of dissolution experiments
- A formulation rationale based on physicochemical data of the drug substance
- Optimisation of predictability of *in vitro* tests

Drug formulation development is more than tablet design and optimization. It requires a sure instinct regarding composition, process and analytics. Once an appropriate composition of excipients is defined, decisions about formulation type and process parameters have to be made. The formulations will be tested *in vitro* and *in vivo* to assess their suitability. Although the dissolution profiles of the different formulations do not necessarily predict *in vivo* profiles, they might point out the advantages of one formulation over others. Finally, only the best formulations will reach the market and be available to society to tackle ill-health and disease.

"A constant companion" in all these steps of development are the analytics:
Before formulation studies begin, the potential candidate is completely characterized and the data are provided to pharmaceutical R&D to indicate first directions to formulation strategy. A knowledge of the physicochemical properties is indispensable for finding the most suitable excipients. Early dissolution screening explores the necessity for excipients which can improve the release into solution. Formulation prototypes are also screened *in vitro* prior to their utilization in preclinical studies. Later, the influence of changes in processing, e.g. during scale up, as well as the influence of storage on the final stability and performance of the formulation, have to be analyzed by quality control dissolution testing.
Dissolution is therefore one of the most important tools in new drug development, starting from early preclinical development and continuing through to the market. Dissolution studies are typically carried out or supported by pharmaceutical R&D.

Dissolution in the context of quality control means release of the active pharmaceutical ingredient (API) from a mixture of substances, typically a solid or semi-solid drug formulation, into a buffered solution. Usually, dissolution testing for these purposes is conducted according to the United States Pharmacopeia (USP) requirements.[2,3] That means apparatus, media, agitation and test conditions are more or less prescribed by the USP or other relevant pharmacopoeia.

Dissolution in pharmaceutical development is used to fulfil various needs but has one common requirement: the dissolution methods are designed to discriminate among formulations. Goals of dissolution in development on dissolution are dependent on the status of the project:

- In early drug characterization, intrinsic dissolution is used to estimate dissolution behaviour of pure drug and to define drug solubility at different pH values.

→ standard pharmacopoeial devices and methods or standardized high-throughput methods are used to provide consistent and comparable data

- In preformulation, dissolution is used to screen suitability of drug and excipient mixtures.

  → solubility testing is commonly used for preformulation studies. Additionally, dissolution profile studies, especially in form of high-throughput methods, is becoming more and more common in the pharmaceutical industry. At this early stage of development a large number of combinations are tested to increase the probability of formulation success.

- Early formulation development utilizes dissolution to mimic *in vivo* conditions and predict formulation behaviour is used to lead the way to a suitable clinical formulation.

  → at this stage, standard pharmacopoeial devices and methods compete against high-throughput dissolution methods. Biorelevant media are used to closely simulate gastrointestinal tract (GIT) conditions to predict *in vivo* behaviour, including bioavailability and food effects on absorption.

- Dissolution in late stage development is used to determine influence of scale-up, process changes and storage.

  → dissolution in this stage of development must meet pharmacopoeial requirements, such as configuration of apparatus, media properties and sink conditions, since results have to be presented and defended to the regulatory authorities.

Dissolution achieved its central position in pharmaceutical development relatively recently. Identification of dissolution as the prerequisite step for drug absorption was recognized in the early 60s by Wagner and since then it has become one of the most important methods in formulation characterization.[4-6]

1 History of dissolution

In 1897, Noyes and Whitney[7] first described a law about the dissolution rate of solid substances in their own solution. Therewith, the fundamentals for understanding dissolution were founded. But in the pharmaceutical area dissolution was disregarded for a long period of time.

Instead, disintegration, which was introduced 1934 by the Pharmacopoea Helvetica, was used for quality control. Disintegration was postulated to indicate the ability of a drug to form a solution and therewith the time after which drug absorption could be achieved. It therefore became the mandatory method for quality control. Not until 1962 was dissolution recognized

to be one of the most important prerequisites for drug absorption. This progress was mainly driven by improvements in analytical techniques, which enabled precise determination of the amount of drug dissolved. In the following decades dissolution became a routine analytical method in pharmaceutical development and quality control. Early on, dissolution testing was already used as predictive tool for *in vivo* data. But from the early 70s until today dissolution underwent substantial changes to become an informative tool for quality control like batch to batch repeatability or generally for SUPAC aspects (scale up and post approval changes).[8] Depending on the drug and formulation characteristics, dissolution testing can also be used to allow waiver of *in vivo* bioequivalences studies: to do this, drugs are first classified depending on solubility and permeability characteristics. If correlation of *in vitro* and *in vivo* data is possible (IVIVC), a biowaiver may also be granted on this basis for some formulations. Table 1.1 shows categorization of drugs by the BCS and expectations as to possible IVIVC.

Table 1.1 Possibilities for biowaiver, as suggested by Lobenberg and Amidon[3]

I high solubility high permeability IVIVC **if** the dissolution rate is slower than the gastric emptying rate (typically controlled release dosage form)	**II** low solubility high permeability IVIVC **if** the *in vitro* dissolution rate is similar to the *in vivo* dissolution rate, unless dose is very high
III high solubility low permeability Absorption (permeability) is rate determining and limited or no IVIVC	**IV** low solubility low permeability Limited or no IVIVC expected

2 Dissolution today

Dissolution testing in the pharmaceutical field is nowadays still mainly located in quality control. Testing conditions and material considerations are described closely in various pharmacopoeia. The USP (United States Pharmacopoeia), representing the leading pharmacopoeia in the Western world, describes seven different dissolution devices, suitable for testing of solid oral, rectal and vaginal dosage forms as well as for semisolid and transdermal formulations. Several of the official USP apparatus are also approved by other pharmacopoeia (see Table 2.1).

In addition to the pharmacopoeial regulations, other regulatory authorities such as the FDA (Food and Drug Administration of the U.S. Department of Health and Human Services) or FIP (International Pharmaceutical Federation) have also published guidances for the industry about dissolution testing.

Table 2.1 Official devices for dissolution described by the USP and other pharmacopoeia (PhEur-European Pharmacopoeia, BP-British Pharmacopoeia, JP-Japanese Pharmacopoeia)

apparatus qualification	name	also approved by
USP I	basket	PhEur, BP, JP
USP II	paddle	PhEur, BP, JP
USP III	reciprocating cylinder	PhEur
USP IV	flow-through cell	PhEur, BP, JP
USP V	paddle over disk	PhEur
USP VI	rotating cylinder	PhEur, BP
USP VII	reciprocating holder	

The official methods are designed for quality control and therefore are most suitable for these purposes. However, the methods often do not reflect relevant physiological conditions: one of the most important aspects besides the device in official dissolution testing is the maintenance of sink conditions. Sink conditions, however, are not necessarily reached *in vivo*: the BCS introduced by Amidon[1] assumes 250 ml to be the prevailing volume in the human GIT. For comparison Schiller et al.[9] and Weitschies et al.[10,11] measured mean volumes of 45 and 105 ml in fasted stomach and intestine, respectively, and 686 and 54 ml under fed conditions. For predicting formulation performance in man *in vitro* dissolution conditions should be as close as possible to *in vivo*. Biorelevant media reflecting buffer strength, pH and composition of physiological fluids represented a first attempt to more closely resemble the GIT conditions.[12] In recent years, adjustment of volumes to physiological values has been frequently discussed for dissolution testing of immediate release formulations: volumes proposed generally vary between 100 and 250 ml.[13]

3 Biorelevance in dissolution testing

3.1 The GI Tract

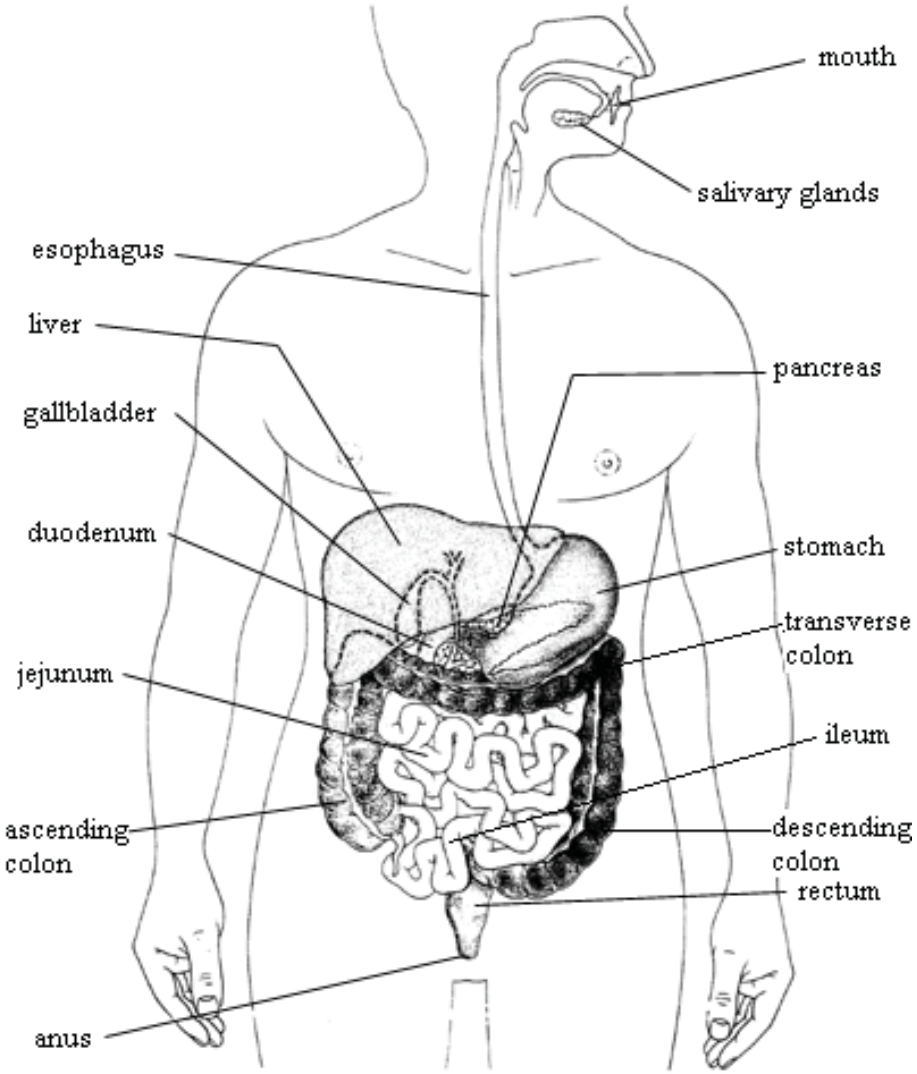


Figure 3.1 Anatomy of the human digestive tract

Figure 3.1 schematically shows the human digestive tract. The stomach and intestines are referred to as the gastrointestinal tract (GIT), in which digestion and assimilation of the aliment takes place. Organs supporting the digestive process are teeth, tongue, salivary glands, pancreas, liver and gall bladder. Secretion of the bile and the digestive enzymes produced in the pancreas occurs continuously but is more pronounced after intake of food. The majority of ingested food and xenobiotics is absorbed in general in about the first 80 cm

of the small intestine.[14]

Orally administered drugs can pass through the GIT en bloc (e.g. matrix tablets), can become dispersed or can dissolve. Fluids are mainly presented to the dosage form / drug intermittently in the intestine (as opposite to the continous contact in standardized dissolution tests).[9]

The processes of digestion and absorption of foodstuffs are enormously efficient.

Bile secreted by the liver is released directly into the gallbladder and into the GIT at a lecithin to bile salt ratio of about 1:1. Concentrations of bile salts in the hepatic bile are low, but a 10 to 25 fold concentration takes place in the gall bladder. Whereas 50 % of hepatic bile is continuously secreted into the GIT, the gallbladder bile is stored until emptying is induced by food intake.[15,16] The major difference between the composition of the hepatic bile and the luminal contents is the higher concentrations of Na^+, Cl^- and HCO_3^-.[17] The typical composition of gall bladder bile is given in Table 3.1.

Table 3.1 Composition of gall bladder bile (percentage of lipid [$^{mol}/_{mol}$])

concentration	ion		
200 mM	Na^+		
10 mM	K^+		
3.5 mM	Ca^{2+}		
50 mM	Cl^-		
150 mM	bile salts	65 mM	glycine conjugated
		34 mM	taurine conjugated
15 g/dl	total lipid	72 %	bile salts (54% cholic acid, 25% chenodeoxycholic acid, 21% deoxycholic acid, 0.2% lithocholic acid)
		17 %	phospholipids
		9 %	cholesterol
50 mM	phospholipids (7 types of lecithin, whereof 90% phosphatidylcholine)		

The metabolic half-life of cholic acid and taurocholic acid is three times higher than that of the glycine conjugate. However, synthesis of the glycine conjugate is higher, leading to relatively higher concentrations of this conjugate.

Bile composition of the postprandial upper intestinal contents is roughly equivalent to that of the gallbladder bile, with a ratio of lecithin to bile salt of 1 to 3.5. In the fasted state however, due to the constant secretion of hepatic bile, the relative concentrations of lecithin to bile salt are about 1 to 5.[18] The most prevalent bile salts NaGC (sodium salt of glycocholic acid) and NaTC (sodium salt of taurocholic acid), are both soluble at intestinal pH values. The bile salts are dissociated due to their low pk_a values of about 4 for glycine and about 2 for taurine

conjugated cholic acid.[19] Mixed micelles are formed consisting mainly of bile salts and lecithin, which, due to the highly lipophilic core, are able to incorporate lipophilic substances like lipids (e.g. cholesterol and acylglycerides) or lipophilic drugs.[16]

Exact determination of the intralumenal composition is difficult. Human data can only be obtained by aspiration of the intestinal fluids. The process of aspiriation however, causes irritation to the GIT, which again can lead to increased secretion and therewith overestimation of concentrations. Also challenging is the handling of these highly complex samples, e.g. in terms of the qualitative and quantitative identification of salts or the concentration of gases like CO_2 and subsequently their ionized counterparts like bicarbonate.[20] In fact, bicarbonate is the predominant buffer in the fasted small intestine, secreted continuously to neutralize acidic gastric juice and to protect the epithelium against digestion[21]. Secretion from epithelial cells and glands is regulated in order to maintain a pH[22] which is more or less stable against changes in acid or base concentration entering the small intestine. Postprandially, a variety of buffer species originating from food components as well as indigenous ones contribute to the overall buffer capacity.[23]

The complex and changing composition combined with high interindividual variations of human fluids leads to differing data about physiological composition in the literature. In most studies glycocholic acid was measured to be the most prevalent bile salt, wheras in 2006 Perez de la Cruz Moreno et al.[24] calculated a much higher concentrations of taurocholic acid in intestinal aspirates. Human studies conducted in the early 70s generally report higher concentrations of various components compared to recent studies.[25,26] In the intestine pH values in the fasted state were expected to be high (near neutral), whereas in the fed state the lower pH was assumed to reflect the influence of chyme emptying from the stomach.[27,28] Table 3.2 summarizes some of the most important studies about intestinal fluid composition.

Table 3.2 Characterization of intestinal fluids originating from the duodenum (duo.) or jejunum (jej.)

sample source	Kalantzi[29] *3	Perez de la Cruz[30]		Pedersen[31]		Lindahl[32]	Rune[21]	Dam[25] *2	Fordtran[33]	Evans[34]
sampling area (distal to pylorus [cm])	30	5 - 10	100	60		60	10	30-60	40	30-60
intestine	duo.	duo.	jej.	jej.		jej.	duo.	jej.	jej.	jej.
fasted state				fresh	frozen					
pH	5.7	7	6.8	6.1	6.7	7.1	6.2			6.63
buffer cap [mM/ΔpH]	7.8									
osmol [mosmol/kg]	188.2	137	200	272	278	271.0				
γ[mN/m]	32.9				33.7					
BS concn [mM]	2.1	2.6	3.5	2.0	1.5	2.9				
lecithin concn [mM]					<0.2 *1					
Na^+ concn [mM]						142.0				
fed state										
pH	6.6						5.7 - 5.8	5.5	7.3	
buffer cap [mM/ΔpH]	27.7								280	
osmol [mosmol/kg]	406.1						200- 375			
γ[mN/m]	28.5									
BS concn [mM]	8.7							38.9	11.9	
lecithin concn [mM]								11.1		
Na^+ concn [mM]										

*1 not determined in fresh fluid, only frozen -> as bile salt concentration decreased, probably lecithin concentration did as well

*2 collected after injection of cholecystikinin

*3 30 min after administration of water or nutrient drink

Knowledge about the GIT physiology is necessary to understand formulation behaviour in man, since dissolution conditions which do not reflect *in vivo* behaviour might lead to choice of suboptimal formulation. Physiological dissolution testing e.g. using biorelevant media is one way of gaining better prediction of *in vivo* behaviour of the probable formulations and hence to better results in development.

Even so, not only the composition but also movements of the gut wall and further factors such as enzymatic activity will cause interindividual variations, making a one to one copy *in vitro* to *in vivo* impossible. A general prediction of *in vivo* data from dissolution testing will therefore always be limited to estimating the average result.

3.2 Simulation of *in vivo* conditions

Many efforts have been made to establish systems simulating *in vivo* behaviour of formulations. Some systems, like the TIM model of TNO Pharma[35], attempt to exactly mimic *in vivo* conditions. But the high complexity of the TIM model makes it impractical for routine dissolution testing. More simple and correspondingly easier to set into practice is the use of simulated gastrointestinal media in a standard dissolution apparatus. Knowledge of the composition and physicochemical properties of gastrointestinal (GI) fluids combined with consideration of motility in the upper GI tract in the design of the dissolution test should lead to a better prediction of *in vivo* performance. These arguments apply especially to compounds belonging to Class II & IV of the BCS (Biopharmaceutical Classification System), since the absorption of these compounds is likely to be limited by dissolution[1]. Oversimplification of the dissolution conditions, as for example with compendial media, often leads to profiles which do not reflect the formulation's *in vivo* behaviour.

To simulate the *in vivo* conditions more nearly than just approximating the pH and volume, substances that occur in the GI fluids can be added to the media. Several media simulating the GI fluids have been proposed, including FaSSIF and FeSSIF[12], fed HIF[36] or SEIF[37].

Biorelevant media, which are designed to closely simulate physiological effects, aim to better link *in vitro* with *in vivo* performance. Dressman et al.[12] created dissolution media through consideration of the main factors which are generally expected to influence dissolution *in vivo*. Values of lecithin, taurocholic acid, osmolality, surface tension, buffer capacity and pH were adjusted to physiological values. To reflect differences between the fasted and fed state, different media were established: FaSSIF (Fasted State Simulating Intestinal Fluid), FeSSIF (Fed State Simulating Intestinal Fluid) and FeSSIFplus (similar composition to fed HIF)[31]. These fluids simulate intestinal composition by using concentrations of key substances similar to those given in Table 3.2. Usually taurocholic acid and phosphatidylcholine from egg are used as bile salts and lecithin components, respectively. To represent triglycerides and fatty acids glycerol monooleate and sodium oleate are commonly used. Osmolality is adjusted to physiological values with NaCl. But even though the media simulate most relevant characteristics, such as concentration of solubilising substances, buffer capacity, pH and solubilisation capacity of drugs[38], they are not a one-to-one copy of gastric or duodenal juice. For example, pH and buffer capacity vary considerably as a function of digestion and as a function of location within the intestine. Additionally, FaSSIF is based on a non-physiological buffer system, phosphate buffer (0.029 M, pH 6.5) whereas FeSSIF and FeSSIFplus are based

on a partly physiological acetate buffer (0.144 M, pH 5.0). In FeSSIFplus, citrate buffer is used as an alternative to acetate buffer.

Interest in using biorelevant media in dissolution testing has increased about 10fold (based on number of publications) over the last ten years (see Figure 3.2). Establishment of biorelevant dissolution testing has made prediction of or at least a ranking of *in vivo* formulation behaviour possible[12].

However, this "simple" idea of improving predictability shows some disadvantages: The conventional preparation method of these fluids is time-consuming, requires organic solvents, requires daily preparation and sometimes does not result in a usable medium.

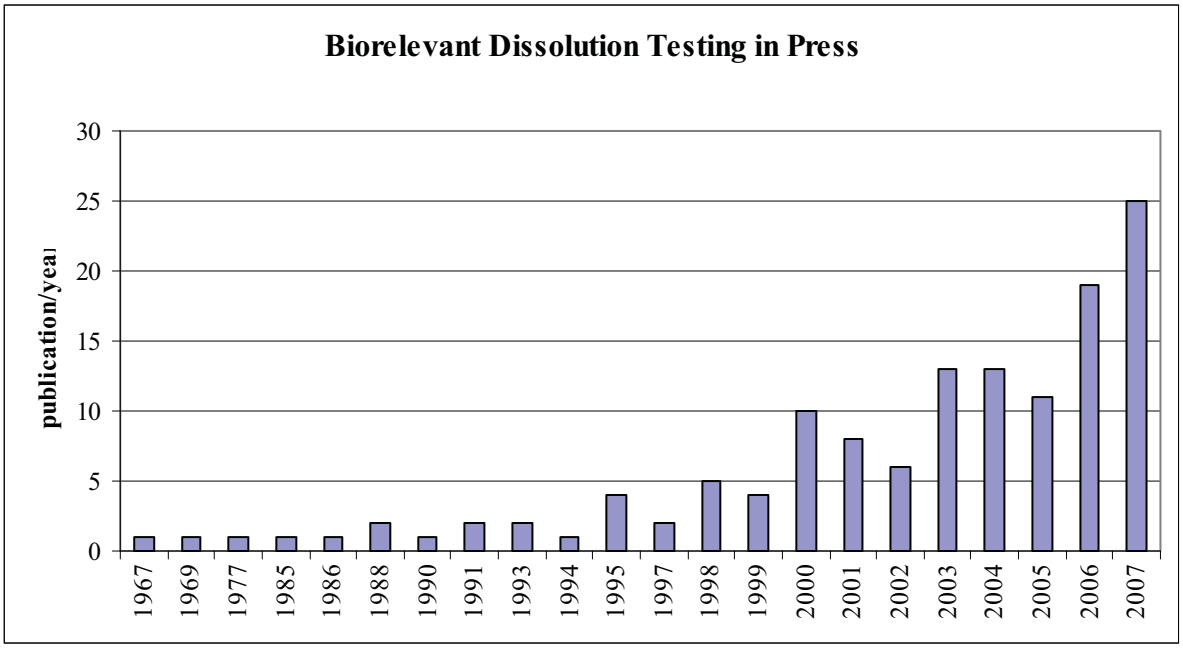


Figure 3.2 Number of publications per year relating to biorelevant dissolution testing (publications listed in this figure are referenced in Table 5.1 in appendix)

II Aims of thesis

The aims of this thesis were to (a) reduce time consumption regarding preparation of biorelevant dissolution media, (b) increase biorelevance of the media FaSSIF and FeSSIF by substituting the non-physiological buffer systems with bicarbonate and (c) to increase throughput by miniaturization of the dissolution test devices

1 Improvement of preparation of biorelevant media

The objectives of this part of the thesis work were to develop an "instant" dissolvable powder for fast and economic preparation of biorelevant dissolution media. Investigations on the feasibility of freeze-drying biorelevant media FaSSIF, FeSSIF and FeSSIFplus were carried out.

Various compositions of solutions were tested. Effects on the freeze-drying process and cake formation of additives like buffer salts, addition of sodium chloride for optimization of osmolality and addition of mannitol as bulking agent were examined. Additionally, the use of tert-butyl alcohol in order to reduce the production time was investigated.

Process parameters for optimal freeze-drying were investigated, taking into account both cake formation and energy consumption.

Further, appropriate packaging materials were sought to facilitate easy application in dissolution testing.

The stability of the lyophilised products under various storage conditions was studied. Reconstitution of the lyophilisate was observed, with particular attention to (a) preparation time, (b) handling and (c) reproducibility.

The reconstituted media was compared to media prepared by the conventional methods. Physical properties like osmolality, surface tension, particle size, density, viscosity as well as effects on solubility and dissolution of various drugs were examined. Finally, the "instant" media manufactured by freeze-drying were compared with other "instant" products introduced to the market during the time-frame of the thesis work.

2 Use of physiologic buffer systems

The objective of this part of the thesis work was to investigate an alternative buffer composition, which more closely resembles the intestinal fluids. The standard laboratory buffer systems phosphate and acetate were replaced by bicarbonate buffer, which is the most prevalent buffer system in the GIT. Attempts were made (a) to prepare a stable bicarbonate buffer suitable for dissolution testing, (b) to compare two different methods for preparation of bicarbonate buffer; and (c) to compare media based on bicarbonate buffer to the standard biorelevant media and pharmacopoeial media in terms of their suitability for dissolution testing.

The various media were compared with regard to ease of preparation, robustness, reproducibility of composition and reproducibility of dissolution profiles.

3 Miniaturization of dissolution testing

The objective of this part of the thesis work was to develop an automated dissolution testing device for small scale dissolution testing. A miniaturized dissolution apparatus was established to (a) perform dissolution testing proportionally downscaled from standard testing, (b) to enable fast and efficient screening of excipients for preformulation and early formulation (c) to increase throughput of dissolution testing for early stage formulation development, and (d) to create a tool for high throughput buffer screening with the aim of improving IVIVC.

III Manufacture of "instant" biorelevant media by freeze-drying

1 Introduction

Freeze-drying (lyophilization) is one of the oldest techniques known to man for preserving biological products from decomposition. It was already being used for food conservation by central Asian tribes more than 10,000 years ago.[39] Nowadays, freeze-drying is often used for production of parenteral products and for preservation of sensitive materials. Modern freeze-drying was introduced in 1890 by Richard Altman, who was trying to stabilize tissue cultures. However, until of the mid-20th century, freeze-drying was largely disregarded as a manufacturing technique. The advent of the first World War made it necessary to provide the fields hospitals with blood, which could only be made possible by using industrial-scale freeze-drying.[40] During the second World War the process was developed commercially when it was used to preserve newly isolated and purified penicillin (1941).[41] A a result freeze-drying became a leading technology. [39,42] Nowadays freeze-drying is a commonly used technique for the preparation of both pharmaceuticals and food products. In the pharmaceutical industry, the main area of application is the biotechnology products due to the low stability of proteins in aqueous solutions.

Freeze-drying is a gentle method for drying, applied mostly to aqueous solutions under reduced pressure at temperatures below the freezing point. The solvent is removed from the solute forming a highly porous cake, that can be reconstituted easily.

1.1 Reasons for Lyophilisation

Lyophilisation means transformation of a product (e.g. mixture of API and excipients) to a highly hydrophilic solid form. Usually lyophilisation can be obtained by amorphization e.g. by freeze-drying or spray-drying of a solution or dispersion. Excipients like stabilisers in case of protein products or matrix-forming agents are often necessary. Lyophilised products often show good stability if stored dry.

Advantages and disadvantages of lyophilisation (by Snowman)[43] are given in Table 1.1.

Table 1.1 Pros and cons of lyophilisation

advantage	disadvantage
- minimizes degradation of thermolabile material - creation of porous and fragile structures - fast and complete reconstitution	- high energetic consumption (3000 kJ to freeze 1 kg of water to – 40 °C and to sublimate it) - time consuming process - product is sensitive to storage conditions
rational use in case of	
- unstable, especially thermolabile substances - expensive materials - low amounts of substance available	

Lyophilisation by freeze-drying consists of three main process steps: freezing, primary drying and secondary drying. During freeze-drying the solution is converted into an elegant porous structure, which is easy to reconstitute. Lyophilisation by freeze-drying can be used to (a) increase the solubility of a substance by amorphization, (b) formulate pressure and temperature sensitive products and (c) improve the stability of water-sensitive products during storage. Formulations free of water improve the poor stability of natural products by avoiding enzymatic decomposition, hydrolysis and/or denaturation (b and c). Many proteins are marketed as freeze-dried formulations in the pharmaceutical sector, e.g. recombinant antibodies, insulin and interleukins. Freeze-dried herbs, fruits or so-called "globetrotter food" are examples from the food sector.

Increasing solubility by freeze-drying is less common for orally applied formulations but still used in the pharmaceutical industry. Solubility can be increased by incorporation of API into mixed micelles[43]. Marketed oral products prepared by freeze-drying containing mixed micelles include Konakion® MM and Valium® MM, both from Roche - Hoffmann-La Roche AG and Cernevit®, Baxter Deutschland GmbH.

1.2 Requirements for freeze-drying components

Solutions prepared for freeze-drying have to fulfil the following general properties: (i) the amount of solvent used in the process should be as low as possible in order to save energy, (ii) the solvent has to be non-toxic and (iii) it must be possible to remove all of the solvent so that none remains in the final product.[44] In addition, the solutes must be able to form a stable and highly porous cake. Moreover, the filling height must be low enough to facilitate fast drying yet high enough to allow formation of a homogeneous solid.

API (active pharmaceutical ingredient)

Because of the relatively high costs of the lyophilisation, it is not the method of choice for most solid product manufacturing. However, some APIs are not stable enough to survive conventional preparation processes. Instability at high temperatures (e.g. during melt extrusion) or in aqueous solutions (e.g. during wet granulation) leads to consideration of more gentle preparation methods like lyophilisation. Stability in solution must be adequate for the short time required for dissolution, filtration, filling and freezing. If necessary, water can be substituted by organic solvents like ethanol or *tert*-butyl alcohol. So even very sensitive APIs can be developed to useful products. Lyophilised products are commonly used to process products for parenteral use, such as hydrolysis-sensitive antibiotics, as “powders for reconstitution to injections or infusions”.

Solvents

Solvents that can be used to prepare freeze-dried products must fulfil the following properties: high vapour pressure, melting point around room temperature, high viscosity and low toxicity. A rapid and complete evaporation from the product is absolutely necessary. Residual solvents could lead to reduced stability of the product or even lead to side effects caused by toxic residues or decomposition products. The evaporated solvent should leave a highly porous cake which is easy to reconstitute. Water, ethanol, *tert*-butyl alcohol, glacial acetic acid or DMSO are widely used solvents in freeze-drying as they can provide the necessary manufacturing properties[44,45]. Solvents with high viscosity tend to reduce collapse by inhibiting viscous flow during formation of the cake. The high viscosity of *tert*-butyl alcohol (3.62 mPas compared to 1 mPas for water) and the high vapour pressure (41.25 mmHg compared to 23.78 mmHg for water) combined with low toxicity makes it an interesting solvent to reduce production times in freeze-drying. In addition, formation of long needles

during freezing create a high surface and therewith a highly porous cake after deposition of the solvent.

Solutes

To obtain a highly porous and rapidly dissolving cake, additional excipients like stabilisers or bulking agents are often necessary. These additives help to decrease the density of the product, to produce highly amorphous cakes and, combined with the increased surface area, to improve ease of reconstitution.

Bulking agents, which should be crystallised in the frozen state, include mannitol, hydroxy-ethyl-cellulose, gelatine, maltodextrose and xanthan. Stablilisers like buffering agents however, should be retained amorphous to avoid pH shifts during crystallisation[46]. pH shifts can lead to chemical decomposition of structures and substances.

Applications

Freeze-drying is mainly used to stabilise various products regarding biological or chemical degradation. Well known examples in the food industry are herbs and fruits. Freeze-dried products are storable and often the taste is intensified due to the high degree of concentration. In the pharmaceutical field freeze-drying is used especially hydrolysis-proune drugs, the cake being redissolved immediately before administration.

1.3 Freeze drying of biorelevant media

Interest in biorelevant media in dissolution testing increased about 10fold (based on publications) over the last ten years. Establishment of biorelevant dissolution testing has made it possible to predict or at least rank *in vivo* formulation behaviour[12]. Although biorelevant dissolution media like FaSSIF and FeSSIF are not a one-to-one copy of the human intestinal fluid, they attempt to simulate the most relevant parameters regarding pH, osmolality, surface tension and solubilisation capacity of drugs[38].

The conventional preparation method of these fluids is time-consuming, requires organic solvents, makes daily preparation necessary and sometimes does not result in an usable medium. FaSSIF and FeSSIF are buffered solutions of mixed micelles consisting of bile salt and lecithin. The composition of the biorelevant media is similar to some pharmaceutical products that are prepared by freeze-drying. Therefore, attempts were made to prepare storable biorelevant media by freeze-drying. The reconstituted media should be comparable to conventionally prepared media regarding composition and physicochemical properties. However, residual from organic solvents, like those used during conventional preparation, should be avoided so that adverse effects on physicochemical properties and dissolution behaviour can be prevented.

Beside good storability characteristics, the freeze-dried media should be instantly dissolvable and the resultant media highly reproducible, making them a attractive alternative to the conventional preparation method.

Early in 2007, a commercially available instant FaSSIF and FeSSIF came onto the market.[47] The Phares SIF™ powder is a hygroscopic powder and therefore delivered in amber glass containers with a desiccant sachet enclosed. Storage at 2-8 °C allows a limited shelf life of one year. Media composition differs from those discussed here, in that a phosphatidylcholine from soy (S-PC) was substituted for phosphatidylcholine from egg. Costs are about 3 fold higher compared to raw material only, not taking into account costs for preparation of conventional vs. freeze-dried media.The SIF powder is prepared without chlorinated solvents but still contains residual organic solvents which might have an impact on the media properties. Dissolution results obtained with our novel freeze-drying preparation method were compared to those obtained with the Phares SIF™[48] product.

Elaborate preparation and the high direct costs of the solubilising agents are reasons why

biorelevant media are not used for routine quality control. In earlier studies it was attempted to substitute the solubilising excipients by less expensive, less pure materials. However, differences in dissolution and concerns about reproducibility led to a recommendation to use purer grades.[49] Highly pure sodium taurocholate can be obtained from alternative suppliers to reduce costs, and such a product was tested in these studies to further reduce costs of the media. Media using the various bile salts were prepared by the conventional and the freeze-drying method and compared regarding physicochemical properties and dissolution behaviour. The commercially available SIF™ media were also compared regarding these aspects.

1.4 Lyophilisation – Physical basics

1.4.1 Process of lyophilisation

The freeze-dryer represents a closed system consisting of three major compartments: a freeze-drying chamber, a vacuum pump and a compressor to refreeze the evaporated water. The concentrated solutions are introduced into the freeze-drying chamber at room temperature and are subsequently frozen below the eutectic temperature. Reduction of pressure leads to evaporation of the solvent without increasing the product temperature. Then, the temperature is carefully increased to initiate and accelerate the process of drying. During processing, the solid to liquid phase transition (thawing) must be avoided. Figure 1.1 demonstrates the progression of temperature and pressure throughout the process.

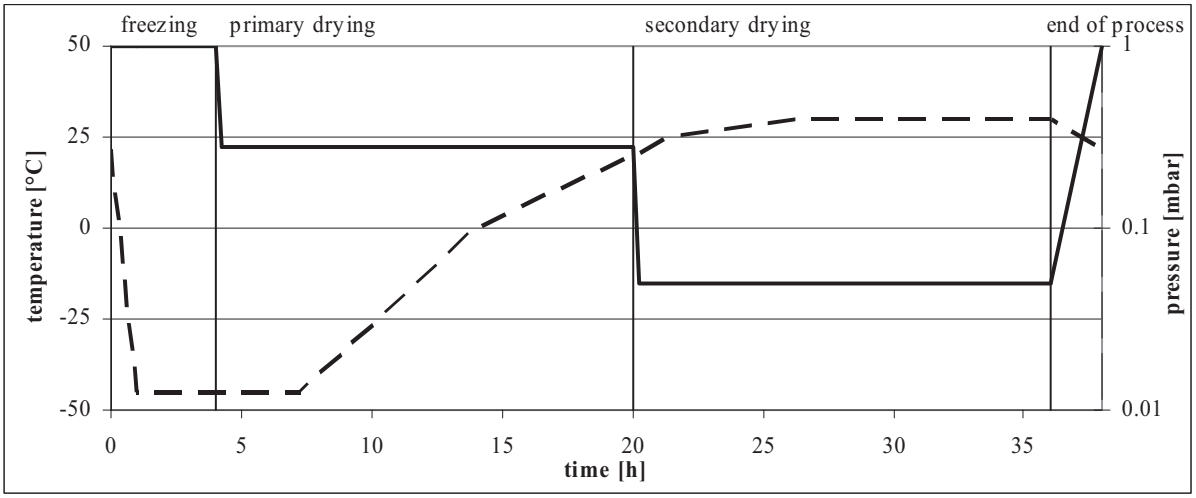


Figure 1.1 Freeze-drying process (schematically) with temperature (– –) and pressure (——) curve over time

Removal of solvent can be divided into primary and secondary drying. First, the pressure is reduced by applying a vacuum, inducing primary drying (sublimation), then in the secondary drying phase desorption takes place by increasing the temperature. Changes of aggregate states during the freeze-drying process are shown in Figure 1.2 (green line).

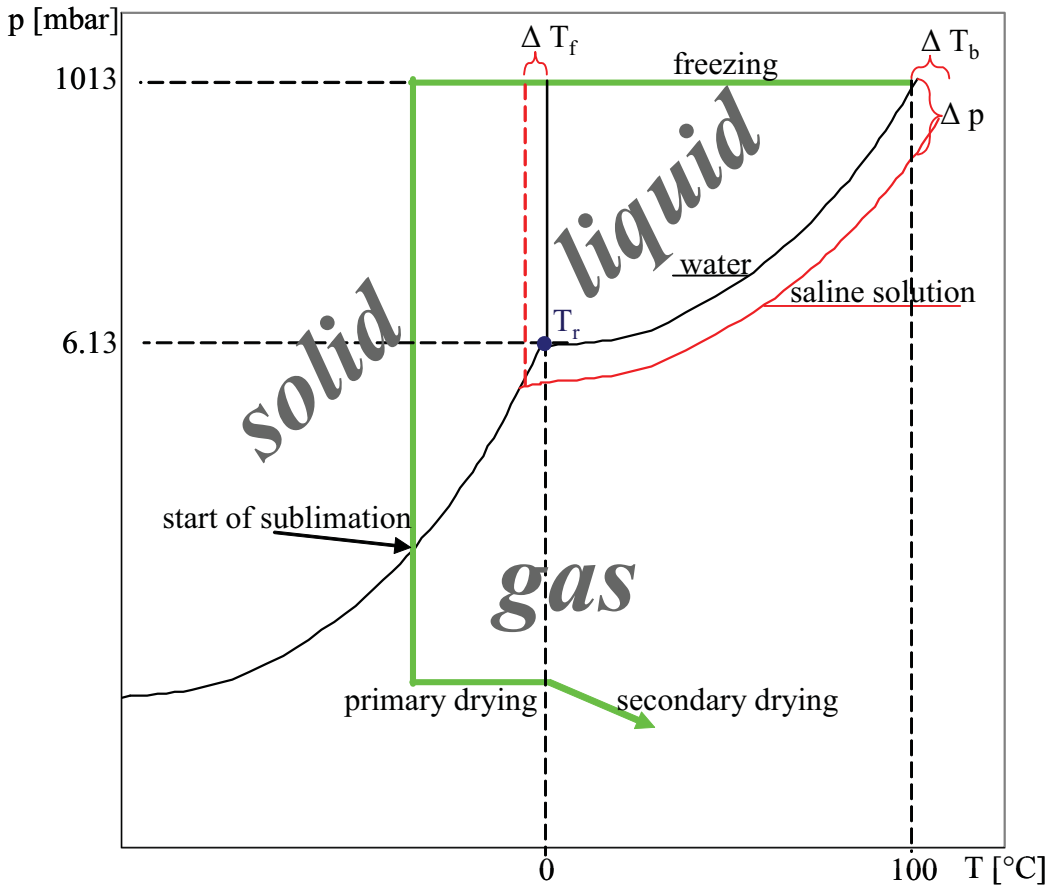


Figure 1.2 Phase transitions during freeze-drying

During the freeze-drying process the product must be kept below a critical temperature to avoid collapse of the structure. The collapse temperature, Tc, is several degrees below the glass transition temperature Tg'. Above Tc, the solute phase undergoes viscous flow, leading to a decrease in the cake's porosity[50]. The glass transition temperature defines a phase transition in which a supercooled melt yields a glassy structure and cristalline like properties.

1.4.2 Freezing point

The solid, liquid and gaseous states all play an important role in freeze-drying. The three states of water and their corresponding phase boundaries, converging at the triple point, are demonstrated in Figure 1.3. At 6.13 mbar and 273.16 K, the triple point of water, the three states coexist. The phase diagram depicts the effects of pressure and temperature on the state of water. The curves represent equilibrium between two phases as a function of pressure and temperature. The vapour pressure curve shows the intersection of liquid and gas. This terminates at the critical point (221 bar, 374 °C) above which the gas can not be liquefied any more. The sublimation curve indicates the vapour pressure of the solid as it sublimes at

different temperatures. For most substances, an increase in pressure favours the mainly more dense solid phase. However, frozen water shows a lower density than the liquid state and therefore increasing pressure will favour the liquid state (lower compactness of the molecules). At higher pressure the required temperature for melting is usually increased, whereas for ice it is decreased. Therefore, unlike most compounds, the melting curve of water slopes to the left.

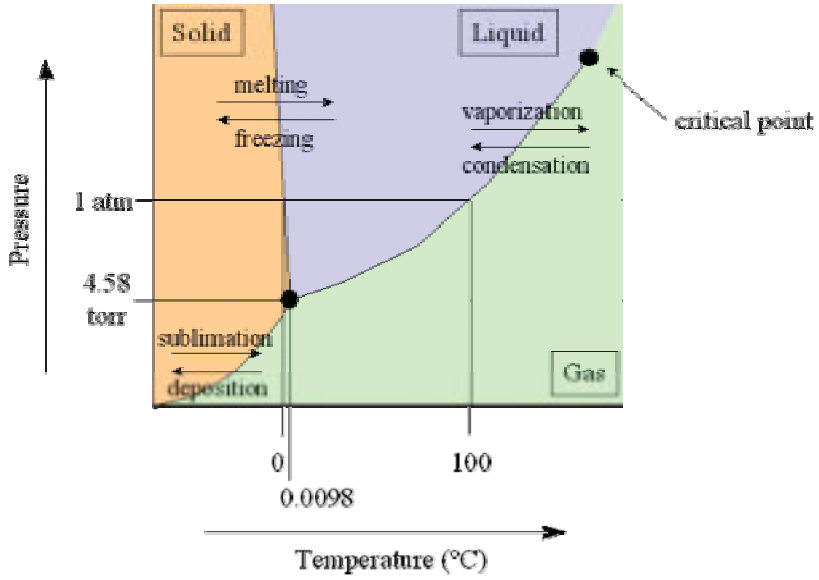


Figure 1.3 Phase diagram of water

At 100 °C the vapour pressure is 1 bar, thus at this temperature water will boil if it is at 1 atm of pressure. At pressures below 0.006 bar (4.58 torr), water will be present as either a gas or solid but no liquid phase exists.

Dissolved substances generally decrease the freezing point of water. The freezing point depression is a colligative property and thus independent of the substance characteristics. Raoult's law (see Equation 1.1) describes the freezing point depression, ΔT, as the ratio of the cryoscopic constant of a solvent to the concentration of dissolved solute (molal concentration n [mol/kg]).

$$\Delta T_f = \frac{K_f}{n}$$

Equation 1.1 Raoult's law (K_f cryoscopic constant of water: 1.86 K*kg/mol)

The process of freezing is identified as the transformation from the liquid to the solid state by

decreasing the temperature. Generally, liquids are supercooled below the freezing point (see Figure 1.2). Freezing starts with ice nucleation followed by ice crystal growth. The ice nucleation temperature is stochastic and dependent on a number of process and formulation variables[45]. The freezing point is only dependant on the number of molecules in solution. Therefore the freezing point depression can be used to calculate osmolality of a solution. Figure 1.4 shows the temperature curves of pure water and of a solution. After supercooling and the onset of nucleation, the temperature is increased by liberation of the heat of crystallisation, which results in a return to the freezing point temperature. Only after complete solidification, can the temperature be further decreased.

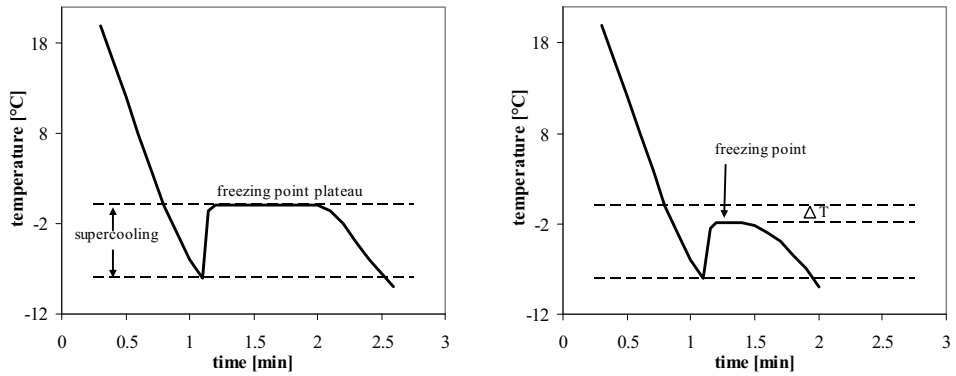


Figure 1.4 Temperature-time-curves for pure water and sample solutions

Initialisation of the crystallisation in aqueous solution is accelerated compared to in pure water. Pure water can be supercooled to about – 49 °C until homogeneous, spontanous ice nucleation occurs. Filtered solutions e.g. these used for the production of pharmaceutical injectables are generally supercooled in a range of 10 to 15 °C below the equilibrium freezing point[51]. The dissolved or dispersed particles support the energetically favoured heterogeneous nucleation. Eutectic mixtures are obtained by reconcentration. Solute and solution or solvent and solution are frozen separately until the eutectic state is reached. Subsequently the solution is frozen without a change in the composition (see also Figure 1.5).

1.4.3 Freezing process of solutions

Particles in colloidal solutions or dispersions accelerate crystallisation and lead to heterogeneous nucleation. Figure 1.5 shows the freezing process of a saline solution schematically. Cooling down a solution leads to changes in the concentration of the solution. After reaching the eutectic point there are no more changes in concentration and the solution starts to freeze. The eutectic temperature (T_{eut}) is the lowest temperature of thermodynamic

equilibration between solid and liquid. Below this temperature at the so-called glass transition temperature (T'_g), solvent and solute are crystallising homogenously. The glass transition temperature formed during freezing to the amorphous concentrate T'_g is different from T_g of the final cake[52]. Since the glass transition temperature is a function of moisture content, it changes sharply with a decrease in water content.

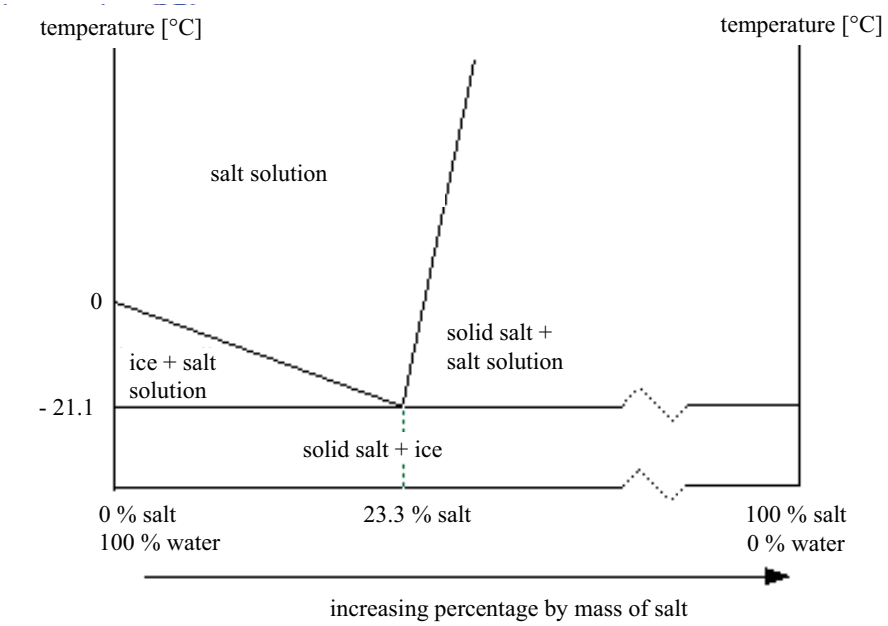


Figure 1.5 State diagram of a saline solution during freezing

In the case of buffer solutions, this phenomenon may lead to hysteresis of the pH. pH dependent changes in solubility can therefore lead to precipitation or to breaking of dispersions and emulsion. Therefore the freezing behaviour of the liquid has to be characterized before starting the freeze-drying process. Supercooling of the liquid takes place in the absence of nucleation. With the beginning of crystallisation the released heat of solidification leads to inhomogeneous ice formation. These inhomogeneities lead to formation of channels during the subsequent sublimation, facilitating evaporation of gas and generating a highly porous cake. The glass transition temperature, which in most cases is a few degrees below the eutectic point, needs to be reached in order to avoid collaps of the cake. Generally a temperature 4 to 5 °C below the eutectic point after freezing and during primary drying should be attained within the product[53]. Supercooling to a temperature below the glass transition temperature causes almost complete kinetic immobilisation of molecules. As a result, energetic transfer during the drying process by sublimation of gas is minimised and further reconcentration can be avoided. The glass transition temperature can be determined by differential scanning calorimetry (DSC).

1.4.4 Freezing time

The freeze-drying time influences formation and structure of the solid tremendously. Whereas fast freezing leads to small crystals, slow freezing allows formation of large crystals.

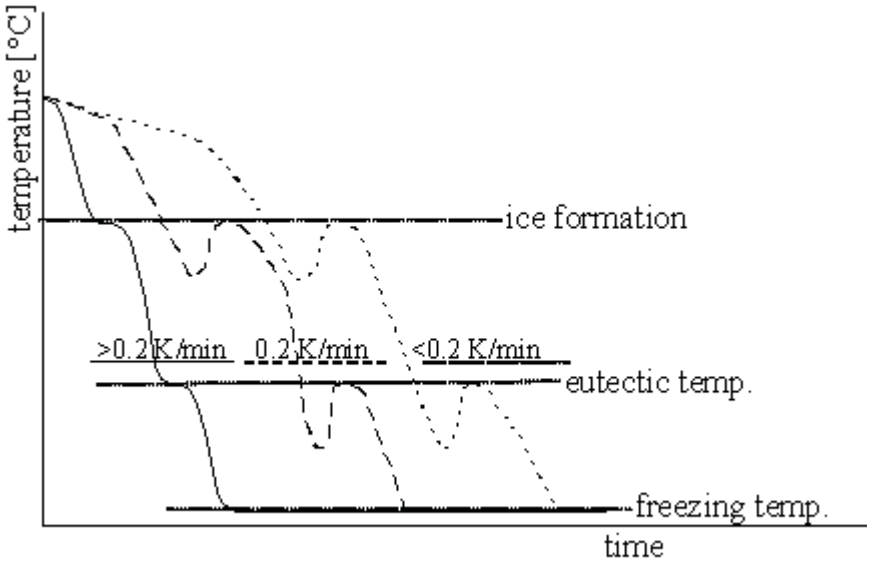


Figure 1.6 Influence of freezing temperature on solid composition

Figure 1.6 explains the influence of changing the freezing time. Fast freezing will lead to homogenous solids but, due to the lack of time for crystallisation growth, small crystals are obtained. Small solvent crystals create only narrow channels during sublimation. Secondary drying will be hindered, resulting in a high moisture content of the lyophilisate. Slow freezing, on the other hand, will lead to reconcentration and growth of crystals. Inappropriate cake formation may result. Only an optimum freezing temperature will lead to sufficient solvent crystal growth for creation of channels yet retain homogenous cake formation. The freezing temperature and rate must be optimised for each product e.g. for liposomal formulations a freezing rate of 10 K/min[43] is recommended.

