Investigation of biorelevant *in vitro* models for the characterization of the dissolution and precipitation behavior of poorly soluble pharmaceutical compounds

I n a u g u r a l d i s s e r t a t i o n

zur

Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Universität Greifswald

vorgelegt von

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Greifswald, 07.10.2022

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Tag des Promotionskolloquiums: 16.03.2023

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List of abbreviations

API	Active Pharmaceutical Ingredient
AUC	Area Under the Curve
BCS	Biopharmaceutics Classification System
c_{max}	Maximum Concentration
DLS	Dynamic Light Scattering
DoE	Design of Experiment
FaSSIF	Fasted State Simulated Intestinal Fluid
FeSSIF	Fed State Simulated Intestinal Fluid
GI	Gastrointestinal
HIF	Human Intestinal Fluids
HPMCAS	Hydroxypropyl Methylcellulose Acetate Succinate
LC	Liquid Chromatography
MS	Mass Spectrometry
SGF	Simulated Gastric Fluid
SIF powder	FaSSIF/FeSSIF/FaSSGF Powder (biorelevant.com)
(U)HPLC	(Ultra-) High Performance Liquid Chromatography
USP	United States Pharmacopeia

1. Introduction

1.1 Purpose

A crucial prerequisite for the absorption of an active pharmaceutical ingredient (API) through the mucosal membrane in the gastrointestinal (GI) tract is the release of the drug from its formulation and subsequent dissolution in the GI fluids, because only dissolved API can be absorbed. However, many new drug molecules suffer from limited aqueous solubility. In 2006, 30-40% of the top 200 orally administered drugs in the United States, Great Britain, Spain, and Japan were classified as practically insoluble, indicating a solubility below 0.01 mg/mL in water (Takagi et al. 2006). Taking into account that most pharmaceutical products are administered orally (Zhong et al. 2018), the solubility and dissolution of a drug are key biopharmaceutical determinants. As drug development is a cost-intensive and time-consuming process, it is of high importance to assess risks of insufficient drug absorption as early as possible to either exclude potential candidates from further development, or to define suitable formulation strategies. Certainly, *in vivo* data from preclinical species or even human studies would be of highest relevance during drug development, however, *in vivo* studies are costly and bear considerable ethical hurdles. Therefore, a combination of *in vivo*, *in vitro,* and *in silico* approaches may be used instead to assess the performance of a drug candidate or drug product early in development (*Fig. 1*). Using *in vitro* assays, the various phases of drug absorption can be investigated, such as the compound's dissolution at physiological pH levels and its permeation through cell layers. In such assays, the conditions are typically well defined. However, the relevance of *in vitro* assays for simulating the actual *in vivo* conditions is often limited, indicating the need for translational tools to extrapolate from *in vitro* to *in vivo*, like *in silico* absorption models (Miller et al. 2019).

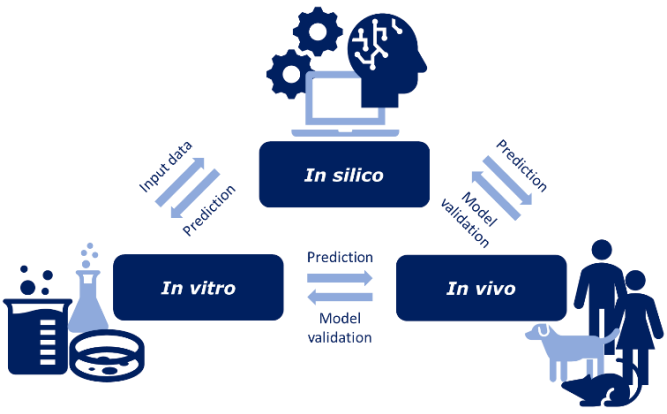

Fig. 1: Interplay between in vitro assays, in silico modelling approaches, and in vivo studies.

Consequently, biopharmaceutical *in vitro* assays have a high relevance during drug discovery and development to directly assess the performance of a compound, or as a source for generating input parameters for *in silico* modelling. In turn, this comes with the need of high-quality assays. High quality is defined by the predictive power and reproducibility of the assay, but also a thorough mechanistic understanding of the underlying processes happening *in vitro*, and their main influencing factors are of relevance. Therefore, this thesis aimed to investigate the impact of a specific parameter of such biopharmaceutical assays, namely the biorelevant medium, on *in vitro* solubility, dissolution, and precipitation testing.

1.2 *In vitro* models for solubility and dissolution testing

Solubility and permeability have long been identified as key parameters defining oral drug absorption. Consequently, the biopharmaceutical classification system (BCS) categorizes drugs as high or low solubility and high or low permeability drugs (Amidon et al. 1995).

Accordingly, API candidates are typically tested in terms of their solubility. However, the solubility of a drug can be determined in several ways and therefore, the assay conditions should be selected based on the properties of the investigated drug and the purpose of the experiment. In general, solubility is defined as the amount of a solute (e.g. drug particle) that can be dissolved in a specific volume of a defined solvent (Alsenz and Kansy 2007). The applied solvent system can yield different solubilities, such as the unbuffered, buffered, and intrinsic solubility of ionizable compounds in aqueous media (Alsenz and Kansy 2007). Thus, for bio-predictive solubility testing, the solvent system needs to be selected carefully. In addition to the equilibrium solubility of a compound, the rate at which a compound is released from its formulation and dissolves is of interest for forecasting its behavior in the GI tract.

Dissolution testing can be performed at different levels of complexity. For instance, dissolution testing of a solid oral dosage form can be performed using compendial apparatuses as described in the United States Pharmacopeia (USP) with known hydrodynamic conditions and rather simple buffer systems (USP 2006). Using this approach, the unknown experimental factors are reduced to a minimum, and so is its biorelevance. In addition to compendial apparatuses, more biorelevant set-ups such as biphasic and two-stage dissolution testing, or even more complex set-ups like the TNO gastro-Intestinal model have gained increased interest during the past years (Butler et al. 2019; Dickinson et al. 2012). While these assays aim to resemble the *in viv*o conditions more closely than their compendial counterparts, this also comes with an increase in complexity, and it

may be difficult to assess the most important biopharmaceutical factors for drug absorption of a specific drug product. Consequently, it is important to select the testing conditions and the complexity of a set-up based on the required output data. With respect to basic compounds, for instance, their risk for drug precipitation in the small intestine is of high interest, which suggests the use of the so-called transfer model (Fiolka and Dressman 