1.4.5 Primary drying / Sublimation

Primary drying is frequently the most time-consuming step in the freeze-drying process. In this phase, the pressure needs to be decreased through the application of a partial vacuum. The vacuum is a prerequisite to sublimation. Application of a slight increase in temperature intensifies this direct change from solid to vapour. Still, the temperature of the solid needs to be low enough to avoid transition into a liquid phase. Evaporation of the sublimated water creates pores and channels, leading to a low density cake and therewith a high surface area.

The necessary energy is transferred to the system by convection and radiation. In the beginning, convection is the more important means of supplying energy. But with increasing height of the dry solid, energetic transfer is decreased and radiation of heat becomes the main energy transfer process. Temperature differences between shelves and product of up to 30 °C can occur because of the high energy consumption required by sublimation. The cooling produced by rapid sublimation also helps to prevent the collapse of the cake, since the temperature of the cake is maintained below the collapse temperature. Although an increase of temperature would accelerate drying, melting and collapse of the product have to be avoided. Therefore, the vacuum and temperature have to be adjusted during processing to achieve the maximum sublimation rate at which the temperature can be maintained below collapse temperature of the product. After sublimation, cooling effects are reduced and the product temperature approaches shelf temperature. After the primary drying is finished, water can no longer act as plasticizer and generally no collapse will occur. About 5-30 % solvent remains in the product at this point and further drying can only be initiated by moving to secondary drying.

1.4.6 Secondary drying / Desorption

By further increasing the vacuum, noncrystallisable water can also be removed from the product. This unfrozen water may be absorbed on crystalline solids or in the solute phase, either as hydrate or dissolved in amorphous solids, forming a solid solution[45]. Desorption of this water requires higher energy which can be supplied by concomitantly increasing vacuum and shelf temperature. Desorption is completed when no more solvent evaporates from the product, as demonstrated by the constancy of pressure in the closed freeze-drying chamber.

Over the complete drying process a cold condenser chamber provides a surface on which the sublimated water can resolidify.

Lyophilised products are generally highly hygroscopic and therefore dense packaging material is required. Sealing subsequent to drying is a widely used technique to avoid remoistening of the product. Use of vials that can be closed by direct stoppering is a useful approach. After finishing secondary drying, the vacuum is reduced by inflow of an inert gas like nitrogen until a pressure of about 900 mbar is reached. The stopper is pushed pneumatically into the vial and subsequently capped by an aluminium cap to optimize density. The slight negative pressure within the vial prevents spontaneous opening.

1.5 Lyophilisation – Technical Basics

Three main parts constitute a freeze-drying machine: the drying chamber, containing the product on shelves which can be cooled or warmed; the condenser, where sublimated gas is refrozen, and the vacuum pump for reducing the pressure within the drying chamber to enable evaporation at low temperatures. Figure 1.7 illustrates the construction of a lab scale freeze-dryer.

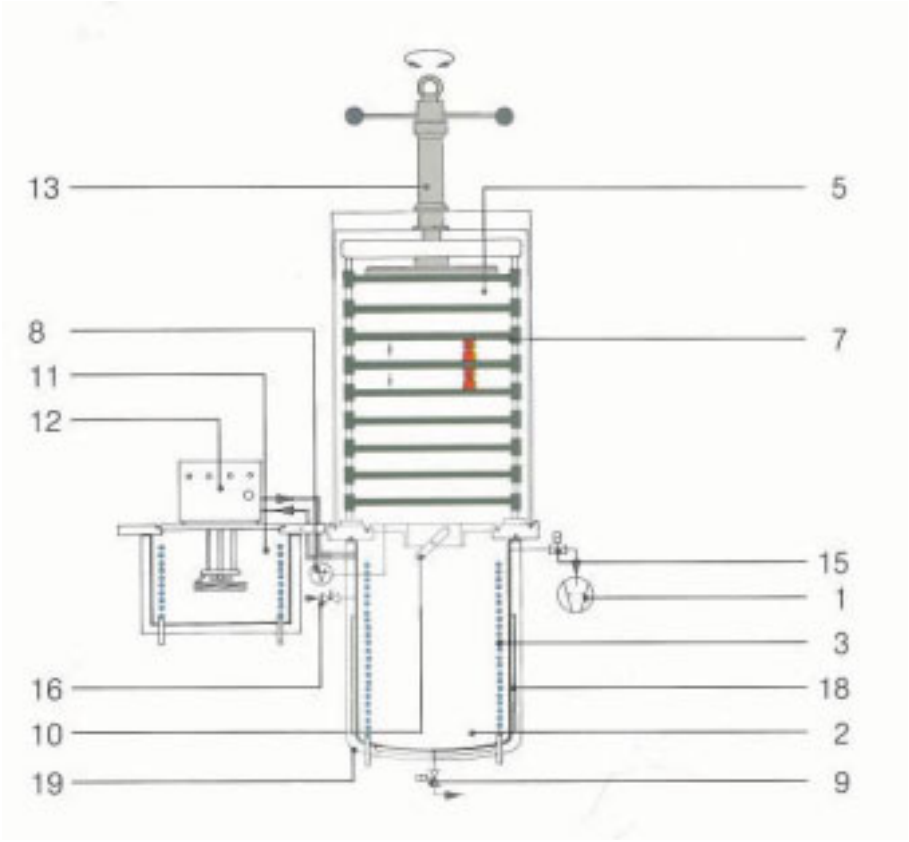


Figure 1.7 **Schematic drawing of a freeze-dryer** (1-vacuum pump, 2-condensing chamber, 3-condenser, 5-drying chamber, 7-temperature controlled shelves, 8-vakuometer, 9-exhaust valve, 10-motor-driven intermediate valve, 11-heating bath, 12-recirculation pump, 13-closing device, 15-pressure control valve, 16-micro air-release valve, 17-rubber valve, 18-defrosting device, 19-isolation; 4, 6 and 14 are external parts and not shown here)

The drying chamber is equipped with temperature controlled shelves on which the product is loaded. Energy can be removed from or applied to the product during freezing and drying via the shelves. By closing the chamber and valve a closed system can be obtained. The shelves are cooled down to about – 40 to – 50 °C, mainly by liquid circulation.

The vacuum pump allows a decrease in pressure from 1 to 10^{-3} mbar, creating a high vacuum within the drying chamber. The evaporated solvent is transferred to the condenser chamber to

be frozen. To indicate when the drying process is complete, the chambers are isolated by a vapour valve. The pressure increases within the drying chamber while solvent is still evaporating from the product. But when drying is complete the pressure remains constant. Interval isolation is done about three hours before expected process finish to check on the state of the product. After finishing the drying process, the drying chamber is again isolated from the freezing chamber and vacuum is broken by introducing nitrogen. After sealing and removal of the product, the condenser is defrosted and dried. Operation and control of the systems are mainly by computer although some lab scale dryers still are controlled manually.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and formulations

All chemicals and formulations used are given in Table 2.1.

Table 2.1 Reagents for preparation and characterization

substance	specification	lot	provider
acetonitrile (ACN)	HPLC grade		Merck AG, Darmstadt, Germany
ammonium acetate (NH_4CH_3COO)	analytical grade		Merck AG, Darmstadt, Germany
BIXX-F	40 % BIXX		Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
calcium chloride	analytical grade		Sigma Aldrich, Steinheim, Germany
Dantrol®	100 mg capsules	ZP291	Sanofi-Synthelabo, Switzerland
danazol	98%	035K1173	Sigma Aldrich, Steinheim, Germany
danazol		7216E	ICB Biochemicals, Inc., Eschwege, Germany
ethanol (EtOH)	analytical grade		Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
glycerol monooleate, Rylo MG 19	pharma	173403	Danisco, Braband, Denmark
glycerol monooleate, Rylo MG 20		2202/63	Danisco, Braband, Denmark
hydrochlorid acid (HCl)			Merck AG, Darmstadt, Germany
isopropanol	HPLC grade		Sigma Aldrich, Steinheim, Germany
Lipoid E PC	> 98% phosphatidylcholine	105026-1 / 911	Lipoid GmbH, Ludwigshafen, Germany
Lipoid E PC S	> 98% phosphatidylcholine	108015-2 / 935	Lipoid GmbH, Ludwigshafen, Germany
methanol (MeOH)	HPLC grade		Mallinckrodt Baker B.V., Deventer Netherlands
methylene chloride	HPLC grade		Merck AG, Darmstadt, Germany
monosodium phosphate (NaH_2PO_4)	analytical grade		Sigma Aldrich, Seelze, Germany
SIF™		PHA S0704011	Phares AG, Switzerland

sodium acetate ($NaOOCH_3$)	analytical grade		Sigma Aldrich, Seelze, Germany
sodium chloride (NaCl)	analytical grade		Merck AG, Darmstadt, Germany
sodium choleate	crude ox bile extract	113K0953	Sigma Aldrich, Steinheim, Germany
sodium hydroxide (NaOH)	analytical grade		Merck AG, Darmstadt, Germany
sodium oleate	analytical grade	51110	Sigma Aldrich, Seelze, Germany
sodium taurocholate	> 99%	2001030070; 2006040099	Alfred Tiefenbacher GmbH&Co, Hamburg, Germany
sodium taurocholate	> 99%	2107021	CPC, New Zealand
tert-butyl alcohol $(CH_3)_3COH$	analytical grade		Sigma Aldrich, Seelze, Germany
α-phosphatidylcholine, type X-E	> 60%	024K043	Sigma Aldrich, Steinheim, Germany
Zorbax Extend-C18	4.6x75 mm 3.5 µm		Agilent Technologies, Bensheim, Germany

To characterize the prepared media the physicochemical properties were studied and the solubilising properties for two model drugs were tested. The saturation solubility of danazol and the dissolution behaviour of formulated BIXX were the two model systems chosen.

Danazol was used to observe the influence of sodium taurocholate and phosphatidylcholine on solubility. This steroidal BCS II (see Figure 2.1) drug is a neutral and lipophilic compound (logP = 4.2) which is practically insoluble in water (solubility in water = 1 µg/ml). Since danazol is not ionisable, its solubility is not pH dependent. Danazol has been used as an effective treatment for endometriosis since the early 1980s, but its use declined markedly after the introduction of the GnRH agonists in the late 1980s and early 1990s.

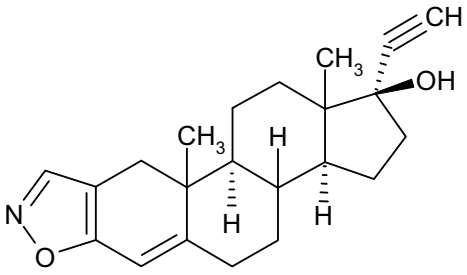


Figure 2.1 Chemical structure of danazol

BIXX is classified as BCS II drug with pH dependent, low solubility. It is a weakly basic BCS II drug with solubility decreasing from 0.082 mg/ml at pH 4.0 to 0.001 mg/ml at pH 6.5.

The formulation used in these studies is not yet commercially available and will therefore be referred to as BIXX-F.

2.1.2 Devices for production and testing

All devices used for the preparation and analytic of the media are given in Table 2.2.

Table 2.2 Devices used for preparation, packaging and characterization

equipment / instrument	type	use	provider
aluminium caps	double layer 20.3x7.7 mm	packaging	Helvoet Pharma, Alken, Belgium
aluminium foil bottom	AA 8021 (B) (=AlFe1,5)	packaging	ALCAN PACKAGING, Singen, Germany
aluminium foil lidding	DIN EN 573-3,12/94: EN AW-AlFe1Si/8079	packaging	ALCAN PACKAGING, Singen, Germany
analytical balance	AX 205 Delta Range	preparation media	Mettler Toledo, Schwerzenbach, Switzerland
balance	PG 5002-S Delta Range	preparation of buffer	Mettler Toledo, Schwerzenbach, Switzerland
dissolution tester	AT 7	dissolution	Sotax, Allschwill, Switzerland
bubble pressure tensiometer	DynoTester	characterization	SITA Messtechnik GmbH, Germany
cellulose filters	Minisart NML 0.2µm 26mm		Sartorius AG, Goettingen, Germany
DSC	DSC821e	characterization (glass transition temperature, melting point)	Mettler Toledo, Schwerzenbach, Switzerland
densimeter	DMA 4500 (harmonic oscillation)	characterization	Anton Paar, Graz, Austria
elastomer stoppers	20 mm	packaging	West Pharmaceutical Services, Eschweiler, Germany
fluorimeter	SFM 25		Kontron Instruments, Germany
freeze-dryer	delta 1-24 KD (LPC-16 software)	preparation of media	Martin Christ, Osterode, Germany
freeze-dryer	LYOVAC GT-6 (Falco software)	preparation of media	Amsco/Finn-Aqua, Hürth,Germany
GHP filters	Acrodisc 0.2 µm 13mm	sampling	Pall Life Science, Ann Arber, USA

GHP filters	Acrodisc 0.45 µm 13mm	sampling	Pall Life Science, Ann Arber, USA
glass vials			
HPChemStation	Rev.A.10.02	HPLC analysis	Agilent Technologies, Bensheim, Germany
HPLC column oven	series 1100, G1316A	HPLC analysis	Agilent Technologies, Bensheim, Germany
HPLC degasser	series 1100, G1322A	HPLC analysis	Agilent Technologies, Bensheim, Germany
HPLC detector	series 1100, G1315A	HPLC analysis	Agilent Technologies, Bensheim, Germany
HPLC injector	series 1100, G1313A	HPLC analysis	Agilent Technologies, Bensheim, Germany
HPLC pump	series 1100, G1311A	HPLC analysis	Agilent Technologies, Bensheim, Germany
hygrometer	ciSorb (Sorp software)	characterization	C.I. Electronics Ltd., Salisbury, UK
KFT	Titrino 720 KFS	characterization	Metrohm
magnetic stirrer	RCT basic	preparation	IKA Works Inc., Wilmington, USA
magnetic stirrer multi plate	Variomag	dissolution	Dr. Hoiss + Partner GmbH, Berlin, Germany
membrane filter	cellulose acetate filters, 0.2 µm	degassing and high pressure filtration	Sartorius AG, Goettingen, Germany
multipipette	Multipette stream	volumetric partiononing	Eppendorf, Hamburg, Germany
osmolality	Osmomat 030-D	characterization	Gonotec GmbH, Berlin, Germany
particle size measurement	Zetasizer Nano-S (DTS software)	characterization	Malvern Instrument Limited, Malvern, UK
PE flasks		packaging	
pH electrode	Inolab	preparation	Mettler Toledo, Schwerzenbach, Switzerland
pH meter	CG 840	preparation	Schott, Hofheim/Ts., Germany
pressure filtration device	16249	preparation	Sartorius Membranfilter, Goettingen, Germany
pump	CY 7-50	dissolution	Sotax, Allschwill, Switzerland
pump	CP 7	dissolution	Sotax, Allschwill, Switzerland
refrigerator	CP 3501-Ex	storage	Thalheimer, Ellwangen
rheometer	DV-III Ultra (Rheocalc software)	characterization	Brookfield, Middlboro, USA
rotavapor	RE 111	preparation	Buechi, Switzerland

scanning electron microscope	SUPRA 55 VP	characterization	ZEISS NTS, Oberkochen, Germany
shaker	MS1 Minishaker	homogenisation	IKA Works Inc., Wilmington, USA
shaker	DTS-2	well-plate-shaking	neo-Lab Migge, Heidelberg, Germany
slinging thermostat	thermomix 1441	dissolution	Braun Melsungen AG, Germany
tablet press	FlexiTab	preparation	Röltgen, Solingen, Germany
thermomixer	Thermomixer comfort		Eppendorf, Hamburg, Germany
thermostat	RML 6	temperature control viscosity	Lauda, Lauda-Königshofen, Germany
ultrasonic bath	Sonorex RX 103 H	preparation	Bandelin electronic, Berlin, Germany
UV/VIS spectrophotometer	Lambda 2S	dissolution	Perkin Elmer, Ueberlingen, Germany
UV/VIS spectrophotometer	8453	dissolution	Agilent Technologies, Bensheim, Germany

2.2 Methods

To study feasibility and compatibility of the freeze-drying method, the instant media were compared to conventional media prepared according to Galia et al.[38] Composition of the media differed in buffer salt selection from these presented by Galia: Potassium salts were substituted by sodium salts, since sodium is more physiological relevant and avoids solubility problems in the dissolution testing of HPMC and gelatine capsules.

All measurements regarding preparation and physicochemical properties were run at least in triplicate.

2.2.1 Compositions of solutions for freeze-drying

In order to change only one parameter per experiment, conventionally prepared FaSSIF and FeSSIF media were used in the early feasibility studies of freeze-drying experiments. The instant media obtained by this method showed big advantages in terms of practicability in the dissolution laboratory: Preparation of FaSSIF and FeSSIF starting from the freeze-dried media was much less time consuming compared to the conventional preparation method. However, using a standard lab-scale freeze-dryer it took two to three weeks to lyophilise the media. The high amount of water combined with suboptimal freeze-drying vessels led to the unacceptably long process time. Therefore, the next series of experiments focussed on options

for optimizing the freeze-drying method.

Five different types of solutions were tested, all containing bile salt and lecithin but varying in solvents and supplementary buffer salts. The media were additionally prepared at different dilutions. Table 2.3 shows composition and molar ratio of the solutes. Dilutions were calculated by dividing the final volume by volume of the concentrated solution (see Table 2.4. for final concentrations).

Table 2.3 Molar ratios and dilutions of solutions for freeze-drying

(NaTC purified sodium taurocholate, NaTCc crude sodium taurocholate, PC purified phosphatidylcholine, PCc crude phosphatidylcholine, GMO glycerol monooleate, NaO sodium oleate, NaCl sodium chloride, NaPh sodium phosphate, NaAc sodium acetate, tBA *tert*-butyl alcohol)

medium	NaTC	NaTCc	PC	PCc	GMO	NaO	NaCl	NaPh	NaAc	solvent	dilution to FaSSIF	dilution to FeSSIF	dilution to FeSSIFplus
1	1	-	0.25	-	-	-	-	-	-	water	50 - 300	10 - 50	-
2	1	-	0.25	-	-	-	35.28	9.68	-	water	10 - 30	-	-
3	1	-	0.25	-	-	-	13.54	-	9.6	water	-	5 - 15	-
4	-	1	-	0.25	-	-	-	-	-	water	50 - 300	-	-
5	1	-	0.25	-	-	-	-	-	-	tBA	50 - 300	10 - 50	-
6	1	-	0.27	-	0.67	4	-	-	-	water	-	-	10 - 30

2.2.2 Preparation

Six different solution types were prepared: FaSSIF/FeSSIF for reconstitution with buffer solutions, FeSSIFplus for reconstitution with buffer solutions, FaSSIF for reconstitution with water, FeSSIF for reconstitution with water, all without use of organic solvents during preparation, and FaSSIF / FeSSIF for reconstitution with buffer solutions prepared with an organic solvent. The concentrated solutions were prepared under strong stirring at room temperature using a magnetic stirrer in either pure water, pure *tert*-butyl alcohol or mixtures of both. Different concentrations were prepared by subsequent dilution of the highly concentrated solution. The various concentrations were prepared to determine what amount of solvent would lead to homogenous cakes with the lowest energy consumption.

Table 2.4 Composition of buffer solution and final concentrations of components in the reconstituted media

biorelevant solute concentrations	**FaSSIF**	**FeSSIF**	**FeSSIFplus**
sodium taurocholate [g/l]	1.65	8.25	4.12
phosphatidylcholine [g/l]	0.59	2.954	1.573
glycerol monooleate [g/l]	-	-	1.783
sodium oleate [g/l]	-	-	3.044
buffer concentrations			
phosphate [mmol/l]	29	-	-
acetate [mmol/l]	-	144	144
NaCl [g/l]	6.186	11.87	11.87
pH	6.5	5	5

2.2.2.1 Preparation of FaSSIF and FeSSIF for reconstitution with buffer solutions

Water as solvent:

To enable use of pure water as the solvent, sodium taurocholate was first dissolved in pure water at room temperature. Phosphatidylcholine was subsequently added. Two hours of stirring led to a slightly turbid suspension. A clear yellowish emulsion was obtained by subsequent ultrasonification for about 15 min in a ultrasonic bath at room temperature. For a final volume of 12 litre, 19.8 g of sodium taurocholate and 7.08 g of phosphatidylcholine were suspended in 40 ml of pure water. Aliquots of the solution were further diluted to obtain a series of concentrations. 0.5 ml aliquots were diluted 1:0.2, 1:0.82, 1:1, 1:1.4, 1:2, 1:3 and 1:5. Concentration factors of the solutions were 300, 250, 165, 150, 125, 100, 75 and 50 compared to the final FaSSIF concentrations (see Table 2.4 for final concentrations).

Tert-butyl alcohol as solvent:

For the preparation with pure *tert*-butyl alcohol, phosphatidylcholine was dissolved in the fluid solvent (melting point of this alcohol is 25 °C). Subsequently sodium taurocholate was dispersed into the solution. For a final volume of 1 litre, 0.59 g of phosphatidylcholine and 1.65 g of sodium taurocholate were dispersed in 5 ml of pure liquid *tert*-butyl alcohol, which corresponds to a concentration factor of 200. The dispersion was stirred at room temperature for two hours and ultrasonificated for 30 min. 0.5 ml aliquots were diluted 1:1, 1:2 and 1:4 with *tert*-butyl alcohol to obtain final concentration factors of 200, 100, 66.7 and 50, respectively.

Mixture of *tert*-butyl alcohol and water (1:1) as solvent:

This method was done following Li et al.[54] by mixing the alcoholic and water phase to obtain a clear isotropic liquid. For the preparation with diluted *tert*-butyl alcohol, phosphatidylcholine was dissolved in pure *tert*-butyl alcohol and sodium taurocholate in water. For a final volume of 1 litre, 0.59 g of phosphatidylcholine and 1.65 g of sodium taurocholate were dissolved in 2.5 ml of pure liquid *tert*-butyl alcohol and 2.5 ml of pure water, respectively (concentration factor of 200). The clear solutions were mixed and stirred at room temperature for 4 hours. After ultrasonification for 30 min 0.5 ml aliquots of the dispersion were diluted 1:1, 1:2 and 1:4 with 50 % *tert*-butyl alcohol in water to achieve final concentration factors of 200, 100, 66.7 and 50, respectively.

The reconstitution solvents for the lyophilisate in each case are the buffer solutions corresponding to the compositions of FaSSIF and FeSSIF. The pH values of 6.5 (FaSSIF) and 5.0 (FeSSIF) have to be controlled and if necessary adjusted after dissolution of the lyophilisate is complete.

2.2.2.2 Preparation of FeSSIFplus for reconstitution with buffer blank

The concentrated solutions were prepared at room temperature under strong stirring using a magnetic stirrer. To prepare the FeSSIFplus concentrates, lower concentrations of solutes than for FeSSIF were chosen. Sodium taurocholate and sodium oleate were dissolved in pure water, phosphatidylcholine and glycerol monooleate were subsequently added. The solution was stirred at room temperature for two hours and ultrasound then was applied for about 15 min. For a final volume of 12 litre, 49.44 g of sodium taurocholate and 36.63 g of sodium oleate were dissolved in 400 ml of pure water. 18.88 g of phosphatidylcholine and 21.4 g of glycerol monooleate were subsequently suspended in the solution. 0.5 ml aliquots of the dispersion were diluted 1:0.5 and 1:2 with water to achieve final concentration factors of 30, 20 and 10, respectively compared to the final concentration (see Table 2.4 for final concentrations).

The reconstitution solvent for the lyophilisate is the buffer solution corresponding to the compositions of FeSSIFplus. The pH value 5.0 has to be checked and if necessary adjusted after dissolution of the lyophilisate is complete.

2.2.2.3 Preparation of FaSSIF and FeSSIF for reconstitution with water

For the preparation of concentrates containing buffer salts, sodium chloride or mannitol, these hydrophilic materials were first dissolved in water. Sodium taurocholic acid and phosphatidylcholine were then dispersed in the aqueous solutions. To determine the maximum possible concentration factors for FaSSIF and FeSSIF "complete", highly concentrated buffer blank solutions were prepared and diluted. In case of FaSSIF, mono- and dibasic phosphate salts were mixed to obtain a fixed pH value after reconstitution. To prepare FeSSIF complete, sodium acetate was used instead of glacial acetic acid in order to maintain buffer concentration during lyophilisation. The pH was not adjusted to pH 5.0, in order to avoid formation of acetic acid. Preparation of the most concentrated solutions possible was achieved as follows:

FaSSIF: 54.3 g of dibasic sodium phosphate dihydrate and 74.23 g of sodium chloride were dissolved in 400 ml of water. 19.8 g of sodium taurocholate and 7.08 g of phosphatidylcholine were added to the solution. 0.5 ml aliquots of the dispersion were diluted 1:0.5 and 1:2 with water to achieve final concentration factors of 30, 20 and 10, respectively compared to final concentration (see Table 2.4 for final concentrations).

FeSSIF: 141.75 g of sodium acetate and 142.44 g of sodium chloride were dissolved in 800 ml of water. 99 g of sodium taurocholate and 35.45 g of phosphatidylcholine were added to the solution. 0.5 ml aliquots of the dispersion were diluted 1:0.5 and 1:2 with water to achieve final concentration factors of 15, 10 and 5, respectively compared to final concentration (see Table 2.4 for final concentrations).

For reconstitution of these lyophilisates, pure water can be used. However, the pH values of the solutions have to be checked and adjusted to the desired value after reconstitution.

2.2.3 Freeze-drying

The solutions were freeze dried as follows. Firstly they were either poured into trays for bulk drying or, for pre-dosed preparations, distributed into vials or blister cavities. Distribution was done volumetrically after determination of the density of the solution for those studies.

Different filling heights were tested to observe influence of this parameter on cake formation. Filling heights of 3 to 20 mm were tested.

The trays, vials or blisters were then loaded into the freeze-drying chamber at room temperature. The freezing rate was varied from 0.5 to 3 K/min and the final temperature to

– 40 or – 50 °C, depending on the freeze-dryer used, at atmospheric pressure. The final temperature was held for 0.5 to 3 hours to enable complete freezing of the solution.

Primary drying was carried out under a vacuum of 0.28 mbar for 5 to 15 hours. The temperature was then increased to 0 °C over 2 to 5 hours and then further to 20 °C over 3 to 10 hours. Completion of the primary drying process (sublimation) is tested by determining the pressure rise within a given time-frame. Typically, the chambers were isolated for 120 seconds during a pressure rise measurement, and these were investigated two hours into primary drying. The sublimation was continued until the rise in pressure did not exceed 2.5 mbar/min in the 120 second observation time.

To obtain sufficient desorption of the solvent during secondary drying, the vacuum was increased to 0.03 mbar for 5 to 10 hours. Temperature was increased to 30 °C over 5 hours and held at 30 °C until the final product was obtained. Completion of secondary drying was also determined by pressure rise testing, beginning after two hours. Complete drying was indicated by a pressure rise below 5 mbar during 240 seconds of chamber isolation. Subsequently the temperature was reduced to room temperature and the vacuum was reduced by inflow of nitrogen to enable closing (in case of vials) and prepare for removal of the product from the chamber. Figure 2.2 illustrates temperature and pressure time-lines in a freeze-drying process.

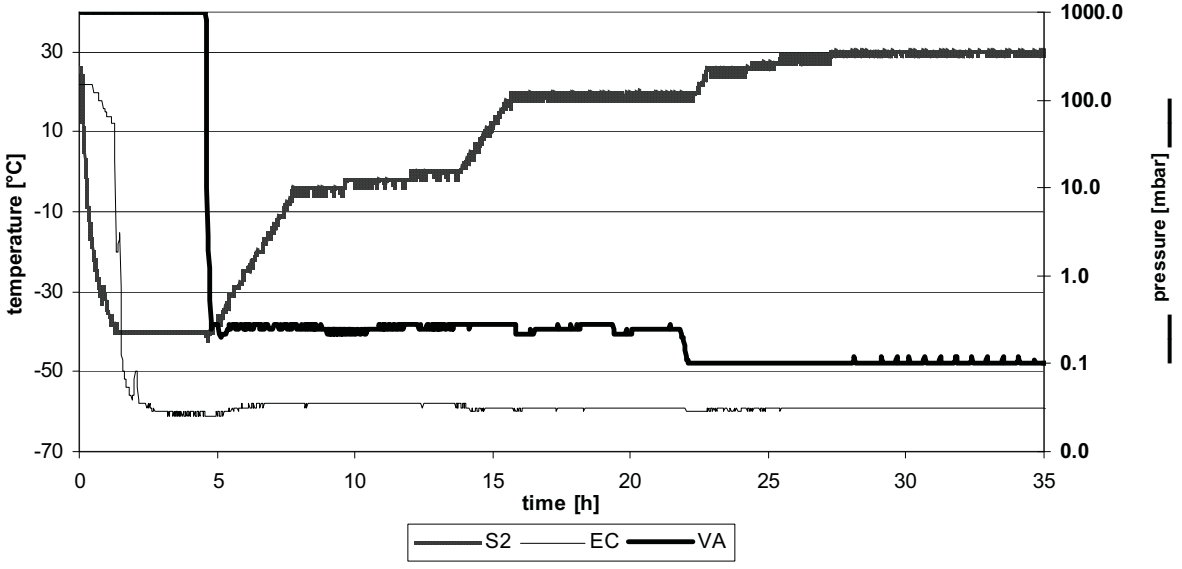


Figure 2.2 **Sample freeze-drying parameters:** shaded line (S2) is the shelf temperature [°C], the thin line (EC) is the temperature of the condenser [°C] and the bold line (VA) is the pressure [mbar]

2.2.4 Packaging and storage

Tests on variation of packaging material and storage were carried out with instant products containing FaSSIF and FeSSIF without buffers. The instant form of FeSSIFplus (without buffers) was prepared and stored in standard glass vials, closed with stoppers and caps, whereas the instant products containing the complete set of FaSSIF and FeSSIF components were prepared on trays and packaged as bulk material in polyethylene (PE) flasks with $CaCl_2$ as the desiccant.

Lyophilisation and packaging were conducted either in one production step or the lyophilised material was further treated / packaged in a separate step.

For packaging of bulk material, the concentrated solutions were lyophilised on trays and transferred in a separate step into PE-flasks. Calcium chloride was added as a desiccant to better avoid moisture build-up during storage.

For packaging the pre-dosed units, several materials and methods were compared:

(a) to enable easy preparation and reduce the number of production steps, the concentrated solutions were directly lyophilised in commonly used freeze-drying vials and closed under inert conditions (nitrogen) as the final step of the freeze-drying process. The stoppers were afterwards clamped with aluminium caps.

(b) the lyophilised media were removed from the freeze-dryer and further dried under low humidity conditions (21 °C and <12 % RH). Aliquots of the highly voluminous powders obtained were weighed to correspond to a given final desired volume of biorelevant media and compressed to FaSSIF and FeSSIF instant tablets. The tablets were filled into PE-flasks with calcium chloride as desiccant.

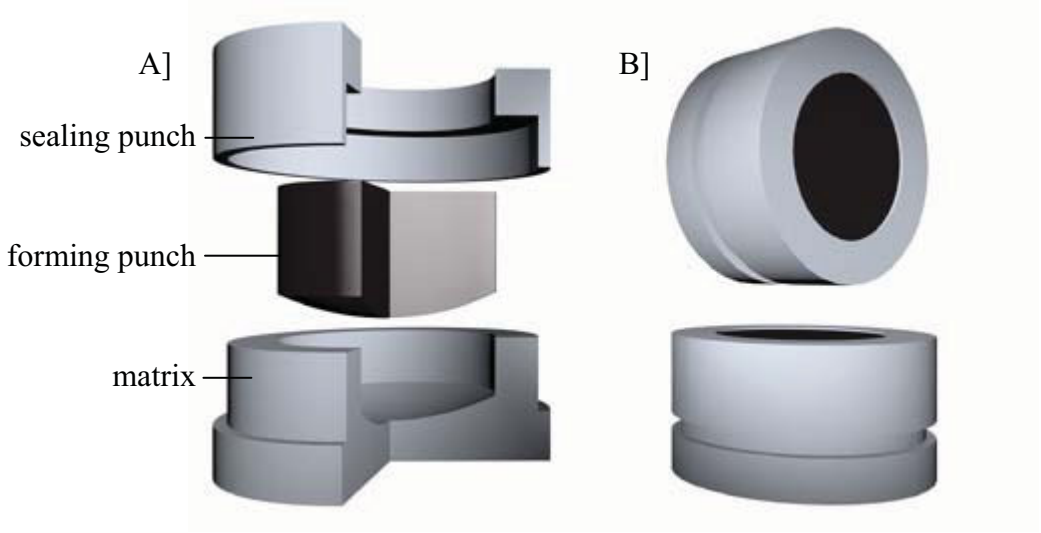


Figure 2.3 Special device for large volume blister containing of matrix, forming and sealing punch given exploded in A] and closed for blister forming in B]

(c) standard peel-push and push blisters as well as specially developed blisters were used to increase throughput and allow high volume dosing. The blister sealing was conducted in a separate step at reduced humidity conditions (21 °C and <12 % RH). Two different blister sizes were used for freeze-drying of low and high volumes. The low volumes (1 – 3 ml) were done in standard blisters to fit capsule size 0. To allow freeze-drying and packaging of 20 – 30 ml of concentrated solution, blister cavities with a diameter of 60 mm were used. Figure 2.3 shows a special developed device for creating the cavities from the bottom foil. The bottom foil was clamped between the matrix and the sealing punch and the cavity was formed by pushing the forming punch towards the matrix through the sealing punch recess. The blisters were sealed under dry room conditions directly after freeze-drying as follows: the blisters were inserted into the matrix and covered with sealing foil. The sealing punch was preheated to 250°C and pressed onto the matrix and blister for 5 - 10 seconds (the optimal sealing time of the newly developed blisters was chosen by testing sealing at 250 °C for 1, 5, 10 and 15 seconds).

For sealing standard blisters a temperature of 250 °C was also used, but the sealing time could be reduced to 0.2 seconds. The sealing method was validated by density testing under vacuum in a solution of methylene blue. Figure 2.4 shows a sealed blister with instant FaSSIF media corresponding to a reconstituted volume of 6 litre.

Figure 2.4 Large volume blister sealed (left) and opened (right, 1 €-cent for size comparison), prepared by the special device

Storage conditions for stability testing of the packaged products were 25 °C / 60 % RH, 40 °C / 75 % RH and cooled at 5 °C.

2.2.5 Reconstitution

Reconstitution of the lyophilisate was done with either water or buffer solution. Powders obtained from solutions prepared according to section 2.2.2.3 were dissolved in water, forming a clear solution. The pH was then checked and adjusted if necessary. Powders prepared according to section 2.2.2.1 and 2.2.2.2 were dissolved in buffer solutions corresponding to the desired medium composition (see Table 2.4). Buffer solutions were prepared by dissolving all solutes in pure water. pH values of the solutions were adjusted before dissolution of the lyophilised powders. The amount of reconstitution media was determined by either pre-dosing or by weighing the bulk powder. Preparation of FaSSIF and FeSSIF from freeze-dried media prepared according to section 2.2.2.1 was done by dissolving 2.24 g or 11.204 g of powder per litre of buffer, respectively. 12.28 g/l of powder according to the method outlined in section 2.2.2.2 was dissolved to prepare FeSSIFplus.

2.2.6 Characterization of physicochemical properties

Physicochemical properties were measured to test interchangeability of media prepared freshly or by reconstitution and to test stability of the freeze-dried media. FaSSIF and FeSSIF were prepared following the conventional method and compared to redissolved instant media to evaluate interchangeability of the methods.

The stability of the instant media was tested both in the solid state and after reconstitution.

Differential scanning calorimetry was used to determine glass transition temperature of the concentrated solutions. These tests were carried out in the analytical department. Aliquots of the solutions were filled into 40 µl aluminium pans and thermal analysis was performed under nitrogen atmosphere with the thermal gradients listed in Table 2.5. Differential scanning calorimetry (DSC) was also conducted on the lyophilised samples.

Table 2.5 Thermal gradient during differential scanning calorimetry of concentrated solutions and of lyophilisates

concentrated solution	total time [min]	change [K/min]	temp. [°C]	lyophilisate	total time [min]	change [K/min]	temp. [°C]
	0	0	25		0	0	25
	7.5	10	-50		6.75	20	160
	17.5	0	-50		11.75	0	160
	27.5	10	50		18.5	20	25
	-	-	-		23.5	0	25
	-	-	-		24	10	30

Lyophilised media were tested regarding optical appearance, SEM, glass transition temperature and moisture content.

The lyophilised products were reconstituted according to section 2.2.5 and the resulting biorelevant media tested for viscosity, osmolality, particle size, density, surface tension and solubilisation properties.

Viscosity: For the determination of viscosity a rotational viscometer with coaxial-cylinder geometry, which operates according to the Searle principle (measurement of torque required to rotate an immersed element (spindle) in a fluid). Small volume measurement of the low viscous fluids was possible using spindle and chamber diameters of 25.47 and 27.62 mm, respectively. The medium was equilibrated for 15 min to achieve a constant temperature of 20 °C. Viscosity was measured at 20 °C over a range of shear rates from 25 to 250 reciprocal seconds.

$$\eta = viscosity\,[P] = \frac{\tau}{\gamma} = \frac{shear\ stress\ [N/m^2]}{shear\ rate [1/s]}$$

Equation 2.1 calculation of viscosity

Osmolality: Osmolality was measured by freezing point depression. 50 µl of the media were cooled below the freezing point using a Peltier trap. The temperature of freezing was measured within the sample using a thermistor (temperature-dependent resistor) and used to calculate osmolality.

Particle size: Particle size distribution and polydispersity index were measured by photon correlation spectroscopy (PCS). The average particle diameter and size distribution of the solutions were measured at 20 °C ± 2 °C measuring backscattering at a detection angle of

173 °. A red He-Ne laser with a wavelength of 633 nm was used. Scattering signals were correlated by the non-negative least squares method (NNLS) and the characteristics were calculated according to the Mie theory. To carry out these calculations, the refractive index of the blank buffer was first calculated and a fictitious refractive index of 1 was assumed for the mixed micelles.

Surface tension: Surface tension was measured at 22 °C ± 2 °C and a bubble development time of 120 ms. Mean values were calculated from 10 measurements.

Solubilisation: Two different methods for measurement of solubilisation of danazol were used: Since the orbital shaker broke down after two months' storage, the methodology of the 3 and 6 months samples differed for solubility determinations directly after preparation of the freeze-dried media.

Preparation of test tubes was identical for all measurements: Since solubility can be dependent on the excess of substance used[55], danazol was first dissolved in MeOH and dispensed volumetrically into 2 ml tubes to obtain 100 µg per tube. The tubes were filled with 1.8 ml of reconstituted media.

Directly after freeze-drying the orbital shaker could be used to shake the sample tubes at 37° C for 24 hours at 450 rpm. Measurements with media reconstituted after 3 and 6 months of storage were done by keeping the sample tubes at 37 °C for 22 hours without shaking and subsequent shaking for 2 hours at 37° C.

After 24 hours of solubilisation, samples were filtered (0.45 µm GHP filters) and diluted 1:1 with methanol. All syringes, filters and needles used were preheated to 37 °C in an oven.

Determination of the dissolved concentration of danazol was performed by HPLC-UV-VIS analysis using a mobile phase of water/acetonitril 62:38 and a column temperature of 45 °C. After a retention time of 1.8 min, danazol absorbance was determined at a wavelength of 286 nm.

Dissolution of BIXX: *In vitro* dissolution was tested in the USP basket method (Apparatus I) in media deaerated according to the USP[56] method for deaeration of solutions. The dissolution data were generated at 37 ± 0.5 °C in 200 ml dissolution medium using the basket rotating at 100 rpm. Samples were taken automatically and measured via UV/VIS at 330 nm. pH was monitored before and after dissolution testing. Measurement of BIXX via HPLC-UV/VIS was conducted using a mobile phase of 0.05 M ammonium acetate pH 4.4 and acetonitrile.

Dissolution of danazol from Danatrol® capsules: *In vitro* dissolution data were generated at 37 ± 0.5 °C in 500 ml dissolution medium using the USP dissolution apparatus II with the paddle rotating at 75 rpm. Sinkers were used to prevent capsules from floating. Samples were

taken automatically and measured on-line via UV/VIS.

Standard curves for danazol and BIXX were run every analytical day.

All dissolution experiments were run in triplicate.

Miniaturized dissolution of BIXX: *In vitro* dissolution data were generated at 37 ± 0.5 °C in 20 ml dissolution medium using a miniaturized dissolution apparatus with paddles rotating at 100 rpm. Samples were taken automatically and measured on-line. pH was measured before and after each dissolution. A standard curve for BIXX was run every analytical day. All dissolution experiments were run in triplicates. Analysis of variance for repeated measurements ($ANOVA_{rep}$) was used to statistically assess the effect of preparation method and storage on the buffer as well as time interactions for each pair of dissolution profiles. A Bonferroni post hoc test was performed for pairwise comparisons between buffers at each time point. All statistical comparisons were performed using GraphPad Prism Vs. 5 (GraphPad Software, Inc., San Diego, USA).

2.2.7 Comparison of commercially available and freeze-dried instant media

To prepare media by the conventional and the freeze-drying method, pure phosphatidylcholine from egg (PC) and pure sodium taurocholate (NaTC) from PCA, Italy and CPC, New Zealand were used. SIF™ instant biorelevant media was purchased from Phares AG, Switzerland.

Media

Buffers corresponding to FaSSIF and FeSSIF were used for preparation of the various media. These "blank" buffers were adjusted to pH 6.5 in case of FaSSIF (0.029 M phosphate, 0.106 M NaCl) and to pH 5.0 in case of FeSSIF (0.144 M acetate, 0.203 M NaCl). 2.24 g/l and 11.2 g/l of SIF™ instant powder were dissolved in the corresponding buffers to prepare FaSSIF-SIF™ and FeSSIF-SIF™. The standard / conventional method (CM) introduced by Galia et al.[38] was used to prepare FaSSIF-CM and FeSSIF-CM. Freeze-dried (FD) media, FaSSIF-FD and FeSSIF-FD were prepared from aqueous solutions following method III2.2.2.1. 2.24 g/l or 11.2 g/l of lyophilisate were reconstituted in the corresponding buffers to prepare FaSSIF and FeSSIF, respectively.

Characterization of physicochemical properties

The various dissolution media were characterized according to III2.2.6.

Dissolution tests

Dissolution of Danatrol® capsules and BIXX-F capsules was conducted in all biorelevant

media and their corresponding buffers.

In vitro dissolution data were generated at 37 ± 0.5 °C in 500 ml dissolution medium using the USP dissolution apparatus II with the paddle rotating at 75 rpm. Samples were taken automatically and measured on-line via UV/VIS. Standard curves for danazol and BIXX were run every analytical day. The pH was measured after dissolution of BIXX-F.

All dissolution experiments were run in triplicate.

To check of the on-line UV/VIS measurements, some samples were additionally removed manually at predetermined time points and analysed by HPLC to verify the online UV/VIS dissolution results. The verification samples were filtered through 0.45 µm Sartorius® membrane filters and subsequently diluted 1:1 with methanol. Analysis was carried out by HPLC-UV/VIS measurement using a reversed phase column and pure water/acetonitril (68/32) as mobile phase for danazol and 0.05 M ammonium acetate pH 4.4 and acetonitrile as mobile phase for BIXX.

3 Results

The methods to prepare biorelevant media for reconstitution with either buffer blank or water achieved varying degrees of success. Preparation with water according to methods described in 2.2.2.1 and 2.2.2.3 was unproblematic with respect to solution preparation, freeze-drying and reconstitution using concentrates ranging from a factor of 5 to 165 fold compared to the final concentration in the reconstituted medium. The preparation with *tert*-butyl alcohol (tBA) according to methods described in 2.2.2.1 or with water according to 2.2.2.2 proved to be problematic in terms of reproducibility. Freeze-drying was either not possible or not economically viable. Table 3.1 summarizes the results for the viability of preparation of freeze-dried biorelevant media.

Table 3.1 Viability of various preparation methods for manufacture of freeze-dried biorelevant media

method	solvent	preparation	freeze-drying	reconstitution	recommended concentration factor
2.2.2.1	water	facile	facile	fast and complete	165 (FaSSIF) 33 (FeSSIF)
2.2.2.1	tBA	no dissolution of solutes	-	-	not recommended
2.2.2.1	tBA-water	not possible to obtain a clear solution	possible	acceptable	not recommended
2.2.2.2	water	very good	very good	very good	20
2.2.2.3	water	possible	possible	possible	15 but not recommended

3.1 Preparation and lyophilisation of concentrated solutions

3.1.1 Preparation

Preparation of FaSSIF and FeSSIF in water for reconstitution with buffer solutions:

Preparation following method 2.2.2.1. Sodium taurocholate completely dissolved at even the highest concentration of 50 % ($^{w}/_{v}$). Phosphatidylcholine, which has to be stored frozen, was thawed, weighed and added to the solution under strong stirring using a magnetic stirrer. The bigger pieces of the solid phosphatidylcholine were shredded by the stirring and dispersed within about 1 hour. After 2 hours a highly viscous but clear solution was obtained. No visual

change could be observed after subsequent ultrasonification. Depending on the concentration factor, the solutions were slightly yellowish (highest concentration) to colourless (low concentrations). The density of the solutions was close to that of water, 1.005 to 1.05 g/cm³.

Preparation of FaSSIF and FeSSIF in pure *tert*-butyl alcohol for reconstitution with buffer solutions:

No clear solution could be obtained with method 2.2.2.1 using *tert*-butyl alcohol. Phosphatidylcholine dissolved in fluid *tert*-butyl alcohol even at highest concentration of 12 % ($^{w}/_{v}$) but addition of sodium taurocholate led to a milky and highly turbid dispersion. Solution could not be completed even after 2 hours of stirring and ultrasonification for about 60 min. Further dilution with *tert*-butyl alcohol to a two-, three- or even fivefold volume did not lead to clarification. Therefore pure *tert*-butyl alcohol is not suitable as a solvent for the preparation of concentrated FaSSIF and FeSSIF solutions.

Preparation of FaSSIF and FeSSIF in *tert*-butyl alcohol / water mixtures for reconstitution with buffer solutions:

According to method 2.2.2.1, the clear solutions of 24 % ($^{w}/_{v}$) phosphatidylcholine obtained in *tert*-butyl alcohol and 66 % ($^{w}/_{v}$) sodium taurocholate in water were mixed under stirring. This led to immediate precipitation. The precipitate could not be dissolved either by 2 hours of stirring and ultrasonification or by fivefold dilution with 50 % *tert*-butyl alcohol. By stirring the solution for additional 12 hours some clarification could be achieved but the solution was still milky. A density of the 1.057 g/cm³ was measured for this solution.

Preparation of FeSSIF plus for reconstitution with buffer solutions:

Preparation of solution for FeSSIF plus was carried out according to method 2.2.2.2. Sodium taurocholate and sodium oleate were completely dissolved in water even at the highest concentration of 22 % ($^{w}/_{v}$). Phosphatidylcholine and glycerol monooleate were weighed and added at room temperature to the solution under strong stirring using a magnetic stirrer. The solids were shredded and dispersed within about 1 hour of stirring, leading to slightly turbid or opalescent dispersions. The solution was stirred at room temperature for an additional 60 minutes resulting in a highly viscous, ochre-colored but clear solution, whose appearance did not change during a further 15 min of ultrasonification. The solution with a concentration 32 % ($^{w}/_{v}$) had a density of 1.026 g/cm³. The ochre color observed at a concentration factor of 30 faded to yellowish when the solution was diluted by a factor of 20 or 10. The clear tawny

solutions were filled into vials according to a final volume of 200 ml of reconstituted FeSSIFplus.

Preparation of FaSSIF complete:

Sodium taurocholate was dispersed into a concentrated solution of sodium phosphate and sodium chloride in water according to method 2.2.2.3. The pH value of the solution changed slightly (0.2 values) by the addition of the bile salt. Within seconds the solution started to form a gelatinous liquid containing small, clot-like semi solids. By dilution with a threefold amount of water, the jelly was redissolved, forming a clear yellowish solution.

To avoid clot formation, variations in the sequence of the preparation steps were investigated. Separate concentrated solutions of each solute in water were prepared. The concentrated sodium taurocholate solution was stirred at room temperature and the concentrated solutions of the buffer salt and NaCl were added successively: Sodium taurocholate was dissolved in water at a concentration of 18 % ($^{w}/_{w}$) forming a clear solution. After addition of concentrated solutions of Na_2HPO_4 and NaH_2PO_4 in water no change in clarity occured. A 30 % ($^{w}/_{w}$) solution of NaCl was subsequently added. After 10 minutes the solution started to precipitate. A fiber-like solid phase was formed, resulting in a jelly-like mass after about one hour. Precipitation could not be avoided by a change of mixing order. In all cases, fine fiber-like precipitation followed by gelatinisation occurred. The final concentration of the solution was sodium taurocholate 4.88 %, Na_2HPO_4 8.52 %, NaH_2PO_4 4.87 % and NaCl 18.14 %, all ($^{w}/_{w}$). Photomicrographs of precipitates from both preparation methods are shown in Figure 3.1.

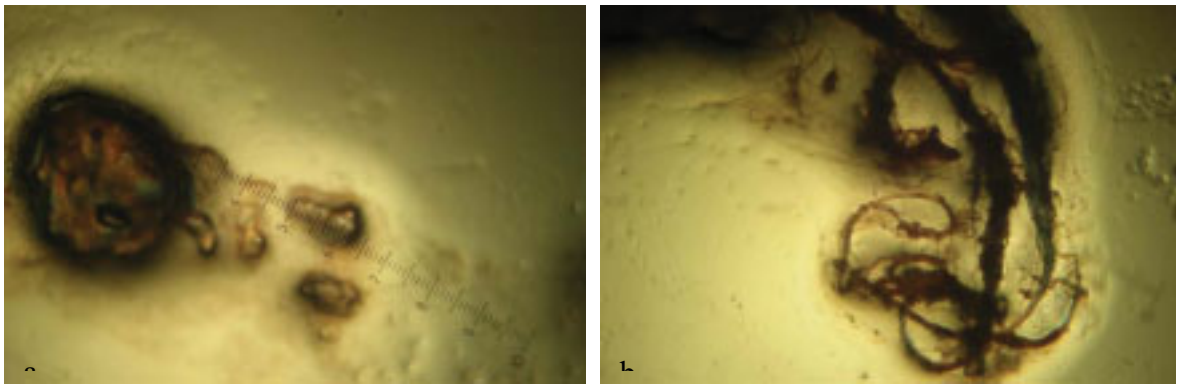


Figure 3.1 **Precipitates** obtained from **a)** dispersion of sodium taurocholate in the concentrated salt solution and **b)** successive addition of salts to a solution of sodium taurocholate in water

The precipitate could be redissolved within 10 minutes by dilution with water to about threefold the original volume. Lecithin could then be dispersed in this solution. The final molar concentration of phosphate in the concentrated solution was 430 mM (factor 15). The

pH of the resulting solutions was 5.5 ± 0.2. However the low maximum concentration factor attained makes freeze-drying by this method unacceptable from the economic point of view.

Preparation of FeSSIF complete:

The concentrated solution of FeSSIF was prepared according to method 2.2.2.3. Dissolving sodium taurocholate in the concentrated buffer solution resulted in precipitation. At concentration factors higher than 30 precipitation occurred. After dilution from factor 30 to factor 15 (compared to the reconstituted solution), the precipitate redissolved. Subsequently phosphatidylcholine was dissolved, forming a clear, slightly yellowish solution. To enable preparation of a factor 30 concentrated solution, a further experiment was run in which each solute was dissolved separately, and mixed separately. With this method a slight, fine particular precipitation occurred that could only be dissolved by dilution to factor 15, 10 or 5. The clear, slightly yellowish solutions were poured into trays for bulk freeze-drying.

Filling: After determination of the solution's density, the glass vials were filled volumetrically. Densities varied between 1.2 and 1.6 g/cm³ depending on the concentration factors. The filling height varied between 1 and 15 mm depending on the concentration and the target final volume. At concentration factors of 100 to 300 and with filling heights of 5 to 15 mm, freeze-dried cakes with a highly amorphous appearance were obtained. Filling heights below 3 mm led to non confluent cake formation consisting of a solid ring with a higher density at the edges of the glass vials.

FaSSIF and FeSSIF for reconstitution with buffer blank

The highest feasible concentrations factors were 165 and 33 in case of FaSSIF and FeSSIF, respectively. Highly porous and rapidly dissolving cakes were obtained at these concentration factors. Solutions at lower concentration factors also led to homogenous cake formation with rapid dissolution. However, the correspondingly greater filling heights caused prolonged drying times.

Dispersions obtained with of *tert*-butyl alcohol did not form homogenous cakes.

FeSSIFplus for reconstitution with buffer blank

Homogenous cake formation was only obtained from factor 20 and factor 10 concentrates. The factor 30 concentrate led to a sticky, packed cake that dissolved only slowly when buffer solution was added.

FaSSIF and FeSSIF complete for reconstitution with water

Homogenous cakes of complete FaSSIF and FeSSIF could not be obtained by freeze-drying these solutions. During freezing and primary drying, incrustation, puffing, breaking and finally agglutination of the broken cakes was observed. The strong puffing caused the freeze-drying glass tray to fracture. Figure 3.2 shows FaSSIF dried from a concentrated solution containing phosphate buffer and sodium chloride. Before reconstitution, the cake was ground and weighed according to the target reconstitution volume. Per litre of water, 12.3 g and 44 g of the freeze-dried and ground powder were dissolved for the preparation of FaSSIF and FeSSIF, respectively. In both cases reconstitution was slow and the desired pH values could not be achieved.

Figure 3.2 Freeze-dried FaSSIF (complete composition, 41 fold concentrated)

Best results were obtained from concentrated solutions without use of buffer salts or sodium chloride. Therefore lyophilisates obtained using this method were studied further in terms of physicochemical properties and stability of the freeze-dried biorelevant media.

Concentration factors were 165, 33 and 20 for FaSSIF, FeSSIF and FeSSIFplus, respectively. The optimum compositions of the concentrates are given in Table 3.2.

Table 3.2 Compositions of concentrate solutions for lyophlisation of biorelevant media

	FaSSIF	FeSSIF	FeSSIFplus
sodium taurocholic acid (NaTC) [g]	272.25	272.25	82.4
phospahtidylcholine from egg (PC) [g]	97.35	97.35	31.47
glycerolmonooleat (GMO) [g]	-	-	35.67
sodium oleate (NaO) [g]	-	-	61.05
H_2O [ml]	1000	1000	1000
concentration factor	165	33	20

3.1.2 Lyophilisation of various concentrates

Feasibility studies for lyophilisation of the various concentrates were carried out using a standard freeze-drying program for mixed micelle solutions. A freezing rate of 2 K/min was used to reach - 45 °C. In the first experiments, primary and secondary drying was conducted slowly over a period of 48 hours, this was later shortened to 30 hours without resulting in collapse of the structures. The most suitable concentration ranges of the solutions for obtaining homogenous and rapidly dissolving freeze-dried FaSSIF and FeSSIF are given in Table 3.3. The given concentrations are low enough to produce homogenous cakes but high enough to facilitate efficient freezing times and therewith reduce energy consumption.

Table 3.3 Preferred concentrations of solutions for preparing freeze-dried biorelevant media

media	FaSSIF	FeSSIF	FaSSIF	FaSSIF	FeSSIF plus	FaSSIF complete	FeSSIF complete
solvent	water	water	tBA	tBA	water	water	water
concentration factor	165	33	-	-	20	15	15

At concentrations higher than these mentioned above, homogenous cakes could not be obtained. The cakes obtained from higher concentrated solutions exhibited inhomogeneities, with incrustations on top and a strongly puffed-up solid. By lowering the filling heights, the extent of foaming could be reduced but even then it was not possible to form a fluffy cake. Further reduction of the filling height resulted in a discontinuous fluid level due to an insufficient amount of liquid. As a result, solid rings with slow dissolving rates were obtained. Low concentrations combined with usual filling heights led to homogenous cake formation. However, such concentrations necessitate high volumes and thus increase processing time. To

maintain efficiency of the preparation process, the amount of solvent should be reduced to a minimum.

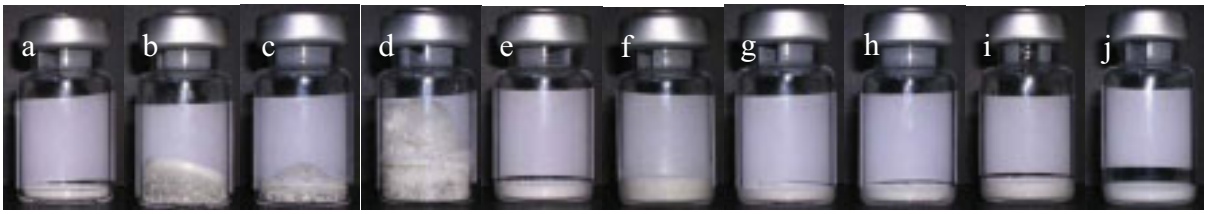


Figure 3.3 Freeze-dried media from solutions of different concentrations and filling heights

Figure 3.3 shows the influence of the concentration factor and filling height on cake formation. The corresponding concentration factors and reconstitution volumes are given in Table 3.4. A minimum filling height of 4 mm resulted in homogenous cake formation. A 20 mm fill height proved to be the maximum possible using the standard freeze-drying process.

Table 3.4 concentration factors and filling heights corresponding to Figure 3.3 for FaSSIF

Figure 3.3	**a**	**b**	**c**	**d**	**e**	**f**	**g**	**h**	**i**	**j**
concentration factor relative to FaSSIF	300	300	300	300	165	150	125	100	100	50
corresponding reconstitution volume [ml]	20	100	200	500	40	100	20	20	100	20
filling height [mm] (approximately)	<1	3	5	11	4	6	<1	1	5	3

3.1.3 Freeze-drying process

Solutions were cooled down from 22 °C to -40 or -50 °C at freezing rates of 0.55, 1.1 and 1.7 K/min. Freezing rates of 0.55 and 1.1 K/min resulted in homogenous, highly porous cakes, which were easily redissolvable in blank buffer solutions. More rapid freezing at 1.7 K/min, however, led to very compact cake formation, incrustation on the surface and partial puffing.

The minimum temperature was maintained for at least two hours for every experiment. The pressure during primary drying was reduced to 0.28 mbar in each experiment. Temperature controlled shelves were warmed up to -5 °C over 3 hours. A further increase to 20 °C was performed in one or two steps over a period of 10 to 24 hours. Single step heating was performed over 14 hours leading to compact cakes with melt-like parts. Single step heating over 24 hours led to similar structures but with less distinctive phases. Two step heating was

done by increasing the shelf temperature to 0 °C over 5 to 10 hours and then to 20 °C over 5 to 14 hours. In all cases homogenous and highly porous cake formation was obtained.

The pressure during secondary drying vacuum was further decreased to 0.05 mbar. Shelves were warmed up to 25 or 30 °C over 1 or 5 hours and the temperature then maintained for additional 5 to 20 hours. Secondary drying led to a final moisture content of less than 3 % in all cases. Stepwise increase of temperature to 25 °C over 1 hour and to 30 °C over 5 hours with an additional drying time of 10 hours enabled a moisture content of less than 1 %, when the solid was sealed in the freeze-dryer. Figure 3.4 shows the freeze-drying process which proved optimal for the preparation of lyophilisates of freeze-dried biorelevant media FaSSIF and FeSSIF for the subsequent characterization of the physicochemical properties and for stability studies.

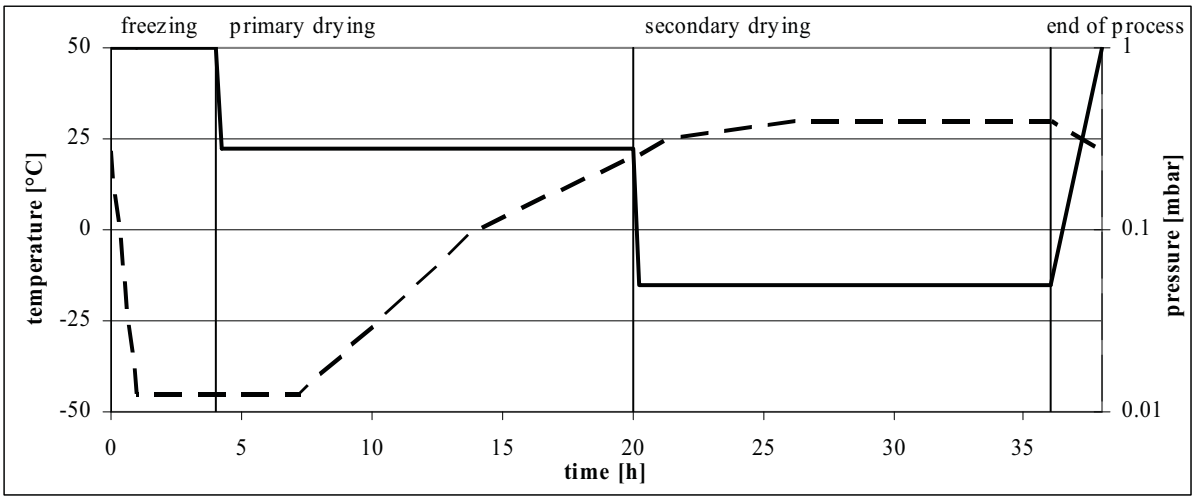


Figure 3.4 Optimized freeze-drying process regarding to temperature (- -) and pressure (–) , used for preparation of freeze-dried biorelevant media

3.1.4 Packaging

The lyophilised media were packaged dry to avoid moisture uptake and hence decomposition of the structure and substances. All packing materials proved to be humidity resistant over a period of at least 6 months. The content of water did not increase by more than 2 % over a period of six months when packaging was conducted according to methods for bulk packaging, and methods b) and d) for individual "dose" packaging. For in-process sealing (according to method c) no increase in moisture during storage was observed. For detailed data see results obtained by Karl-Fischer Titration given in chapter 3.2.4.

To seal lyophilised media in vials using method c), the vacuum was reduced to 0.8 mbar by

inflow of nitrogen$_{(g)}$ after finishing the freeze-drying process. The stoppers were pushed into the vials by manually screwing down the shelves. The vials were afterwards removed and clamped with aluminium caps.

In all other cases, the lyophilised powder was removed from the freeze-dryer and stored in a dessicator over silica gel until further processing. For bulk packaging and manufacture of tablets b) freeze dried product was ground using a mortar and pestle. Bulk was then filled into flasks. To prepare tablets, a 17 mm oblong punch was used. The highly porous media had to be compressed in repeated compaction steps with repeated die fillings. To compress powder for preparation of 1 l of FaSSIF seven filling and compression steps were necessary. Precompression was done at 1 kN, whereas 12 kN were necessary for the final compression step. A more realistic production technology approach would be to increase the bulk density of the powder e.g. by roller compaction as a preliminary step.

Packaging in blisters was done according to method 2.2.4 (c). Sealing of the blisters was done under standard conditions (250 °C, 0.2 seconds) using a standard blister/sealing machine. Newly developed blisters proved to be adequately sealed after a sealing time of 5 seconds at 250 °C. Blisters sealed for < 1 second or > 10 seconds did not show sufficient resistance to dye uptake in the methylene blue bath.

3.1.5 Reconstitution

pH adjusted buffers were used to prepare the instant media from FaSSIF, FeSSIF and FeSSIFplus obtained following method 2.2.2.1 (with water as solvent) and 2.2.2.2. These freeze-dried media could be reconstituted within seconds, forming clear solutions. Depending on the amount of powder, the slowest dissolving time was 30 seconds. The dissolution progress could visually be separated in two steps: First, a syrup-like solution was formed. Over a time of about 15 seconds, which could be accelerated by shaking, dilution of this "syrup" occurred, resulting in a clear aqueous solution. FeSSIFplus showed a slightly yellowish coloration. pH values of the solutions were checked and there was no need to adjust pH in any of the reconstituted solutions.

Complete instant media obtained by method 2.2.2.3 were dissolved in pure water. Reconstitution of the media was slower compared to instant media for reconstitution in buffer, and shaking was necessary to dissolve solid residuals. As a result, the pH deviated sharply from the target pH and adjustment of the pH was necessary. There was a threefold increase in preparation time compared to the instant media for reconstitution in buffers. Therefore freeze-drying of **complete** media was not further investigated.

In situ reconstitution of bulk and pre-dosed powder inside the dissolution vessel:

Preparation of FaSSIF, FeSSIF and FeSSIFplus from the instant media obtained from 2.2.2.1 and 2.2.2.2 was carried out by redissolving the lyophilised powder via addition of the appropriate buffer solutions (FaSSIFblank, FeSSIFblank or FeSSIFplus blank). The powder was weighed into vessels according to 2.2.5 and dissolved in the corresponding media (pre-warmed to 37 °C).

In situ reconstitution of tablets inside the dissolution vessel:

Preparation of biorelevant media from tablets was carried out directly in the dissolution vessels. The buffer blank was pre-heated, degassed according to the USP method and filled into the dissolution vessels. The tablets were added under constant stirring using the USP paddle apparatus II at a stirring rate of 50 rpm. The tablets were dissolved within 25 ± 5 seconds leading to clear solutions. Samples were taken after 5 minutes at different positions within the vessel to test the homogeneity of the solution regarding physicochemical properties.

Physichochemical properties of the solutions are given in III3.4.2.

3.2 Characterization of Lyophilisates

3.2.1 Appearance

Solid cakes obtained from freeze-drying of the concentrated solutions using methods 2.2.2.1 and 2.2.2.2 were white in the case of FaSSIF and FeSSIF and creamy-colored in case of FeSSIFplus. No speckles or other inhomogeneous coloration was observed. Visually, a highly porous, fine spongy interior body was revealed when the cake was broken. Ground lyophilisate was of low density and electrostatically charged.

Filling heights exceeding a liquid level of 15 mm led to less amorphous cakes. Visually recognizable changes in density, increasing from the bottom towards the surface, could be observed. At moisture contents above 5 % the cakes lost their spongy, voluminous shape and started to shrink and stick during storage.

Homogenous cakes were not obtained when method 2.2.2.3 was used. The surface of the cakes burst open and the solids exhibited visually different phases, with incrustations on top and rough sponge-like bodies. The colour of the solids was inhomogeneous and speckled, differing in areas of creamy white, yellowish and brownish (see Figure 3.2). The ground solids showed slight brownish colorations. The powder was of high density and showed good flow properties.

3.2.2 SEM (Scanning Electron Microscopy)

SEM was also used to observe the influence of the formulation's composition and of the freeze-drying process on the product. Using this method, observation of structures in the size range 10 to 20 nm are observable. The infrastructure observed by SEM was determined by the size and distribution of ice crystals in the solid solute matrix formed during freezing.[57].

Solid structures obtained from complete media and media for reconstitution with buffer blank had different infrastructures.

Micrographs of FaSSIF and FeSSIF instant media for reconstitution with buffer are given in Figure 3.5. Cakes were homogenous and the surface appeared smooth, with no crystallinity. The pores were numerous and homogenously distributed.

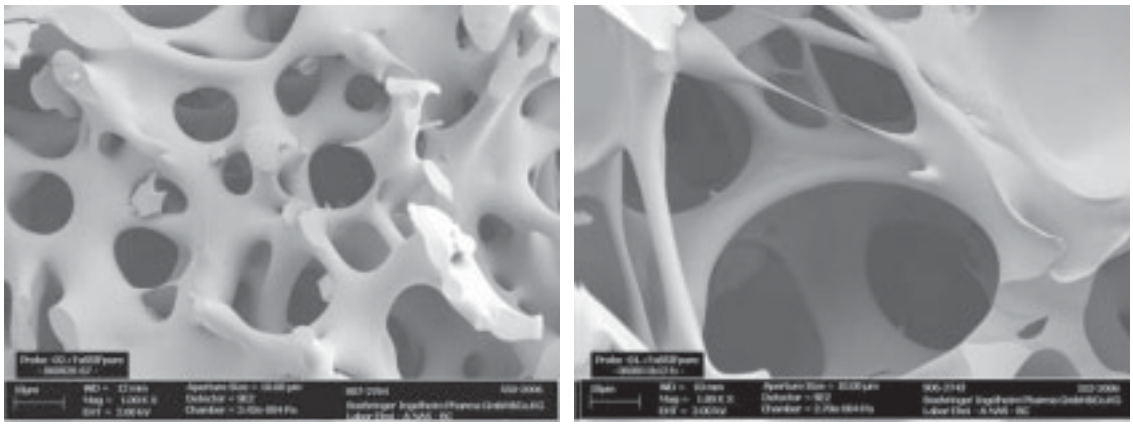


Figure 3.5 Freeze-dried FaSSIF and FeSSIF (bile salt and lecithin only) on a scale of 1:1760

Figure 3.6 shows freeze-dried FeSSIFplus, containing bile salt, lecithin, glycerol monooleate and sodium oleate but no buffer salts. The surface appeared smooth, with no crystalline regions. Porosity was similar to media without GMO and NaO.

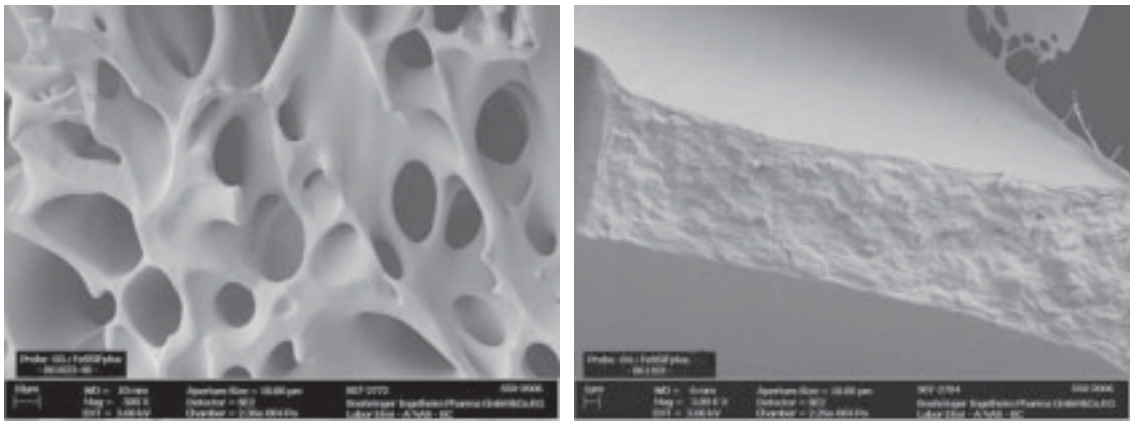


Figure 3.6 Freeze-dried FeSSIFplus on a scale of 1:880 and 1:5200

Figure 3.7 and Figure 3.8 are micrographs of complete FaSSIF and FeSSIF. The lyophilised media showed partial crystallinity. No smooth surfaces or homogenous pores were visible. The structures were fissured and no homogenous cake formation could be observed.

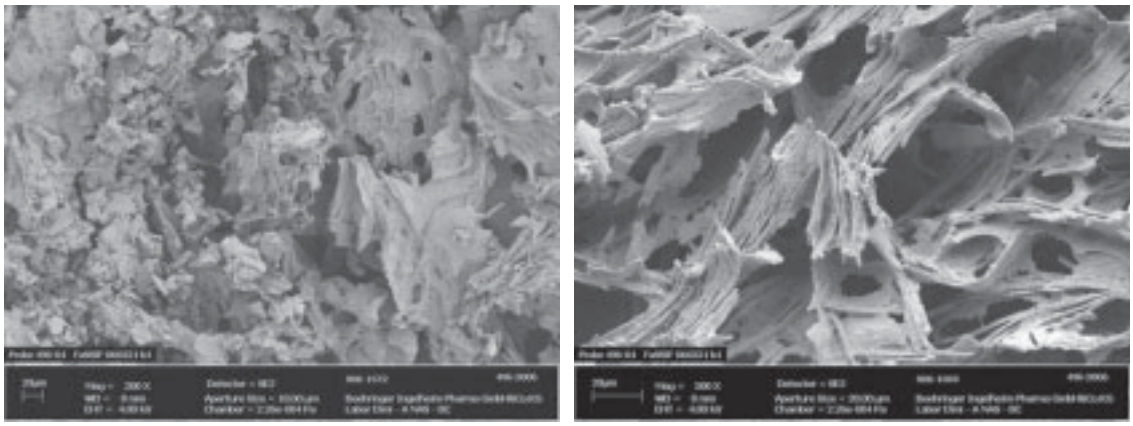


Figure 3.7 Freeze-dried FaSSIF (complete composition: phosphate buffer and NaCl) on a scale of 1:355 and 1:880

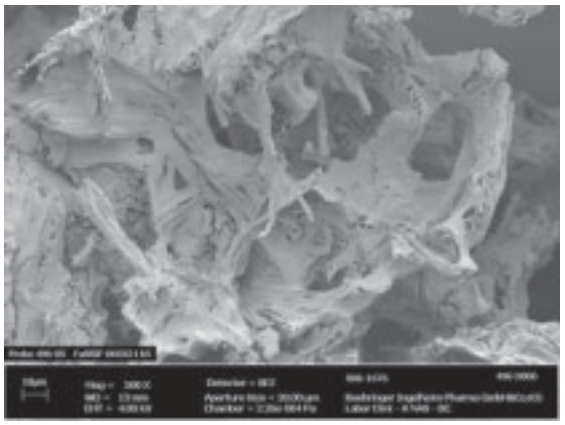


Figure 3.8 Freeze-dried FeSSIF (complete composition: NaAc and NaCl) on a scale of 1:880

3.2.3 Hygroscopicity

All freeze-dried products presented in this work showed high hygroscopicity. Lyophilisates left at room temperature started to deliquesce if not stored in sealed packages. Measurements of the adsorption isotherm were conducted with FaSSIF and FeSSIF for reconstitution with water and with complete FaSSIF and FeSSIF.

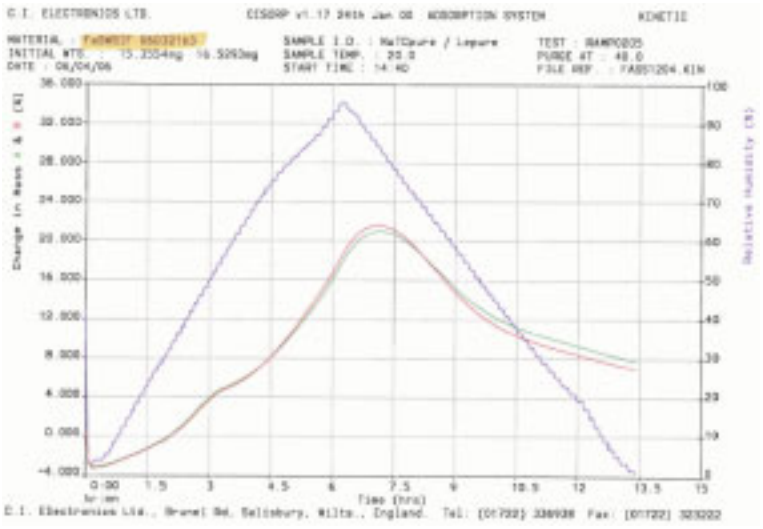


Figure 3.9 Adsorption isotherm of freeze-dried FaSSIF/FeSSIF for reconstitution with buffer

FaSSIF and FeSSIF for reconstitution with buffer blank:

The solid structure was destroyed within 8 hours. After 24 hours at 25 °C / 40 % the media were completely dissolved forming a highly viscous solution. Figure 3.9 shows the adsorption isotherm for freeze-dried FaSSIF. The media started to adsorb water quickly with increasing humidity. A maximum water adsorption of 20 % was measured at a humidity of 95 %.

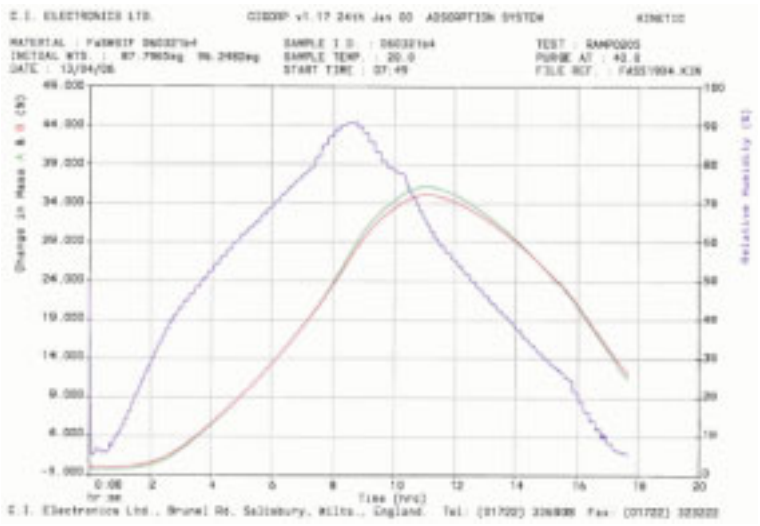


Figure 3.10 Adsorption isotherm of freeze-dried complete FaSSIF

Complete FaSSIF and FeSSIF for reconstitution with water:

The solid structure was destroyed within 24 hours. After 48 hours at 25 °C / 40 % the media was completely dissolved forming a highly viscous solution. The adsorption isotherm of complete FaSSIF is given in Figure 3.10: During measurement, complete FaSSIF showed even higher hygroscopicity compared to FaSSIF for reconstitution with buffer blank. However, the media started to adsorb water with a delay of 2 hours. The maximum value in mass change of about 35 % after being exposed to 95 % humidity was higher than for FaSSIF and FeSSIF for reconstitution with buffer.

3.2.4 Karl-Fischer-Titration

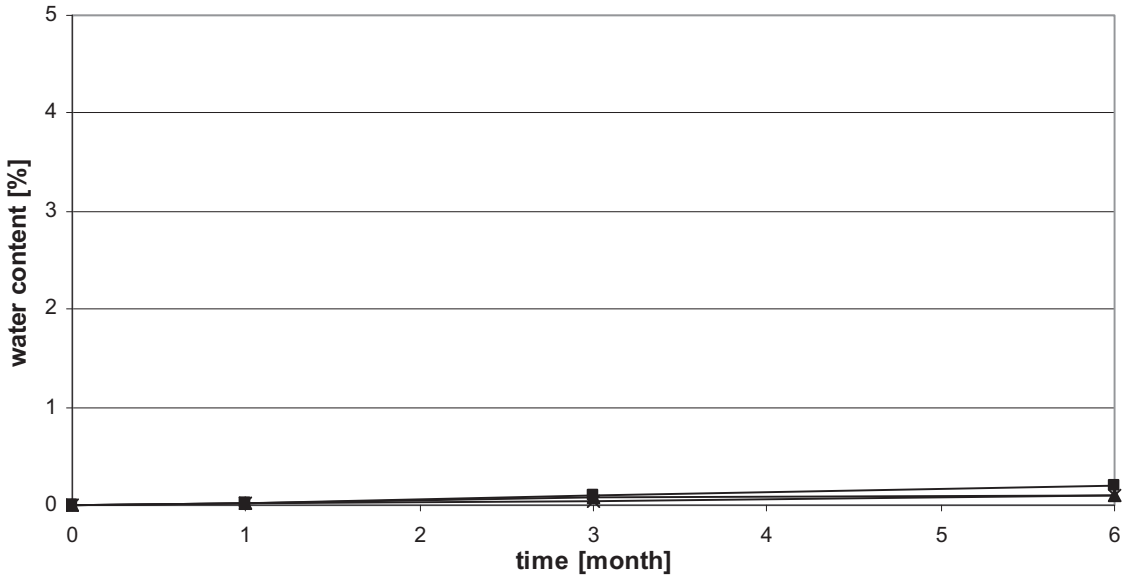


Figure 3.11 Water content of FaSSIF (for 500 ml) in sealed glass vials stored under different conditions: x cooled, ■ 25°C / 60% RH and ▲ 40°C / 75% RH

Water content of the lyophilised media (FaSSIF - for 500 ml) was maintained below 0.2 % over a period of at least six months in sealed glass vials. No changes in weight could be observed after storage under the various conditions.

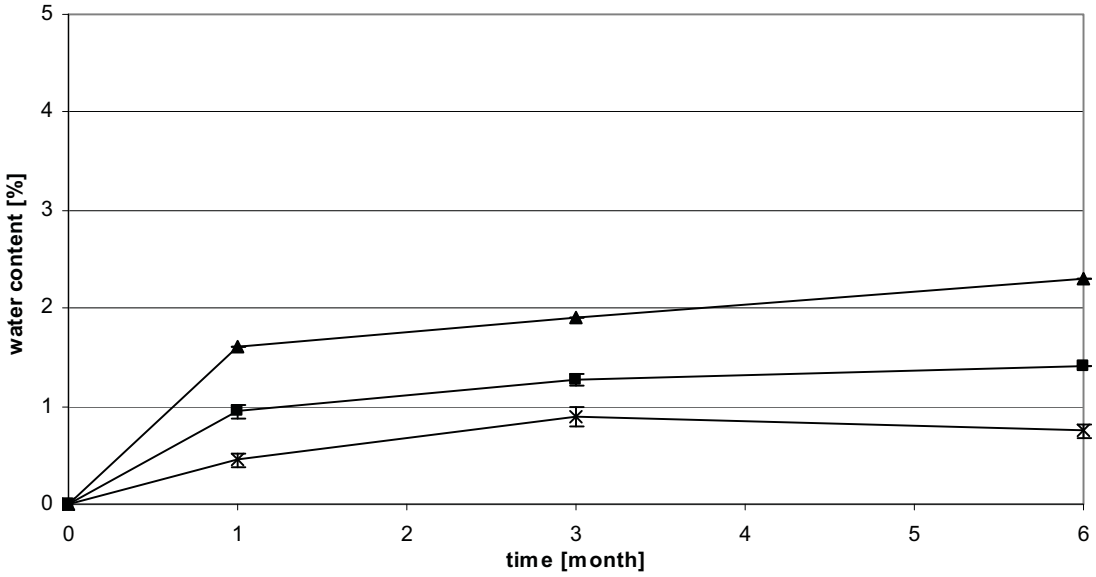


Figure 3.12 Water content of FaSSIF (for 40 ml) in sealed glass vials stored under different conditions: x cooled, ■ 25°C / 60% RH and ▲ 40°C / 75% RH

Water content of FaSSIF (for reconstitution to 40 ml) packaged in sealed glass vials was more sensitive to storage conditions. Uptake of water was highest at 40°C / 75 % RH. Within the first month the average uptake was highest with an increase of weight of 1 %. Over the following two and five months, average uptake decreased to 0.36 % and 0.13 %, respectively. The higher uptake of water compared to FaSSIF (500 ml) is caused by the higher surface to volume ratio. Water easily adsorbs at the surface, wheras penetration into the cake is slow.

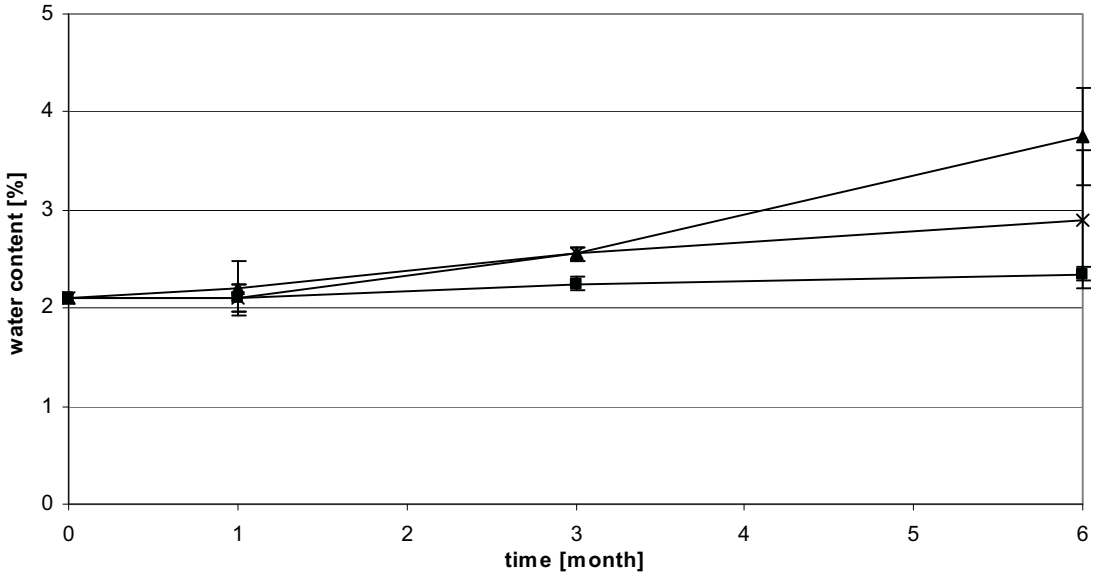


Figure 3.13 Water content of FaSSIF (for 30 ml) in standard Blister (peel-push) stored under different conditions: x cooled, ■ 25°C / 60% RH and ▲ 40°C / 75% RH

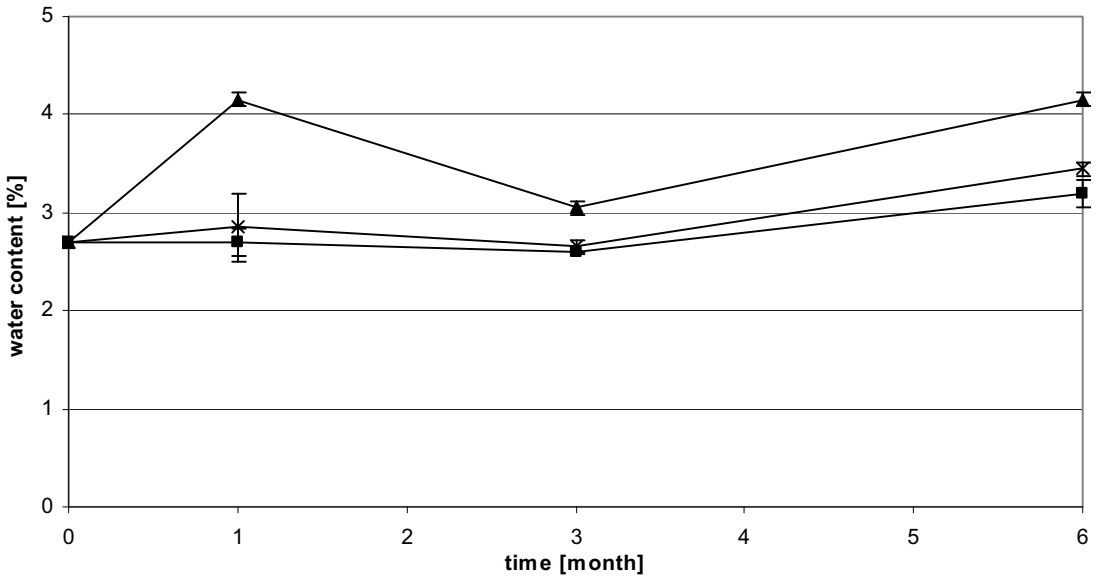


Figure 3.14 Water content of FaSSIF (for 30 ml) in standard Blister (push) stored under different conditions: x cooled, ■ 25°C / 60% RH and ▲ 40°C / 75% RH

The water content of FaSSIF packaged in blister was higher. The 0 month data was determined immediately after sealing the blisters at dry room conditions. The moisture content was already high, with values of 2.1 and 2.7 % for the peel - push (Figure 3.13) and the push blister (Figure 3.14), respectively. Values obtained from the peel – push blister were lower compared to the push blister throughout storage, with media stored in blisters at 25 °C / 60 % RH showing best performance.

In all cases storage under 40 °C / 75 % RH showed highest uptake of water.

Media stored in newly developed large size blisters (data not shown) showing a moisture content of 2.3 ±0.2 % at all measured time points.

3.2.5 DSC (Differential Scanning Calorimetry)

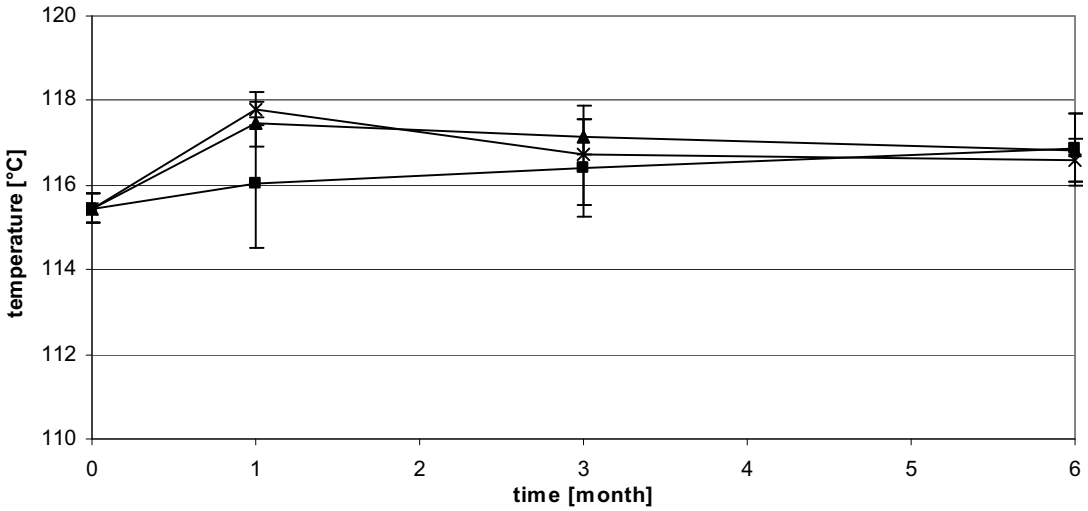


Figure 3.15 Glass transition temperature of FaSSIF (for 40 ml) in glass vials stored under different conditions: x cooled, ■ 25°C / 60% RH and ▲ 40°C / 75% RH

The glass transition temperature increased within the first month of storage by 1.4 % on the average and then decreased again. A relative increase of 1.1 % of the glass transition temperature after six months' storage was measured. No differences between storage conditions could be observed at this time period.

3.3 Properties of reconstituted vs freshly prepared media

Physicochemical properties of the instant biorelevant media were compared with conventionally prepared media. Solubility of danazol was also determined, as a measure of the solubilisation capacity of the media. Further, dissolution of the acidic formulation BIXX-F in both kinds of media was carried out using the miniaturized equipment described in chapter V of the thesis.

For physicochemical determinations, the solutions were given about 15 min to thermally equilibrate after preparation.

Measurement of particle size distribution, surface tension and viscosity were conducted at 22 °C ± 1 °C, density was measured at 20.0 °C. The solubility of danazol was measured at 37 °C.

Particle size distribution of FaSSIF is given in Figure 3.16. A polydispersity index (PdI) of

0.12 was calculated for conventionally prepared FaSSIF, whereas for instant FaSSIF a PdI value of 0.05 could be obtained. The low PdI value of 0.05 indicates that the particle size distribution is narrow and monomodal. In the case of conventionally prepared media, the PdI was reduced to 0.1 after 24 hours. Average diameter (Z_{ave}) values of 88 and 89 nm were calculated for conventionally prepared and instant FaSSIF, respectively. In the case of instant FaSSIF, micelles remained stable at a particle size of 89 nm. However, in conventionally prepared FaSSIF changes in particle size over time were observed: Directly after preparation a bimodal distribution was measured with a calculated Z_{ave} of about 60 nm (major peak), with a second peak at about 200 nm. The particle size increased to a Z_{ave} of 88 nm within one day, with the second peak (200 nm) becoming less pronounced, explaining the reduced PdI values of conventional FaSSIF one day after equilibration.

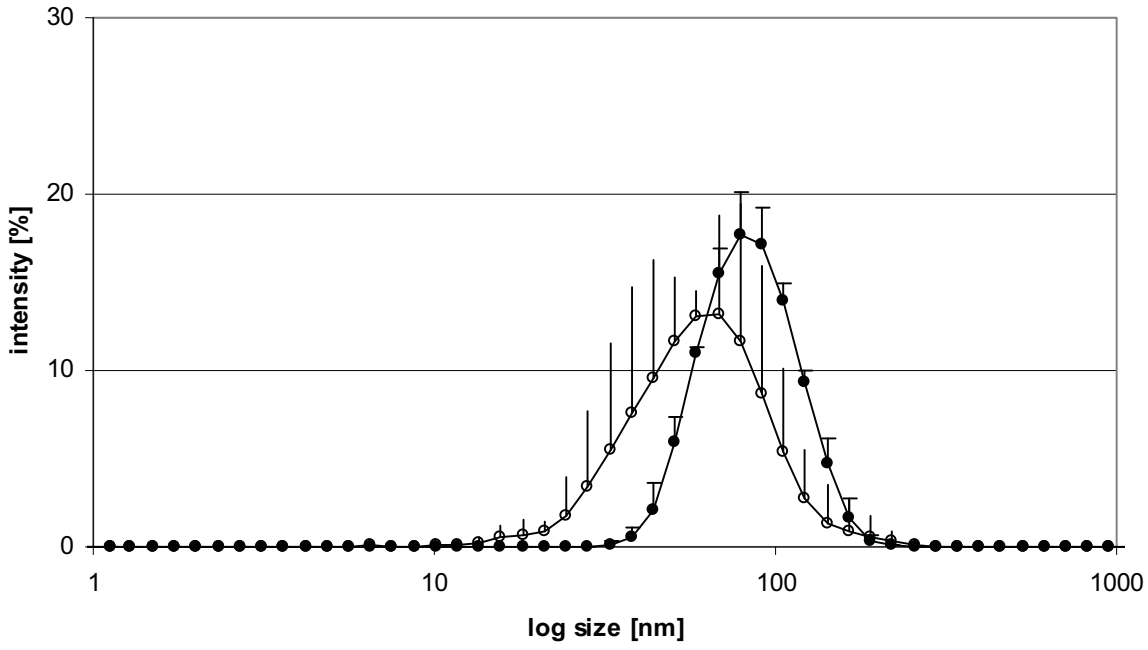


Figure 3.16 Particle size distribution of FaSSIF (n=9) prepared by the conventional (○) and the freeze-drying (●) method. Bars indicate standard deviations (— for conventional, ⊢ for freeze-dried)

Particle size distribution of FeSSIF is given in Figure 3.17. A polydispersity index (PdI) of 0.4 was calculated for conventionally prepared FeSSIF, whereas for instant FeSSIF a PdI value of 0.1 was obtained.

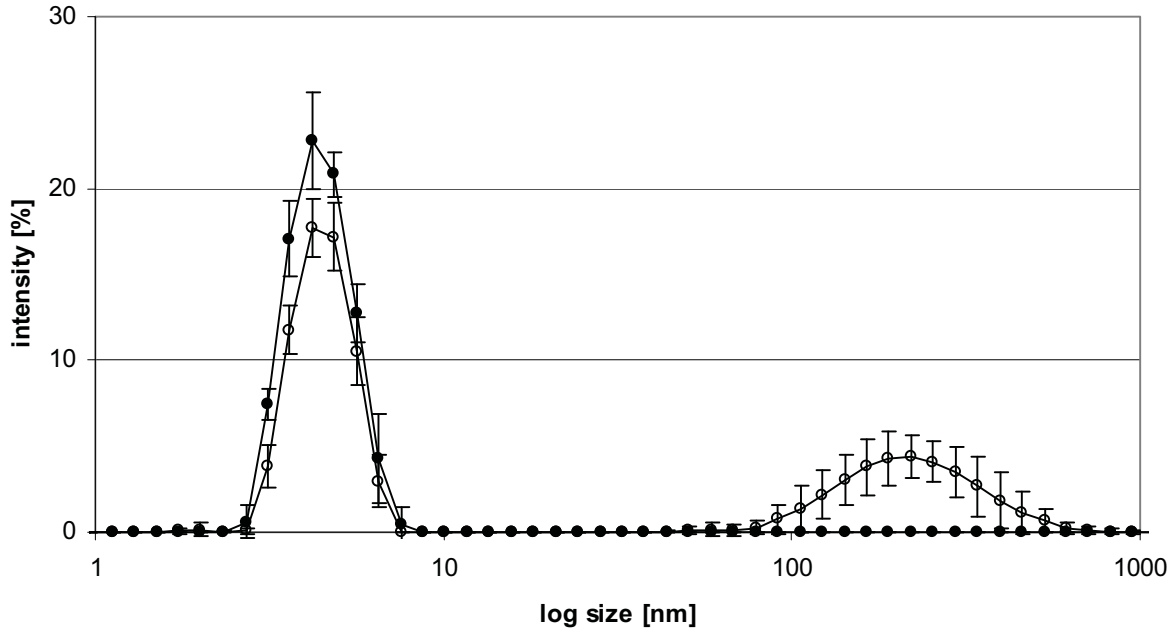


Figure 3.17 Particle size distribution of FeSSIF (n=9) prepared by the conventional (○) and the freeze-drying (●) method. Bars indicate standard deviations

Average diameter (Z_{ave}) values of 8 and 6 nm were calculated for conventionally prepared and instant FeSSIF, respectively. The slight difference in Z_{ave} for FeSSIF was not statistically significant. A narrow and monomodal distribution for instant FeSSIF was calculated, whereas conventionally prepared FeSSIF exhibited a broad and bimodal distribution with a second peak at 200 nm, leading to the increased Z_{ave} of 8 nm. The 200 nm peak was not reduced in size over time.

The calculated values for surface tension, osmolality, solubility of danazol, viscosity and density are given in Table 3.5 for FaSSIF and in Table 3.5 for FeSSIF. All measurements were run with n=9.

Table 3.5 Physicochemical properties of conventionally prepared and instant FaSSIF

	conventional mean	**conventional SD**	**instant mean**	**instant SD**
surface tension [mN/m]	54.8	0.2	54.9	0.1
osmolality [mosmol/l]	268	1	263	0.9
viscosity [cP]	1.014	0.08	1.019	0.02
density [g/cm³]	1.00615	$1*10^{-5}$	1.00618	$3*10^{-5}$
solubility of danazol [µg/ml]	8.4	0.7	8.4	0.7

Table 3.6 Physicochemical properties of conventionally prepared and instant FeSSIF

	conventional mean	conventional SD	instant mean	instant SD
surface tension [mN/m]	48.6	0.1	48.8	0.1
osmolality [mosmol/l]	707	2	707	3
viscosity [cP]	0.98	0.06	0.998	0.01
density [g/cm³]	1.01482	$4*10^{-4}$	1.01484	$1*10^{-4}$
solubility of danazol [µg/ml]	33.8	1	34.9	1

Dissolution profiles of BIXX-F in FaSSIF and FeSSIF are presented in Figure 3.18 and Figure 3.19. Dissolution data is presented as % released vs. time curves, with means and standard deviations indicated for each time point. In FaSSIF, maximum percentage released values of 15.4 and 16.4 % in conventional and freeze-dried media, with T_{max} at 40 min in both cases, were attained. The high initial solubility is driven by excipients like supersaturizing agents or pH modifiers, that effect supersaturation and cause a delay in reprecipitation. Final percentage release is defined by the low saturation solubility of BIXX at higher pH values.

In FeSSIF, release continued throughout the 120 min test period. After 40 minutes, 22.3 (conventional) and 23.4 % (freeze-dried) of BIXX were dissolved. According to the two-way ANOVA test for repeated measurement and the post hoc Bonferroni test, no significant difference in the dissolution profiles was observed. However, standard deviations of the mean dissolution values in conventional media were generally slightly higher.

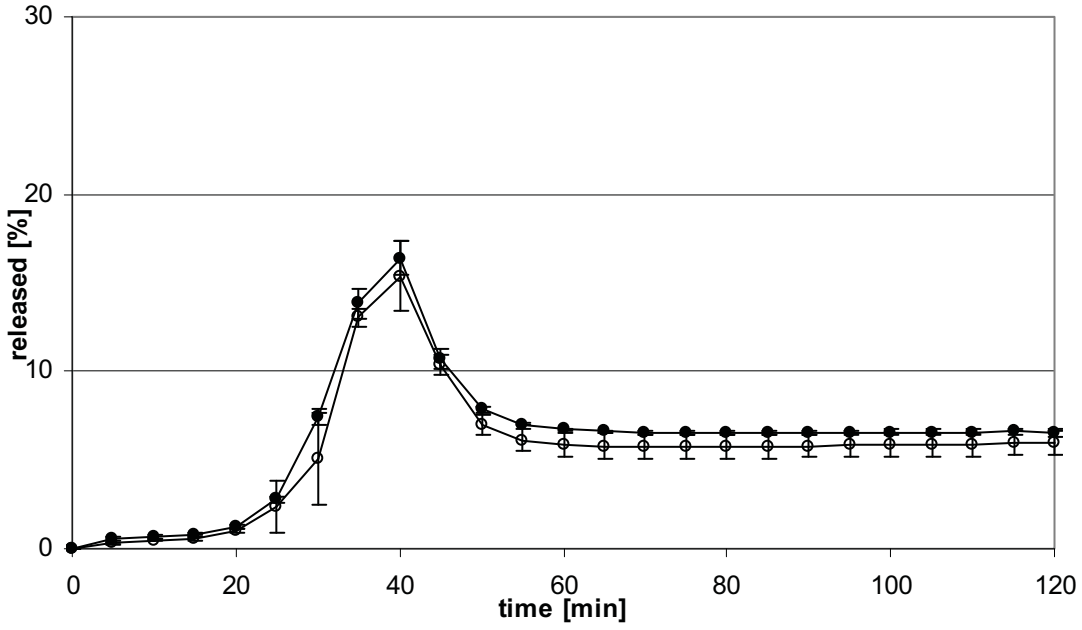


Figure 3.18 Dissolution of BIXX-F in FaSSIF prepared by the conventional (○) or freeze-drying method (●)

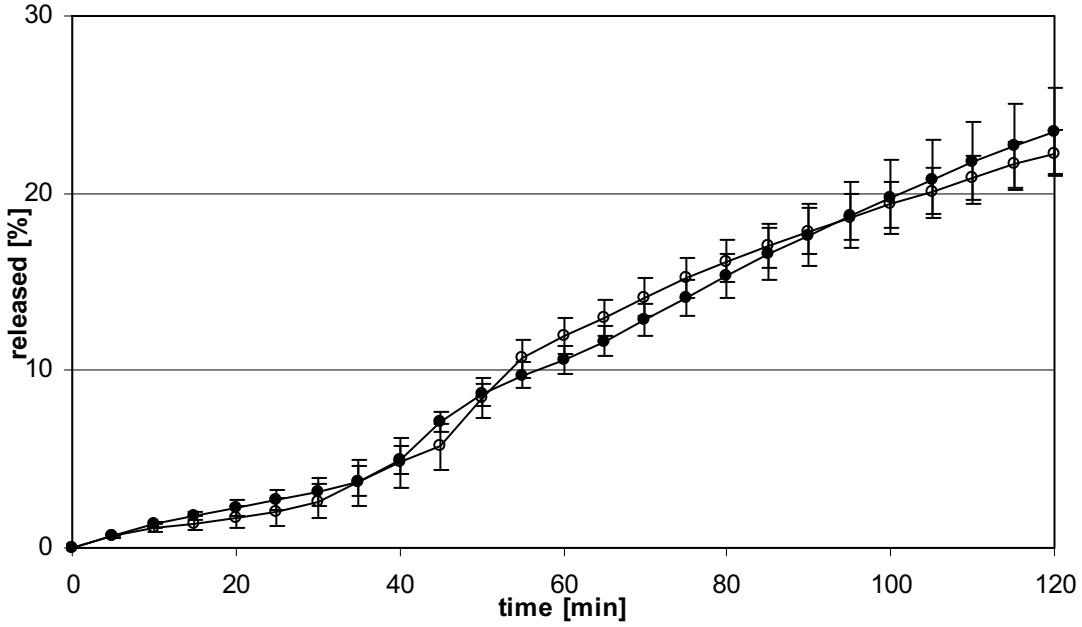


Figure 3.19 Dissolution of BIXX-F in FeSSIF prepared by the conventional (○) or freeze-drying method (●)

Since BIXX shows pH dependent solubility, the influence of adding pH modifiers in the formulation was observed in the profiles. Acidifying excipients in the formulation cause a decrease in pH, depending on the buffer capacity of the medium. In the weaker FaSSIF buffer a greater decrease of pH and thus a higher dissolution compared to rates in the FeSSIF buffer was observed. In FaSSIF a pH drift to 6.14 was observed whereas in FeSSIF the pH remained stable at pH 5.0. In the corresponding buffers a decrease to 6.18 and 4.9, respectively was measured. The stable pH in FeSSIF buffer led to a final % released of 2 %. The strong solubilising effect of the bile salts and lecithin in FeSSIF itself, however, led to a threefold % released (compared to FaSSIF) with still increasing progression. A final release of 40 % after 4 hours was reached (data not shown).

3.4 Characterization of the redissolved media after storage

Physicochemical properties of instant FaSSIF, FeSSIF and FeSSIFplus were compared to these of media prepared by the conventional method (dissolution of bile salt in buffer blank, addition of a solution of lecithin in methylene chloride with subsequent evaporation of the organic solvent and final dilution to the desired concentration) (see 3.4.2 to 3.4.2.6). The freeze-dried media were redissolved in the corresponding buffer and given time to equilibrate for at least 15 minutes.

Only FaSSIF and FeSSIF for reconstitution with buffer were tested regarding stability.

3.4.1 Optical appearance

All solutions obtained from instant FaSSIF and FeSSIF were clear and did not show turbidity. During dissolution (maximum dissolution time was 30 seconds) of the instant powder, striae originating from the concentration gradient within the foming solution occured. Shaking led to homogenous dispersion and thus to a clear solution. Dissolution of FeSSIFplus led to a slightly yellowish solution showing an insignificant turbidity. The visual appearance is summarized and compared to conventionally prepared media in Table 3.7.

Table 3.7 Appearance of the biorelevant media after complete dissolution

	appearance instant	**appearance conventional**	**colour instant**	**colour conventional**
FaSSIF	low viscosity clear solution	low viscosity clear solution	colorless	colored
FeSSIF	syrup-like	slightly turbid	-	whitish
FeSSIF plus	syrup-like	slightly turbid	slightly yellowish	slightly yellowish

3.4.2 Physicochemical properties

Physicochemical properties were characterized to compare the freeze-drying method with freshly prepared biorelevant media (see III3.3) and to observe stability in the lyophilised state.

3.4.2.1 Density

Density values of the reconstituted solutions were 1.00617 ± 0.00002 g/cm³ and 1.015 ± 0.0001 g/cm³ for FaSSIF and FeSSIF directly after freeze-drying and after storage of 3 and 6 months. Means and standard deviations are given in Figure 3.20 and Figure 3.21 for FaSSIF and FeSSIF. Coefficients of variation (CV) were below 0.01 % for FaSSIF and 0.04 % for FeSSIF. No statistical difference could be detected with the t-test or the F-test.

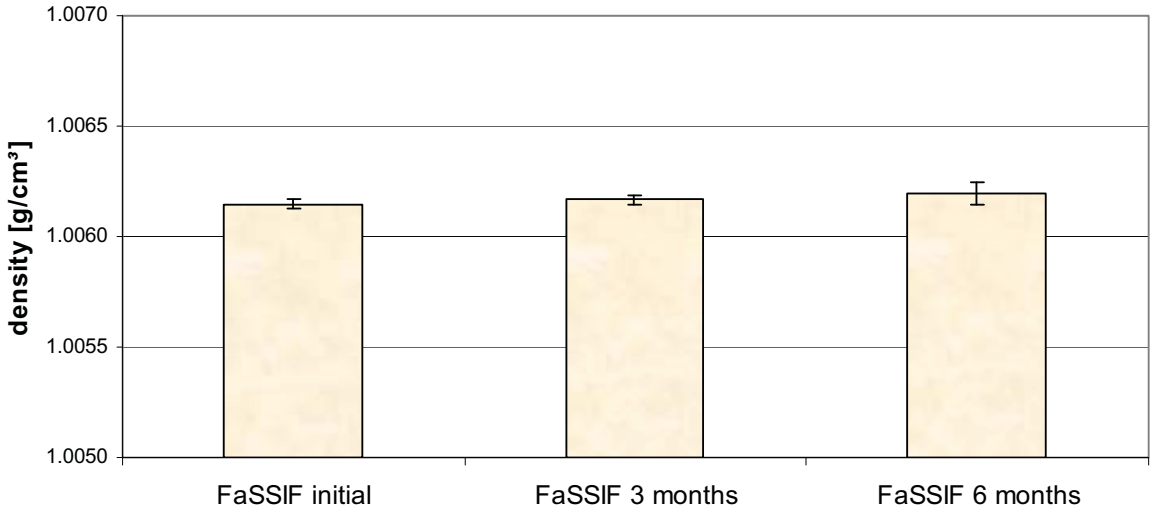


Figure 3.20 Density of reconstituted instant FaSSIF media after freeze-drying: initially and after storage

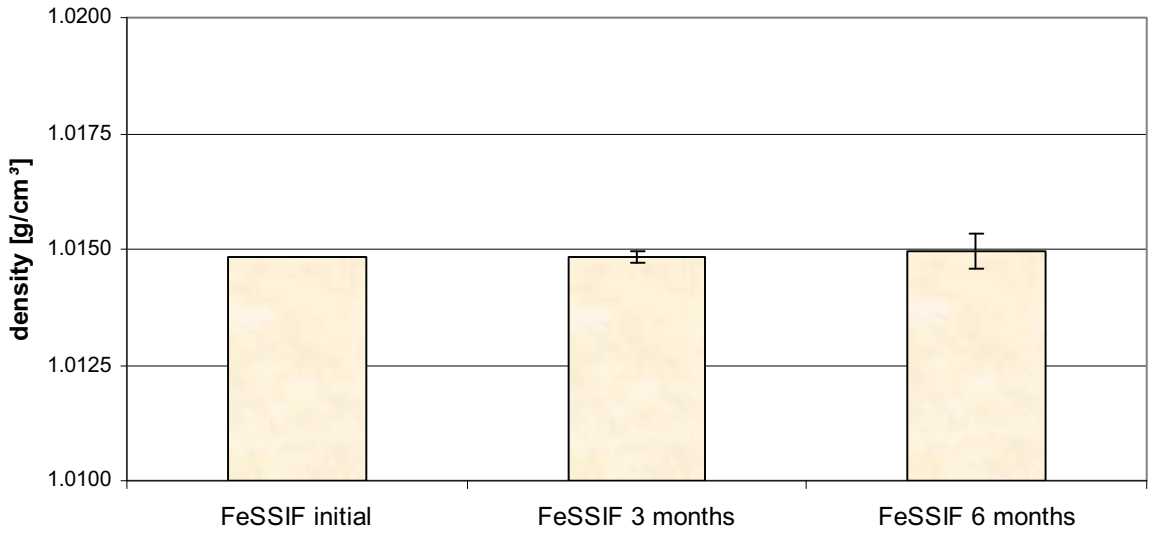


Figure 3.21 Density of reconstituted instant FeSSIF media after freeze-drying: initially and after storage with standard deviations below 0.0004 in all cases

3.4.2.2 Osmolality

No statistically difference regarding osmolality could be determined following t-test and F-test for both media. Means and standard deviations are given in Figure 3.22 and Figure 3.23 for FaSSIF and FeSSIF. Coefficients of variation (CV) were below 0.25 % for FaSSIF and 0.45 % for FeSSIF.

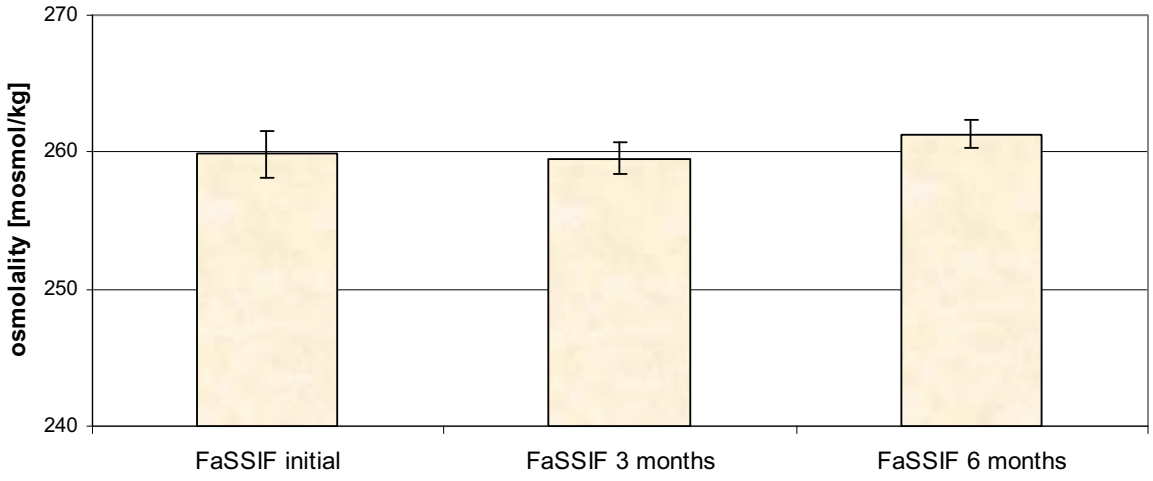


Figure 3.22 Osmolality of of reconstituted instant FaSSIF media after freeze-drying: initially and after storage

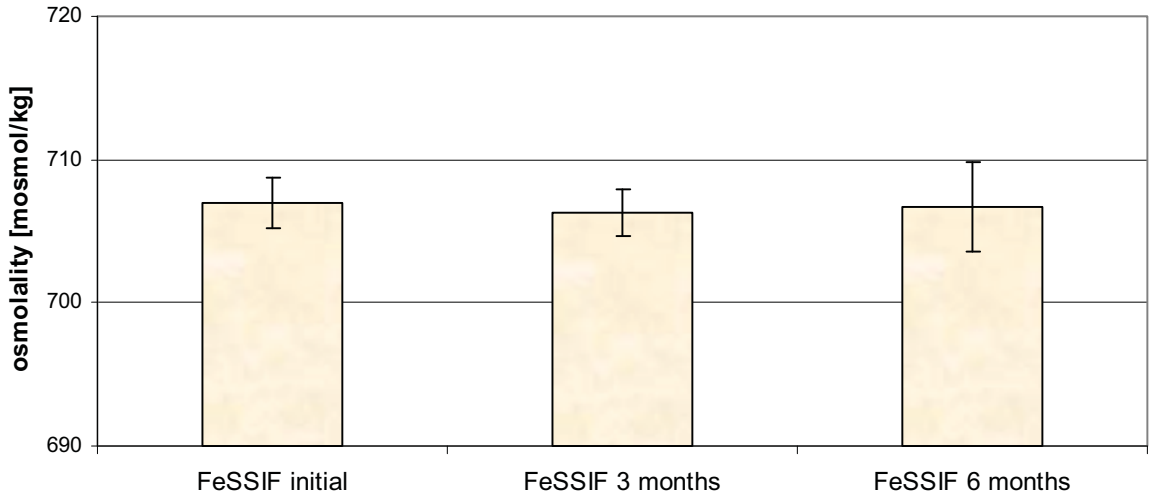


Figure 3.23 Osmolality of of reconstituted instant FeSSIF media after freeze-drying: initially and after storage

The buffer solution used for the reconstitution of FaSSIF had an influence on the osmolality of the resulting media. Whereas for the 0 and 3 month FaSSIF, buffer with an osmolality of 258 mmosmol/kg was used, the buffer used for the 6 month measurement had an osmolality

of 261 mosmol/kg. These data are reflected in the higher values for the solutions reconstituted after 6 months storage. The small variations in osmolality of the buffer are most-likely related to varying quality of the demineralised water.

3.4.2.3 Viscosity

Viscosity values for reconstituted FaSSIF and FeSSIF are given in Figure 3.24 and Figure 3.25. t-test and F-test showed no significant changes with storage.

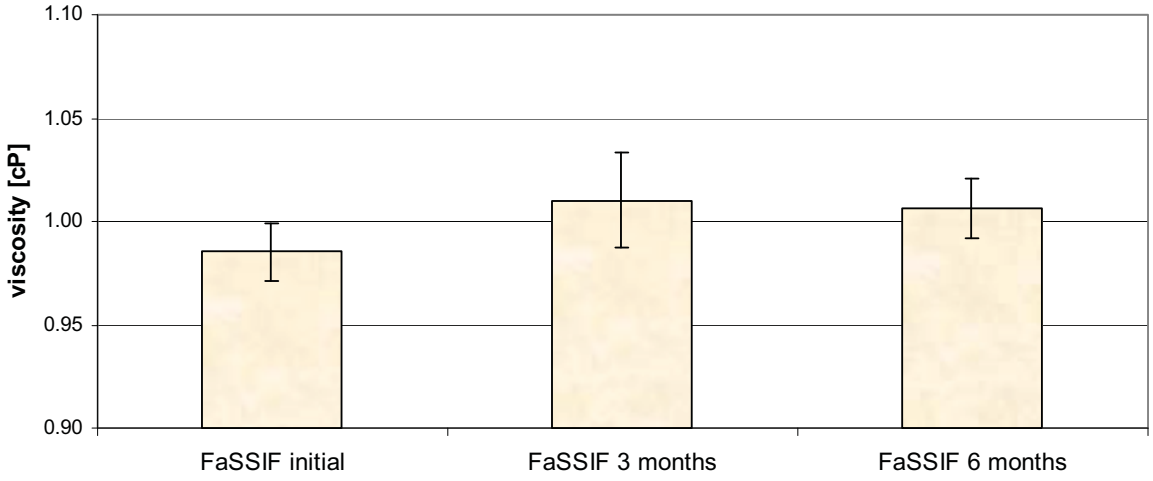


Figure 3.24 Viscosity of reconstituted instant FaSSIF media: initially and after storage

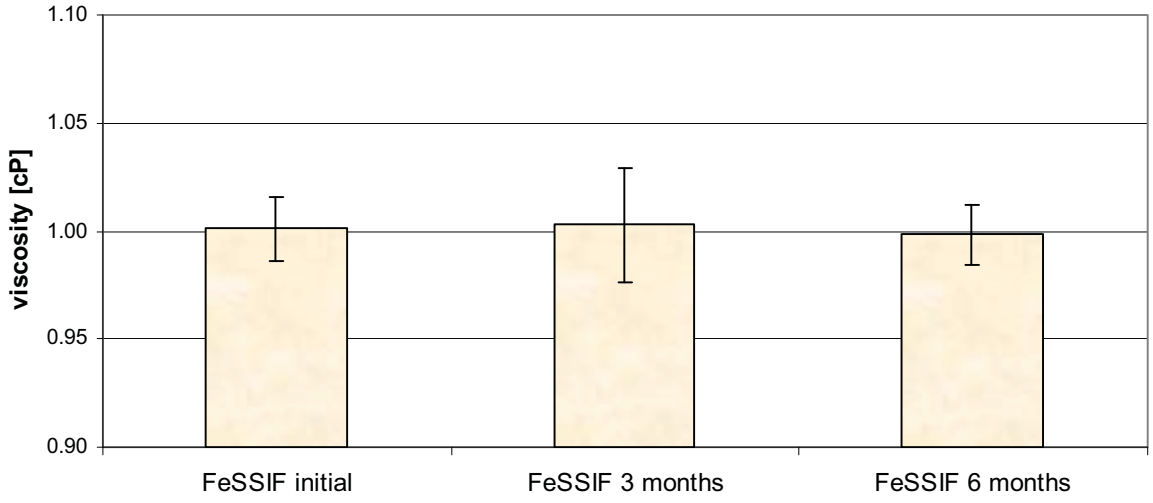


Figure 3.25 Viscosity of reconstituted instant FeSSIF media: initially and after storage

3.4.2.4 Surface tension

There were no statistically significant changes in surface tension of reconstituted media with storage (t-test and F-test). Means and standard deviations of surface tension are given in Figure 3.26 for FaSSIF and in Figure 3.27 for FeSSIF.

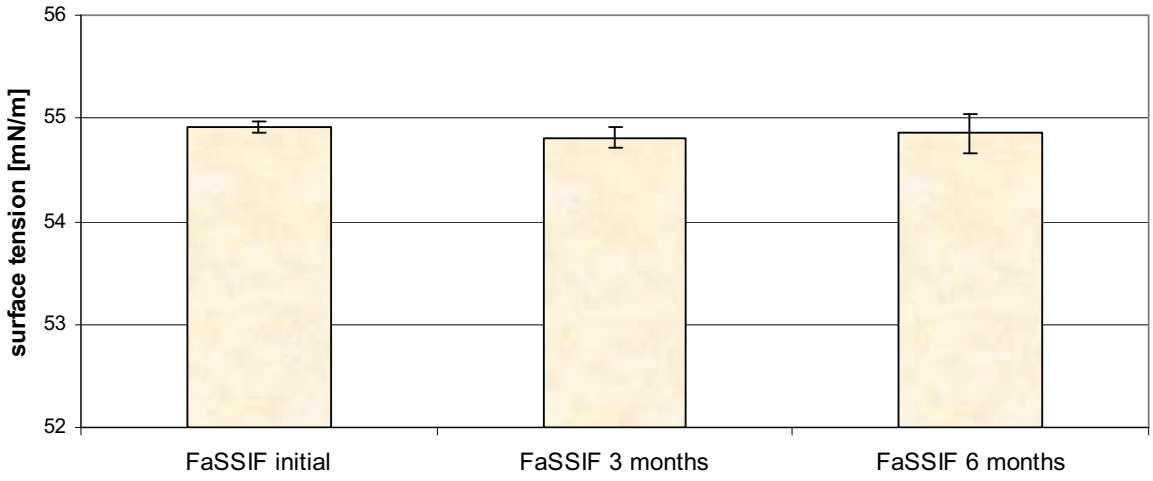


Figure 3.26 Surface tension of reconstituted instant FaSSIF media: initially and after storage

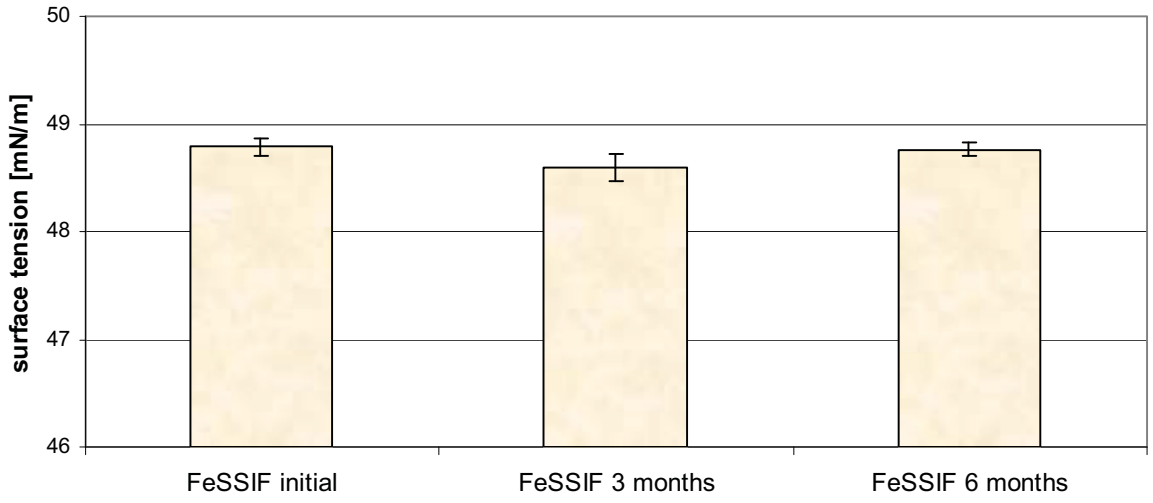


Figure 3.27 Surface tension of reconstituted instant FeSSIF media: initially and after storage

3.4.2.5 Solubility of danazol

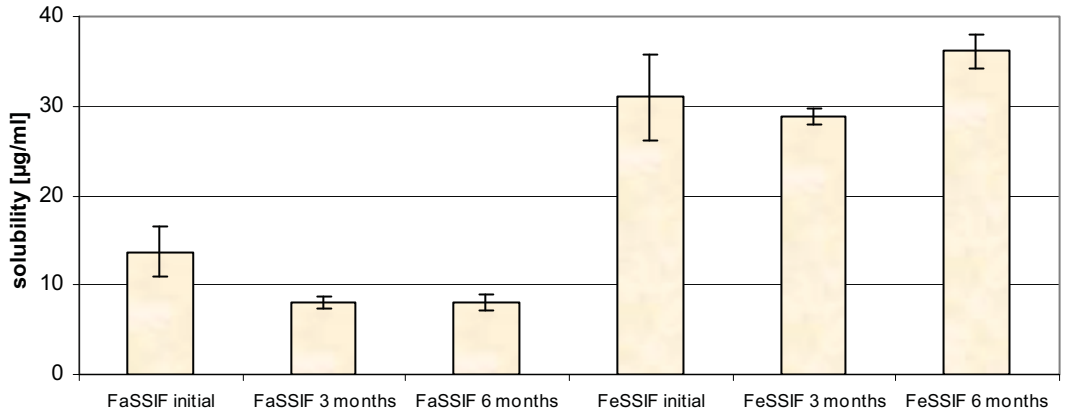


Figure 3.28 Solubility of danazol in redissolveld instant media FaSSIF and FeSSIF

Differences in solubility of danazol were not significant in FaSSIF and FeSSIF with storage. Standard deviations in media reconstituted directly after freeze drying are higher compared to stored media. The higher deviations might result from different methods used for the measurements (the saturation solubility of danazol was investigated using 2 different methods (see III2.2.6)). The reduced shaking time used at 3 and 6 months explains the lower solubility of danazol in case of FaSSIF, whereas in FeSSIF no influence of shaking rate could be observed.

3.4.2.6 Dissolution of BIXX-F

The mean dissolution data of BIXX-F in reconstituted media showed no statistically significant differences with storage ($ANOVA_{rep}$. and post hoc Bonferroni test). In FaSSIF mean maximum concentrations were 15.5 ±1% released at a T_{max} of 40 minutes at all storage times and conditions (only selected data are given in Figure 3.29). A final dissolution value of 7.5 ±1 % was reached at 120 min. In FeSSIF concentrations increased over the first 120 min. At 120 min 23 ±0.5 % BIXX were dissolved with 40 % release after 4 hours.

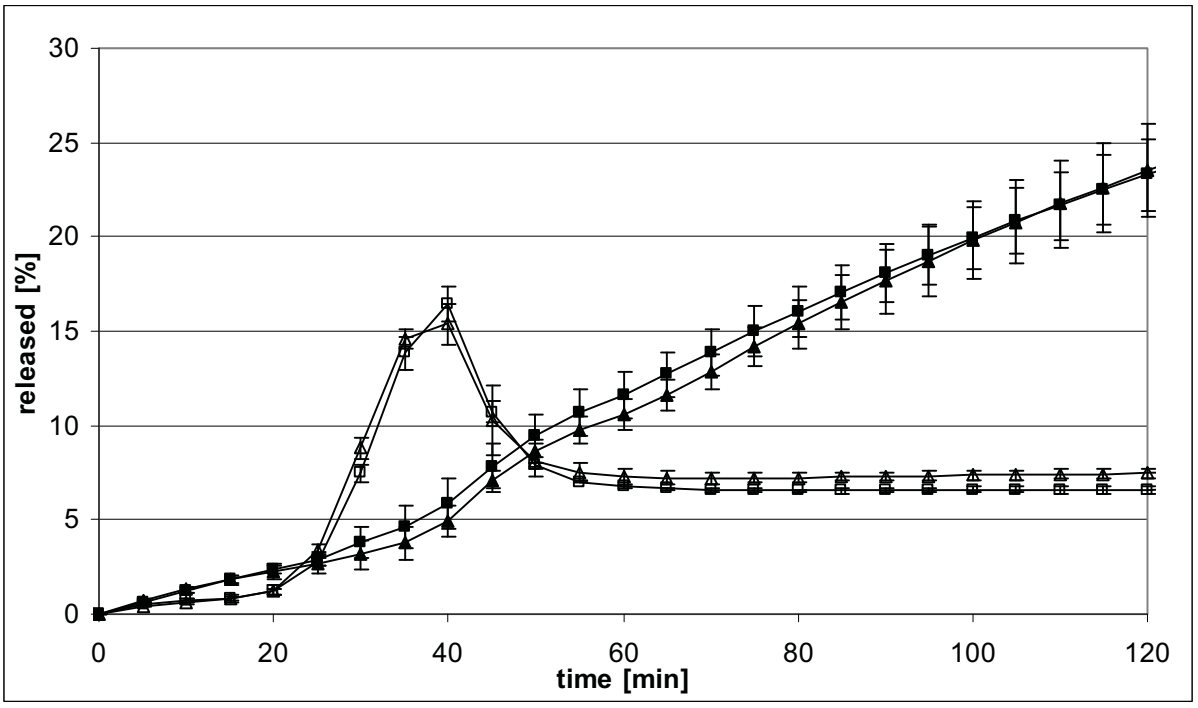


Figure 3.29 Dissolution of BIXX-F in reconstituted FaSSIF (open symbols) and FeSSIF (solid symbols) prepared from freeze-dried media directly after preparation (□) and after 6 months storage at 25 °C / 60 % RH (Δ)

Since BIXX shows pH dependent solubility, the pH of the media was measured after 120 min. In FaSSIF, a pH drift to 6.2 was observed whereas in FeSSIF the pH remained stable, in the corresponding buffers, decreases to 6.2 and 4.9, respectively were measured. No significant difference in pH behaviour was observed after the various storage conditions and time.

3.4.2.7 Particle size distribution and polydispersity index

An overview of the different particle size distributions of the mixed micelles in FaSSIF, FeSSIF and FeSSIFplus is shown in Figure 3.30. Medium diameters of the mixed micelles of the media were 90, 6 and 11 in FaSSIF, FeSSIF and FeSSIFplus, respectively.

FaSSIF and FeSSIF:

The biorelevant media contain mixed micelles consisting of bile salt and phosphatidylcholine, only. The ratio of concentrations (bile salt to phosphatidylcholine) is identical in both media. However, salt concentration in solution is about 3fold lower in FaSSIF compared to FeSSIF. In FaSSIF, the high repulsion forces in the micelle shelf cannot be reduced so far, due to the lack of counterions. Higher repulsion forces between the molecules of the micelles create longer distances and therewith lead to bigger micelles. Under addition of counterions,

repulsion forces are lower and molecule distances within the micelle can be reduced.

FeSSIFplus:

Reduced repulsion forces by counterion alignment allows tight packing of the mixed micelles compared to FaSSIF media. Electrostatic repulsion between molecules is lower caused by the "neutralization" by alignement of the counterion. However, incorporation of GMO and sodium oleate leads to increase of micelle size. The additional molecules are arranged between the bile salt and lecithin molecules. Thus distend of the micelles is caused.

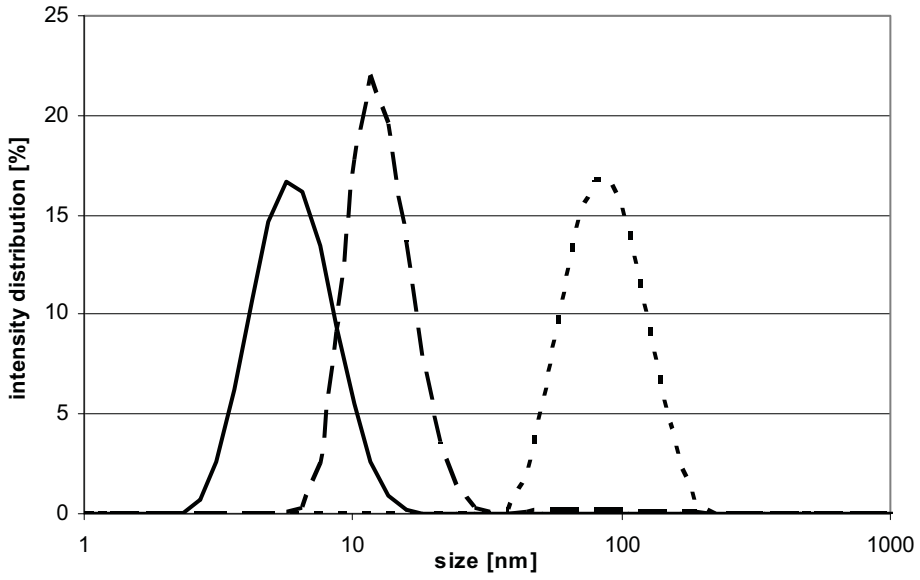


Figure 3.30 Particle size distribution of mixed micelles in FaSSIF (dotted line - right), FeSSIF (solid line - left) and FeSSIFplus (dashed line - middle) prepared by reconstitution of freece-dried media.

The polydispersity indicates are < 0.1 for all media. The low PdI indicate a monodisperse distribution or, close to 0.1 to a narrow distribution.

3.5 Comparison of commercially available and freeze-dried instant media

3.5.1 Physicochemical characterization

Physicochemical properties of media prepared by either method showed no significant differences in surface tension, osmolality and density. Surface tension was 53 ± 0.4 and 47.4 ± 0.3 mN/m for FaSSIF and FeSSIF. Osmolality was determined to be 296 ± 3 and 706 ± 9 mosmol/l, respectively. Density of all FaSSIF and FeSSIF solutions were 1.00693 ± 0.00004 and 1.01484 ± 0.00015 g/cm³.

Results obtained from particle size measurements are given in a mean value of 6.4 nm. However, in case of conventionally prepared FeSSIF, a broad and bimodal distribution with a second peak at 200 nm was observed leading to an increased Z_{ave} of 8 nm. A narrow and monomodal distribution for instant FeSSIF-SIF™ and freeze-dried FeSSIF was calculated.

Results obtained from particle size measurements are given in Table 3.8. Average diameter (Z_{ave}) values of FeSSIF did not show significant differences with a mean value of 6.4 nm. However, in case of conventionally prepared FeSSIF, a broad and bimodal distribution with a second peak at 200 nm was observed leading to an increased Z_{ave} of 8 nm. A narrow and monomodal distribution for instant FeSSIF-SIF™ and freeze-dried FeSSIF was calculated.

Table 3.8 Mean values and standard deviation of D90 and polydispersity indices (PdI) of various FaSSIF and FeSSIF (FD-freeze-dried; CM-conventional; SIF-commercial instant, CPC-bile salts from CPC, New Zealand, PCA-bile salt from PCA, Italy)

	mean Z_{ave} [nm]	SD Z_{ave}	mean PdI	SD PdI
FaSSIF FD CPC	89	2	0.046	0.015
FaSSIF FD PCA	89	1	0.038	0.012
FaSSIF CM CPC*	88	11	0.124	0.019
FaSSIF CM PCA**	88	15	0.038	0.012
FaSSIF-SIF™	45	2	0.025	0.008
FeSSIF FD CPC	6.4	0.1	0.071	0.013
FeSSIF FD PCA	6.4	0.3	0.130	0.066
FeSSIF CM CPC	8 (6 and 200)	0.4	0.404	0.071
FeSSIF-SIF™	6.4	0.1	0.090	0.015

*2 hours and **24 h after preparation

Particle size of FaSSIF showed higher variance: In the case of freeze-dried FaSSIF a stable particle size of about 90 nm was obtained. A PdI of below 0.05 indicated a narrow, monomodal distribution. The particle size in instant SIF™ is only half that of reconstituted FaSSIF but also shows a narrow, monomodal distribution. The smaller particle size might be due to substitution of phosphatidylcholine from soy for that from egg: different fatty acids in S-PC and higher concentration of the less polar colamin-kephalin allows a more compact alignment within the mixed micelles. Chemical structures of phosphatidylcholine and colamin-kephalin are given in Figure 3.31 and Figure 3.32. FaSSIF prepared by the conventional method shows a bimodal distribution with a Z_{ave} of 88 nm. The particles are however not stable. Directly after preparation a Z_{ave} of about 60 nm was calculated with a second peak at about 200 nm. Particle size increases to a Z_{ave} of 90 nm within one day under reduction of intensity of the 200 nm peak. It can be assumed that equilibrium is achieved at a particle size of 90 nm in micelles using E-PC and 45 nm using S-PC. The delayed equilibrium makes conventional FaSSIF less reproducible. Also the high standard deviations in solubility and physicochemical parameters with conventional FaSSIF can be explained by different rates in approach to equilibrium in the test solutions. Using instant SIF™ or freeze-dried media, equilibrium was reached immediately after preparation.

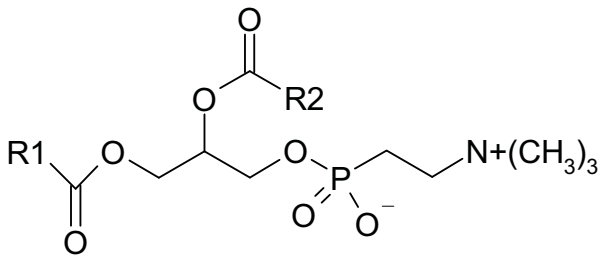


Figure 3.31 Chemical structure of phosphatidylcholine (R1 and R2 are alkyl-substitutes)

Solubility of danazol in FaSSIF and FeSSIF was 15 and 49 µg/ml in conventionally prepared media. In the freeze-dried media, 14 and 48 µg/ml were dissolved. Use of bile salts from different suppliers had no impact on results. The slightly higher concentrations in conventionally prepared media may result from residual organic solvent, which could cause an increased solubility of danazol. In the SIF™ media solubility of danazol was marginally lower with values of 13 and 47 µg/ml. The tighter mixed micelles formed with soy lecithin might show a slightly reduced capacity for solubilising additional molecules. However, for all solubility measurements, no significant differences were calculated using the F and t-tests.

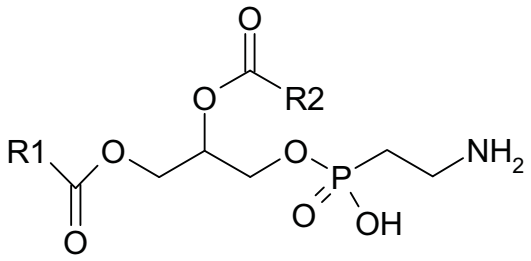


Figure 3.32 Chemical structure of colamin-kephalin (R1 and R2 are alkyl-substitutes)

3.5.2 Dissolution results

Danazol. Figure 3.33 shows mean dissolution data for danazol from Danatrol® capsules. Profiles obtained in blank buffers are not included since danazol barely dissolves in the buffers.

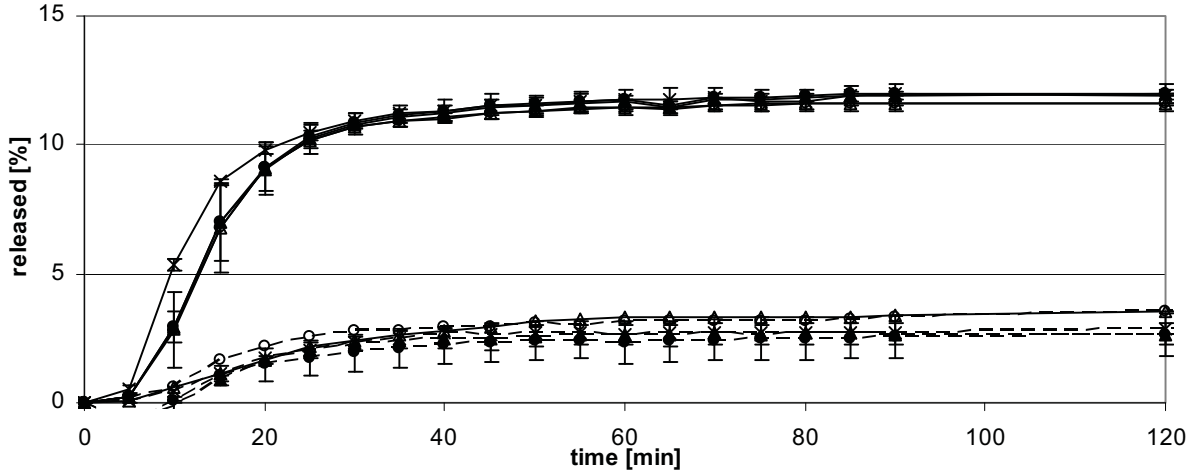


Figure 3.33 Mean dissolution values and standard deviations of Danatrol® in FaSSIF (- -) and FeSSIF (—): x SIF™, ▲ FD CPC, Δ FD PCA, ● CM CPC and ○ CM PCA

Dissolution of danazol from Danatrol® capsules in FeSSIF was about 3 fold higher compared to FaSSIF. In blank buffers, no differences could be observed indicating that the effect on solubility is purely surfactant dependent. For FaSSIF no significant difference in dissolution profile could be detected by two-way ANOVA for repeated measurements for the various media. Dissolution in FeSSIF also showed no significant difference between the freeze-dried and conventionally prepared media. However, for FeSSIF prepared using instant SIF™ powder a significant difference for time points between 10 and 20 minutes could be detected using the post-hoc Bonferroni test. Dissolution was slightly faster but resulted in identical

maximum concentrations and T_{max}.

BIXX. Figure 3.34 shows mean dissolution data for BIXX from BIXX-F in various FaSSIF media. The source of bile salt did not result in differences. T_{max} in conventionally prepared media was 35 minutes whereas the maximum concentration achieved in the freeze-dried and instant SIF™ FaSSIF was reached at 40 minutes. Mean maximum concentrations were 15.3 % in conventionally prepared media, 16 % in freeze-dried and 18 % in instant SIF™ FaSSIF. However, due to high standard deviations, differences were not statistically significant. In all FaSSIF solutions an initially higher % released fell away to a final value of 7.5 ± 0.5 % at 120 min.

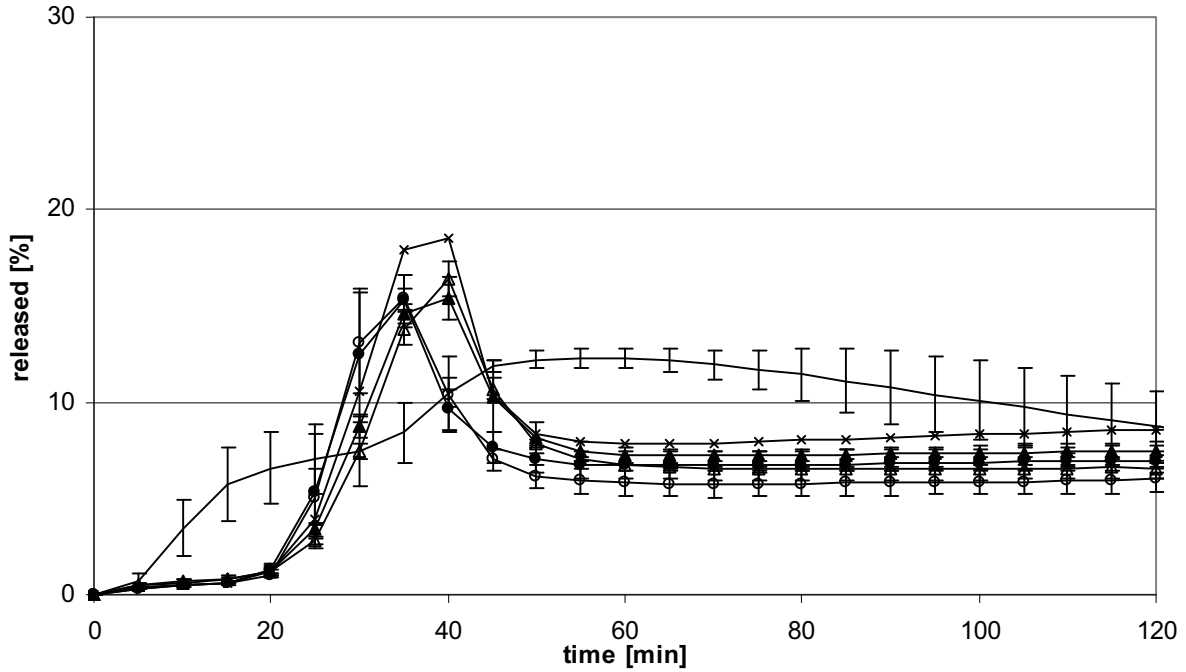


Figure 3.34 Mean dissolution values and standard deviations of BIXX-F in FaSSIF: x SIF, ▲ FD CPC, Δ FD PCA, ● CM CPC, ○ CM PCA and buffer (no symbol)

Figure 3.35 shows mean dissolution curves for BIXX-F of BIXX in the various FeSSIF media. No significant differences were observed among the profiles.

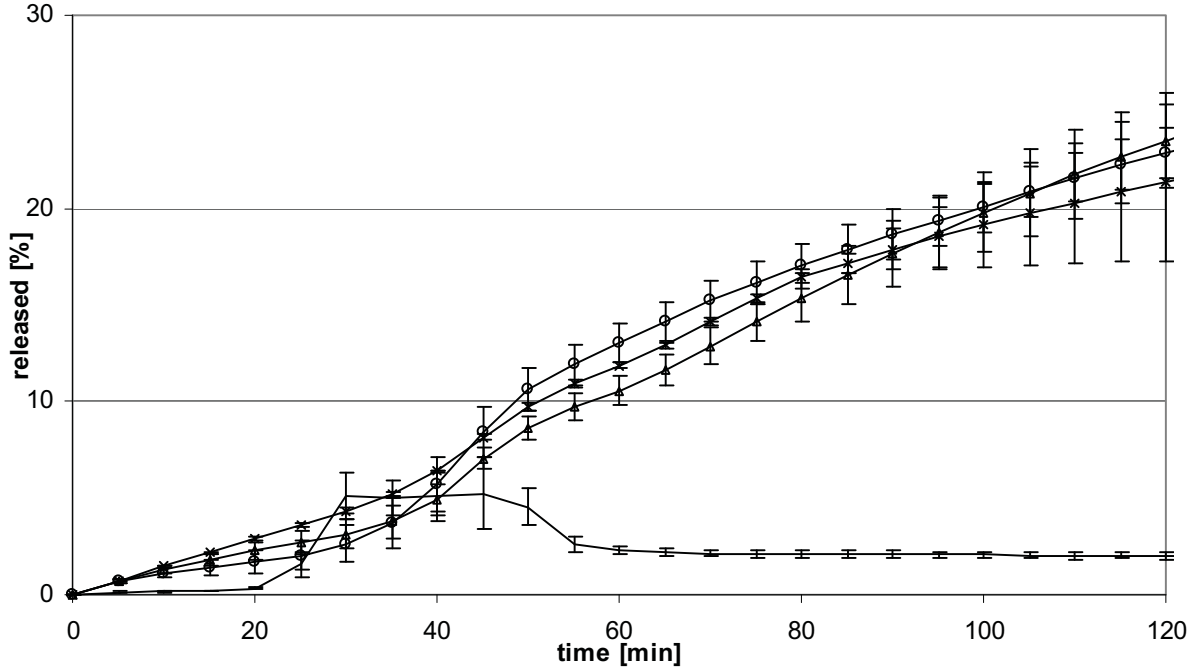


Figure 3.35 Mean dissolution values and standard deviations of BIXX-F in FeSSIF: x SIF, ▲ FD CPC, Δ FD PCA, ● CM CPC and buffer (no symbol)

4 Discussion

4.1 Preparation of the lyophilised media

4.1.1 Preparation and freeze-drying of concentrated solutions

FaSSIF, FeSSIF and FeSSIFplus for reconstitution with buffers

The clear solutions prepared to manufacture "instant" FaSSIF and FeSSIF for reconstitution with buffers are mixed micelle solutions[58]. Sodium taurocholate dissolves in water by forming simple micelles, which then act as a solubiliser for the subsequently added solid phosphatidylcholine. Incorporation of phosphatidylcholine leads to mixed micelles. The clarity of the solution indicates a micelle size below 500 nm[59]. The concentrated FeSSIFplus solution also appears clear, so the additional fatty acid and monoglyceride are either dissolved in solution or more probably incorporated into the non-visible micelles. Events during preparation of the concentrated solutions can be explained by a characterization of the different kind of solutes:

Bile acids are insoluble amphiphiles that neither swell in water nor form micelles in bulk solution and therefore only can be found at the air/liquid interface. Bile salts however, are soluble amphiphiles, which do not exhibit lyotropic mesomorphism. They are present in water as unstable monolayers in the bulk solution at low concentrations, whereas above the critical micelle concentration (cmc) they spontaneously form micelles[60]. The polar groups of the bile salts are distributed along one side of the molecule making them bean-shaped. Only small aggregates of about 2 to 15 nm, are formed consisting of about 2-9 molecules[61,62]. As such, they can act as solubilisers for as a variety of compounds. Insoluble amphiphiles can be solubilised by soluble amphiphiles, forming a stable emulsion. Depending on the type of amphiphile as well as on the temperature and degree of ionization within the solution, an equilibration time of about 0.5 and 2 hours is observed. Examplary, chemical structure of sodium taurocholate (NaTC) is given in Figure 4.1.

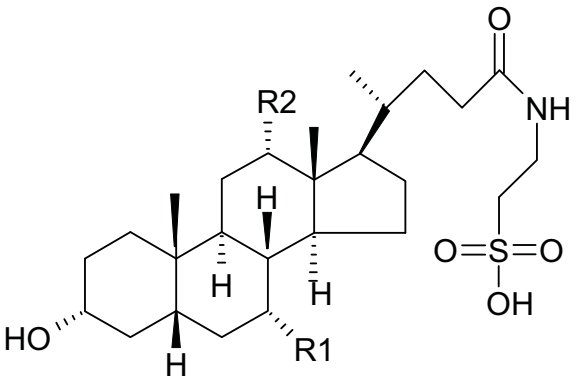


Figure 4.1 Chemical structure of sodium taurocholate

Long chain phosphatidylcholines are insoluble but swelling amphiphiles, zwitterionic at all physiological pH values. They are present at the air/liquid interface as well as in the aqueous bulk solution, forming liquid lamellar crystals under swelling and hydration[60,63]. At higher concentrations phosphatidylcholines form bilayered liposomes. Mixing of a detergent, like bile salts, and a phosphatidylcholine solution leads to disruption of the phospholipid bilayer with subsequent phase transition into mixed micelles with a nearly spherical shape. One mole of bile salt can solubilise up to 2 moles of phosphatidylcholine[16]. At a molar ratio of NaTC to PC of 4 to 1 in FaSSIF and FeSSIF, no free phosphatidylcholine can be found, whereas monomers of sodium taurocholate are still present in bulk solution. Micelle size, weight, aggregation number and cmc of the two components is strongly dependent on temperature, pH, ionic strength, concentration of various additives as well as the concentrations and molar ratio of the two amphiphiles. The micelles show a hydrophobic core of liquid-like hydrocarbon with polar headgroups and counterions presented at the surface.[60] With increasing detergent concentration, the phospholipid bilayers are completely disrupted and the monomers are included into the bile salt micelles. The cmc of the two amphiphile system is reduced compared to that of a pure bile salt solution.[19] An increase of the % detergent incorporation leads to a reduction of the average diameter of the mixed micelles[64,65]. By contrast, mixed micelles swell under addition of phosphatidylcholine.[37]

Sodium oleate is an emulsifying agent, that, dissolved with sodium taurocholate during preparation of FeSSIFplus, is incorporated into the forming mixed micelles.

Glycerol monooleate (GMO), with a melting temperature of 35 °C, is solid at room temperature. The nonemulsifying type (n/e) of GMO with a HLB value (hydrophil-lipophil balance) of 3.3 is often used to stabilise oil-in-water emulsions[63]. In excess water GMO gels

by forming a highly ordered cubic phase. During preparation of concentrated FeSSIFplus, GMO is incorporated in mixed micelles of the dissolved taurocholate and sodium oleate, here acting as an emulsifier. The charged GMO takes up more space in the micelle causing swelling of the cubic phase, explaining the higher micelle size in comparison to FeSSIF[37].

Feasible concentrations for lyophilisation:

To obtain the best freeze-drying results, the concentrations of sodium taurocholic acid and phosphatidylcholine in water were optimized. Concentrates for the production of instant FaSSIF and FeSSIF proved optimal at 165fold and 33fold of the final concentration in the reconstituted medium, respectively. In case of FeSSIFplus, the maximum possible concentration factor was 20.

Cakes obtained from freeze-drying of such concentrates are homogenous in appearance, stable upon storage (provided they are adequately protected from moisture) and rapidly dissolving. Less concentrated solutions show similar advantages, but due to the increased volume, the processing is less efficient. The higher volumes require a higher energy consumption due to prolonged process time, as well as reducing throughput and batch size.

The solutions should contain only the lipid components: sodium taurocholate and phosphatidylcholine in case of FaSSIF and FeSSIF, with additionaly sodium oleate and glycerol monoleate in the case of FeSSIFplus. No bulking agents like mannitol are necessary to obtain a homogenous and highly porous cake. Filling heights by freeze-drying in vials should not exceed 20 mm.

Complete FaSSIF and FeSSIF (with buffer substances and NaCl) for reconstitution with water

Preparation of complete instant FaSSIF for reconstitution with water did not offer any advantages. Major disadvantages are occurred during (a) preparation of the concentrated solutions and (b) freeze-drying of the solutions. As coherent cakes can only be prepared from low starting concentrations, this preparation option was abandoned.

(a) preparation of the concentrated solutions

Clear concentrated solutions of complete FaSSIF and FeSSIF were only obtained at concentration factors below 15. At higher concentrations gelatinous precipitation of the solutes occurred. The gel formation was not caused by a pH change: the bile salts used in the compositions are generally much better soluble than their corresponding acids.[66] The taurine conjugated salt shows a pKa of ~2 and is therefore soluble over an even broader pH range

than e.g. the glycocholate. In concentrated phosphate or acetate solutions sodium taurocholate is soluble at least over a pH range of 2-13. Changes in pH could not therefore have caused or influenced formation of the jelly-like precipitate. Precipitation was more likely caused by salting-out of sodium taurocholate solution by the addition of the highly concentrated sodium chloride. Salting-out is initiated by dehydratation of the bile components, explaining the slow fiber like precipitation by successive addition of sodium chloride to the sodium taurocholate solution in phosphate buffer. The slow addition of sodium chloride to the mixed micelle solution caused a slow dehydratation: when the dissolved bile salt was added quickly to the saline phosphate solution, precipitation occurred immediately. (Figure 3.1b).

(b) lyophilisation of complete FaSSIF and FeSSIF

FaSSIF complete:

Coherent cakes could not be obtained by freeze-drying of complete FaSSIF and FeSSIF. The non-coherent structure of FaSSIF was induced by phase separation during freezing[67]. This is caused by the different solubilities of the buffer salts NaH_2PO_4 and Na_2HPO_4 in solution during freezing:

The dibasic phosphate salt shows lowest solubility with about 0.82 M among the four phosphates[68]. Therefore it precipitates faster during freezing leading to a shift in pH value by changing ratio between Na_2HPO_4 and the more acidic NaH_2PO_4. Gomez et al.[67] studied pH changes of phosphate buffered solutions during freeze-drying. The freezing rates used in their experiments were 0.5 K/min and thus comparable to freezing rates used in our experiments. They observed a downwards pH drift, from 0.5 pH units at the start of supercooling to 1 pH unit at the onset of freezing. A sharp drop in pH of 3.4 units was observed in the frozen solution. The magnitude of the pH drop depends very highly on the initial pH value. At high pH, the molar ratio between NaH_2PO_4/Na_2HPO_4 is small, so the change during freeze-drying and the corresponding pH drop is high. The change in molar ratio is driven by solubility and moves towards the composition of the ternary eutectic of $H_2O/NaH_2PO_4/Na_2HPO_4$. The freezing process starts with concentration of the phosphates, due to ice (water) formation, until the saturation solubility of the eutectic is reached. The eutectic temperature is - 9.9 °C for the NaH_2PO_4/Na_2HPO_4 mixture and it theoretically freezes at a molar ratio of 3.42 to 0.06. In practice, however, no ternary eutectic could be observed.[68] This leads to the assumption that ice is formed first, leading to concentration of the phosphates. Na_2HPO_4 then crystallises as $Na_2HPO_4 \times 12\ H_2O$ in a binary eutectic mixture, leading to a shift in the molar ratio in favour of NaH_2PO_4 and therewith to a decrease in pH. NaH_2PO_4, on the other hand, is likely to be directly transformed into solid amorphous states[68]. At higher phosphate concentrations, as used in our experiments, crystalline $Na_2HPO_4 \times 12\ H_2O$ can be dehydrated and transformed to an amorphous state during drying[69]. A pH of 3.5 is reached before complete freezing when the sodium salt of phosphate is used. The behaviour of the potassium salt is just the opposite: monobase salt shows lower solubility leading to a pH value of 7.5 to 8 before complete freezing.[43]

The separated precipitations lead to heterogeneous nucleation and hinder homogenous cake formation. The craggy cakes obtained by freeze-drying of complete FaSSIF can be explained by the disparate behaviour of the buffer species during freezing.

In addition, by decreasing the pH, the solubility of sodium taurocholate is lowered. With a

pK_a ~2 it starts to precipitate at about pH 3[19]. Precipitation during freeze-drying of the components of the FaSSIF complete concentrate in turn caused in phase separation and inhomogeneous cake formation. Lueckel et al.[52] reported that by far-from-equilibrium freezing, the crystallisation of $Na_2HPO_4 \times 12\ H_2O$ and the aligned pH shift can be avoided, which implicates sample volumes of below 1 ml. However, since the maximum concentration for a complete FaSSIF solution is 15 fold of final concentration, a minimum volume of 33 ml must be used to prepare an instant media which can be reconstituted in 500 ml, a typical dissolution test media volume.

In addition, reconstitution of the complete instant media did not result in the target pH due to transformation of mono to bibasic sodium phosphate caused by the different solubilites of the phosphates. A possible way to hinder phase separation during freezing to optimize crystallisation and cake formation would be the addition of bulking agents. However, the bulking agents, like mannitol, might influence of dosage forms dissolution in the reconstituted media. As the freeze-drying of media without buffer salts represents a highly suitable alternative, use of buffered concentrated FaSSIF for preparation of instant biorelevant media is not recommended.

FeSSIF complete:

FeSSIF buffer is usually prepared using glacial acetic acid. At a pressure of 1.3322 mbar the acid has a boiling temperature of – 17.5 °C. To avoid evaporation of glacial acetic acid and therewith loss of buffer capacity, sodium acetate was used instead. To enable maintenance of sodium acetate, pH was not adjusted. The acetate stays dissociated and no evaporation occurs. As a result, adjustment of pH is necessary after reconstitution, obviating the advantages envisaged for freeze-drying of the complete medium. No benefit is gained by freeze-drying complete FeSSIF. On the contrary, the unsatisfactory cake formation makes freeze-drying of complete solutions for reconstitution with water disadvantageous and its use is not recommended.

4.1.1.1 Freeze-drying process

The lyophilisation process was optimized to allow homogenous ice and cake formation. The optimized process is summarized in Table 4.1.

For all steps, the processing time was minimized as far as possible to save time and process costs. Shortening the processing time, however, is limited by the threat of inhomogeneous cake formation, which can be explained as follows:

Excessive freezing rates led to inhomogeneous ice formation. The surface and outer parts of the liquid are frozen first, forming a smooth shell enclosing a still liquid core. During subsequent freezing of the inner parts two phenomenon can be observed: (a) the resulting volume expansion leads to breakage of the smooth ice surface or (b) the ice surface is too solid to allow necessary expansion. Lyophilisates exhibiting property (a) consisted of inhomogeneous cakes with areas of crushed solids. In case (b) the cakes obtained were sticky, because channels allowing sufficient sublimation and desorption of the solvent could not be formed. As consequence the drying process remained incomplete, leading either to poor storage stabilities, if the drying process was appropriately prolonged, or to poor reconstitution rates due to the low surface area / porosity.

Maintenance of the freezing temperature for a minimum of 2 hours was necessary to guarantee complete freezing. Shorter times and premature heating caused collapse of the structure or even partial melting. As a result porosity was decreased or the water was not removed.

At moderate increases in temperature and sufficient vacuum, the water could sublime easily, leaving channels for further sublimation / evaporation. Maintaining the freezing temperature for at least 2 hours was also necessary to ensure that the solid was below its glass transition temperature.

Early and fast initiation of desorption during secondary drying by too rapid increase in temperature also caused cake collapse. Cakes still containing water show a reduced glass transition temperature. They started to fluidize, destroying the porous matrix. But when the increase in temperature during secondary drying was slow, the water was given time to desorb almost completely from the solid. Increasing the temperature under reduced humidity, circumvented a phase transition from the glassy to the fluid state.

Table 4.1 Optimized lyophilisation process

process	duration [h]	temperature [°C]	pressure [mbar]
start of freezing process	1	from RT to -40	atm
freezing	3	-40	atm
primary drying	3	-40	0.28
	7	from -40 to 0	0.28
	6	from 0 to 20	0.28
secondary drying	1.5	from 20 to 25	0.05
	5	from 25 to 30	0.05
	till end	30	0.05

4.1.1.2 Packaging

No increase of moisture in vials was observed since the closing step was conducted under reduced pressure. Under the partial vacuum the stoppers were "stuck" to the vials and after the stoppers were capped with aluminium, no inflow of water vapour could occur. In all other cases the increase in water content within the first time-frame can be explained by the contact of the product with moisture in air during removal from the freeze-drying and during packaging. The blister packaging developed as part of this project (see chapter III2.2.4) prevented any increase of moisture content during storage. The sealing contact area of the new blisters was about 10fold higher compared to the standard ones, reducing chance of channelling during spreading of the adhesive. The manual process of pressing the heated sealing ring on the matrix using a bench vice proved sufficient. These large size blister sealed well enough to enable storage of lyophilised media for at least six months without detrimental changes in properties. For industrial production, tools and standardised sealing conditions would be necessary to prevent the channel formation which can occur when the power supply during sealing is uneven.

The tablets were not tested regarding moisture uptake but a similar trend as for powder in flasks (a) is expected since identical ground raw and packaging material was used. For detailed moisture content data see 3.2.4.

4.1.1.3 Reconstitution

FaSSIF, FeSSIF and FeSSIF plus for reconstitution with buffers:

Reconstitution of the instant media was complete within seconds. This can be explained by the high surface area formed during evaporation of the solvent during freeze-drying. Sodium taurocholate and phosphatidylcholine did not cause a pH change in the reconstituted solution. No further treatment of the instant media was necessary (except when deaeration is necessary for the dissolution test).

FaSSIF and FeSSIF for reconstitution with water:

In case of FaSSIF and FeSSIF for reconstitution with water the surface area created was much lower. The clumpy solid was therefore not so "wettable" and time of reconstitution was longer. In addition, the buffer system was modified by salt transformation (FaSSIF) or acetic acid evaporation (FeSSIF), making pH adjustment necessary after reconstitution. In the case of FeSSIF the buffer capacity was also weakened by the evaporation of the acetic acid. Therefore this is not an appropriate product concept.

4.1.2 Comparison of freeze-dried with freshly prepared media

Physicochemical properties of the media prepared by the two methods proved to be similar. Better reproducibility of the "instant" media was demonstrated by the generally smaller standard deviations in all parameters. Preparation following the conventional method (CM) depends more on the skill of the user and higher day to day variability is likely. Partly subjective decisions about how well the organic solvent has been removed and possible loss of buffer and solute during processing results in higher variability in all physicochemical properties. The slightly higher surface tension, solubility of danazol and dissolution profile of BIXX in CM media can be explained by presence of small amounts of residual organic solvent (methylene chloride). The particle size of instant media shows a more narrow and strictly monodispers distribution. Higher polydispersity indices for the freshly prepared medium result from variance during micelle formation, which takes place during solvent evaporation, and by delayed equilibrium of the micelle formation. It can be assumed that the state of equilibrium has been attained when the micelles in FaSSIF and FeSSIF are 90 and 6 nm in diameter, respectively. Using instant media particle size equilibrium is reached quickly after preparation, but in the case of conventionally prepared media equilibrium is delayed. The high polydispersity indeces of 0.12 and 0.4 observed initially after preparation for CM FaSSIF and FeSSIF, respectively, were reduced by ongoing micelle formation over the ensuring 24 hours. The incomplete mixed micelle formation might also be the reason for the slightly higher osmolality values: Na^+ from NaCl acts as counterion of the bile components. During micelle formation in conventional media Na^+ ions gradually become ionically more "shielded", leading to the higher osmolality values measured directly after preparation. From this arguments, conventionally prepared media should be equilibrated for at least 24 hours, making the preparation even more time-consuming. However, this is contrary to the recommendations for preparation on the day of use, which is based on the short shelf-life of the product.

In summary, the investigated methods to prepare biorelevant dissolution media from instant FaSSIF, FeSSIF and FeSSIFplus represent three major advantages: improved reproducibility, a more simple and faster preparation, and better storage properties.

4.1.3 Characterization of the lyophilisate

4.1.3.1 Appearance

The cakes derived from preparation of instant media for reconstitution with buffers led to homogenous and highly porous cakes. The solvent (water) could be removed easily, forming channels as it sublimed during primary drying. Solvent desorption during secondary drying was therewith facilitated, leading to a dry and stable cake. No stickiness or breaking of the surface occurred. The high surface area created enables easy and rapid dissolution.

By contrast, "complete" FaSSIF and FeSSIF for reconstitution with water led to poor homogeneity in the cakes caused by a variety of phenomenons, as explained in III3.1.1. Due to the dissatisfying results and lack of any advantages, use of complete FaSSIF and FeSSIF was not pursued.

4.1.3.2 SEM (scanning electron microscopy)

FaSSIF, FeSSIF and FeSSIFplus for reconstitution with buffer:

The micrographs confirmed visually recognized properties. The high pore formation and lack of crystal parts explain the good reconstitution and stability properties.

Complete FaSSIF and FeSSIF for reconstitution with water:

The stickiness of the cake and the crushed surfaces are visible on the µm level (see Figure 3.7 and Figure 3.8). The structures indicate that pore formation during primary drying is inadequate. Subsequently, water could not evaporate properly, leading to partial melting during heating and therewith total collapse of the structure. Crystallisation of the buffer salts in this process explain the rough structure. The compact cake structure results in small surface areas, which in turn result in slow reconstitution.

4.1.3.3 Hygroscopicity

Because of the high hygroscopicity of the lyophilised media, dry storage conditions are mandatory. Even at low humidity values, the media start to adsorb water. With increasing moisture content the solid cakes partially dissolve and the cake structure is lost. In addition phosphatidylcholine is hydrolysed in the presence of water. Therefore it is necessary not only to completely dry during the freeze-drying process and to ensure dry storage.

The delayed start of absorption during measurement as well as during open storage in case of

complete FaSSIF and FeSSIF is caused by the reduced surface area, which causes longer penetration times of water into the cake.

4.1.3.4 Moisture content (Karl-Fisher-Titration)

Increase of moisture content did not exceed 3 % for any (sealed) packaging and storage conditions.

Media directly packed in the freeze-dryer showed the smallest water content and smallest increase during storage. High fill levels showed best results because of the better surface to volume ratios generated.

In cases where packaging / further processing of the media (blister sealing, grinding or tabletting) was carried out after the freeze drying process, partial water penetration occurred during the extra step, as reflected in the high initial moisture content of media packaged in blisters. Since the small increase in moisture content did not influence physicochemical properties, it seems that packaging can be done in either vials or blisters.

In all cases storage at high humidity values resulted in a higher moisture uptake. Therefore storage of the product at room temperature in a dry place is preferable.

4.1.3.5 Glass transition temperature (Differential Scanning Calorimetry)

An initial increase of the glass transition was explained by maturation of the amorphous state of the solid. The overall increase of 1.1 % and the absolute value of 117 °C indicates a stable product. This glass transition temperature value theoretically enables stability during storage as long as temperatures are kept below 80 °C and media is well sealed by the packaging.

4.1.4 Characterization of redissolved media

4.1.4.1 Appearance and particle size

All solutions are visually clear since the micelle size is smaller than 500 µm. The high solute concentrations in FeSSIF and FeSSIFplus cause an increase in the solution density. Refraction of light causes a syrupy appearance. The slightly yellowish coloration of FeSSIFplus is caused by sodium oleate.

Mixed micelle size and aggregation number: Pure phosphatidylcholine micelles are formed by swelling and hydration of the zwitterions.[60,63] At higher concentrations bilayered liposomes are formed. Addition of bile salt leads to formation of mixed micelles, which are smaller in size (see III4.1.1).

By contrast, pure micelles of bile salts show high ionic repulsion of anionic groups and therefore have a limited aggregation number of 2-9 molecules. Increase of counterion concentration leads to reduction of repulsion and therefore to decrease of cmc[65] and micelle size[62]. Addition of the blank buffer with a high concentration of NaCl therefore leads to a decrease in micellar size although more bile salt monomers are driven from the solution into the mixed micelles[65].

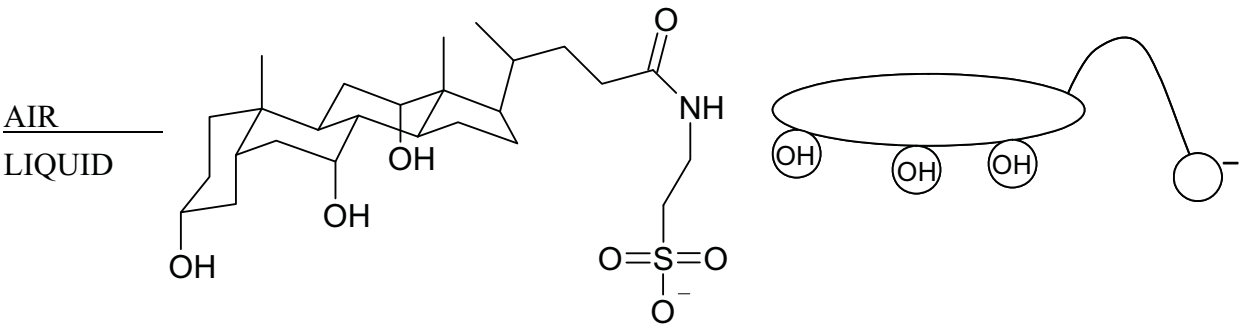


Figure 4.2 Chemical structure and positioning of sodium taurocholate in water

By increasing counterion concentration, hydrophilic repulsion forces are reduced and bile salt molecules are able to move closer together. As a result hydrophobic interactions (the van der Waals and London forces) between the bile salt steroid backbone and the hydrocarbon part of the phospholipids are favored. In short, the mixed micelle size is reduced with increasing counterion concentrations. That explains the smaller micelle radii in FeSSIF compared to FaSSIF: FeSSIF, with double the NaCl concentration of FaSSIF, shows a reduction in micelle size from 90 to 7 nm. Higher concentrations of the bile salt counterion Na^+ causes reduction of repulsion. Thus, a higher aggregation number can be achieved[70] and more bile salt molecules are incorporated into the mixed micelles. Molecular arrangement of the micelle former becomes more tight and the micelle diameter decreases. Both solutions appear optically clear due to the obtained stable isotropic mixed micelles.[60] In Figure 4.3 a bile salt rich mixed micelle is given.

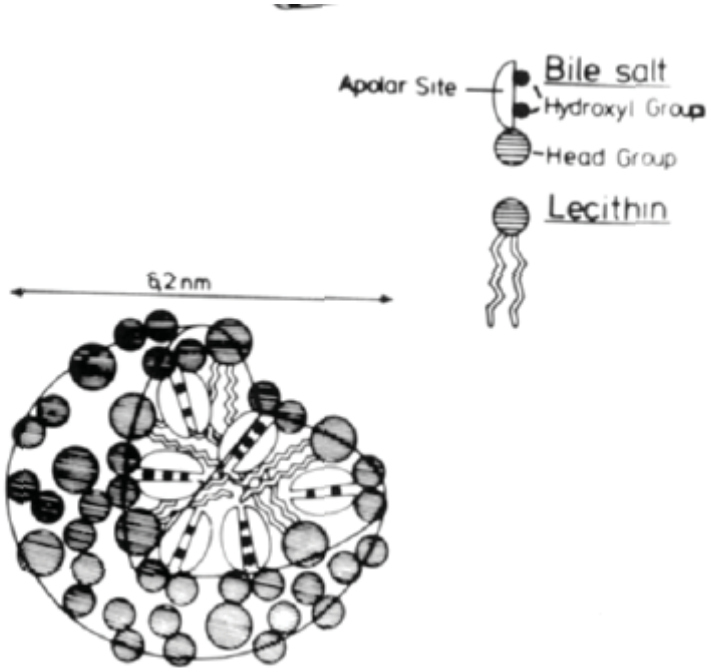


Figure 4.3 Bile salt rich mixed micelle (Muller,K. Structural dimorphism of bile salt/lecithin mixed micelles. A possible regulatory mechanism for cholesterol solubility in bile? X-ray structure analysis,1981[71])

In FeSSIFplus solutions the ionic strength is even higher. However, the addition of further components disables compact molecular arrangement. The 11 nm micelles are bigger than the 6 nm micelles in FeSSIF, based on the same blank buffer. In comparison with 100 nm micelles in FaSSIF however, the mixed micelles are smaller due the increased ionic strength and thus repulsion within the mixed micelle "shelf".

4.1.4.2 Physicochemical properties of reconstituted media

Regarding physicochemical properties of the reconstituted media prepared after various storage intervals, no significant differences in surface tension, viscosity, osmolality, density and solubilisation capacity could be observed. Solubility of danazol showed some differences, however these were not statistically significant due to the high standard deviations in values obtained with the conventionally prepared FaSSIF and FeSSIF media.

The reproducible properties of the solutions are reflected in the similarity of the % released and pH profiles during dissolution testing of BIXX-F. Mixed micelles in both media help to solubilise BIXX and keep it in solution. The similar profiles between initial and stored media indicate a reproducible mixed micelle formation and also indicate a stable composition with storage. The pH change in reconstituted FaSSIF (all storage times and conditions) and its corresponding buffer was from 6.5 to 6.2. In the case of FeSSIF, the high amounts of solubilising agents influence the pH stability. Whereas in the corresponding buffer solution a reduction to pH 4.9 was measured, a stable pH in all reconstituted FeSSIF (all storage times) was maintained.

Since no changes with storage occurred, hydrolysis and other decomposition processes appear not to be an issue with the instant media.

4.1.5 Stability, storage and packaging

The media proved to be stable for at least 6 months, with best storage conditions observed at room temperature. The surface to volume ratio of the media should be reduced to a minimum to reduce moisture uptake. Due to the high hygroscopicity of the lyophilisate, the media have to be stored dry. Preparation in vials or subsequent packaging into blisters or flasks with desiccants is necessary. In case of bulk product, the freeze-dried media should be purged with $nitrogen_{(g)}$ to protect from moisture uptake. During further processing like tablet pressing or blister sealing, moisture uptake can occur, necessitating dry room conditions and short process times.

4.1.5.1 "Pre-dosed" packaging vs. bulk product

Pre-dosed: The pre-dosed media allow very fast preparation. In case of vials, the excellent seal of the packaging increases the shelf-life. However, flexibility is reduced and inappropriate pre-dosing might lead to production of unnecessarily large volumes of expensive media. Moisture uptake during blister sealing and especially during tablet compression is associated with a risk of batch failure.

Bulk: Bulk instant media has very little contact to humid air during preparation and filling. It enables flexible media preparation and is therefore, in principle, cost saving. On the other hand the media is stressed with humid air during weighing, leading to reduced shelf life. Inappropriate re-closure of the container may lead to rapid decomposition.
The user should decide whether flexible preparation or standard procedures are more important in the daily lab work.
Preparation of tablets requires an additional compacting step but may be preferable due to the more convenient handling compared to bulk material.

4.1.6 Applicability in an industrial setting

Freeze-dried media are easy to prepare and enable rapid preparation of biorelevant media:
1) The concentrates can be prepared in under two hours in simple preparation steps. 2) A standard freeze-drying process developed for liposomes can be used, making the freeze-drying process feasible for many laboratories. 3) The media can be stored in their solid form. 4) Omission of buffer substances makes the lyophilisate a flexible starting material for various modifications of biorelevant media. 5) Only one powder is necessary for the preparation of FaSSIF and FeSSIF. 6) Reconstitution is rapid, very simple and leads to highly reproducible media. 7) The instant media shows identical composition, physicochemical and release properties to the conventional, freshly prepared, FaSSIF and FeSSIF media.

4.2 Comparison of commercially available and freeze-dried instant media

Substitution of sodium taurocholate (NaTC) from CPC for NaTC from PCA:
The less expensive sodium taurocholate from CPC had no impact on physicochemical properties, on solubilizing effects or dissolution behaviour. Therefore substitution of NaTC from CPC for NaTC from PCA is possible and recommended.

Suitability of freeze-drying method vs. conventional preparation:
The freeze-drying method shows identical properties but higher reproducibility (see chapter III). Failure of batches can be avoided, leading to indirect cost saving of the expensive solubilising agents. In addition, the freeze-drying method is time-saving in terms of preparation and therefore it is recommended to use this novel preparation method.

SIF™ vs. conventional:

Early in 2007 a commercially available instant FaSSIF and FeSSIF came onto the market.[47] The Phares SIF™ powder is a hygroscopic powder and therefore delivered in amber glass containers with a desiccant sachet enclosed. Storage at 2-8 °C allows a limited shelf life of 1 year. Media composition differs from these discussed here in that a soy (S-PC) rather than egg (E-PC) phosphatidylcholine is used for preparation. Costs are about 3 fold higher compared to raw material only, not taking into account costs for preparation of conventional or freeze-dried media.

The SIF™ media containing phosphatidylcholine from soy instead of E-PC led to different particle size. S-PC shows a different fatty acid composition and a higher concentration of unsaturated acids. In addition, a higher concentration of colamin-kephalin in S-PC can be found. The different chemical structures in soy lecithin allow a more compact alignment within the mixed micelles compared to phosphatidylcholine. However, the solubilisation capacity in case of danazol and dissolution of BIXX was similar in SIF™ to conventionally prepared media. Dissolution of danazol in FeSSIF was initally faster but reached the same maximum value. Therefore it is recommended to test possible influence of SIF™ media on dissolution of novel entities before substituting them for conventional or freeze-dried media.

5 Summary and conclusion

To prepare easy and ready to use FaSSIF, FeSSIF and FeSSIFplus, a method for lyophilisation of concentrated solutions was investigated. To effectively conduct the process, the maximum possible concentration (concentration factors 165, 33 and 20 compared to the reconstituted product, respectively) was determined and filling heights optimized at between 0.5 and 2 cm. After lyophilisation the media can be stored at room temperature until use for at least 6 months. (According to international conference on harmonisation (ICH) Guideline Q1A(R) – "Stability Testing of New Drug Substances and Products" a storage time of 12 months can be assumend after successful stability under stress conditions (40 °C/75 %RH).) The instant media can be dissolved with the blank buffer within about 15 sec and can be used immediately. This means that the preparation time for a dissolution test can be reduced by 2 to 3 hours compared to the conventional preparation method. For dissolution testing degassing following USP[2] is recommend. Thus particle size equilibration can be accelerated. Physicochemical properties are the same for media prepared by the conventional or the freeze-drying method, except for micelle formation, which was more reproducible with the instant media.

6 Acknowledgement

This chapter was published in part by Boni et al.[72]

7 Standard operation procedure (SOP) for the preparation of freeze dried FaSSIF and FeSSIF

7.1 Composition

Table 7.1 Composition of concentrates and freeze-dried media per 1 l-vial

	substance	FaSSIF g / 1 l-vial	FeSSIF g / 1 l-vial	FeSSIFplus g / 1 l-vial
(01)	sodium taurocholate	1.65	8.25	4.12
(02)	sodium oleate	-	-	3.044
(03)	H_2O	10	35	50
(04)	phosphatidylcholine	0.59	2.954	1.573
(05)	glycerol monooleate	-	-	1.783
	total (dried)	**2.24**	**11.204**	**10.52**

7.2 Instant media

White to slightly yellow dry powder, rapidly dissolving in water or aqueous buffer solutions.

7.3 Preparation

Instant media is prepared by freeze drying of concentrated solutions of biorelevant media. Buffer solutions and NaCl are not added to the concentrates but are used for the reconstitution of the lyophilisate.

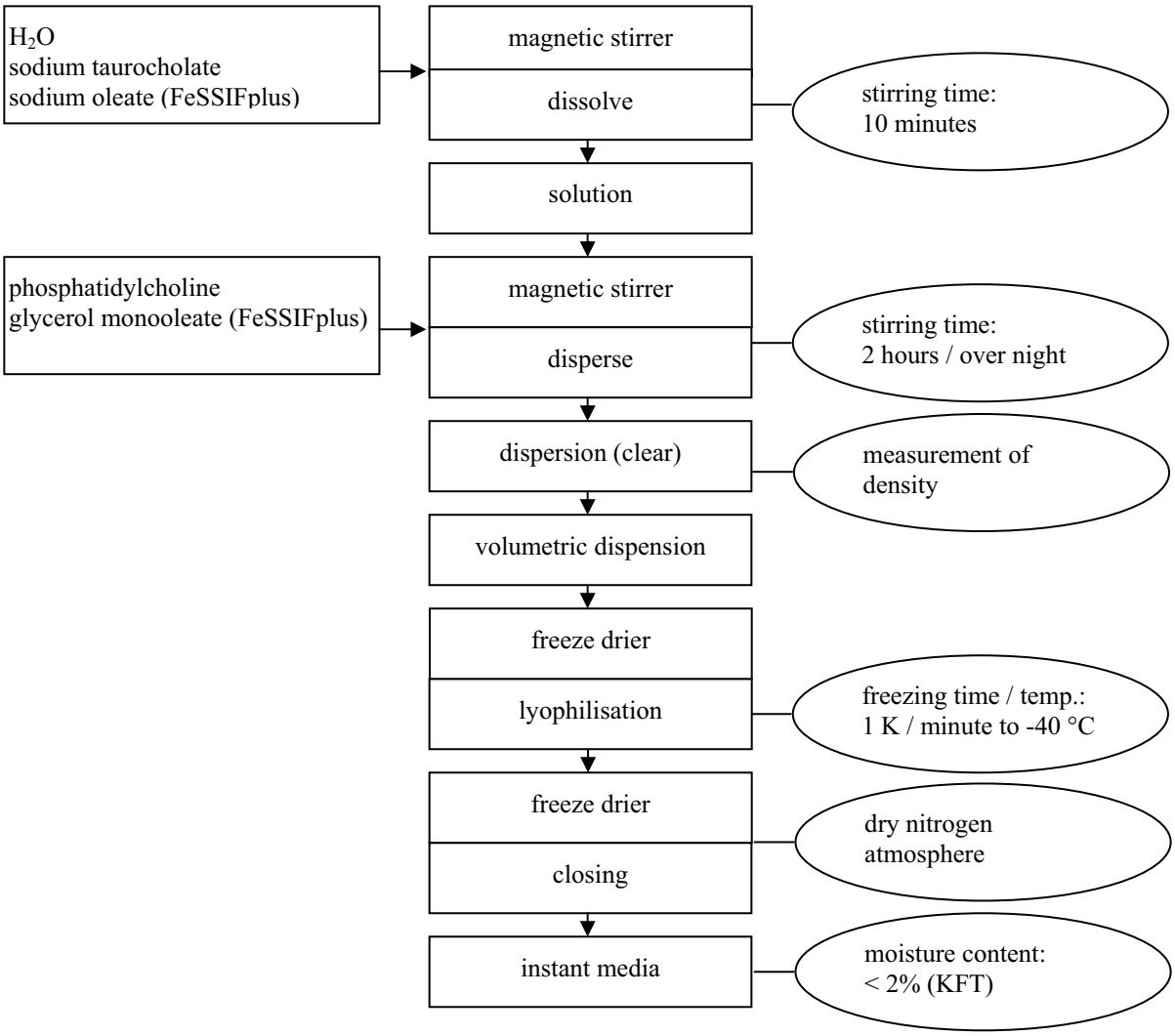
To prepare FaSSIF, FeSSIF and FeSSIFplus sodium taurocholate and sodium oleate (FeSSIFplus, only) are dissolved in water. The solution is stirred at room temperature for about 15 minutes until clear solution. Phosphatidylcholine and glycerolmonoleate (FeSSIFplus, only) are added under strong stirring for about 2 hours until a clear dispersion is obtained. Acceleration of dispersion can be obtained by ultrasonification or high shear mixing.

Density of the solution is measured and the solution volumetrically dispensed into glass vials.

The solutions are cooled to -40 °C at 1 K/min. Primary and secondary drying strongly depends on filling rates of vials and freeze dryer capability.

After freeze drying the vials have to be closed densely under dry atmosphere.

Storage time of the instant media is expected to be 2-3 years at room temperature.



7.4 Storage

The instant media is highly hygroscopic and therefore has to be stored dry.

Closing under nitrogen or addition of desiccant is recommended.

At room temperature a storage time of 36 months is assumed.

IV Improvement of biorelevance by substituting convention buffer salt for bicarbonate buffer in biorelevant media FaSSIF and FeSSIF

1 Introduction

In vitro dissolution testing of pharmaceutical formulations is one of the most informative tools in drug development, with formulations often optimised according to the desired release profile.

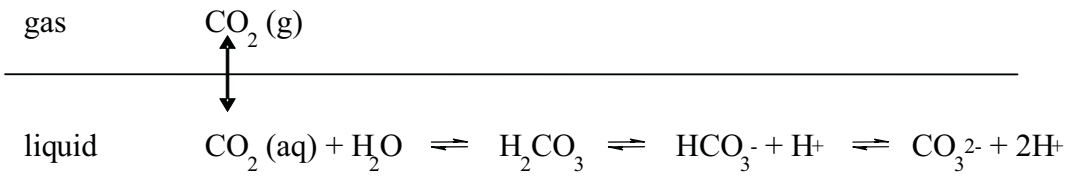
Many efforts have been made to establish *in vitro* systems predicting *in vivo* behaviour of formulations. Some systems, like the TIM model of TNO Pharma[35], attempt to exactly mimic *in vivo* conditions. But the high complexity of the TIM model makes it impractical for routine dissolution testing. More simple and correspondingly easier to set into practice is the use of simulated gastrointestinal media in standard dissolution apparatus. A knowledge of the composition and physicochemical properties of gastrointestinal (GI) fluids combined with consideration of motility in the upper GI tract in the design of the dissolution test should lead to a better prediction of *in vivo* performance. These arguments apply especially to compounds belonging to Class II of the BCS (Biopharmaceutical Classification System), since the absorption of these compounds is likely to be limited by dissolution[1]. On the other hand, oversimplification of the dissolution conditions, as for example with compendial media, often leads to profiles which do not reflect the formulation's *in vivo* behaviour.

Biorelevant media, which are designed to simulate physiological effects more closely, aim to better link *in vitro* with *in vivo* performance. Galia et al.[38] created dissolution media through consideration of the main factors which are generally expected to influence dissolution *in vivo*. Values of lecithin, taurocholic acid, osmolality, surface tension, buffer capacity and pH were adjusted to physiological values. To reflect differences between the fasted and fed state, two media were established: FaSSIF (Fasted State Simulating Intestinal Fluid) and FeSSIF (Fed State Simulating Intestinal Fluid). But even though they simulate the most relevant characteristics such as concentration of solubilising substances, buffer capacity and pH, they are not a one-to-one copy of gastric or duodenal juice. FaSSIF is based on a non-physiological buffer system, phosphate buffer (0.022 M, pH 6.8) and FeSSIF on a partly physiological

acetate buffer (0.144 M, pH 5.0). In fact, bicarbonate is the predominant buffer in the fasted small intestine, secreted continuously to neutralise acidic gastric juice and protect the epithelium against digestion[21]. Secretion from epithelial cells and glands is regulated in order to maintain a pH[22] which is more or less stable against changes in acid or base concentration entering the small intestine. Postprandially, a variety of buffer species originating from food components as well as indigenous ones contribute to the overall buffer capacity.

Many orally administered drugs are dissolved and absorbed in the upper small intestine, where bicarbonate is the predominant buffer system in the fasted state. By contrast, most buffer solutions used to prepare dissolution media (compendial and biorelevant) are based on phosphate buffers. The phosphate buffers have been traditionally favoured as they provide good buffer stability[73] during dissolution. Since dissolution profiles can be dramatically influenced by buffer type and strength[74], it is of considerable importance to optimize the buffer system. Especially for ionisable drugs, variations in buffer may increase or decrease dissolution, lead to crystallisation, salting out of the drug or have other arteficial effects. Thus, if practicable, it would be preferable to use a bicarbonate buffer for experiments simulating the fasted state dissolution of drugs.

Equation 1.1 shows schematically generation of bicarbonate buffer by dissolution and dissociation of CO_2 in aqueous solutions. $CO_{2(g)}$ partially dissolves into the solution and hydrates to carbonic acid (H_2CO_3). Carbonic acid dissociates to bicarbonate (HCO_3^-) or even carbonate (CO_3^{2-}), depending on the pH of the solution:



Equation 1.1 Formation of carbonate from bicarbonate in aqueous solution

Because of the high pK_{a2} of 9.9 at 25 °C, CO_3^{2-} is of little relevance at intestinal pH. Bicarbonate (pK_{a1} = 6.1 at 25 °C) is the dominant ion at pH 6.5. With increasing partial pressure of $CO_{2(g)}$ above solution, more CO_2 will be dissolved and the equilibrium will be shifted, leading to higher concentrations of carbonic acid and bicarbonate, and a decrease in pH. Addition of NaOH to the solution leads to an equilibrium shift and a higher buffer capacity.

In contrast to the stable pH *in vivo*, which is held constant by pancreatic, bile and intestinal secretion, it is difficult to maintain a stable bicarbonate buffer *in vitro*[75]. Evaporation of $CO_{2(g)}$ with time leads to an increase in pH and changes in the buffer strength. The first step to establish a stable bicarbonate buffer is, therefore, to maintain equilibrium between $CO_{2(aq)}$ in solution and $CO_{2(g)}$ in the atmosphere.

Some attention to this subject has already been given in the literature. Fadda et al.[76] studied dissolution results in physiological buffers like Hanks or Krebs buffer containing bicarbonate as additional buffer system. McNamara[20] made efforts to establish a stable and pure bicarbonate buffer for application in dissolution. In this method, a saline solution was used as starting material. $CO_{2(g)}$ was supplied continuously and the target pH was maintained by adding 1N NaOH. During dissolution, supply of CO_2 was regulated to maintain the buffer pH. However, addition of both CO_2 and NaOH during the experiment will result in a change in buffer capacity, which is not the case in the physiological situation[77]. Therefore the McNamara method was attempted to be modified to create a more stable and reproducible bicarbonate buffer system which could be used as the basis for preparing dissolution media analogous to those currently used. Dissolution profiles of three formulations of a typical BCS Class II compound in these bicarbonate-based media were then compared to profiles in their compendial and biorelevant counterparts, to determine whether bicarbonate buffer lends itself to routine dissolution testing.

2 Materials and methods

2.1 Materials

2.1.1 Chemicals and formulations

Sodium phosphate and sodium bicarbonate, both analytical grade, were purchased from Riedel de Haen (Seelze; Germany). Sodium chloride and sodium hydroxide, also analytical grade, were purchased from E. Merck, Darmstadt (Germany). Crude sodium cholate and L-α-phosphatidylcholine (min. 60 % TLC) were purchased Sigma-Aldrich, Steinheim (Germany). Carbon dioxide (type 3.5 purity 99.9 5% CO_2) was purchased from Sauerstoffwerke Friedrichshafen (Germany).

BIXX formulations were provided by Boehringer-Ingelheim Pharma GmbH & Co. KG (Germany). BIXX is a weakly basic BCS II drug. Its solubility decreases from 0.082 mg/ml at pH 4.0 to 0.001 mg/ml at pH 6.5. Three formulations of BIXX were used to compare dissolution in the various media[78]. The formulations are not yet commercially available and are therefore referred to as BIXX-A, BIXX-B and BIXX-C. BIXX-A consists of spherical pellets containing an acidifying excipient, whereas BIXX-B and BIXX-C are multiparticulate matrix formulations with no pH-modifying excipients.

2.1.2 Media

Six different buffer systems were compared: bicarbonate buffer and phosphate buffer, each at two buffer strengths simulating the fasted (10 mmol/l/ΔpH) and fed (30 mmol/l/ΔpH) state in the duodenum[79]; fasted state simulating intestinal fluid (FaSSIF)[73] and a modified FaSSIF based on bicarbonate buffer. The pH was adjusted to pH 6.5 in each case. Identical pH values of FaSSIF and FeSSIF were chosen to rather compare influence of buffer strength on dissolution than pH influence. Furthermore, with decreasing pH values a less strong bicarbonate buffer could be created: the lowest pH value at which the high FeSSIF buffer capacity of 30 mmol/l/ΔpH could obtained was 6.5. At pH 6.0 and pH 5.5 highest adjustable buffer capacities were 10 and 5 mmol/l/ΔpH. To reduce expenses crude phosphatidylcholine and sodium cholate were used in this study[49].

2.1.2.1 Bicarbonate buffer according to McNamara[20]

$CO_{2(g)}$ is supplied continuously into 1 litre of 0.9% (w/v) NaCl. Mixtures of $CO_{2(g)}$ in air with fixed partial pressures of 5, 10, 15 and 20% $CO_{2(g)}$ are used for this method. To adjust the pH to 6.5 a mixture of carbon dioxide and air is supplied and 1 N NaOH is added until the pH is stable. After 30-40 min of stirring and sparging with $CO_{2(g)}$, it is assumed that the solution has equilibrated. The medium is then transferred to USP apparatus I dissolution vessel. During thermal equilibration and the dissolution test $CO_{2(g)}$ is supplied continuously into the medium. Since protons are generated by the ongoing dissolution of the CO_2, the pH has to be adjusted continuously with 1 N NaOH to maintain the desired value.

2.1.2.2 Bicarbonate buffer: Modified method

In the modified method, the concentration of NaOH in the medium is kept the same from run to run to achieve a reproducible buffer capacity. $CO_{2(g)}$ is supplied at a constant rate into a saline solution (0.9% NaCl (w/v)) containing the appropriate concentration of NaOH. CO_2 is partially dissolved, forming bicarbonate buffer, at an equilibrium between the concentration of CO_2 dissolved and its concentration in air. This procedure leads to a defined, reproducible concentration of carbonate in the medium[80]. If higher amounts of NaOH are added (keeping the $CO_{2(g)}$ supply constant), more CO_3^{2-} is generated, enabling a higher buffer capacity to be achieved. For example, a buffer capacity of 10 mmol/l/ΔpH at pH 6.5 can be achieved with 12 mmol/l NaOH. To establish bicarbonate buffer with a capacity of 30 mmol/l/ΔpH at pH 6.5, 42 mmol/l NaOH has to be added. The NaOH/NaCl solution is thermally equilibrated to 37 ± 0.5 °C. $CO_{2(g)}$ is directly supplied into the bulk solution using a glass frit (porosity grade 3) until pH 6.5 is achieved. Using a flow rate of 400 ml/min, the target pH and buffer capacity is obtained within about 20 min. After equilibration, the medium is transferred into the dissolution vessels. To maintain buffer capacity, a constant CO_2 partial pressure has to be maintained above the solution thereafter (see Table 2.1).

Table 2.1 CO_2-flow to maintain constant pH at defined buffer capacities

buffer capacity [mmol/l/ΔpH]	volume [ml]	medium-air-interface [cm²]	CO_2-flow [ml/min]
30	500	45	370
10	500	45	370
10	200	10	55

To avoid overt bubble formation in the medium $CO_{2(g)}$ is supplied above, rather than into, the medium. Importantly, the flow rate of $CO_{2(g)}$ has to be adjusted appropriately for the surface area, buffer concentration, pH and volume of media[21]. The experimental set-up for the CO_2 inflow is given in Figure 2.1. To obtain constant pressure independently from bottle filling a second needle valve is used. It is important to use tubes of identical length to maintain identical pressure above the solutions.

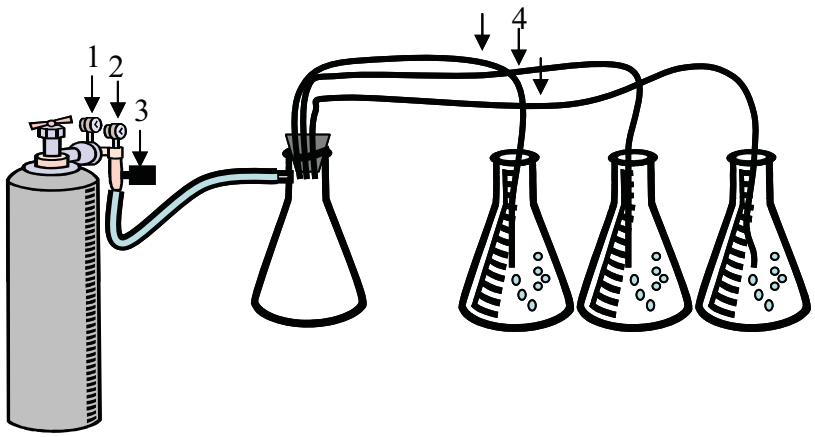


Figure 2.1 Bicarbonate apply with 1-pressure of bottle, 2-exhaust valve, 3-needle valve for flow adjustment, 4-gas tubes to vessels

2.1.2.3 FaSSIF, and FaSSIF based on bicarbonate buffer

The preparation of FaSSIF and FeSSIF differed somewhat from recent publications[73,81], in that crude cholate was first dissolved in 0.9 % NaCl solution. Thereafter a solution of crude phosphatidylcholine in methylene chloride was added. Subsequently methylene chloride was evaporated to create the basis for the medium. For the versions based on bicarbonate buffer, 12 mm/l NaOH or 42 mmol/l (for FaSSIF and FeSSIF respectively) were added to reach the required buffer capacity and the pH was adjusted by supplying 120 ml/min or 370ml/min $CO_{2(g)}$ to the medium using a glass frit (with porosity grade 3). After equilibration, the buffer was transferred to dissolution vessel and $CO_{2(g)}$ was supplied above solution to maintain a constant pH.

In total, six different media were tested:

Phosphate buffer at pH 6.5 and a buffer capacity of 10 mmol/l/ΔpH.

Bicarbonate buffer at pH 6.5 and a buffer capacity of 10 mmol/l/ΔpH.

Phosphate buffer at pH 6.5 and a buffer capacity of 30 mmol/l/ΔpH

Bicarbonate buffer at pH 6.5 and a buffer capacity of 30 mmol/l/ΔpH.

Phosphate buffer at pH 6.5 and a buffer capacity of 10 mmol/l/ΔpH, containing "fasted state" amounts of phosphatidylcholine and sodium cholate.

Bicarbonate buffer at pH 6.5 and a buffer capacity of 10 mmol/l/ΔpH, containing "fasted state" amounts of phosphatidylcholine and sodium cholate.

3 *Methods*

Buffer capacity measurement: Buffer capacity was measured by titrating 20 ml of the buffer system with 0.1 M HCl (n=3). In the case of bicarbonate buffer, liquid paraffin was added to seal the surface of the medium and inhibit the loss of carbon dioxide. Buffer capacity (ß) (mmol/l/ΔpH) was then calculated using the following equation, where ß is the buffer capacity, and ΔB is the amount (mmol) of HCl required to produce a change of one pH value (ΔpH)[82].

$$\beta = \frac{\Delta B}{\Delta pH}$$

Equation 3.1 equation for calculating buffer capacity.

3.1.1 Dissolution Tests

In vitro dissolution data were generated at 37 ± 0.5 °C in 500 ml dissolution medium using the USP dissolution apparatus I. For all dissolution tests a SOTAX® USP Apparatus I dissolution tester (model AT 7, Sotax, Switzerland)) with the basket rotating at 100 rpm was used. Samples were taken automatically using a SOTAX® piston pump (model SOTAX CY-7-50) and measured on-line using a Perkin Elmer® UV/VIS spectrophotometer (Lambda 2S, Perkin-Elmer, Überlingen, Germany). A standard curve for BIXX was run every analytical day. pH was monitored during dissolution using a standard pH meter CG840 from Schott (Hofheim/Ts., Germany) and Inolab® electrode of Mettler Toledo (Schwerzenbach, Switzerland). All dissolution experiments were run in triplicate.

Although no interference was expected in the on-line UV measurements, some samples were additionally removed manually at predetermined time points to verify the online UV/VIS dissolution results by HPLC. These samples were filtered through 0.45 µm Sartorius® membrane filters (Minisart, Sartorius, Göttingen, Germany) and subsequently diluted 1:1 with methanol. Analysis was carried out by HPLC-UV/VIS measurement (Agilent® HPLC1100, Waldbronn, Germany) using 0.05 M ammonium acetate pH 4.4 and acetonitrile as mobile phase.

3.1.2 Statistical Methods

Dissolution data is presented as % released vs. time curves, with means and standard deviations indicated for each time point (with the exception of Figure 1, where individual curves are shown for the bicarbonate buffer data). pH drift data is presented the same way.
Analysis of variance for repeated measures ($ANOVA_{rep}$) was used to statistically assess the effect of buffer (bicarbonate vs. phosphate) as well as time interactions for each pair of dissolution profiles. A Bonferroni post hoc test was performed for pairwise comparisons between buffers at each time point. All statistical comparisons were performed using GraphPad Prism Vs. 5 (GraphPad Software, Inc., San Diego, USA).

4 Results and discussion

4.1 Preparation of bicarbonate buffers

The preparation of bicarbonate buffer by the McNamara and the modified method are summarised in Table 4.1 .

Table 4.1 Comparison of preparation methods for bicarbonate buffers

Parameters	McNamara method	Modified method
adjustment temperature	room temperature	37 ± 0.5 °C
buffer capacity determined by	% atm CO_2	concentration NaOH
time of adjustment	30 min ?	20 min
CO_2 supply during dissolution	into solution (10 µm glass frit)	above solution
reproducibility of buffer	?	good
reproducibility of dissolution results	poor	poor

4.1.1 McNamara Method

McNamara assumed that only the partial pressure [% atm] of the supplied $CO_{2(g)}$ will define the resulting buffer capacity, with smaller partial pressure leading to lower buffer capacity. According to this method NaOH should be added until equilibrium is reached. The media is then transferred and thermally equilibrated under constant CO_2 supply. Several problems occurred when this method was implemented:

Estimation of time of equilibrium: Using gas mixtures with 5-20% of CO_2, the time to reach equilibrium is shorter as the concentration increases. Using a standard equilibration time can lead to misidentification of equilibrium and inappropriate addition of NaOH. Since the concentration of NaOH is a determining factor for formation of CO_3^{2-}, the buffer capacity cannot be reproducibly achieved or maintained using this method.

Effect of transfer on pH: Using the McNamara method, bicarbonate buffer is equilibrated at room temperature before transferring it to dissolution vessel. Discontinuity of the $CO_{2(g)}$ supply during transfer and the transfer of the fluid itself causes loss of CO_2 resulting in an increase in pH. Readjustment to the desired pH with additional NaOH leads to a decrease in the buffer capacity. The increase of pH in the absence of CO_2 sparging was verified by comparing bicarbonate buffer solutions in open and covered vessels. The pH shift of solutions with a buffer capacity of 30 mmol/l/ΔpH at pH 6.5 was followed over a period of 3 hours. In the open vessel, the pH was elevated to pH 7.7 whereas in the covered vessel the pH changed

to 6.8.

Effect of thermal equilibration on buffer capacity: The temperature change from room temperature to 37°C lowers the solubility of $CO_{2(aq)}$.[83] Thus, warming the medium will lead to a lower buffer capacity compared to the pre-transfer value.

Bubble formation: direct supply of $CO_{2(g)}$ into the dissolution medium results in the formation of bubbles (see Figure 4.3) which can lead to changes in dissolution rate and poor reproducibility of results.

In summary, the McNamara method is time consuming, expensive (due to the CO_2 quality specified) and reproducibility of the resulting medium is not guaranteed.

4.1.2 Modified Method

The modified method leads to more reproducible buffer systems and is cost-saving compared to the McNamara method, but is still time-consuming.

Estimation of time to equilibrium: In the modified method, the buffer capacity was adjusted primarily by the concentration of NaOH. Higher flow rates, and thus corresponding higher partial pressure of $CO_{2(g)}$, can be used to generate a lower pH. Provided no further NaOH is added, the buffer capacity is not changed when the pH is adjusted by changing the flow rate of CO_2. The correlation between the amount of added NaOH [mol/l] and resulting buffer capacity [mmol/l/ΔpH] was linear over a range of 5 to 37 mmol/l/ΔpH at pH 6.5 (regression coefficient = 0.998). In Figure 4.1 buffer capacity in buffer solutions of pH 6.5 with different concentration of NaOH is given. Coefficient of correlation was 0.9982.

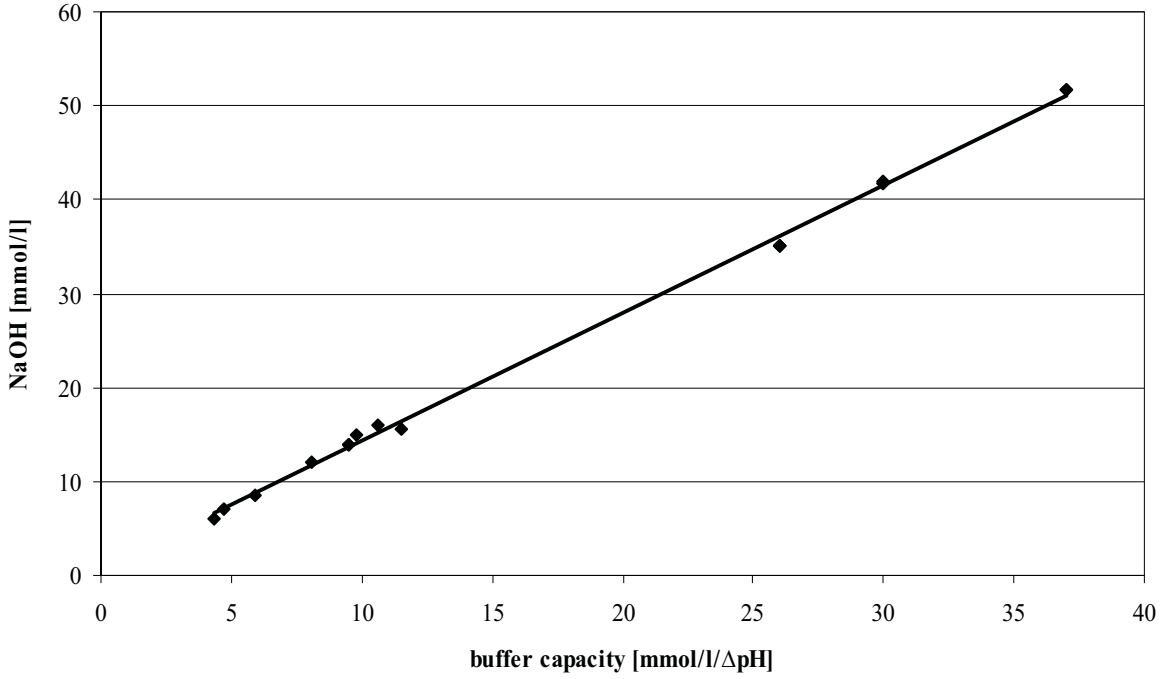


Figure 4.1 Correlation of concentration of NaOH on buffer capacity at pH 6.5

After generation of the bicarbonate buffer system with CO_2 a constant partial pressure of $CO_{2(g)}$ above solution is needed only to maintain, rather than generate, a constant pH value.

Effects of thermal equilibration after transfer: Since the solubility of CO_2 is temperature-dependent, the pH is adjusted after the final temperature has been reached: $CO_{2(g)}$ is supplied into the alkaline saline solution at 37°C. After transferring the medium to the dissolution vessel, a short period of equilibration may be required due to the lack of $CO_{2(g)}$ supply during the transfer process. This is done solely by supplying $CO_{2(g)}$ (without adding NaOH), leading to a buffer capacity identical to the pre-transfer value.

Maintenance of pH: To maintain a stable pH, the partial pressure of $CO_{2(g)}$ has to be adjusted according to the media volume and the vessel geometry. In the modified method this is done by adjusting the flow rate of pure $CO_{2(g)}$, rather than using expensive gas mixtures (the cost of the gas mixture is about tenfold higher compared to that of pure CO_2). The only advantage of using gas mixtures is their robustness against changes in the flow rate. Although the flow rate of pure $CO_{2(g)}$ required will depend on the desired buffer capacity and pH, saline concentration, the volume of medium and even the vessel shape, it can be accurately adjusted by using valve controlled gas outlets. Therefore, the use of pure $CO_{2(g)}$ is cheaper and more practical.

During supply, non-dissolved $CO_{2(g)}$ escapes from solution, forming bubbles as it does so. This can have a considerable influence on dissolution. Therefore it is better to supply the CO_2 above the solution after having reached the target buffer capacity and pH. Maintaining a constant partial pressure equilibrium can be achieved and the buffer pH will be stable under these circumstances.

Using the modified method leads to more reproducible buffer capacities since the amount of NaOH and thus the formation of HCO_3^- and CO_3^{2-} is defined beforehand. Maintenance of equilibrium is easier because the pH doesn't have to be adjusted by titrating NaOH during $CO_{2(g)}$ supply.

4.2 Reproducibility of dissolution profiles in media based on bicarbonate and phosphate buffers

As mentioned in the introduction, buffer systems can affect dissolution results by forming salts or incompatibilities due to interactions between drug, excipients and buffer system. The McNamara method used to prepare bicarbonate buffer creates mechanical stress on the tested formulations due to CO_2 supply directly into the medium and subsequent bubble formation as the gas escapes from the medium. Depending on the size and frequency of these bubbles, different extents of perturbation of the liquid flow and buoyancy (floatability) of the formulations occur. Both of these factors are impossible to control and lead to variable dissolution results, as shown in Figure 4.2 for the bicarbonate buffer method and its phosphate equivalent. The disparity in the three individual dissolution curves obtained with bicarbonate buffer illustrate the high variability in results with this buffer system. Variability was especially pronounced in the first 20 minutes, during which it was observed that some of the BIXX-C granules were actually flung off the basket by exiting $CO_{2(g)}$. By contrast, standard deviations for dissolution in the phosphate buffer (n=3) fell within the data symbols.

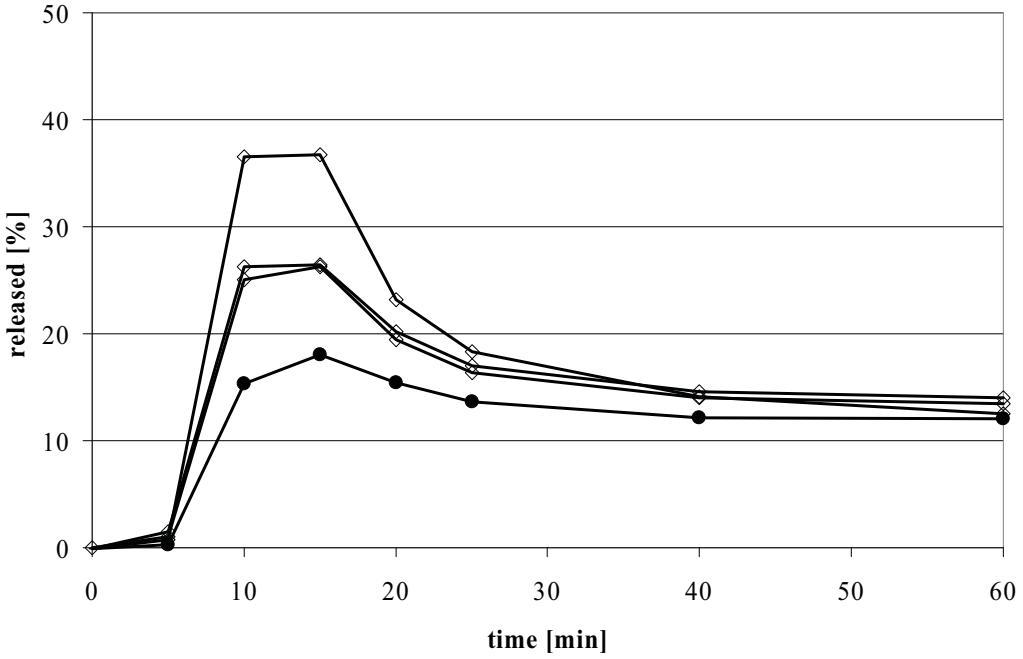


Figure 4.2 Drug release from BIXX-C in bicarbonate buffer prepared according to McNamara (◊) and in phosphate buffer (●), both with a buffer capacity of 30 mmol/l/ΔpH and adjusted to pH 6.5. Individual results are shown for the McNamara bicarbonate buffer, whereas means are shown for the phosphate buffer (error bars fall within the symbols), n=3 in each buffer.

The high mechanical stress in the medium and on the formulation generated by the bubbles could be reduced using the modified method, but it was still of course far higher than in degassed phosphate media. The effect on dissolution results caused by deposition of bubbles can occur in different ways. Bubble formation and transport through the media cause changes in hydrodynamics within the media creating turbulent flow and resulting in more movement of the multiparticulates in the medium and higher shear stress at the drug surface. These effects are expected to lead to faster drug release. On the other hand, if the bubbles adhere to the tablet surface, buoyancy may result. Bubbles can also act as dissolution barriers if they stick to the basket mesh or the tablet surface, leading to lower dissolution results. In preliminary experiments at high CO_2 flow rates, pellets were even observed to "lift-off" from the surface of the medium, so great was the bubble generation. As such events are a matter of chance, results tend to be less reproducible. Bubble size can be reduced by using a gas inlet frit (10 µm glass frit), as was used for the McNamara method. For the modified method, CO_2 was supplied above instead of directly into the medium, which led to slightly longer

preparation times but was able to reduce bubble size. Figure 2 illustrates the generation of gas bubbles under various conditions.

As these effects have been observed to some degree even with standard buffers, the USP[2,56] recommends de-aeration of media before using in dissolution testing. Obviously, this procedure cannot be implemented for buffers based on bicarbonate.

Figure 4.3 Appearance of the medium on the basket a] before supply of CO_2; b] in bicarbonate medium, to which CO_2 is supplied above the solution (modified method); c] in bicarbonate medium, with bubbles resulting from direct sparging (McNamara method); d] size of bubbles reduced by sparging with glas frit of porosity grade 3 (McNamara method).

Comparison of dissolution results of BIXX-B in bicarbonate buffer and degassed phosphate buffer are shown in Figure 4.4. BIXX-B, a formulation that does not contain pH-influencing excipients, showed higher and faster release of active drug substance in bicarbonate buffer. Since there is almost no change in pH during the run (Figure 4.4, right hand axis), the higher dissolution profiles can be attributed to the greater mechanical stress on the formulation.

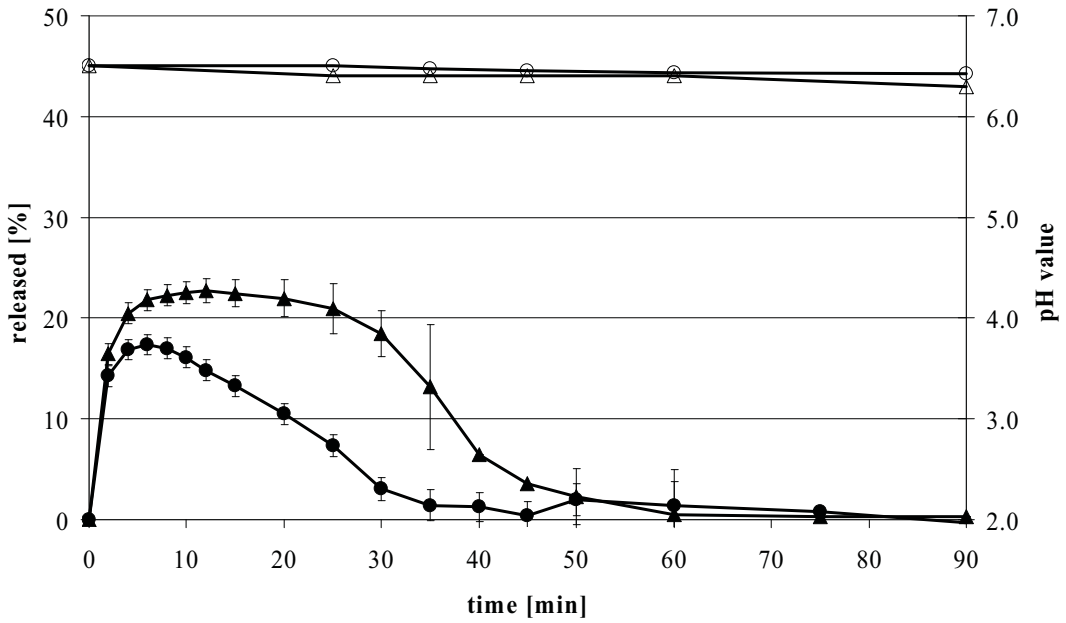


Figure 4.4 Drug release from BIXX-B (solid symbols) and pH profile (open symbols) in bicarbonate (modified method) (▲/Δ) and phosphate (○/●) buffer at pH 6.5 with a buffer capacity of 10 mmol/l/ΔpH, n=3 in each buffer.

4.3 Ability of bicarbonate and phosphate buffers to resist pH change

Despite their nominally equivalent buffer capacities, bicarbonate buffers show lower stability against acid and base compared to phosphate buffers. The effect on the dissolution profile is illustrated in Figure 4.5. The pH profile during dissolution of the BIXX-A formulation (formulated with an acid) demonstrates the poor pH resistance of the bicarbonate buffer. As the acidic excipients dissolve, they tend to lower the pH. Contrary to phosphate buffer, the buffer capacity of the bicarbonate buffer is weakened by the pH drop, due to the creation of carbonic acid, loss of CO_2 and therewith a decrease in buffer strength. This in turn leads to an even lower resistance of the buffer to changing pH so the pH drops faster during BIXX-A dissolution in the bicarbonate than in the phosphate buffer. Because of the lower pH, BIXX, a weak base, is dissolved more efficiently. Therefore dissolution of BIXX from the acid-containing formulation is faster in bicarbonate buffer compared to the phosphate buffer, which remains more stable with respect to pH. Since bicarbonate *in vivo* will be released responsively to maintain a stable pH, it is highly doubtful that the *in vitro* simulation in bicarbonate buffer as described here produces more biorelevant results than buffers like

phosphate that maintain a stable pH.

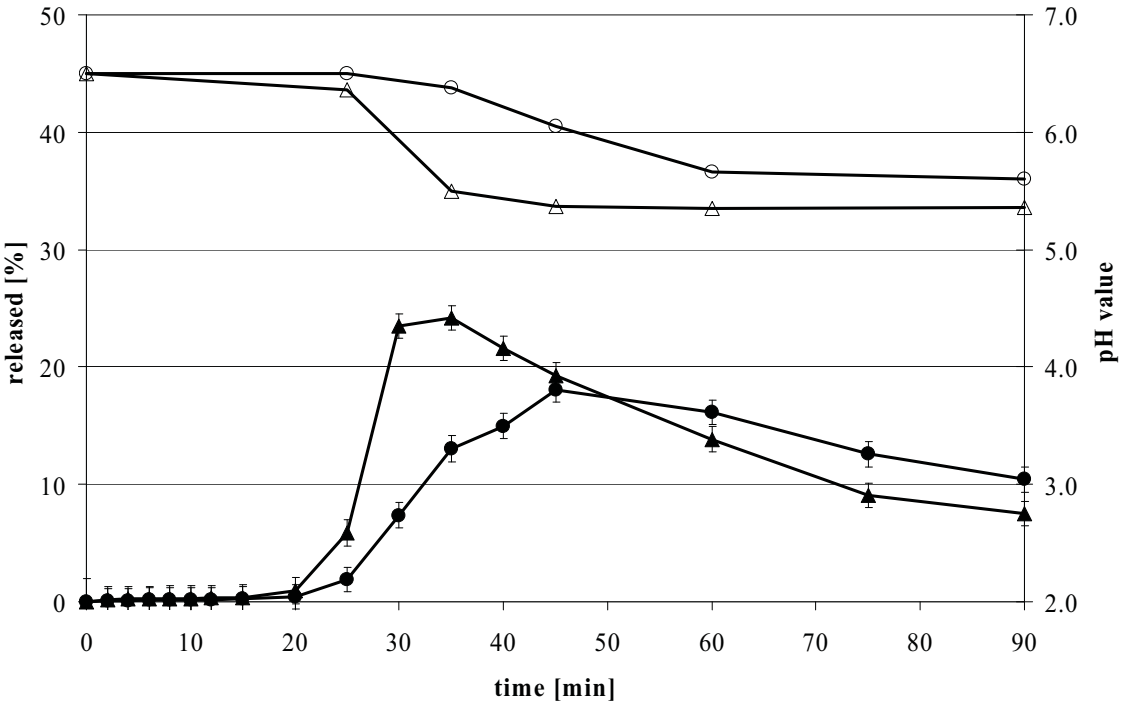


Figure 4.5 Drug release from BIXX-A (solid symbols) and pH profile (open symbols) in bicarbonate (modified method) (▲/△) and phosphate (○/●) buffer at pH 6.5 with a buffer capacity of 10 mmol/l/ΔpH, n=3 in each buffer.

To avoid the influence of pH-shifts on the dissolution profile, higher buffer capacities can be used. The effect of a higher buffer capacity of 30 mmol/l/ΔpH on dissolution results was investigated by comparing phosphate and bicarbonate buffer at pH 6.5. BIXX-A was also used for this part of the investigation. At a buffer capacity of 30 mmol/l/ΔpH, both buffer systems kept the pH comparatively stable, as shown in Figure 4.6. Even so, higher dissolution rates occurred in bicarbonate than in phosphate buffer. The higher dissolution rates can be attributed to the greater mechanical stress generated by carbon dioxide bubble formation.

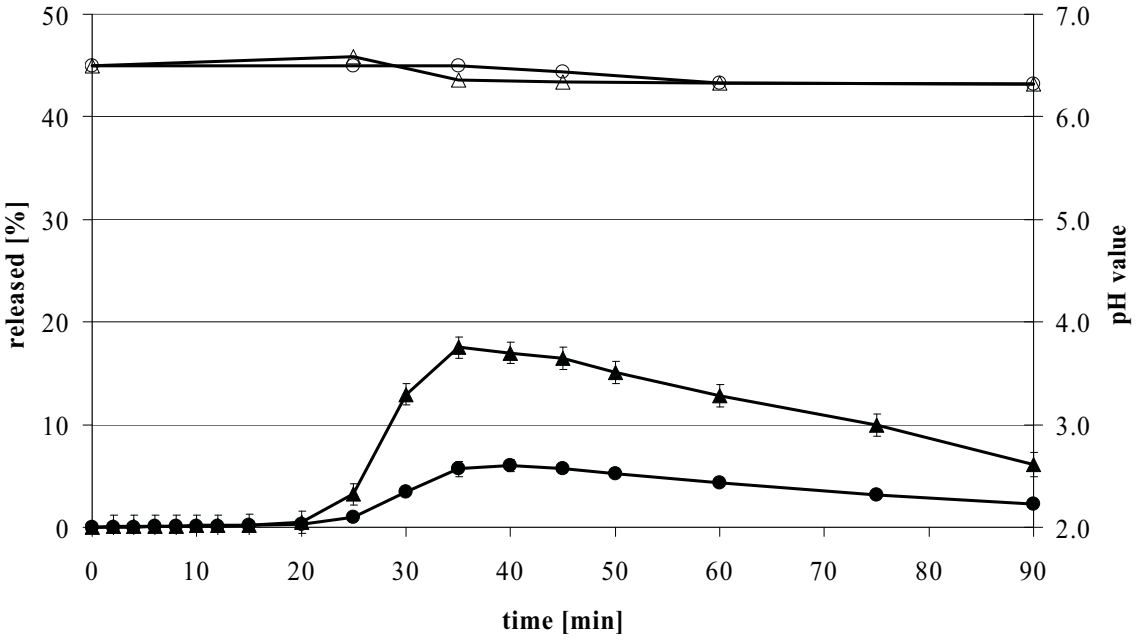


Figure 4.6 Drug release from BIXX- A (solid symbols) and pH profile (open symbols) in bicarbonate (modified method) (▲/△) and phosphate (○/●) buffer at pH 6.5 with a buffer capacity of 30 mmol/l/ΔpH, n=3 in each buffer.

Taking all results together, it is clear that results of dissolution testing in bicarbonate buffer often differ from the commonly used buffer systems. Although theoretically closer to the composition of intestinal fluids, *in vivo* profiles will not be simulated well with a bicarbonate buffer due to practical problems like uncontrollable streaming of gas and the comparatively poor resistance to pH change during the experiment.

4.4 Biorelevant media composed with bicarbonate vs. phosphate

Existing biorelevant media, like FaSSIF and FeSSIF have been shown to predict the *in vivo* behaviour of poorly soluble drugs[73]. Containing physiologically amounts of lecithin and cholic acid derivatives, some aspects of human duodenal juice are simulated. Although buffer strength and pH are adjusted to duodenal values, the media are based on phosphate and acetate buffer. To avoid non-physiological buffer effects, we attempted to replace phosphate and acetate buffer in FaSSIF and FeSSIF.

The dissolution profiles of BIXX-A in FaSSIF based on phosphate and bicarbonate buffer are shown in Figure 4.7. Buffers had been adjusted to pH 6.5 with a 10 mmol/l/ΔpH buffer capacity, and contained 3 mM sodium cholate and 0.75 mM phosphatidylcholine. Supply of $CO_{2(g)}$ caused strong foam formation and it was barely possible to observe the behaviour of formulation BIXX-A. In bicarbonate-FaSSIF buffer, the $AUC_{0\text{-}90}$ was double compared to the results in FaSSIF.

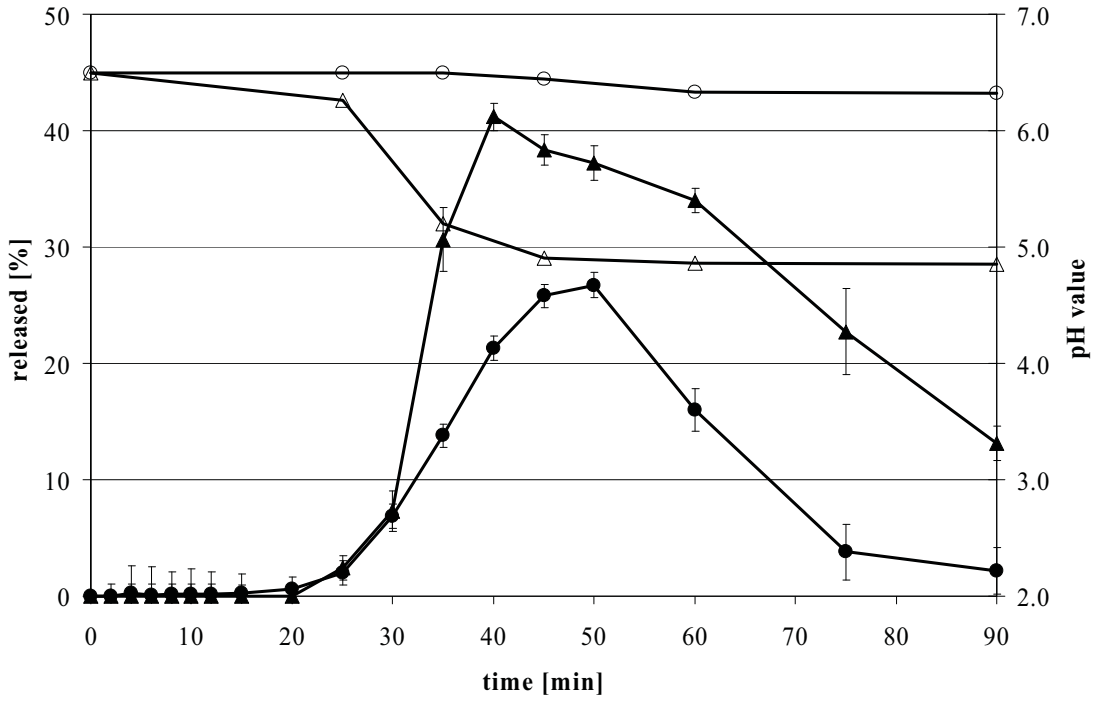


Figure 4.7 Drug release from BIXX- A (solid symbols) and pH profile (open symbols) in bicarbonate (modified method) (▲/△) and phosphate (○/●) buffer at pH 6.5 with a buffer capacity of 10 mmol/l/ΔpH, containing sodium cholate and phosphatidylcholine, n=3 in each buffer

Preparation of bicarbonate-FeSSIF was fraught with practical problems such as the strong foaming caused by inflow of $CO_{2(g)}$ during creation of bicarbonate buffer. During transfer of the media to the dissolution vessel, part of the dissolved $CO_{2(aq)}$ was lost to the atmosphere and the buffer had to be readjusted. The strong foaming associated with sparging $CO_{2(g)}$ above solution made dissolution tests in this media impracticable.

Generally speaking, the attempt to use of bicarbonate buffer in biorelevant media was highly problematic: In FaSSIF, the interaction of evaporating $CO_{2(g)}$ with the surface active bile components causes strong foaming, making it very difficult and time-consuming (approx. 3 hours) to prepare the medium. Standard deviations of dissolution results were generally higher compared to phosphate buffer indicating that dissolution in bicarbonate-based media is less reproducible. Further, as the solubility of CO_2 is different in the presence of sodium cholate and phosphatidylcholine, use of the same % atm of CO_2 above the solutions results in a different equilibrium concentration. Therefore the amount of NaOH and the % atm of CO_2 need to be readjusted for every change in amounts of emulsifier. As the practical problems associated with preparation of bicarbonate-based FaSSIF and FeSSIF media were manifold, further investigations on the use of bicarbonate buffer in FaSSIF and FeSSIF were abandoned.

4.5 Statistical Analysis

$ANOVA_{rep}$ analysis of dissolution in modified bicarbonate and corresponding phosphate buffer showed highly significant results, with P values of <0.0001 in all cases. The Bonferroni post hoc test indicated significance for the following time points: Dissolution of BIXX-B in 10 mmol/l/ΔpH buffers (Figure 4.4) at times between 10 and 45 min.; BIXX-A in 10 mmol/l/ΔpH (Figure 4.5) and 30 mol/l/ΔpH buffers (Figure 4.6) at times after 25 min.; BIXX-A in 10 mol/l/ΔpH buffers containing sodium taurocholate and phosphatidylcholine (Figure 4.7) at times after 35 min.

5 Conclusion

In general, standard buffers used in dissolution testing can be prepared in large amounts and stored for a long period of time. By contrast, a bicarbonate buffer has to be freshly prepared and will be stable only as long as carbon dioxide is supplied to the buffer. Additionally, the preparation of bicarbonate buffer is time-consuming and its stability is poor. As a result of the instability, a major problem with using bicarbonate buffer as a release medium is the generally poor reproducibility of the drug release profiles. Additionally, mechanical stress of forming bubbles on the dissolving dosage form and shifts in pH due to excipients lead to different % release than with phosphate buffers. Both of these effects are artefactual and may lead to inappropriate predictions of *in vivo* performance. Use in biorelevant media like FaSSIF and FeSSIF proved impossible due to strong foaming of dissolution media during supply of carbon dioxide. Since the bile components are necessary to match solubility of poorly soluble compounds in the human intestinal fluids, it appears unlikely that bicarbonate buffers could be used to appropriately simulate *in vivo* conditions. It is concluded that the use of bicarbonate buffer is impractical and that convergence of dissolution media to physiological conditions should not be reached at the expense of an easily manageable and reproducible system.

6 Acknowledgement

This chapter was published in part by Boni et al.[84]

V Miniaturization of Dissolution devices

1 Introduction

Over the last decades solid oral dosage forms have proven to be the most widely used formulation type. Reasons are easy to name: in comparison to other formulation types, tablets are cheap to produce, easy to package and store and show best consumer acceptance. With competition in the pharmaceutical market and increasing numbers of multisource products, ways had to be found to compare and characterize these products.

Besides bioavailability (BA) and bioequivalence (BE) studies, dissolution has become one of the most important tools for comparison of multisource products as well as for scale up and post-approval changes (SUPAC) concerns. Depending on the API (active pharmaceutical ingredient) characteristics (solubility, permeability and therapeutic range), waivers of *in vivo* BA/BE studies can be granted based on dissolution study results. Replacing pharmacokinetic studies with dissolution tests saves human resources and clinical study costs, but it has to show that relation exists between *in vivo* behaviour and *in vitro* results.[85] Such a correlation is, of course, helpful as a tool for efficient development of drug formulations as well as for regulatory purposes.

1.1 Dissolution in the pharmaceutical industries

Devices

In the quality control area, USP dissolution apparatus I-IV are mainly used for testing of solid oral dosage forms. In general vessels, which can hold 500 to 1000 ml are described for apparatus I and II in the pharmacopoeia, although some dissolution equipment manufacturers offer a special 250 ml vessel with a mini paddle for apparatus II. Most dissolution apparatus are equipped with 6 to 8 vessels, limiting the number of dosage units which can be studied in one run. Measurement of dissolved API is conducted online via UV/VIS or samples are taken at pre-determined timepoints to be measured, typically via UV/VIS, HPLC-UV/VIS or HPLC-MS.

Applications

In recent decades high throughput screening (HTS) became predominant in drug substance selection and lead optimization. Partly as a result of the experimental design of HTS, an

increasing number of poorly soluble compounds are being selected for early formulation development[86]. (Lipophilic drugs are more successful in reaching the lipophilic targets in the cell cultures used for high throughput drug substance screening.) Poorly soluble drugs, frequently belonging to the BCS II-class[1] due to their lipophilicity often require sophisticated formulation design and approaches.

Since BCS II drugs by definition show high permeability through gastrointestinal membranes, dissolution of the API is usually the rate limiting factor for drug absorption. Therefore release and dissolution of API from the formulation need to be characterized as closely as possible. The most informative testing about design performance is obviously studies in man but the high costs, enormous lead times and ethical aspects limit their use to confirming performance of already optimized dosage forms. *In vitro* dissolution is often used for early and middle stage characterization. Although *in vivo* behaviour cannot be precisely simulated with dissolution tests, selection of superior formulations can be made. Dissolution testing helps to point out advantages of one formulation over the other and thus supports development of the optimum formulation.

Improvement of biorelevance

Efforts were and still are being made to improve the predictability of *in vivo* behaviour by dissolution testing. Biorelevant dissolution testing should indicate the *in vivo* performance of the formulation and predict oral bioavailability better than commonly used dissolution designs. To meet this claim, dissolution conditions in biorelevant testing are brought as close as possible to *in vivo* situations.

Whereas in quality control establishing sink conditions is a primary concern in choosing the media volume, in pharmaceutical development more physiological volumes can be used. The Biopharmaceutical Classification System (BCS) introduced by Amidon[1] assumes 250 ml being the prevailing volume of fluids available in the upper GI tract in human. Dissolution of the highest dose in man in 200 or 250 ml presumably reflects *in vivo* conditions better than dissolution under sink conditions.

To simulate *in vivo* conditions, buffer capacity and osmolality as well as volume and pH are adjusted to reflect the *in vivo* data. In pharmaceutical development, the use of physiological volumes is becoming more and more relevant. For the GI tract, an estimated volume of

250 ml[87] is chosen to reflect administration of the oral formulation with one glass of beverage. In dissolution testing, a volume of 200-250 ml enables observation of supersaturation effects as well as indicating the least amount of drug dissolved. However, use of this volume in a closed system fails to account for concomitant absorption and dissolution. Sink conditions, by contrast, automatically lead to complete solution of the API.

Formulations which show good dissolution under minimum volume test conditions should enable uptake of the drug at the gut wall. To further challenge formulations of a poorly soluble drug, buffer solutions at the physiological pH values at which the lowest drug solubility is observed can also be used. That said, the buffer has to discriminate among the formulations tested. Ideally, formulations with best results obtained *in vitro* should generate the best performance in man. Real *in vivo* data however, is not always well predicted by dissolution test results. To improve or obtain a rough prediction of *in vivo* behaviour, biorelevant media can be used. Not only pH and osmolality but also physiological components of the gastrointestinal fluids are added to create artificial media closely simulating *in vivo* conditions. Media simulating *in vivo* fluids, such as FaSSIF, FeSSIF[88] and SEIF,[37] have been proposed. A disadvantage of these biorelevant fluids are their expense and the time consuming preparation by conventional methods. By use of instant FaSSIF and FeSSIF and a smaller (BCS) volume, time-saving and reduction of costs can be attained.
As part of the effort to make dissolution more time- and cost-effective, miniaturized dissolution testing equipment was developed and is presented as part of this work. To validate the design a comparison to standard scale equipment was then conducted regarding dissolution results, throughput and handling.

1.2 Standard dissolution testing

Standard dissolution testing is regulated by various pharmacopoeia. One of the most important is the USP. The general chapters 711 and 1092[2,89] address the major issues of dissolution. Dissolution testing is used to observe release of the API from formulations in defined buffer media. To meet qualification settings a certain amount of drug has to be dissolved within a specified time interval. For quality control (QC) purposes, the test volume has to facilitate complete dissolution of the API. These conditions are reflected by the so-called "sink conditions" in dissolution testing, whereby complete dissolution of the API must be possible in in a volume of medium corresponding to one-third of the volume used in the dissolution test. Resulting volumes vary from as little as 500 ml up to 4 l per dissolution

vessel for one dosage unit using the standard USP apparatus I and II and their modifications. By using USP apparatus III and IV even more buffer solution can be used, for example up to 30 l in the flow-through cell for testing modified release dosage forms. Sink conditions can either be obtained by increase of dissolution volumes or by addition of solubilisers like sodium dodecyl sulphate (SDS).
Usually the media are based on simple buffers like phosphate or acetate buffers are used. pH values of 1 to 7 are used, reflecting environmental conditions throughout the gastrointestinal (GI) tract. Samples are taken at a specified zone, located midway between the surface of dissolution medium and top of the rotating basket or paddle, not less than 1 cm from the vessel wall.

Originating from quality control dissolution testing for batch release, these conditions also enable evaluation of SUPAC effects on dissolution performance. Prediction of *in vivo* behaviour, which is necessary for formulation development, is rarely possible with such tests: By testing a drug formulation in such non-physiological high amounts of volume, both dissolution rate and extent can be overestimated, as the dissolved drug in solution cannot reach saturation. *In vivo* however, the available volume for dissolution often precludes complete dissolution of the drug. Advantages of formulation excipients like solubility enhancers or acidic and basic substances for *in vivo* performance might therefore not be detected with usual QC dissolution tests.

1.3 Physiological small volume dissolution testing

In contrast to standard dissolution testing, volumes in physiologically relevant tests are not chosen to obtain sink conditions but rather directed to physiological conditions. An oral drug formulation, usually applied with one glass of water, should release the API into a volume of about 200 – 250 ml of fluid. This amount of fluid can be assumed to be available for the drug dissolution in the stomach. A volume of 100 ml can be assumed to be present throughout the intestine[9].

1.3.1 Choice of volume

As mentioned above, dissolution results can be erroneously high if non-physiological, high volumes are used. Independent of formulation properties, the API will dissolve *in vitro* after a certain time. A formulation showing good *in vitro* performance therefore runs the risk of

failing in man.

A major difficulty to overcome when applying a dissolution volume of 200 ml is the limited release process. At concentrations close to saturation of the surrounding solution, a reduction in the release of the API is observed. To combat dissolution-limited absorption, different types and amount of excipients, like acid / base or other solubility enhancers are added to obtain supersaturation of the API. Consequently, a higher drug fraction absorbed can be achieved.

To test whether supersaturation effects also occur *in vivo*, a physiological relevant volume should be used. Most "challenging" conditions are created by testing the highest dose in man in 200 ml, simulating the ingestion of one glass of water with the intake of the drug. Comparative dissolution conducted in this volume should help to identify most suitable formulation. In For these types of dissolution studies a specific dissolution method should be developed for each API, varying in buffer strength and pH.

1.3.2 Buffer type and strength

The most commonly used buffers in dissolution are phosphate, acetate, citrate buffers, mixtures of them and acidic solutions of hydrochloride acid. These compositions establish stable pH values in the range of 1 to 7. Standard buffer strengths are chosen to obtain a constant pH value throughout the whole dissolution run, a condition favoured by pharmacopoeia and regulatory authorities. Thus, strong buffer solutions are often implemented, impeding comparative dissolution testing. As a result, effect of acidic or basic excipients added to a compound with pH dependent solubility might not be recognized. Therefore, buffer strengths used in comparative dissolution testing are typically reduced to the point where they can maintain a relatively stable pH but still differentiate formulation performance. Commonly used buffer strengths in this method vary between 0.01 and 0.1 mol/l.

1.3.3 pH value

pH values in the human gastrointestinal tract are mainly assumed to be strongly acidic in the stomach and in a range of 6 to 7.8 within the intestine. Low gastric pH values however, are mainly found in young, healthy subjects, whereas in elderly or immuno-compromised humans gastric juice pH is often elevated to values up to pH 4. But also in healthy subjects gastric pH may be elevated to higher values by intake of drugs with water: assuming a gastric volume of about 25 ml and pH 1.7, which is diluted by one glass of water, an almost unbuffered "dissolution media" of pH 2.7 is formed and presented to the drug formulation.

Administration of proton pump inhibitors and histamine blockers (H_2-receptor) can also lead to increase in gastric pH.

To simulate the challenging conditions presented to the formulations, low buffer capacities and pH values at poorest API solubility (still within the physiological pH range) are chosen. As described in 1.3.2 this will help identify the most suitable formulation for presenting the API in an absorbable form.

1.3.4 Mini-Paddle Apparatus

Standard dissolution USP vessels, such as these used in apparatus I and II, are not suitable for 200 ml. Therefore a special “small volume vessel” was developed. Vessel and paddle are smaller in diameter but of similar height dimensions to the standard 1000 ml vessel. Therewith sample taking corresponding to the USP sampling is possible. Rotation speed is still similar to the standard Apparatus, 50 or 75 rpm with paddle, 100 or 150 with basket, mainly. In small scale vessels hydrodynamics, like the extent of media flow, are often reduced, leading to limited movement at the bottom. Coning can be avoided by increased stirrer speeds or use of peak vessels.

1.3.5 Sample removal with the Mini-Paddle Apparatus

Samples can be taken automatically and measured online via UV/VIS, if possible. If HPLC is necessary, samples can be taken automatically or manually, filtered using 0.2 µm membrane filters and measured via HPLC-UV/VIS. Automatic sampling is conducted through a hollow stirrer shaft. To prevent sampling of undissolved components a mesh was applied to the sampling zone.

1.4 Small scale dissolution testing

In early stage development, manufactur of the drug substance and product is still evolving and usually large scale production is not yet possible. Early after synthesizing and identification of a possible candidate, studies on drug characterization, toxicity and pharmacokinetic studies are initiated. Also, concomitant formulation screening is necessary. Since supply of API is limited in this stage, early development has to be carried out with very low amounts of drug substance. To optimize formulation development by testing different types and compositions of formulations, batch sizes therefore have to be as small as possible. Customary techniques and machines, however, typically run at relatively high drug consumption. Therefore, miniaturization of various manufacturing techniques and machines has been generated for early stage formulation screening, for example the Haake Mini Lab (a small scale extruder)

from Haake, the mini-granulator (250 ml) of Diosna or the Micro-Spray (a mini-spray dryer) from Procept. Small-scale production helps to save drug substance and often, the production time is reduced, making formulation screening more efficient. This “high throughput” formulation screening increases demand on analytical testing capacities, which often takes more time than the production itself. Thus, dissolution testing, with its associated analytics, is often the rate limiting factor in formulation testing. Since one standard apparatus enables simultaneous dissolution of only 6 units, several dissolution apparatus may be necessary to screen all developed formulations.

Therefore, down-scaling of the dissolution apparatus by using several designs to address need at various times in the development process was investigated, allowing higher throughput dissolution testing of formulations.

The apparatus is set up as follows:
Dissolution vessels of 20 ml volume are used and stirred at 50 – 300 rpm at 37 °C. To meet or at least approach standard USP conditions, a sampling zone similar to standard scale was established: in case of manual sampling as well as in the automated vial method a stainless steel canula is inserted to about half of the filling height and a distance of about 0.5 cm to the vessel wall. In case of the automated BI miniDiss, sampling is conducted through a hollow shaft.

1.4.1 Manual vial method

The manual vial system used in early experiments has a quite simple construction:
Vials containing 10 - 20 ml of buffer are heated in a dissolution bath to 37 °C and stirred using a multiplate magnetic stirrer. The samples are taken manually and measured using UV/VIS or HPLC-UV/VIS. Depending on the user's experience and the sampling time points, a maximum testing number of up to n=18 can be achieved. The test itself is limited by human resources due to the lack of automation. The short preparation times and high flexibility makes the system a suitable tool to obtain information quickly.

1.4.2 Automated vial system

The automated vial system was developed to optimize user-friendliness and to reduce risk of errors. Automation of the vial method implies on-line measurement of samples. For this purpose, an 8-fold peristaltic pump and a corresponding cell changer were installed to enable for online UV/VIS-measurement. The sampling tubes were inserted and fixed slightly below

the liquid surface to minimize influence on hydrodynamics. To avoid blocking of tubes and cells a conically wire mesh was fixed at the end of the tubes. Continuous pumping was necessary to maintain homogenous mixing of solution in tubes and vials (tube volume: about 2 ml). By the automated vial system simultaneous dissolution of n=8 (limitated by the cell changer of spectrophotometer) can be conducted.

1.4.3 BI miniDiss

Further improvement of the automated vial method was realised by construction of the BI miniDiss: To minimize the high mechanical stress from magnetic stirring, a paddle stirrer was established. Construction is close to standard USP apparatus I and II but miniaturized. The dissolution media is heated using a temperature controlled aluminium block and solutions are stirred by basket or paddle stirrers at 50 to 150 rpm. Automatical sampling is conducted by drawing in the solution through the hollow shaft stirrers, with online UV/VIS analysis. The suction opening of the hollow shaft is covered with wire mesh. A coase filter pre-filtration is used to keep larger and undissolved particles in the dissolution media and to prevent tubes and cells from blocking. As illustrated in Figure 1.2, the paddle / basket is located in the centre of the vial at about one-third of the height. Figure 1.1 and Figure 1.2 show the construction of the apparatus and a small scale paddle. A test number of n=8 can be reached, limited by spectrophotometric set-up.

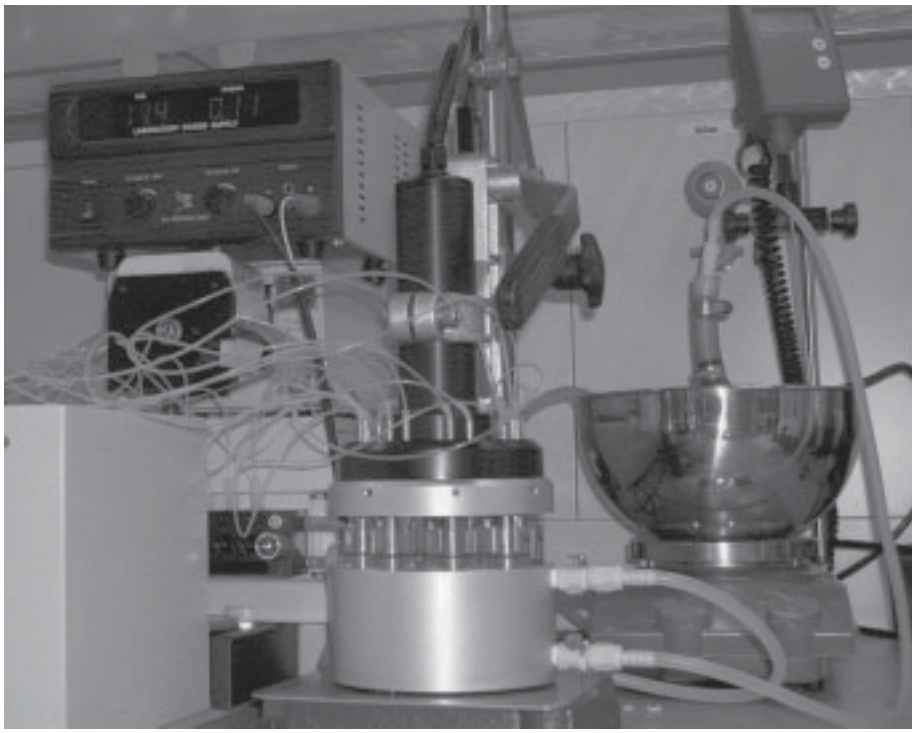


Figure 1.1 Automated BI mini Dissolution

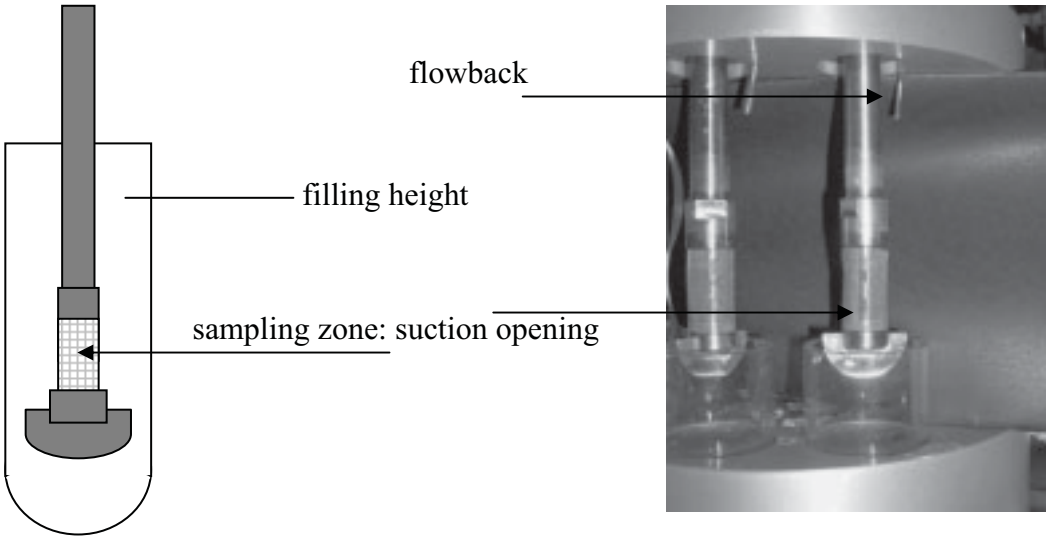


Figure 1.2 20 ml peak-vessel of BI miniDiss

1.5 Well-plate method

A further increase of the throughput in dissolution testing or even high throughput was attempted by using the well-plate method. Here, dissolution is scaled-down to a volume of 2.5 ml or even 1 ml and conducted using microtiter plates of 24 or 96 well design. Each well can be used as a separate dissolution vessel. Testing of formulations however is limited by the significant downscaling factor of about 100. Only multiple unit dosage forms, like pellets,

extrudates or granules (or again, intermediates) can be tested. Miniaturization of tablets is technically feasible but could cause significant changes in dissolution rate compared to standard scale. The well plate method is mainly used for supersaturation experiments in early formulation screening. The mixtures are prepared from stock solutions of drug substance and excipients using a pipetting robot. The solutions are directly added to a buffer solution or dried forming a solid film which is subsequently dissolved in buffer. The well plates are heated at 37 °C and stirred at 300 – 400 rpm using plate shakers. Samples are taken semi-automatically using a pipetting robot and transferred to 384 well measuring plate or, if feasible, directly measured within the dissolution well. A UV/VIS Eliza reader is used for measurement of amount of drug dissolved. Accurate pipetting is necessary to maintain reproducible filling heights in the measurement plate. Figure 1.3 illustrates sampling and UV/VIS absorption measurement.

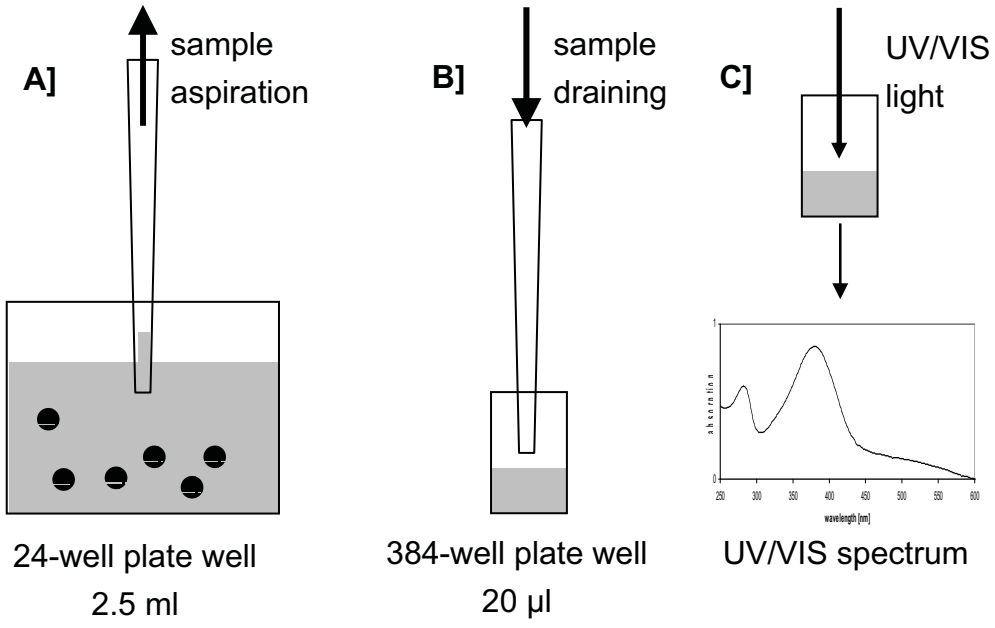


Figure 1.3 Delineation of sampling using the well plate method: A] 20 µl sample aspiration from 24-well plate with formulation / undissolved API (●), B] transfer into 384-well plate and C] UV/VIS absorption measurement within the 384-well using an Eliza UV/VIS reader

The defined thickness of a layer, required for reliable UV/VIS measurement, can be obtained using a specially designed well plate: a central cylinder, with a UV-permeable quartz dine at one end is mounted in each well. The lid for the plate holds the counterpart quartz pane to obtain the defined thickness. Variations in the cylinders' height enable adjustment of the thickness of the layers. A patent has been filed for the well plate method.

1.6 Compatibility of the various methods

To test feasibility of the different systems, formulations were released in various media using all devices. The dissolution profiles, T_{max} and maximum percentage release were compared.

1.7 Correlation of *in vitro* and *in vivo* release

In pharmaceutical development, correlation of *in vitro* and *in vivo* release from formulations help to characterize and optimize formulations. IVIVR obviously can only be calculated after testing the formulation in man (or other species). However, dissolution systems that give rough estimations on the *in vivo* behaviour can be found after early *in vivo* studies and used for further development. Dissolution conditions which best simulate *in vivo* behaviour can then be used to optimize formulations or at least to minimize failures and to identify high potential products.

Early screening of buffers can be helpful to find best test conditions for the prediction of *in vivo* behaviours. To evaluate general buffer suitability, a retrospective correlation of several formulations was conducted. The formulations were tested in rat (BIBF) and man (BIBR) to calculate AUC values. Several buffers and formulations were used for *in vitro* testing and correlation of *in vitro* profiles with AUC data was used to indentify the best buffer for estimation of formulation behaviour *in vivo*.

For a rough estimation of buffer suitability, *in vitro* and *in vivo* AUC values for the formulations were directly compared by caculating coefficient of correlation (R^2) for each buffer. AUC values of test formulations (T_f) were related to the defined reference formulation (R_f), with the formulation A and B as references in the case of BIBF and BIXX, respectively. The obtained AUC ratios *in vitro* (test) were compared to *in vivo* data (reference).

To better assess predictability, similarity factors were calculated for each buffer. Similarity factor (f_2) (see Equation 1.1) can be used to measure closeness between two profiles. To enable simulation of profiles, formulations were assumed as time-points (n) and buffers as formulations (f).

$AUC_{0\text{-}inf}$ were used for *in vivo* data, whereas for *in vitro* results $AUC_{0\text{-}120}$, $AUC_{0\text{-}60}$ and $AUC_{0\text{-}30}$ were calculated and compared.

$$f_2 = 50 \times \log\left\{\left[1 + \left(\frac{1}{n}\right)\sum_{f=1^n}\left(R_f - T_f\right)^2\right]^{-0.5} \times 100\right\}$$

Equation 1.1 silimarity factor f_2 used for *in vitro in vivo* relation

Typically, a similarity factor of 50 and 100 is required to accept similarity of two profiles. It can be used as basis for granting a biowaiver or in general, to prove performance sameness of products to regulatory authorities.

However, our “off-label” use of the similarity tests allowed acceptance at lower f_2 values, as it was primarily used to differentiate between "good" and "poor" IVIVC.

.

2 Materials and methods

To establish and compare the different dissolution devices and techniques various tests were conducted. The small scale vial method was established first. Optimization by changing of stirrer design and speed combined with subsequent automation was conducted by construction of the BI MiniDiss. Dissolution of various formulations in different media were tested in the devices and compared to standard dissolution testing in USP apparatus I and II.

2.1 Materials

2.1.1 Chemicals and formulations

All chemicals and formulations used are given in Table 2.1.

Table 2.1 Chemicals and formulations for preparation and characterization

substance	specification	provider
acetonitrile (ACN)	HPLC grade	Merck AG, Darmstadt, Germany
ammonium acetate (NH_4CH_3COO)	analytical grade	Merck AG, Darmstadt, Germany
BI 20 – F	conventional tablet design	Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
BIBF – A	multiparticulate formulation	Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
BIBF – B	multiparticulate formulation	Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
BIBF – C	multiparticulate formulation	Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
BIBF – D	semi-solid formulation	Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
BIBF – E	semi-solid formulation	Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
BIBF – F	semi-solid formulation	Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
BIMT – B	multiparticulates	Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
BIMT – C	multiparticulates	Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
BIMT – T	conventional tablet	Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
BIXX – A GA1 29/030_08/025	40 % drug load	Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
BIXX – A+ GA1 29/030_08/025	39 % drug load	Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
BIXX – B	20 % drug load	Boehringer Ingelheim Pharma GmbH & Co.

GA1 29/030_11/015		KG, Biberach, Germany
BIXX – B+ GA1 29/030_11/015	19.4 % drug load	Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
BIXX – C ZPO 36003	30 % drug load	Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
BIXX – D ZD 6706	16.5 % drug load	Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
BIXX – E ZP036001	30 % drug load	Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
citric acid ($C_6H_8O_7$)	analytical grade	Sigma Aldrich, Seelze, Germany
ethanol	analytical grade	Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
freeze-dried FaSSIF		prepared according to chapter III
freeze-dried FeSSIF		prepared according to chapter III
hydrochlorid acid (HCl)		Merck AG, Darmstadt, Germany
isopropanol	HPLC grade	Sigma Aldrich, Steinheim, Germany
monosodium phosphate (NaH_2PO_4)	analytical grade	Sigma Aldrich, Seelze, Germany
sodium acetate ($NaOOCH_3$)	analytical grade	Sigma Aldrich, Seelze, Germany
sodium chloride (NaCl)	analytical grade	Merck AG, Darmstadt, Germany
sodium hydroxide (NaOH)	analytical grade	Merck AG, Darmstadt, Germany
Zorbax Extend-C18	4.6x75 mm 3.5 µm	Agilent Technologies, Bensheim, Germany

Drug selection:

BI 20 shows poor and pH dependent solubility. The amount used in the solubility experiment also influences the measured solubility in water, due to API induced changes in pH and micelle formation. Permeability of the substance is good leading to BCS II classification. It is a surface active substance, starting micelle formation at concentrations between 1 and 30 mg/ml. The amphiphilic character of the substance (chemical structure not shown) makes it also possible to form mixed micelles. BI 20 is formulated as a conventional tablet containing pH modifiers.

BIBF was chosen as model drug to establish methods for improved IVIVR. BIBF is a BCS II drug with pH dependent solubility. Solubility is below 0.005 mg/ml over a range of pH 4 to 8 in phosphate and citrate buffers. Solubility increases to about 0.2 mg/ml at pH 3. BIBF is used as an antitumor agent. The formulations were all tested in pharmacokinetic and toxicological studies in various animal models. *In vivo* data from rodents only were used for the calculation of IVIVR. The formulations are not commercially available and therefore referred to as BIBF-A to -F. Formulations A to C are solid multiparticulate formulation, whereas D to F are liquid

or semi-solid drug formulations.

BIMT is a poorly soluble and a weak base with pH dependent solubility. Solubility increases from < 10 µg/ml tp 5 mg/ml at pH 1. A high bioavailability from ral solution makes it a typical BCS II class drug. Expected daily dose is 100 mg. Since formulations are not yet commercially available and therefore referred to as BIMT-B, - C and - T. All formulations were tested in Phase I studies.

BIXX is classified as BCS II drug with pH dependent low solubility. It is a weakly basic BCS II drug with solubility decreasing from 0.082 mg/ml at pH 4.0 to 0.001 mg/ml at pH 6.5. The formulations are not yet commercially available and therefore referred to as BIXX-A to -D. The formulations of BIXX consist of solid multiparticulates and one semi-solid formulation (BIXX-D). All formulations were tested in Phase I studies. The fraction absorbed was calculated by blood and urine samples after single dosing. Every formulation was tested on all patients. Since BIXX is a weak base, it is more soluble at acidic pH. Achloric stomach, which often occurs in elderly or immune deficiency patients, might therefore cause reduced absorption. To simulate such effects the formulations were applied with and without coadministration of pantoprazol. Ratios of resulting absorption AUCwp/AUCwop (with and without coadministration of pantoprazol) were used for calculation of IVIVR.

2.1.2 Dissolution media for compatibility tests

To enable discrimination among the various formulations several dissolution media had been tested in previous studies (data not shown). Compositions of the selected buffers used for the following experiments are given in Table 2.2.

Table 2.2 Composition of the various buffers used for dissolution testing (ph-phosphate, nc-sodium chloride, ac-acetate, NaTC-sodium taurocholate, PC-phosphatidylcholine)

buffer type	molarities [mol/l]	pH values	volumes [ml]
HCl	0.01	2.0	2.5, 20, 200
citrate	0.001	6.25	
phosphate	0.04	6.0	
phosphate	0.007	6.25	
phosphate	0.007	6.5	
FaSSIF blank	0.029 (ph), 0.106 (nc)	6.5	
FaSSIF	0.029 (ph), 0.106 (nc), 3 (NaTC), 0.75 (PC)	6.5	
FeSSIF blank	0.144 (ac), 0.203 (nc)	5.0	
FeSSIF	0.144 (ac), 0.203 (nc), 15 (NaTC), 3.75 (PC)	5.0	

2.1.3 Dissolution media for buffer screening

39 different buffer solutions were tested, differing in strength and pH. Concentration and pH values of citrate and phosphate buffers used are given in Table 2.3.

Table 2.3 Composition of phosphate and citrate buffer regarding strength and pH value

pH values	buffer strength [mmol/l]										
phosphate	1	2	5	6	7	7.5	8	10	20	29	144
5.00					+					+	+
5.50		+						+	+		
6.00			+		+	+		+	+	+	+
6.25	+		+	+	+	+	+	+	+	+	
6.35				+	+						
6.50			+	+	+	+		+	+	+	
6.75			+			+		+	+	+	
citrate	1	1	5	6	7	7.5	8	10	20	29	144
6.25						+		+			
6.35				+							
6.50			+		+						

2.1.4 Devices for dissolution and testing

The devices used for media preparation and dissolution are summarized in Table 2.4.

Table 2.4 Devices for media preparation and dissolution testing

equipment / instrument	**type**	**use**	**provider**
analytical balance	AX 205 Delta Range	formulation weighing	Mettler Toledo, Schwerzenbach, Switzerland
balance	PG 5002-S Delta Range	preparation of buffer	Mettler Toledo, Schwerzenbach, Switzerland
bath	AT 7	dissolution	Sotax, Allschwill, Switzerland
BI miniDiss	BI MD II	dissolution	Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
controller BI miniDiss	EA-PS 2032-050	dissolution	EA Elektro-Automatik GmbH& Co.KG, Viersen, Germany
dissolution software	HP ChemStation Rev. A 10.01	dissolution	Agilent Technologies, Bensheim, Germany
magnetic stirrer	RCT basic	media preparation and dissolution	IKA Works Inc., Wilmington, USA

magnetic stirrer multi plate	Variomag	dissolution	Dr. Hoiss + Partner GmbH, Berlin, Germany
membrane filter	cellulose acetate filters, 0.2 µm	degassing and high pressure filtration	Sartorius AG, Goettingen, Germany
multipipette	Multipette stream	volumetric partiononing	Eppendorf, Hamburg, Germany
pH electroder	Inolab	preparation and dissolution	Mettler Toledo, Schwerzenbach, Switzerland
pH meter	CG 840	preparation	Schott, Hofheim/Ts., Germany
pipetting robot	precision 2000	dissolution	BIO-TEK® Instruments, Inc., Winooski, USA
pipetting software	precision power 3.4 Rev. 18	dissolution	BIO-TEK® Instruments, Inc., Winooski, USA
peristaltic pump	89092A	dissolution	Agilent Technologies, Bensheim, Germany
pump	CY 7-50	dissolution	Sotax, Allschwill, Switzerland
pump	CP 7	dissolution	Sotax, Allschwill, Switzerland
Pump BI miniDiss	aquamotion WP 1800	dissolution bath	Gardena, Ulm, Germany
shaker	DTS-2	well-plate-dissolution	neo-Lab Migge, Heidelberg, Germany
slinging thermostat	thermomix 1441	dissolution	Braun Melsungen AG, Germany
spectrometer control	KC 4 1.10.3	dissolution	BIO-TEK® Instruments, Inc., Winooski, USA
laser photo tachometer	DT-IL	device characterization	Conrad Electronic GmbH, Hirschau, Germany
UV/VIS spectrophotometer	8453	dissolution	Agilent Technologies, Bensheim, Germany
UV/VIS spectrophotometer	Powerwave HT-I	dissolution	BIO-TEK® Instruments, Inc., Winooski, USA

2.2 Dissolution Methods

All dissolution tests were conducted in media heated to 37 °C. Tested amount of formulations was corresponding to the highest dose in man in 200 ml. For BIBF and BIXX concentrations of 0.45 mg/ml and 0.75 mg/ml API, respectively, were used. Dissolution was monitored over 120 min, taking samples after 5, 10, 15, 20, 30, 45, 60, 75, 90 and 120 minutes.

Coefficients of extinction of BIBF and BIXX were measured in each buffer. Calculation of the amount API dissolved was calculated following Beer's law (see Equation 2.1).[90]

$$A\ {}^{1\%}_{1cm} = \frac{A}{c \times d}$$

Equation 2.1 Coefficient of extinction following Lambert-Beers-Law (A-extinction, c-concentration of absorbing substance [%] and length of the path [cm])

Turbidity disturbances were handled by scatter correction of the obtained spectra. Following methods 2.2.1, 2.2.2, 2.2.3 and 2.2.4, polynomial scatter corrections were made over ranges of 500 to 600 nm and 450 to 550 nm in case of BIBF and BIXX, respectively, using the advanced software modus of HP ChemStation Software. Figure 2.1 shows sample spectra for BIBF corrected by polynomial scatter correction.

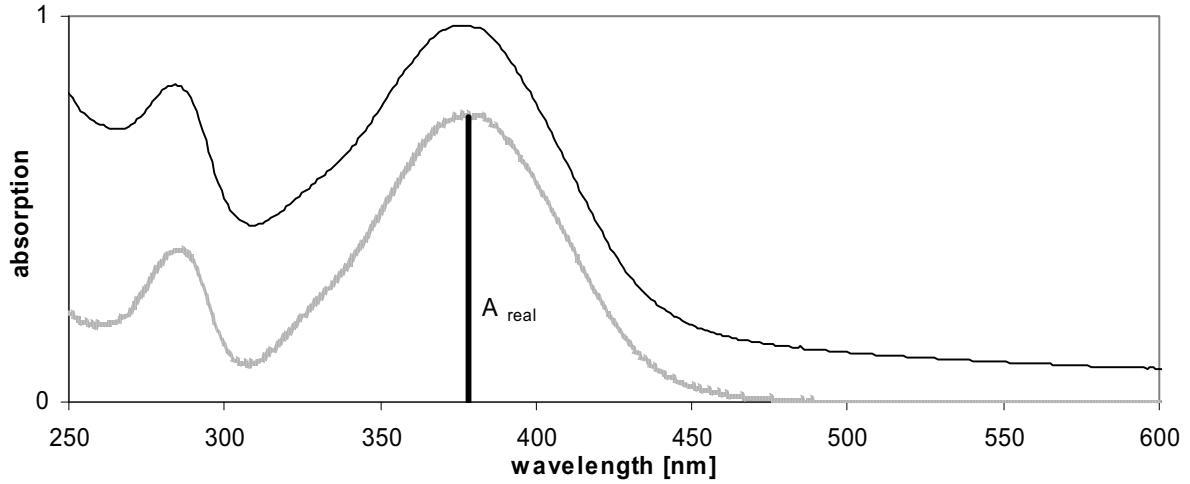


Figure 2.1 Polynomial scatter correction of BIBF (solid line-uncorrected spectrum; knurled line-corrected spectrum)

Following method 2.2.5 a linear scatter correction was conducted after measurement of absorption values at λ_{max}, λ_{450}, λ_{470}, and λ_{550}. The decline of scattering was calculated using Microsoft® Excel 2002. Figure 2.2 shows sample linear scatter corrections for BIBF. Linear regression is done over a range of 450 to 550 nm. Extrapolation to A_{max} enables calculation of real absorption shift.

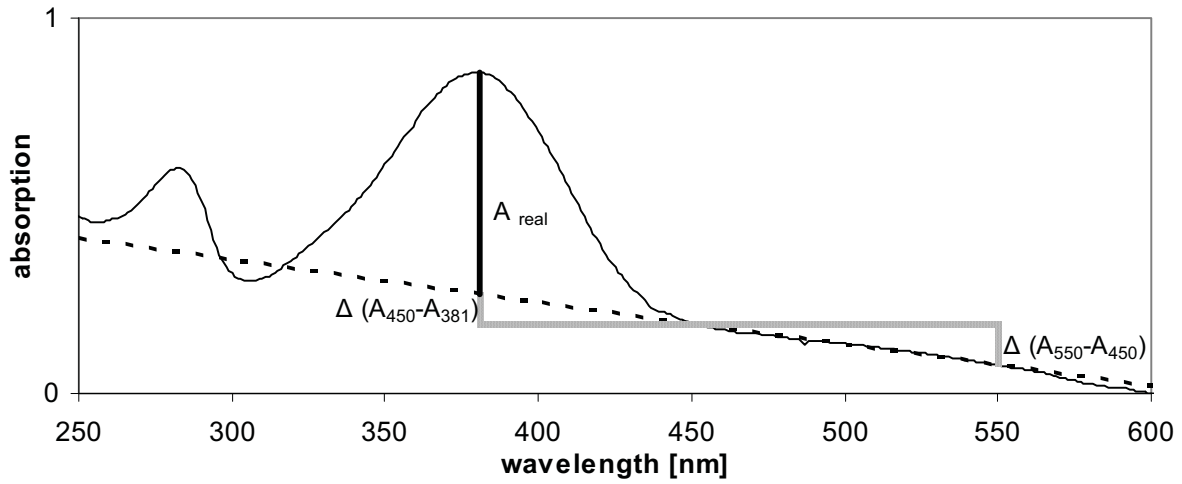


Figure 2.2 Linear scatter correction of BIBF (solid line-uncorrected spectrum; dashed line-linear regression curve)

Mathematical calculation following Equation 2.2 was used to calculate the true absorption values for each sample taken.

$$A_{real} = A_{\max} - (A_{450} + \left(\frac{(A_{550} - A_{450})}{(\lambda_{550} - \lambda_{450})} \times (\lambda_{450} - \lambda_{\max}) \right)$$

Equation 2.2 Linear calculation of A_{real} in turbid solutions

pH of each media was measured after dissolution.

All dissolution test were run in triplicate. Standard deviations are given for each sample time point.

2.2.1 Modified USP apparatus

All tested formulations were tested using the modified USP Apparatus I dissolution tester (AT 7, Sotax, Switzerland) to avoid flotation of the small multiparticulates. The formulations were directly weighed into the baskets, then the baskets were introduced into the dissolution media. A stirring rate of 100 rpm was used. Small volume vessel of 250 ml were used and formulations tested in 200 ml of media. Samples were taken automatically using a piston pump (CY-7-50, Sotax, Switzerland) and measured on-line via UV/VIS (8453, Agilent, Germany). Automatic measurements were controlled by the advanced dissolution modus of HP ChemStation software.

2.2.2 Manual vial method

20 ml vials with a diameter of 26 mm were used in the manual vial method. The solutions were stirred using 0.6 x 15 mm magnetic stirrers. Stirring rates of 250, 300 and 350 rpm were tested. Buffers were pre-heated to 37 °C using a water bath and a magnetic stirrer multiplate. The formulations were directly weighed into the test vials. The buffer was added at t=0 into the formulation vials. Sequential addition of buffer with time intervals of 40 seconds were chosen to avoid delayed sampling and measurements. Samples were taken manually using a 1 ml syringe and needle and measured unfiltered using a 1 mm glass cuvette. Samples were measured via UV/VIS (8453, Agilent, Germany) and analyzed by the advanced modus of HP ChemStation. The short intervals of sampling within the first 30 minutes limited to simultaneous dissolution of n=6.

2.2.3 Automated vial method

Vials, stirrers and media volume were identical to those used following the manual vial system. The formulations were weighed directly into the testing vials and buffer added sequentially to the formulations. Time intervals of 5 seconds were chosen. The unfiltered samples were taken automatically for online UV/VIS-measurement (8453, Agilent, Germany) using 1 mm flow through glass cuvettes. The high proportion of buffer media within the tubes (about 2 ml) made continuous pumping necessary. No interference by media flow during absorption measurement was observed. Samples were analyzed using the advanced modus of HP ChemStation. Simultaneous dissolution of n=6 were conducted.

2.2.4 BI miniDiss

Vials of 26 mm inner diameter and spherical dished bottom were used. Dissolution was conducted in 20 ml buffer media with a stirring rate of 100 rpm. Formulations were weighed into separate vials and added to the pre-heated buffers sequentially, at time intervals of 5 seconds. To maintain homogenous dissolution in tubes and vials, continous pumping was necessary. At pre-determined time points absorption was measured automatically via UV/VIS (8453, Agilent, Germany). Simultaneous dissolution of n=8 was conducted. Automatic measurements were controlled by the advanced dissolution modus of HP ChemStation software.

2.2.5 Well plate

To facilitate high throughput dissolution screening the well plate method was used. Formulations were weighed into 24 well plates. 2.5 ml of pre-heated buffer was added to each well using a multipipette. Four plates were run in parallel with a time shift of 1, 15 and 16 minutes relative to the first plate. Therewith simultaneous dissolution of n=96 could be tested. The plates were shaken at 350, 380 and 400 rpm at 37 °C using a well plate shaker. At predetermined time points 20 µl of each well were taken using a pipetting robot and transferred to a 384 UV-visible well plate to measure absorption at defined wavelengths. Sampling takes about 40 seconds for pipetting and additional 60 second for measuring for each plate. Calculation of the amount dissolved was done using Microsoft® Excel 2002.

2.3 Use of BI miniDiss for buffer screening of BI 20 for the formulation characterization

Suitability of the BI miniDiss for the characterization of formulations was tested by screening buffer influences on BI 20-F. The BI miniDiss was used to conduct experiments with an increased throughput. Equivalents of formulations containing 10 % of highest dose in man were tested. Final pH of the media was not tested in this experiments. To enable dissolution of BI 20-F using the BI miniDiss the tablet was broken down into tenth equivalents. To minimize influence of varying surface areas, only bigger tablet parts were used. In so doing a more or less homogenous surface to volume ratio was achieved. The dissolution was conducted in 20 ml of various media. The paddle method was used with a stirring rate of 100 rpm.

The BI miniDiss was used according to V1.4.3. Samples were taken automatically using a peristaltic pump and measured via UV/VIS measurement (8453, Agilent, Germany).

2.4 IVIVR

The BI miniDiss was used to conduct experiments with an increased throughput. Dissolution volume was 20 ml for all experiments. Formulations A to F of BIBF and A, B, C,D and E of BIXX were tested. Equivalents of formulations containing 10 % of highest dose in man were tested. The BI miniDiss was used according to V2.2.4. Samples were taken automatically using a peristaltic pump and measured via UV/VIS measurement (8453, Agilent, Germany). Calculation of the true absorbance in turbid solutions was calculated using a polynomial

scatter correction. *In vitro* AUC-values at 0-30, 0-60 and 0-90 were calculated from the dissolution results and correlated to *in vivo* data. *In vivo* AUC-values were calculated from blood and urine samples. The correlation coefficients were compared to identify suitable methods for the prediction of *in vivo* data from *in vitro* dissolution results. Similarity factor was calculated as described in V1.7.

Dissolution of six different formulations was tested and correlated to *in vivo* data to identify buffers with highest predictability. Predictability in this context means concurrence of the performance of the formulations *in vitro* and *in vivo*.

3 Results

3.1 Compatibility of methods

The various dissolution methods were compared in separated steps. First, influence of stirring rate on dissolution was investigated using the vial method.
Comparability of automated and manual vial method as well as of BI miniDiss and automated vial method was demonstrated.
Dissolution of the modified USP method with dissolution in 200 ml presenting the standard method was compared to the vial and BI miniDiss method.
Finally dissolution using the well plate method was compared to the BI miniDiss.

To keep results comprehensible, only representative data (chosen from the multitude of dissolution data generated) is presented.

3.1.1 Vial methods

Influence of stirring rate and sampling is given in Figure 3.1.
A delayed onset of 20 and 25 min was observed for the dissolution of BIXX from formulation B+.
At 350 rpm using the automated system, a significant difference in release was calculated after 25 and 30 min compared to all other release profiles.
In the manual system the maximum percentages release were significantly higher than in the automated system.
No significant difference in t_{max} and the maximum percentage release were obtained comparing the automated methods.

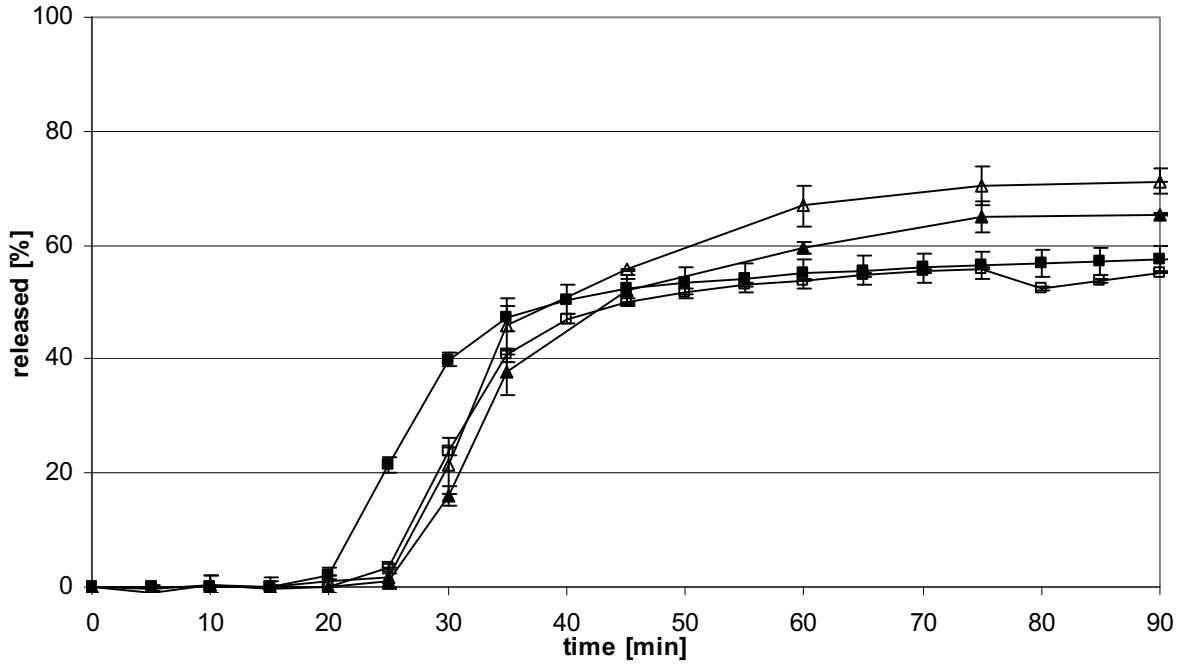


Figure 3.1 Dissolution of BIXX-B+ for comparison of automated and manual sample taking using the vial system and magnetic stirrers: manual 250 rpm (Δ), manual 350 rpm (▲), automated 250 rpm (□) and automated 350 rpm (■)

Multiparticulate formulations were partly destroyed at stirring rates exceeding 350 rpm.
A stirring rate of 250 rpm was identified as most suitable and was therefore used for the following dissolution tests in vials.

3.1.2 Modified USP vs. automated vial method

Dissolution of various formulations in the modified USP and the automated vial system were compared. Exemplary dissolution profiles of BIXX-A and BIXX-B+ are given in Figure 3.2. Dissolution in the modified USP method showed higher release from both formulations. A delay of about 2 minutes was observed between 5 and 15 minutes in case of BIXX-A and between 25 and 35 minutes in case of BIXX-B+. No statistical differences were calculated for t_{max} and the maximum percentage release.

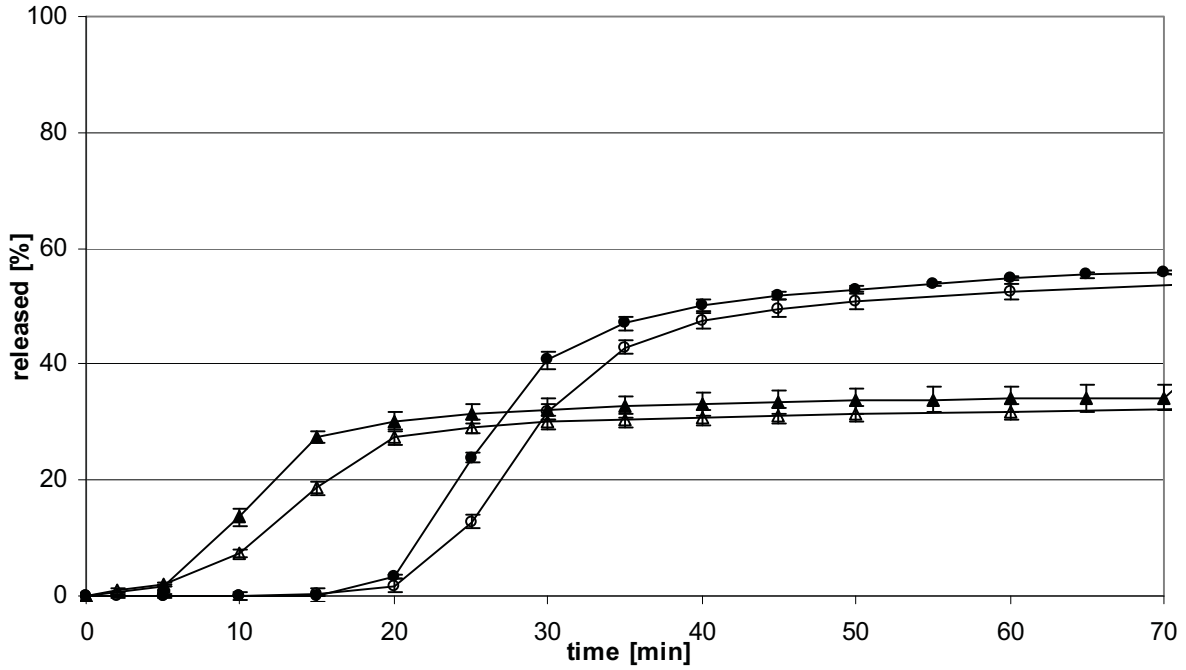


Figure 3.2 Dissolution of BIXX-A (Δ) and BIXX-B+ (○) obtained from USP apparatus I at 100 rpm (solid symbols) and automated vial method at 250 rpm (open symbols)

3.1.3 Modified USP vs. BI miniDiss

Dissolution curves of BIXX (0.75 mg/ml) of formulations BIXX-A, BIXX-A+, BIXX-B and BIXX-B+ obtained from apparatus USP I and BI miniDiss paddle were compared (see Figure 3.3). No statistical difference was observed for BIXX-A. Dissolution of BIXX-A+, BIXX-B and BIXX-B+ showed difference at 10 to 15, 25 to 35 and 30 to 40 minutes, respectively. BIXX- A+ and BIXX-B were slightly faster using the BI miniDiss, whereas BIXX-B+ was faster using USP I. However, t_{max} and the maximum percentage release showed no significant difference for all tested formulations.

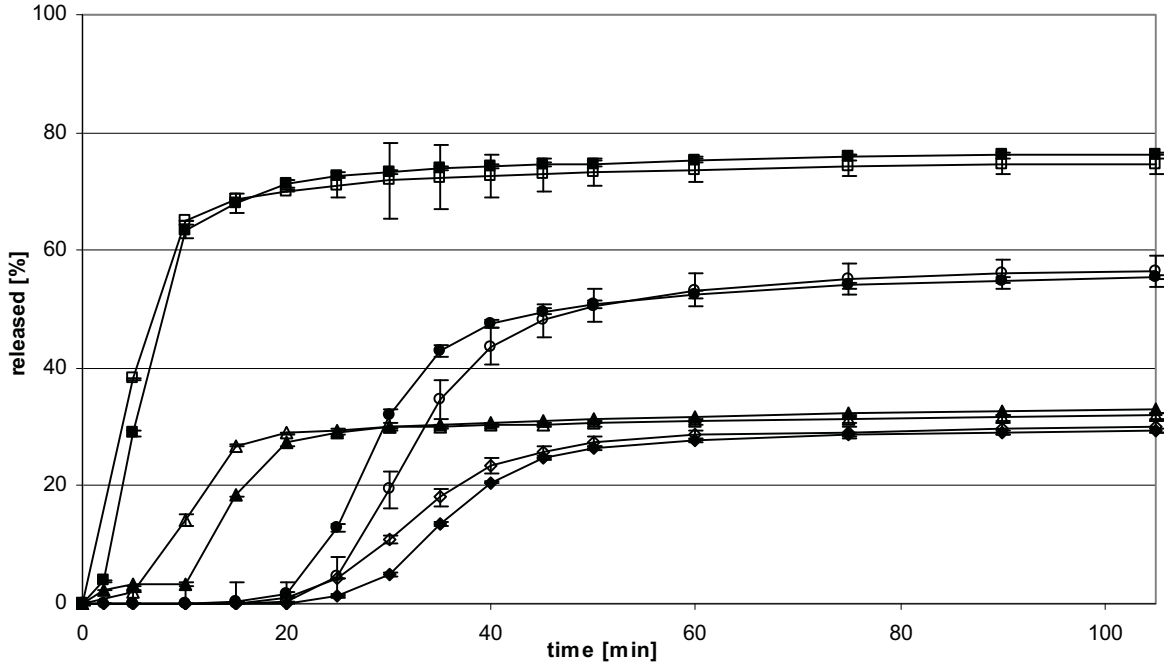


Figure 3.3 Dissolution of BIXX-A (Δ), BIXX-A+ (◊), BIXX-B (□) and BIXX-B+ (○) in 0.04 M phosphate buffer pH 6.5 using USP apparatus I (solid symbols) and BI miniDiss paddle (open symbols) at each 100 rpm

3.1.4 BI miniDiss vs. well-plate method

Determination of shaking rate in the well-plate method:

Influence of shaking rate was tested to optimize the dissolution method in the well-plate method. In Figure 3.4 sample profiles obtained at two shaking rates are shown for BIXX-B, BIXX-C and BIXX-D. The solutions were shaken at 350 and 395 rpm. Release profiles using different shaking rates were statistically significantly different. Dissolution at higher shaking rates were faster and showed higher t_{max} and maximum percentage release values. In case of BIXX-C, faster reprecipitation was observed using a shaking rate of 395 rpm. Standard deviations were generally higher using the well-plates shaken at 350 rpm. Using a shaking rate of 395 rpm, occasional loss of medium could be observed. Spill over was avoided by reducing the shaking rate to 380 rpm. A shaking rate of 380 rpm was identified to be most suitable to obtain low standard deviations and avoid loss of media. All subsequent dissolutions tests were therefore conducted at 380 rpm.

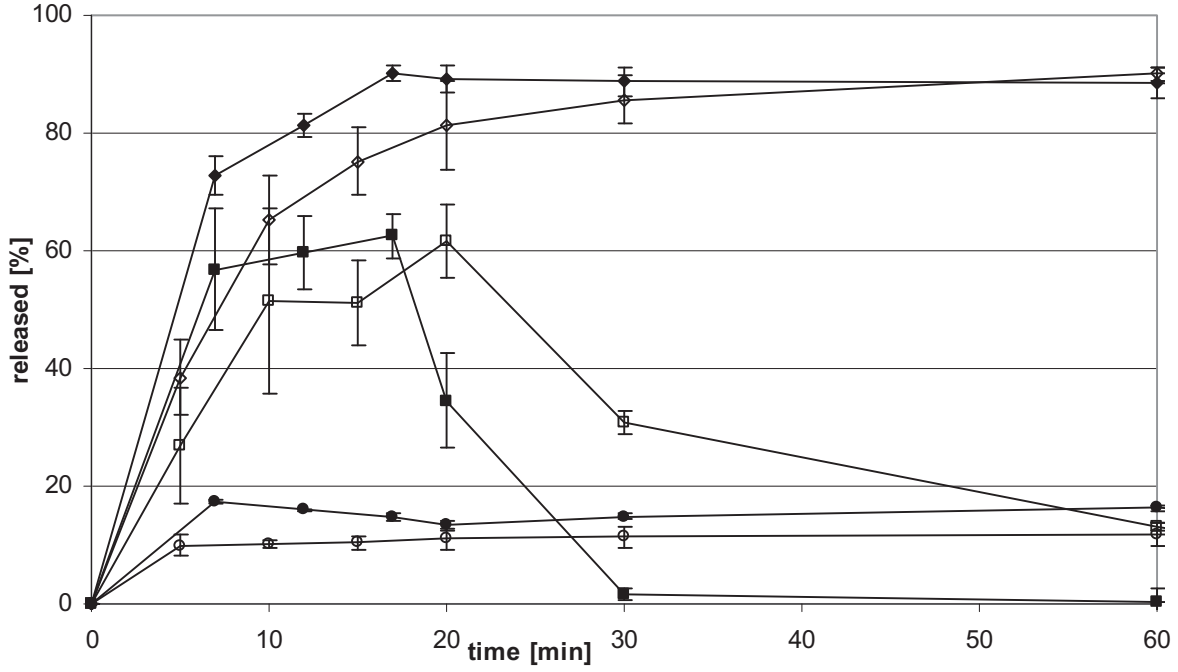


Figure 3.4 Influence of shaking rate on dissolution in well-plates of BIXX-B (◊), BIXX-C (□) and BIXX-D (○) in 0.007 M phosphate buffer pH 6.25 with shaking rates of 395 rpm (solid symbols) and 350 rpm (open symbols)

Comparison of well-plate method to BI miniDiss:

Release of API from the formulation using the well-plate method was compared to dissolution profiles obtained from the BI miniDiss. Dissolution of all formulations was conducted with both methods. Sample release profiles of all BIBF formulations in FeSSIFblank and of BIXX-B in FaSSIF, FeSSIF and the corresponding blank buffers are presented in Figure 3.5, Figure 3.7 and Figure 3.8.

In Figure 3.5 dissolution of the three solid BIBF formulations A to C in FeSSIF using both methods is shown. The well-plate method led to generally higher standard deviations. Statistically significant difference was observed for BIBF-A and B. However, shape of profile and relative ranking of the formulations were similar. Correlation coefficients of 0.95 to 0.98 (see Table 3.1) were calculated for the AUC values obtained from the *in vitro* data from the two methods, indicating that the well plate method and BI miniDiss generally lead to similar profiles for solid dosage forms.

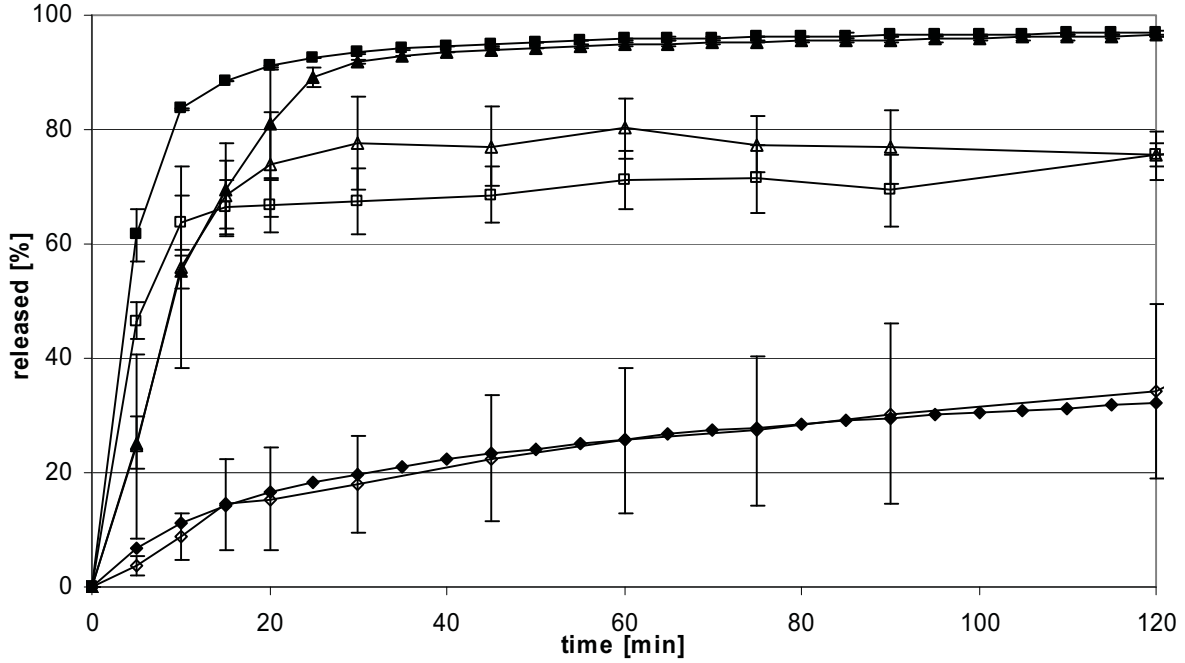


Figure 3.5 Comparison of dissolution of BIBF-A (Δ), -B (□) and –C (◊) in FeSSIF using BI miniDiss (solid symbols) at 100 rpm and well-plate method (open symbols) at 380 rpm

In the case of liquid and semi-solid formulations, no correlation in FeSSIF could be calculated. For example, the dissolution profile of BIBF-F is given in Figure 3.7. Dissolution results in the well-plate method were different in extent and ranking compared to dissolution in the BI miniDiss. The solutions were not homogenously mixed in the well-plate method, which could be visually observed by notifying the differently coloured layers within the wells. Figure 3.6b shows a semi-solid formulation (upper well) and a liquid formulation after 5 minutes of shaking. The semi-solid formulation is not homogenously distributed, which results in large variance in results.

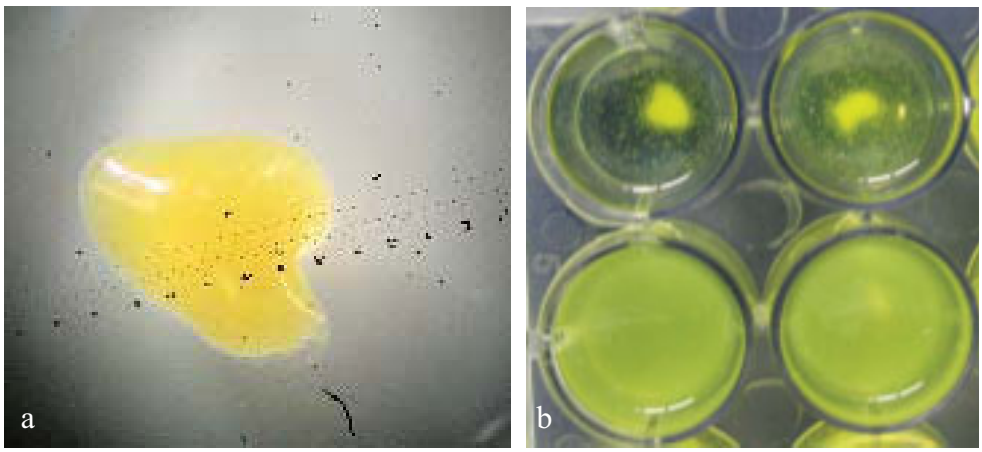


Figure 3.6 a) semi-solid formulation before dissolution and b) after 5 minutes shaking in buffer solution: upper wells: semi-solid formulation, lower wells: liquid formulation

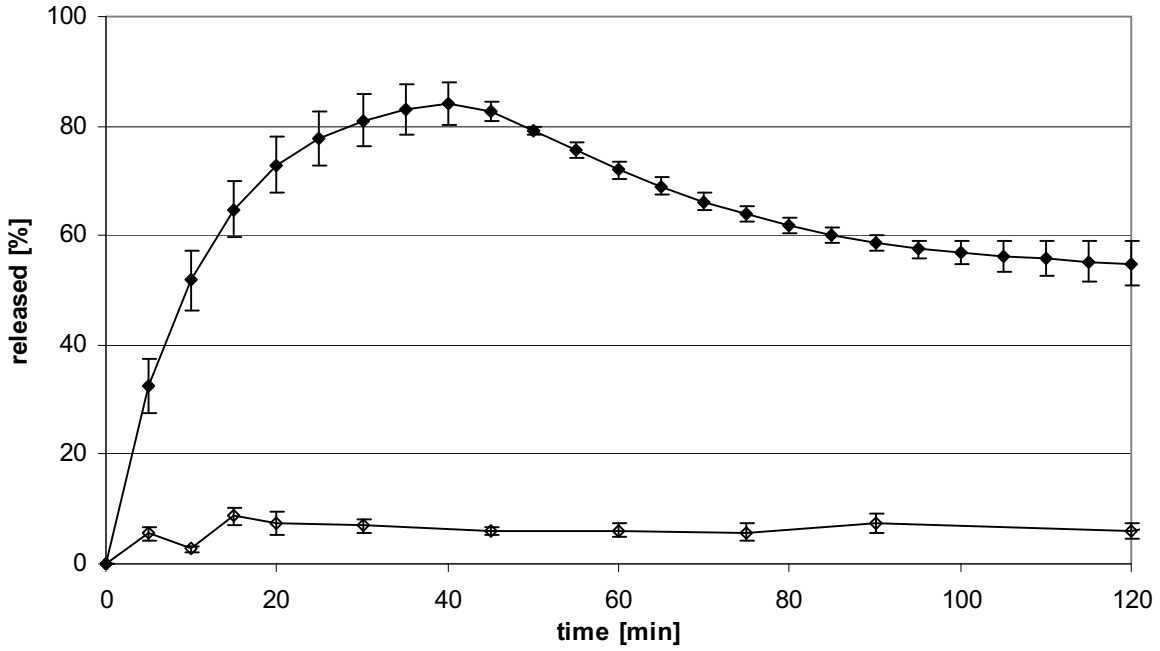


Figure 3.7 Comparison of dissolution of BIBF-F (◊) in FeSSIF using BI miniDiss (solid symbols) at 100 rpm and well-plate method (open symbols) at 380 rpm

Correlation of AUC values following the well-plate and the BI miniDiss methods are summarized in Table 3.1. Release profiles of BIBF in four different buffers is given. Data for semi-solid formulations (D to E) are not given, since no homogenous mixing within the well-plate method could be obtained.

Good correlations could only be obtained for the solid formulations, A to C. The solid formulations showed good correlation in FaSSIF and FeSSIF. No correlation was reached for FeSSIFblank and FaSSIFblank.

The non-solid formulations only showed acceptable correlation (coefficients of 0.998 and 0.995) in FaSSIFblank for AUC_{0-30} and AUC_{0-60}.

Table 3.1 Correlation coefficients (r^2) of AUC values obtained from dissolution of BIBF-A to C using the well-plate and BI miniDiss method

	BIBF	FeSSIF	FeSSIFblank	FaSSIF	FaSSIFblank
r^2 AUC_{0-120}	A-C	0.9775	0.0722	0.7249	0.2841
r^2 AUC_{0-60}	A-C	0.9649	0.0787	0.8160	0.3965
r^2 AUC_{0-30}	A-C	0.9502	0.1296	0.9138	0.4017

Dissolution profiles of BIXX-B in various media using the well-plate and BI miniDiss method are given in Figure 3.8. For all media, statistically significant differences were calculated. In FeSSIFblank higher release was obtained using the well-plate method, whereas in other solutions release was lower.

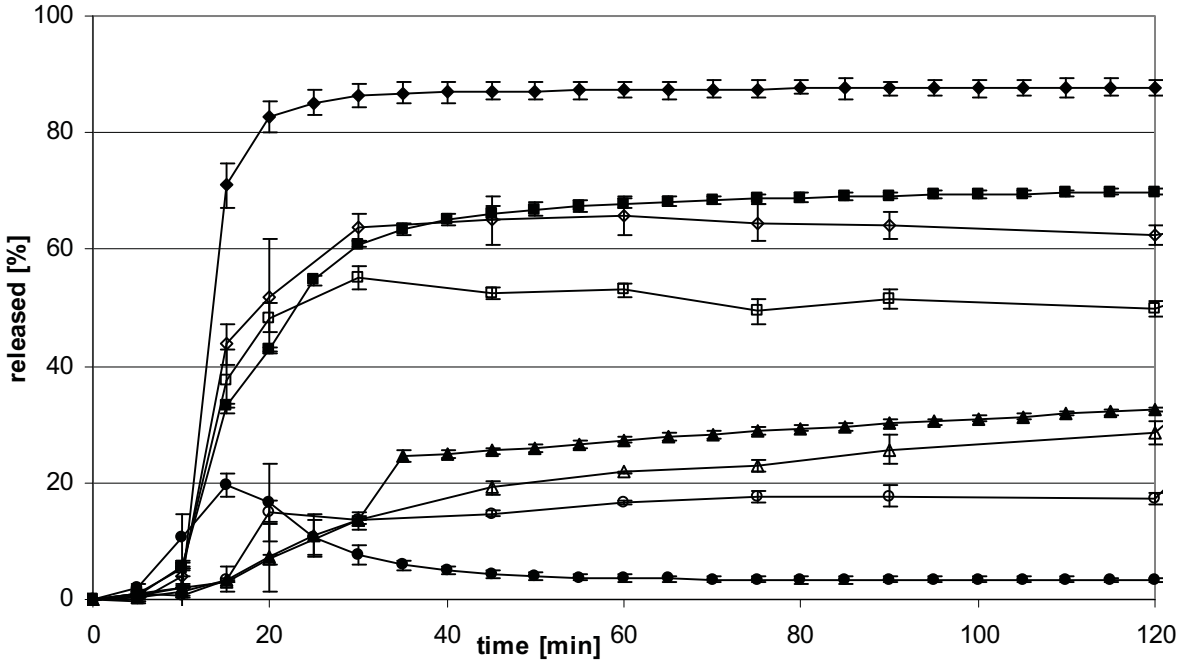


Figure 3.8 Comparison of dissolution of BIXX-B in FaSSIF blank (◊), FeSSIF blank (○), FaSSIF (□) and FeSSIF (Δ) using BI miniDiss (solid symbols) at 100 rpm and well-plate method (open symbols) at 380 rpm

AUC values were similar in both methods. $AUC_{0\text{-}30}$, $AUC_{0\text{-}60}$ and $AUC_{0\text{-}120}$ values from dissolution in well-plates were correlated to the corresponding values obtained in the BI miniDiss. AUC values and correlation coefficients are given in Table 3.2. Good correlation coefficients were obtained for $AUC_{0\text{-}30}$ and $AUC_{0\text{-}120}$.

The high correlation coefficient of 0.98 for $AUC_{0\text{-}120}$ indicate, that both method lead to similar results. The BI miniDiss enables discrimination between all buffers, whereas only a slight discrimation between the blank buffers could be shown using the well-plate method.

Table 3.2 AUC values and correlation coefficients from BIXX-B dissolution in well-plate and BI miniDiss

	AUC_{0-120}	AUC_{0-60}	AUC_{0-30}
well-plate			
FaSSIF blank	6735.1	2893.1	945.6
FaSSIF	5498.1	2452.8	855.1
FeSSIF blank	1698.2	652.6	205.2
FeSSIF	2216.7	702.8	150.6
BI miniDiss			
FaSSIF blank	9291.2	4035.7	1424.7
FaSSIF	6953.2	2805.9	837.9
FeSSIF blank	664.9	458.9	316.4
FeSSIF	2694.1	889.6	150.5
correlation coefficient [r²]	**0.9822**	**0.8829**	**0.9697**

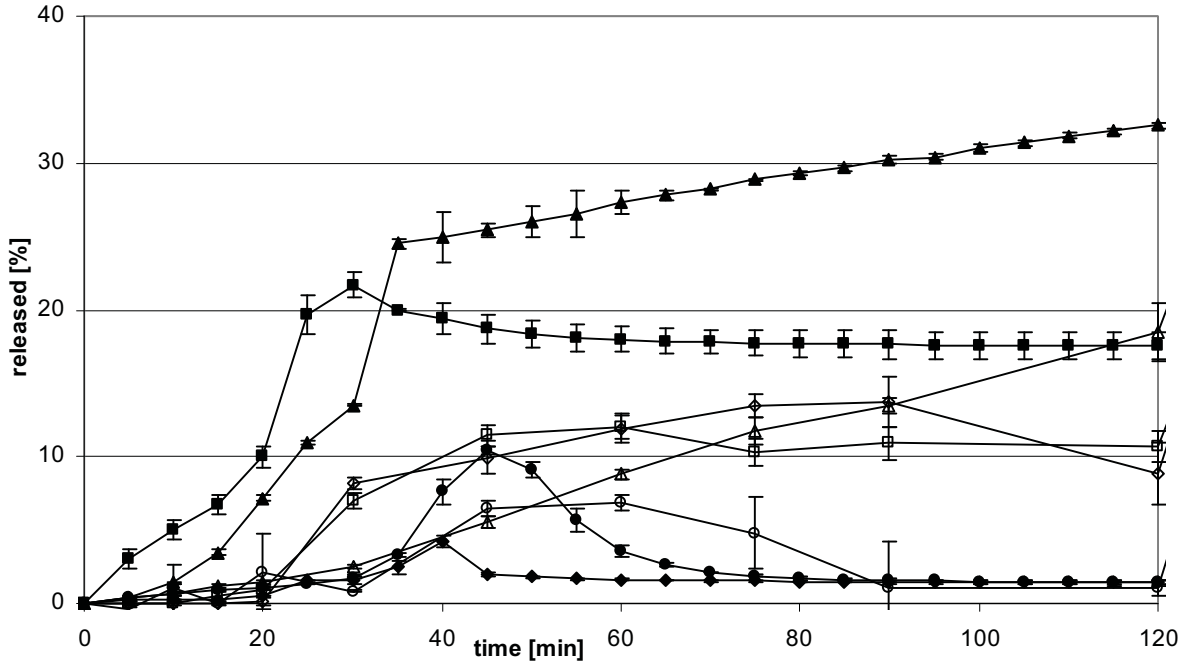


Figure 3.9 Comparison of dissolution of BIXX-A in FaSSIF blank (◊), FeSSIF blank (○), FaSSIF (□) and FeSSIF (Δ) using BI miniDiss (solid symbols) at 100 rpm and well-plate method (open symbols) at 380 rpm

Dissolution profiles of BIXX-A in different media are given in Figure 3.9. Dissolution in FaSSIFblank was higher following the well-plate method whereas for the other solutions higher releases using BI miniDiss were obtained. AUC values of profiles obtained in the

BI miniDiss and the well-plate method were correlated leading to coefficients of 0.887 for AUC_{0-30}, <0.1 for AUC_{0-16} and 0.221 for AUC_{0-120}. Profiles in identical buffers but with different methods showed a similar progression. However, extent of dissolution and relative ranking differed.

3.2 Buffer screening

Dissolution of BI 20 was tested in the BI miniDiss to observe influence of solubilising agents and pH of media. Dissolution curves in various media are given in Figure 3.10 and Figure 3.11. AUC and final pH values are shown in Table 3.3.

In HCl pH 2 and $FaSSIF_{pH\ 5.0}$ (FaSSIF based on FeSSIFblank) almost no dissolution was observed. Maximum percentage release was 1.7 % and 0.8 %, respectively.

A significant increase of dissolution was observed between $FaSSIF_{pH\ 5.0}$ and FeSSIF (maximum percentage release: 10.3 %). Final pH was slightly increased to pH 5.07 in $FaSSIF_{pH\ 5.0}$, whereas in FeSSIF pH remained stable (pH 4.98). Similar trends were observed in $FeSSIF_{pH\ 6.5}$ (FeSSIF based on FaSSIF) and FaSSIF: maximum percentage release values were 52.43 ±1.89 % and 48.29 ±0.77 %. However, dissolution profiles of $FeSSIF_{pH\ 6.5}$ and FaSSIF were not significantly different.

The maximum percentage release in 0.02 M phosphate buffer of pH 6.5 and a final pH of 6.68 was 42.44 ±0.08 %. In FaSSIFblank (similar buffer capacity and final pH value as the phosphate buffer) a significantly lower dissolution was obtained.

Highest amounts dissolved were obtained in $FeSSIF_{pH\ 6.5}$ and purified water, with final pH values of 6.74 and 9.21, respectively.

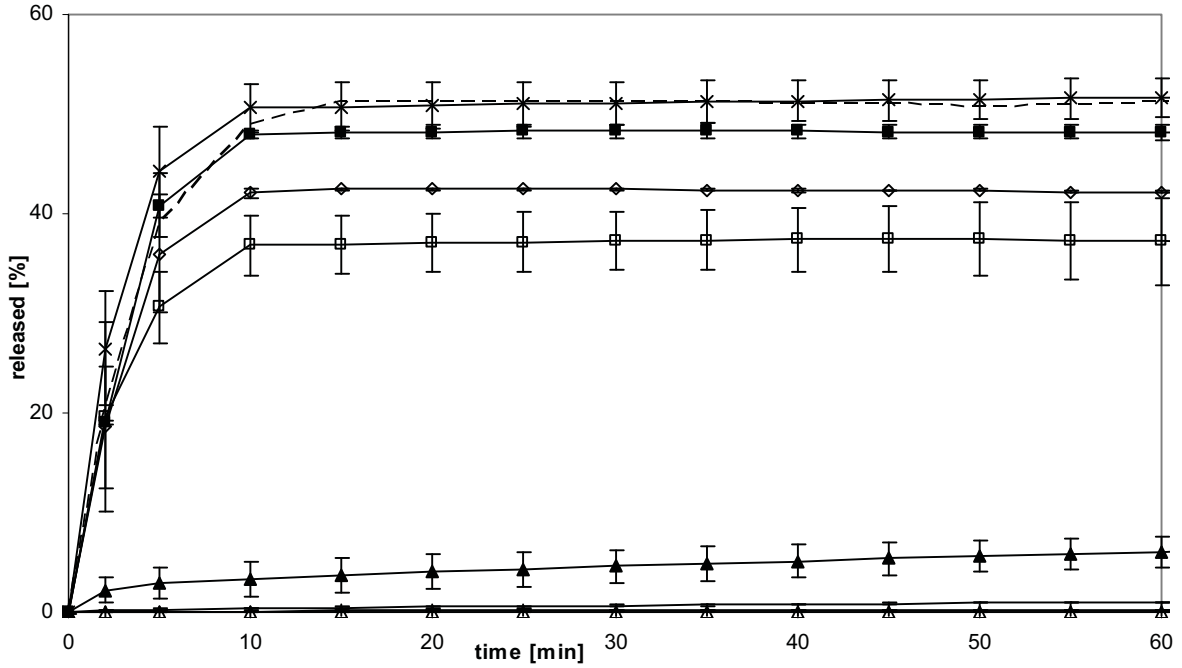


Figure 3.10 Dissolution of BI 20-F in purified water (no symbol, dashed line), 0.01 M HCl (no symbol, solid line), FaSSIF blank (□), FeSSIF blank (Δ), FaSSIF (■), FeSSIF (▲), and FeSSIF based on FaSSIF blank (x), FaSSIF based on FeSSIF blank (+) and 0.02 M phosphate buffer at pH 6.5 (◊) using BI miniDiss at 100 rpm

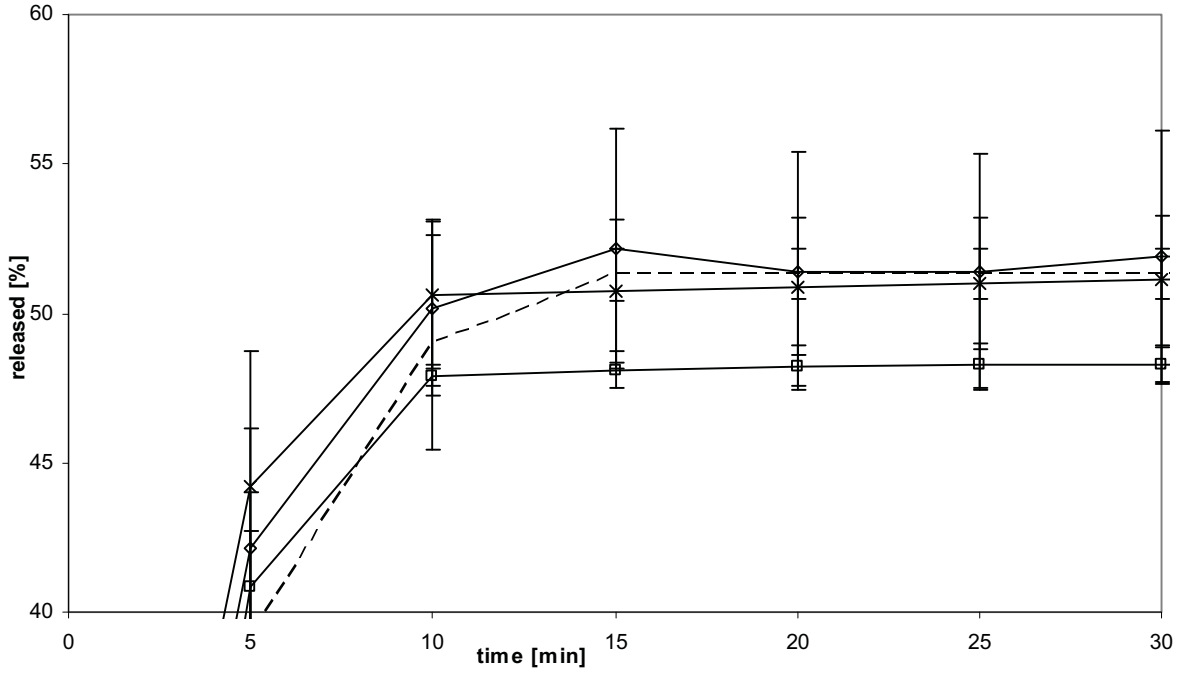


Figure 3.11 Detail of Figure 3.10 showing dissolution profiles of BI 20-F in purified water (no symbol, dashed line), FaSSIF blank (◊), FaSSIF (□) and FeSSIF based on FaSSIF blank (x) using BI miniDiss at 100 rpm

The formulation of BI 20 contains pH modifying excipients leading to an increase of media pH during dissolution. The highest increase of 2.2 pH values was measured for the weak 0.02 molar phosphate buffer pH_{start} 5.5. The strongly pH dependent API shows higher solubility in alkaline solutions. Significant increases in the amount dissolved was observed for pH values above 6.5.

Table 3.3 AUC values calculated for dissolution of BI 20-F using the BI miniDiss

	pH_{end}	AUC_{0-30}	AUC_{0-60}	AUC_{0-120}
0.01 M HCl	1.91	13	38	122
FeSSIF blank	4.98	0.4	1.1	2.7
FeSSIF	4.98	105	265	776
$FaSSIF_{pH\ 5.0}$	5.07	3	9	29
$FeSSIF_{pH6.5}$	6.51	1387	2929	6052
0.007 M citrate buffer pH 6.5	6.62	1443	3056	6261
FaSSIF	6.65	1630	3707	7805
0.02 M phosphate buffer pH 6.5	6.68	1143	2413	4937
FaSSIF blank	6.71	1005	2126	4308
0.002 M phosphate buffer pH 5.5	7.67	1286	2760	5704
purified water	9.24	1351	2883	5964

Table 3.4 shows the solubility of BI 20 in purified water at different concentrations. About 15 % of BI 20 were dissolved when adding below 1 g API per liter, whereas high concentrations dissolved better. At concentrations above 30 mg/ml 100 % of drug was dissolved indicating micelle formation.

Table 3.4 Concentration dependent solubility of BI 20 in purified water

conc [mg/ml]	0.1	0.3	1	3	10	30	100	200
dissolved [%]	13.4	15	15.4	46.6	90	100	100	100

3.3 IVIVR

Correlation coefficients between *in vivo* and *in vitro* AUC values were calculated. In most cases better correlations were obtained for AUC_{0-120} values from *in vitro* data. Figure 3.12 shows a correlation sample of AUC values for BIXX – A, B, C, D and E in 0.001 molar citrate buffer at pH 6.25.

To better compare the dissolution results values visually, AUC ratios were calculated normalizing them to the reference formulation. AUC ratios (test / reference) could then be plotted into one diagram. Results were also compared using the f_2 similarity test.

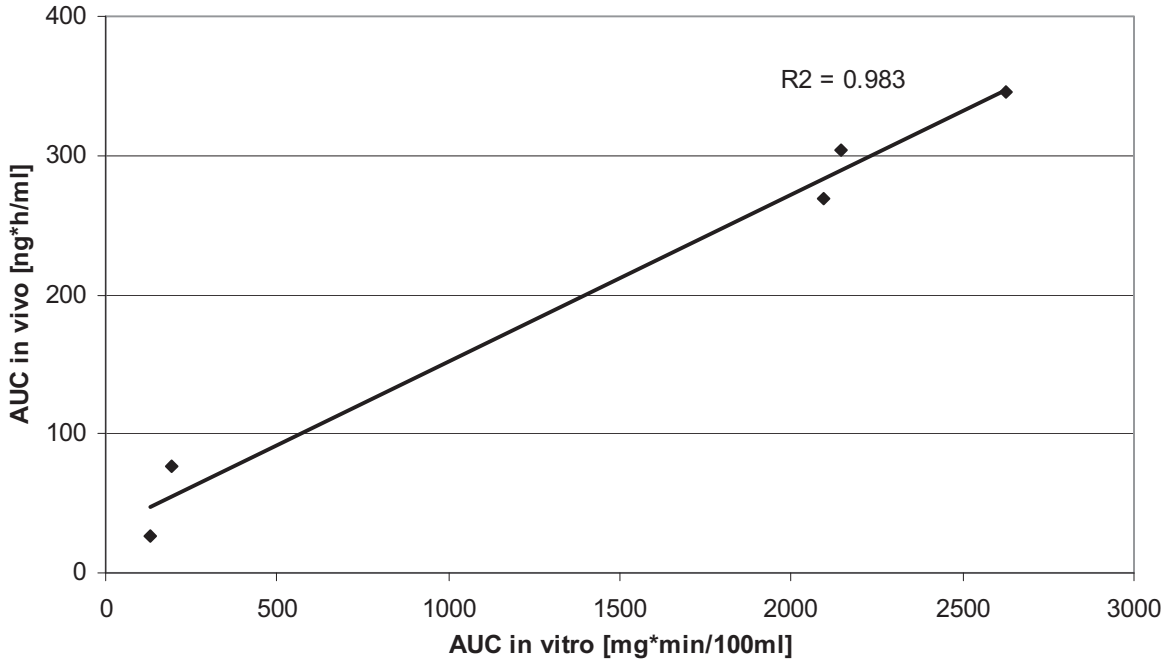


Figure 3.12 Correlation of *in vivo* (0-inf) and *in vitro* (0-30 minutes) (0.001 M citrate buffer pH 6.25) AUC values of BIXX formulations

3.3.1 Results for BIBF

Correlation coefficients of AUC values and similarity factors are given in Table 3.5. Best correlations were obtained in FaSSIF blank (Fab) and 0.002 molar phosphate buffer at pH 5.5. According to similarity factor calculations, best results were obtained in FaSSIF blank. Visually, correct ranking of formulations was obtained in 0.002 M phosphate buffer pH 5.5. Lowest correlation and similarity factors were obtained in FeSSIF blank (Feb) and HCl.

Table 3.5 Correlation coefficient and similarity factor of BIBF formulations A to F

AUC_{0-120}	FaSSIF blank	0.002M Ph 5.5	FaSSIF	FeSSIF	0.007M Ph 6.5	0.001M Ci 6.25	FeSSIF blankb	HCl pH2
r^2	0.9157	0.9181	0.8150	0.6598	0.6918	0.8464	0.3127	0.3316
f_2	55.9	45.1	40.3	29.6	29.4	28.9	20.1	14.9

Fomulation A, which showed lowest absorption *in vivo* was overrated *in vitro* in most buffers (see Figure 3.13).

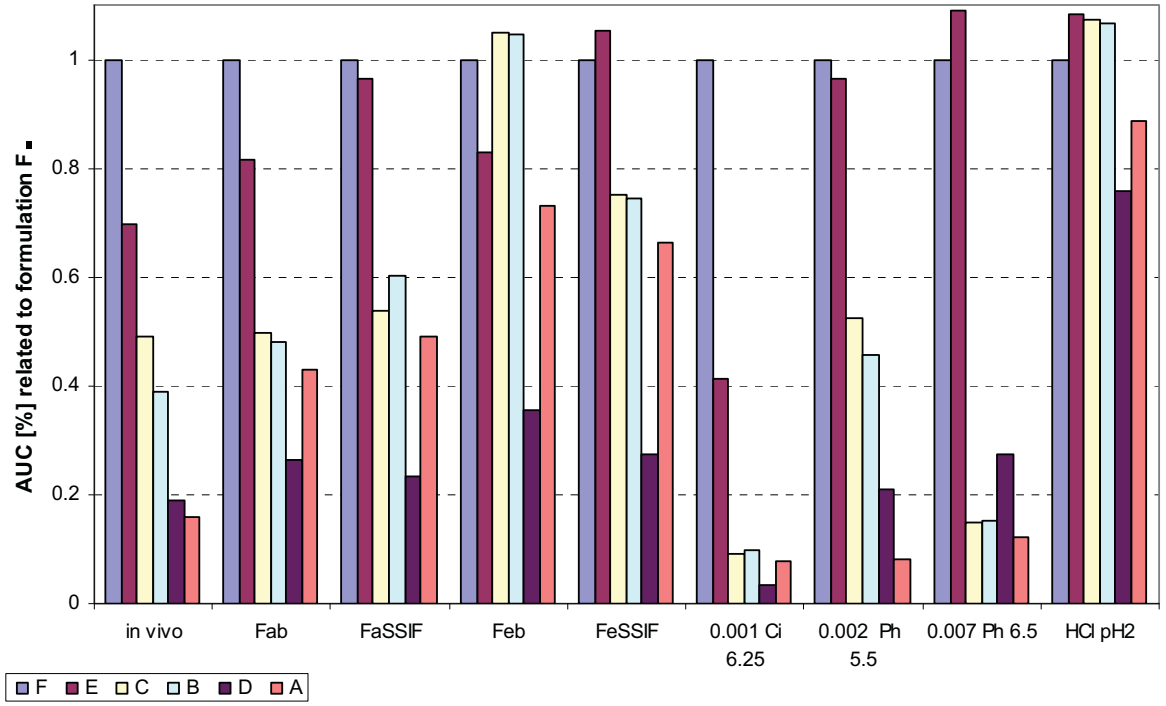


Figure 3.13 Comparison of AUC ratios of BIBF formulations A to F obtained from *in vivo* studies (AUC_{0-inf}) and *in vitro* dissolution in different buffers (AUC_{0-120}) (FaSSIF, FeSSIF and the corresponding blank buffers, 0.001 molar citrate buffer pH 6.25, 0.002 molar phosphate buffer pH 5.5, 0.007 molar phosphate buffer pH 6.5 and 0.01 molar HCl)

3.3.2 Results of BIXX

Solubility of BIXX also shows strong pH dependency. In 0.01 M HCl, at API's highest solubility, all formulations showed high dissolution results.

Table 3.6 Correlation coefficient and similarity factor of BIXX formulations A to E

AUC_{0-120}	Feb	0.001M Ci 6.25	0.007M Ph 6.5	Fab	FeSSIF	HCl	FaSSIF
r^2	0.2933	0.983	0.5057	0.383	0.6867	0.4515	0.4422
f_2	28.8	18.5	18.3	16.7	14.1	14.0	13.5

High dissolution results were also obtained in FeSSIF and the corresponding buffer (FeSSIFblank) at a media pH of 5.0. Correct ranking (regarding *in vivo*) of formulations BIXX-A, B, C and E was predicted by most buffers. However, formulation D was underestimated in all media except 0.01 M HCl.

Similarity was low for all media, whereas a good correlation coefficient of 0.983 was given for 0.001 M citrate buffer at pH6.25 (see Table 3.6).

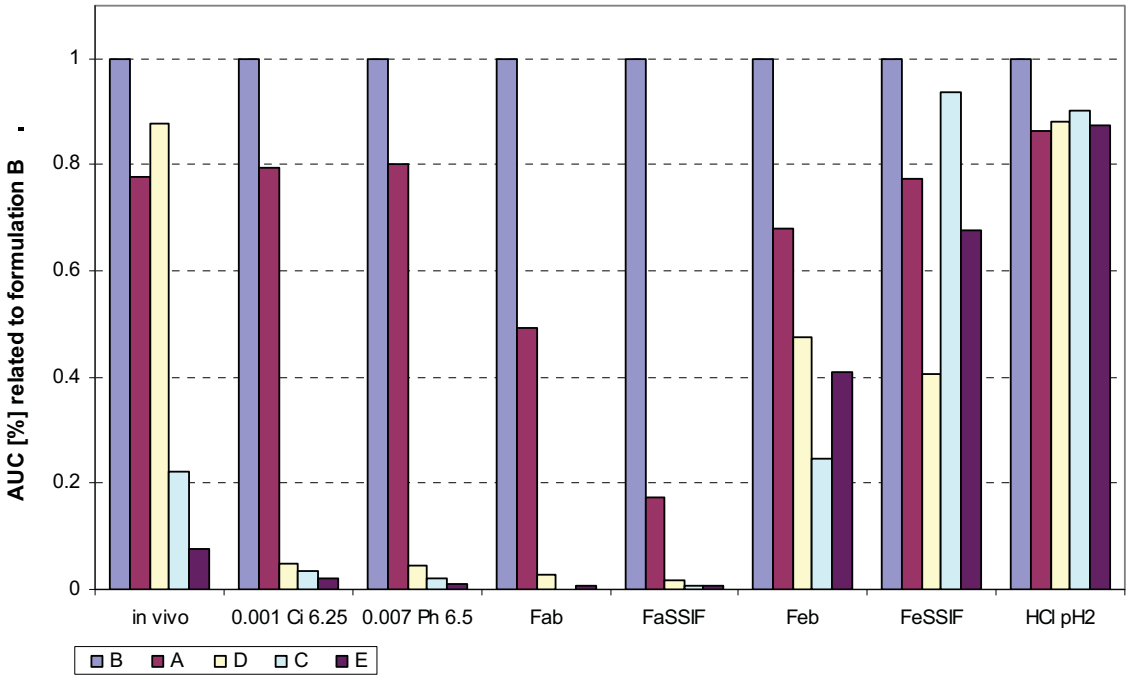


Figure 3.14 Comparison of AUC ratios of BIXX formulations A to E obtained from *in vivo* studies (AUC_{0-inf}) and *in vitro* dissolution in different buffers (AUC_{0-120}) (FaSSIF, FeSSIF and the corresponding blank buffers, 0.001 molar citrate buffer pH 6.25, 0.007 molar phosphate buffer pH 6.5 and 0.01 molar HCl)

4 Discussion

The formulations are placed in 10 – 20 ml buffer media and samples are taken and measured via UV/VIS, if possible. Tested dosage units correspond to the highest dose in man applied in 200 ml. Simultaneous dissolution of n = 8 to 12 can be performed. The reduced scale, factor 10 to 20, limitats the range of testable formulation types. Multiple unit dosage forms like pellets, extrudates, mini tablets or granules can be tested without limitation, whereas single unit dosage forms like tablets, film tablets or matrix tablets need to be downscaled / miniaturized. Miniaturization of formulations may artefactually alter dissolution rate compared to standard scale formulations, e.g. by changing the volume to surface ratio, hardness or film thickness. As an alternative to miniaturization, intermediate products can be used to test suitability of compositions.
In all cases, better understanding of the formulation can be gained.
Small scale dissolution testing by the vial method can be adequately used in early formulation optimization, to indicate the rank order of formulations. In development phases close to the "intended final form", standard dissolution testing should still be utilized.

Further applications of small scale dissolution are preformulation screenings: for this purpose solutions of drug substances and excipients are mixed and added to buffer solutions. The concentration of API dissolved is monitored to observe the occurrence and extent of supersaturation. The most suitable composition can therewith be identified and subsequently used for conventional formulation development.
Buffer type, strength and pH value are chosen corresponding to chapter 1.3.

Apparatus for such small scale dissolution are already on market, like the µDiss[91] from Delphian Technology Inc.. However this system is limited to simultaneous dissolution testing of n=6, only. Stirring is conducted by magnetic stirrers, which might lead to destruction of formulations. The online / in situ measurement is conducted using fiber optic probes which show minimal interference but also limit the possible applications by characteristics of the fiber optics' measurement itself as shown by Lu, et. al[92].

4.1 Compatibility of methods

4.1.1 Vial methods

Dissolution using the manual and automated system showed only slight differences, however these were statistically significant. The faster dissolution of BIXX-B in the automated system is likely a result of the high mechanical stress caused by the magnetic stirrers. Destruction of formulation and thus a higher surface, as well as reduction of the diffusion layer causes facilitated faster release of the API. On the other hand, precipitation from supersaturated solutions might be overestimated due to accelerated exhaustion of the excipients, which in turn would accelerate precipitation. Dissolution conducted in the manual vial method showed generally higher standard deviations (data not shown). Using the most suitable stirring rate of 250 rpm for the vial methods, low mechanical stress and therewith improved reproducibility of dissolution was obtained.

Manual sampling was done without filtration, whereas in the automated system samples were indirectly filtered by passing through the wire mesh. In the unfiltered media, higher extinctions were measured compared to the filtered solutions. The higher values result from UV absorption of small particulates of non-dissolved API explaining the generally higher t_{max} and maximum percentage release values (see Figure 3.1).
Dissolution results were generally similar in both vial methods. However, the reduced time consumption and risk of errors using the automated system combined with the more simple data evaluation provided by the HP ChemStation *Dissolution mode*, makes the automated system the preferred method of the two.

4.1.2 Modified USP vs. vial method

Comparing the modified USP and the vial method, differeces in API release could only be measured within the first 10 minutes. Stronger movement of the media using baskets rotating at 100 rpm in the USP apparatus compared to the magnetic stirrers at 250 rpm explain the faster dissolution. Values of t_{max} and the maximum percentage release, however, show no significant differences. Acceleration of dissolution in the manual vial system could be obtained by increasing the stirrer speed. However, due to the risk of destruction of the formulations combined with accelerated dissolution speed and reprecipitation, stirrer speed should not exceed 250 rpm (see V4.1.1).
In conclusion, the vial method is sufficient to estimate a formulation's dissolution behaviour

but does not correlate directly to the modified USP method.

4.1.3 Modified USP vs. BI miniDiss

Dissolution profiles obtained using the modified USP and the BI miniDiss showed statistically significant difference but were still similar (f_2). Difference were mainly attributable to the different ways of introducing the formulations into the media: Formulations are simultaneously added to the USP apparatus, whereas addition of the dosage unit and therewith the start of dissolution is sequential in the BI miniDiss. This explains the higher inter- and intra-run variations.

Dissolution profiles of the BI miniDiss, given in Figure 3.3, were obtained from different runs: BIXX-A and BIXX-A+, both tested in one run, showed faster release compared to the modified USP method. BIXX-B and BIXX-B+, both tested in one separate run, showed slower release compared to the modified USP method. The delayed release of BIXX-B and BIXX-B+ obviously originate from a delayed addition of drug samples to the vessels. Similar shifts in profiles were observed frequently for other formulations (data not shown). The varying sample addition also explains the generally higher standard deviations obtained in the BI miniDiss. Additionally, deviations in weighed sample (10fold reduced) have a greater influence on results than content uniformity variations have in the larger scale modified USP apparatus.

Still, the formulations showed similar profiles and the same t_{max} and maximum percentage release. In conclusion: Comparison of formulations is feasible using the BI miniDiss. However, further optimization of the starting process is necessary to make the BI miniDiss more precise.

4.1.4 BI miniDiss vs. well-plate method

Shaking rate:

The dissolution behaviour directly correlates with shaking rates: Higher speed of shaking leads to faster process of dissolution as well as reprecipitation from solution. In case of BIXX-B and BIXX-D, no precipitation was observed over 120 minutes and thus only the release profile was influenced. In case of BIXX-D faster and higher dissolution was observed at higher agitation rates combined with faster precipitation. A better mixing and thus a higher shearing caused distruction of the positive microclimate. In case of BIXX-C removal of this microclimate led to precipitation.

The generally higher standard deviations can be explained by insufficient and inconsistent homogenisation of the solutions. Extent of deviations of replicates decreased over time.

Sporadic spill at high shaking rates of 395 rpm made further optimization necessary. However, a further reduction in the volume of dissolution media in combination with lower filling rate was impractical, as these would lead to greater deviations arising from sample weighing. A shaking rate of 380 rpm was identified to enable highest agitation without loss of media.

Dissolution of the semi-solid formulations BIBF-D, C and F (Figure 3.6) showed, that the well-plate method is not generally suitable. Representative samples could not be taken due to inhomogenous sample distribution. However, by carefully watching the experiment, such events can easily be identified visually and time limitations can be set. Generally, the method can be used for early screening of formulation under the limitation, that close observation of the wells is necessary.

Comparison with BI miniDiss:

Using the well-plate method, dissolution was often lower compared to dissolution in the BI miniDiss. Indirect stirring, only by shaking the well-plates, caused lower movement of fluid on the formulations. As a result, shear stress on the formulations surface was reduced and the diffusion layer increased. According to the Noyes-Whitney equation (Equation 4.1) lower dissolution of API as well as functional excipients like solubilising agents or pH modifiers will result. Finally, slower release but also longer extent of supersaturation and thus a higher AUC are obtained.

Stronger hydrodynamic effects by paddle stirring in the BI miniDiss cause higher shear stress and faster removal of saturated solution, explaining the faster dissolution in the BI miniDiss.

$$\frac{dm}{dt} = \frac{D \times A \left(c_s - c_t\right)}{\delta}$$

Equation 4.1 Noyes-Whitney equation for rate of dissolution (dm/dt) controlled by diffusion coefficient (D), saturation solubility (c_s), solubility in bulk (c_t) and particle surface (A), diffusion layer (δ)

Increased stirring also facilitates solubilising effects of the mixed micelles in the biorelevant media. This might be due to more intensive contact of micelles and formulations enabled by the reduction of the diffusion layer. In FaSSIF and FeSSIF, higher dissolution results of BIBF could be obtained compared to the corresponding buffers. The increase in dissolution was higher using the BI miniDiss than the well-plate method. Mean values of extent for FaSSIF to FaSSIFblank were 1.4 and 1.5 for BI miniDiss and well-plate whereas for FeSSIF to

FeSSIFblank mean factors of 8 and 2 were calculated (data not shown).

Regarding dissolution from formulations BIXX-A and BIXX-B compatibility of the well-plate method with the BI miniDiss could not be shown. Although dissolution showed very good correlation in some buffers, there was no general correlation. Still, results appeared on a rank order basis: Formulations leading to good results in the BI miniDiss always showed good results in the well-plate and vice versa. E.g. dissolution of BIXX-B in FaSSIFblank showed very good dissolution in both methods relating to the amount dissolved in the other buffer systems. Similarily formulations with very poor performance, like BIXX-D, showed insufficient dissolution in both methods.

In conclusion, the well-plate method is not a preferred tool for characterization of formulations or to establish qualitative ranking. However, it can be used to gain information on the general suitability of a composition, by helping to select excipients like solubilising agents or pH modifiers for the formulation development. Subsequent dissolution testing of the developed formulations should still be conducted in the BI miniDiss or USP apparatus.

4.2 Buffer Effects

The BI miniDiss proved to be suitable for a fast and effective characterization of formulations. Handling was easy and time-saving with the regard to sample preparation, cleaning and data evaluation. The apparatus can be recommended for screening of formulations.

The amphiphilic character of BI 20 facilitates its uptake into mixed micelles of biorelevant media. Therefore, increase of solubility in such media, containing sodium taurocholate and phosphatidylcholine, is higher compared to the corresponding blank media. Still, pH dependent solubility of the media is the dominant effect: Solubility in FaSSIF with a final pH of 6.65 is about 18 % higher compared to $FeSSIF_{pH\ 6.5}$ with a final pH of 6.51. When pH is held constant by comparison of FeSSIF and $FaSSIF_{pH5.0}$: The higher concentration of solubilising agents is reflected in the higher solubility in FeSSIF, in which a 35fold higher dissolution was obtained.

The higher dissolution in 0.02 M phosphate buffer pH 6.5 compared to FaSSIF blank at similar final pH values (pH 6.68 and 6.71) can be explained by the higher ion concentration in FaSSIF blank: With increasing salt concentration reduction of solubility was observed for BI-20.

The better stability of pH during dissolution in media of high concentrations of solubilising

agents can be explained by the higher concentration of solubilising agents and therewith higher electric loading of mixed micelles and affinity of ions thereto.

Further investigations especially in CMC of BI 20 in different media as well as particle size measurements should be done to better understand interactions between concentration and pH dependent solubility of drugs.

4.3 IVIVR

4.3.1 Results of BIBF

BIBF formulations showed best *in vivo in vitro* correlation regarding ranking of formulations, correlation coefficient and similarity factor in 0.02 M phosphate buffer at pH 5.5. For this buffer both, a high coefficient of correlation (0.9181) and a high similarity factor (45.1) could be obtained. In FaSSIF blank even better results regarding these parameters were obtained. However, ranking of formulations in FaSSIF blank compared to *in vivo* was underdiscriminating, whereas in the 0.02 M phosphate buffer at pH 5.5 in vivo ranking could be reflected. Thus, a suitable buffer for further optimization of BIBF formulations was identified.

Dissolution of all formulations were highest in 0.01 M HCl, caused by the pH dependent solubility of BIBF. Solubility at pH 2 is 100fold higher compared to pH 5 to 7, which in turn might cause low levels of dissolved API in the intestine. Therefore, only formulations releasing dissolved API into the intestine are expected to lead to good bioavailability data: Formulations containing acidifying excipients can help increased absorption by reduction of the microenvironmental pH. Similarly, solubilising agents might help to increase solubility or at least to an extent of supersaturation. The high AUC values in most media can be explained by the functionality of excipients. Formulations BIBF-A, C, D and E contain acidifying or supersaturating agents. Formulation BIBF-B, a conventional formulation without solubility enhancers therefore only shows good dissolution results in acidic media. BIBF-F shows good *in vitro* performance in FaSSIF, FeSSIF and the corresponding blank media. No pH or buffer salt dependency could be observed-. However, contrary to the other formulations, BIBF-F contains solubilisers, which might form micelles. In FaSSIF, FeSSIF and the corresponding buffers, micelle formation could be enhanced by reduction of CMC by the higher sodium

chloride concentrations. Further investigations on CMC and particle size measurements are necessary for a better understanding.

4.3.2 Results of BIXX

Correlation of *in vivo* and *in vitro* results did not lead to good similarity nor correlation coefficient values. However, order of formulations was possible. BIXX-B and A showed good performance *in vitro*, whereas BIXX-C and E showed low release of API. In all media of discriminating pH (pH higher 6) this relation was reflected. The semi-solid formulation BIXX-D however, could not be classified correctly: The high bioavailability obtained *in vivo* could not be reflected by the *in vitro* experiments. In general, effects of formulations or excipients like enhancement of permeation by depression of transporters like P-glycoprotein (P-gp) can not be observed in standard *in vitro* tests. Combination of dissolution and permeation behaviours could be used to further explain the excipients influence. However, set-up and performance of a combined dissolution/permeation experiments, e.g. with caco-2 cells is cost and time demanding.[93-96]

Since BIXX is defined as a BCS II drug (poor solubility, high permeability), bioavailability should not be increased if applied with permeability enhancers. Therefore, further tests on API and excipients characterisation have to be conducted and taken into consideration in dissolution results discussions.

For BIXX formulation, none of the tested buffers accuratly reflected *in vivo* ranking nor showed similar AUC ratios. However, poor and good performers could be identified with most buffers. Best results were obtained in weak citrate and phosphate buffers at pH 6.25 and 6.5, respectively. Thus, both buffers can be used for the identification of probable weak or good performers. For further development however, more buffers should be screened to enable creation of better similarity factors.

5 Conclusion

The miniaturized methods proved to be a suitable method to increase throughput in formulation and buffer screening.

Wheras the well-plate method should only be used for supersaturation screening, the BI miniDiss is feasible for early formulation screening, buffer screening to improve predictability of *in vivo* behaviour as well as for formulation optimization. Compatibility is given for multiparticulate formulations. Limitations are only given by the small scale: Single unit dosage forms can only be tested after miniaturization or after breaking up, which in turn could cause different release profiles compared to standard scales.

The small scale methods help to save consumption of API and excipients as well as expensive buffer substances. Preparation time of dissolution testing and cleaning could bereduced.

In conlusion, a higher number of formulations can be tested within formulation development. Understanding of the formulation and optimization can be relieved.

The BI miniDiss represents a highly economical dissolution method.

VI Summary

Dissolution in different steps of pharmaceutical drug development was considered in this work. Dissolution is used as informative tool throughout the entire development process: After identification of a possible drug candidate, intrinsic dissolution in different buffer media is tested for physicochemical characterization. In galenics dissolution is used to develop and optimize formulations by comparative release studies. During scale-up dissolution testing is used to observe influence of process or parameter changes.
For regulatory affairs all of these dissolution studies are of interest and many have to be presented to the authorities.
Most of the dissolution testing designs in pharmaceutical development are following pharmacopoeial monographs or general chapters and official guidelines. In addition these "official" dissolution testing setups, a progression of more innovative dissolution methods closer to physiological conditions are used. Devices simulating movement and flow of the GIT combined with media simulating the gastrointestinal fluids are often used. Disadvantages of these methods are that they are time-consuming and expensive, both of which limit throughput.

The aims of this thesis were to (a) reduce time consumption regarding preparation of biorelevant dissolution, (b) increase biorelevance of the media FaSSIF and FeSSIF by substituting the non-physiological buffer systems for bicarbonate and (c) to increase throughput by miniaturization of dissolution devices.

To meet the first goal a novel preparation method for the biorelevant media FaSSIF and FeSSIF was established. The conventional method uses chlorinated organic solvent, is time-consuming in preparation (approx. 2 hours) and needs to be done daily. The investigated method uses freeze-drying for the preparation of instant biorelevant media. The instant media only consist of bile salt and lecithin in mixed micelles. *In situ* preparation is done by simply adding blank buffer to the rapidly dissolving lyophilisate. Freeze-dried product gave comparable results to freshly prepared media and improved reproducibility.
Comparison to commercial available instant media indicated superiority of the freeze-drying method.

Next, a buffer system based on the more physiological bicarbonate buffer was investigated. A

method to maintain a stable buffer system throughout the dissolution testing. The buffer therefore was created by sparging carbon dioxide into alkali saline solution to forming carbonate and bicarbonate as buffer system. At equilibrium the media was transferred to the vessels and supply of carbon dioxide continued by sparging the gas above the solution. Therewith bubble formation could be minimized, although not excluded. Only a small range of buffer strength and pH combinations was possible. The lowest pH still providing effective buffer capacity (5 mmol/l/ΔpH) was 5.5. Physiologically relevant buffer capacities of 10 and 30 mmol/l/ΔpH were tested at pH 6.5.
The buffer turned out to be very sensitive against pH modifying agents by loosening its buffer capacity and strength. Standard deviations were generally higher. No superiority over conventional buffer systems like phosphate or acetate buffer regarding IVIVC was given.
Therefore it is concluded that bicarbonate buffer is not a suitable medium for *in vitro* dissolution testing.

Subsequently methods for small scale dissolution testing were established. Improvement of throughput in dissolution testing was achieved. The investigated BI miniDiss method can be used to test release profiles of small particulate formulations or intermediates. High throughput excipient screening for early formulation is possible by using the well-plate method.

In the first series of tests, downscaling by factor 10 was conducted by miniaturizing and automating standard dissolution apparatus. Small vessels of 20 ml volume and paddles of about 8 mm diameter were used. Automating was done by sampling through paddle hollow shafts and online UV/VIS measurement. Since no filtration was possible due to the small sample volume, the true % dissolved was calculated using mathematical scatter correction of spectra from turbid solutions. In this way, release profiles comparable to standard dissolution testing were obtained.
Cleaning and restart is accelerated and therewith throughput increased. The 10fold reduced consumption of drug formulation reduces API consumption, so that a larger variety of formulations can be prepared and tested with the same amount of API.
The BI miniDiss is limited to multiparticulates like pellets, extrudates, minitablets, granules or intermediates. Downscaling of matrix or IR tablets will likely result in different results due to changed surface to volume ratio.

The well-plate method offers a miniaturization of factor 100. Dissolution of multiparticulates showed significant differences compared to standard methods. However, ranking of formulations was possible in several cases. The well-plate method is not suitable for conducting comparative release profiles. However, it can be used for selection of excipients by supersaturation testing. It is an informative tool in early formulation screening helping to optimize formulation of poorly soluble compounds.

As last part of the work, the BI miniDiss was used to screen various buffers to finding the best media for IVIVC, retrospectively. The BI miniDiss proved to be useful as a fast and cost and effective screening method.

In summary, several improvements in dissolution for pharmaceutical development purposes have been developed regarding consumption of API, costs and efficiency. An easy and rapid preparation of biorelevant media was established making their use in pharmaceutical development and routine quality control more feasible. The miniaturized dissolution methods and the improved high-throughput fulfil demands from pharmaceutical industries to facilitate API-saving methods in development.

VII Zusammenfassung

In der vorliegenden Arbeit wurde die Freisetzungsuntersuchung (Dissolution) in den verschiedenen Phasen der Arzneiformanalytik betrachtet. Die Dissolution wird als Hilfsmittel während der kompletten Entwicklung verwendet: Nach der Identifizierung eines Entwicklungskandidaten dient die intrinsische Dissolution in verschiedenen Puffermedien zur Charakterisierung physikochemischer Eigenschaften. In der Arzneiformentwicklung (Galenik) verwendet man vergleichende Dissolution für die Entwicklung und Optimierung von Formulierungen. Während des scale ups unterstützt die Dissolution Identifizierung möglicher Einflüsse von Änderungen der Prozessparameter.
Bei der Einreichung der Zulassungsunterlagen sind all diese Daten von Relevanz und werden in der Regel den Behörden vorgelegt.
Vorlagen für Untersuchungen der Dissolution in der pharmazeutischen Industrie sind meist Arzneibuchmonographien oder entsprechen international anerkannter Richtlinien (Guidelines for Industry).

Neben diesen offiziellen, in Arzneibüchern beschriebenen, Dissolutionsmethoden werden zunehmend innovative Freisetzungsmethoden wie die biorelevante Dissolution angewandt. Um dies zu erreichen wurden Apparate zur Simulation der Motilität des gastrointestinalen Trakts, sowie künstliche Körperflüssigkeiten als Freigabemedien entwickelt. Nachteilig an diesen Methoden ist die zeitintensive Vorbereitung und Durchführung sowie die hohen Kosten, die zu einer Begrenzung des Durchsatzes führen.

Die Ziele der vorliegenden Arbeit waren (a) eine Herstellmethode für lagerfähige und schnell verfügbare biorelevante Medien zu entwickeln, (b) die Biorelevanz der simulierten Dünndarmmedien FaSSIF und FeSSIF zu verbessern (c) einen erhöhten Durchsatz an Dissolution Tests durch Miniaturisierung der Apparate zu verwirklichen.

Im ersten Schritt wurde eine neue Herstellmethode für biorelevante Medien FaSSIF und FeSSIF etabliert. Die konventionelle Methode sieht den Einsatz von chlorierten Lösungsmitteln vor, ist mit zwei Stunden Herstellung sehr zeitintensiv und verlangt zudem eine im Bedarfsfall tägliche Herstellung. Die entwickelten Instantmedien bestehen ausschließlich aus den Grundbestandteilen Gallensalz und Lecithin, welche als Mischmizellen vorliegen und wurden unter Vermeidung organischer Lösungsmittel hergestellt. Die in situ

Herstellung erfolgt durch rasche Auflösung in den entsprechenden Blank Puffern. Es wurde bewiesen, dass die gefriergetrockneten Medien mit konventionell hergestellten vergleichbar sind und zudem eine verbesserte Reproduzierbarkeit aufweisen.
Im Vergleich zu kommerziell erhältlichen Instantmedien zeigte die ermittelte Herstellmethode Überlegenheit hinsichtlich Vergleichbarkeit zu konventionellen Medien.

Im Folgenden wurde ein Puffersystem entwickelt, welches auf dem physiologischen Bikarbonatpuffer basiert. Die Schwierigkeit bestand im Erhalt eines stabilen Puffersystems über den Zeitraum einer Dissolution. Um dies zu gewährleisten wurde Kohlenstoffdioxid in eine alkalische Kochsalzlösung geleitet. Gelöstes Kohlenstoffdioxid diente zur Ausbildung des Carbonat / Bicarbonat Puffersystems. Nach Erreichung des Gleichgewichts wurde die Lösung in Freigabegefäße überführt und zur Erhaltung des Puffersystems ein konstanter Partialdruck von Kohlenstoffdioxid über der Lösung eingestellt. Durch Vermeidung der direkten Begasung konnte die Blasenbildung und damit die Störung der Freigabe, z.B. durch erhöhte Hydrodynamik verringert werden. Die Variabilität der Puffer hinsichtlich Kapazität und pH ist jedoch beschränkt. Um eine noch akzeptable Pufferkapazität von 5 mmol/l/ΔpH zu erreichen kann ein pH von 5.5 nicht unterschritten werde. Deshalb wurden die physiologisch auftretenden Pufferkapazitäten von 10 und 30 mmol/l/ΔpH (nüchtern und postprandial) bei einem pH Wert von 6.5 simuliert.
Es konnte gezeigt werden, dass Bicarbonat basierte Puffer sehr sensible auf pH modifizierende Hilfsstoffe reagieren und dadurch leicht ihre Pufferkapazität verändert wird. Standardabweichungen waren im Allgemeinen höher und zudem konnte keine Überlegenheit gegenüber herkömmlichen Puffern bezüglich *in vitro in vivo* Korrelation gezeigt werden.

Anschließend wurden Methoden entwickelt um miniaturisierte Dissolution Tests zu ermöglichen. Eine Verbesserung des Durchsatzes konnte erreicht werden. Die entwickelte BI miniDiss Methode wurde verwendet um Freigabeprofile multipartikulärer Formulierungen oder von Zwischenprodukten zu testen. Eine Hochdurchsatzmethode konnte durch die Etablierung der Wellplattenmethode erreicht werden.

Im ersten Teil der Miniaturisierung wurde eine Verkleinerung um den Faktor 10 durch die Miniaturisierung und Automatisierung der Standard Dissolution Apparatur verwirklicht. Freigabegefäße im Kleinmaßstab mit 20 ml Fassungsvermögen und Blattrührer mit 8 mm Durchmesser wurden hergestellt. Die Automatisierung erfolgte durch Ansaugen der Proben

durch den hohlen Schaft des Rührers mittels Schlauchpumpe und direkter UV/VIS Messung der Proben in Durchflussküvetten. Eine mathematische Korrektur der getrübten Proben war notwendig, da eine Filtrierung aufgrund des geringen Probenvolumens nicht möglich war. Die erhaltenen Profile waren mit den Standardprofilen vergleichbar.
Die BI miniDiss ist limitiert für Dissolution multipartikulärer Systeme oder von Zwischenprodukten. Ein Miniaturisieren von Single Unit Dosierungen führt durch Veränderungen des Volumen / Oberfläche Verhältnisses zu veränderten Profilen.

Die Wellplattenmethode stellt eine Verkleinerung um den Faktor 100 dar. Die Freigabeprofile von multipartikulären Arzneiformen entsprechen nicht mehr den Standardprofilen. In einigen Fällen konnte jedoch das relative Ranking nachgebildet werden. Für die generelle vergleichende Untersuchung von Arzneiformen ist die Wellplattenmethode nur bedingt geeignet. Ein Einsatz im Screening für geeignete Hilfsstoffe ermöglicht jedoch eine deutliche Steigerung des Durchsatzes. Mit ihrer Hilfe kann die Formulierungsfindung schwerlöslicher Arzneistoffe optimiert werden.

Im letzten Teil der Arbeit wurde unter Verwendung der BI miniDiss ein Pufferscreening durchgeführt um retrospektiv ein geeignetes Medium für eine IVIVC zu finden. Die BI miniDiss eignete sich in diesem Fall um ein schnelles und Zeit-, sowie Kosteneffizientes Screening durchzuführen.

Zusammenfassend lässt sich sagen, dass Verbesserungen in der Pharmazeutischen Entwicklung etabliert werden konnten hinsichtlich Zeitersparnis, Wirkstoffeinsparung und Durchsatzsteigerung. Eine vereinfachte und günstigere Methode zur Herstellung biorelevanter Medien wurde entwickelt und damit Grundstein gelegt um biorelevante Medien in der Routineanalytik zu verwirklichen. Die miniaturisierten Freisetzungsmethoden und der damit gesteigerte Durchsatz entsprechen den verstärkten Anforderungen der Pharmazeutischen Entwicklung nach neuen Wegen zur Einsparung von Wirkstoff.

VIII Appendix

Table 5.1 Publications including biorelevance in dissolution testing

1967	[97]	1990	[98]	1998	[12,38,99,100]	2004	[101-113]
1969	[114]	1991	[115,116]	1999	[117-120]	2005	[121-131]
1977	[132]	1993	[133,134]	2000	[135-144]	2006	[12,145-163]
1985	[164]	1994	[165]	2001	[166-173]	2007	[174-198]
1986	[199]	1995	[200-203]	2002	[204-209]		
1988	[210,211]	1997	[212,213]	2003	[214-226]		

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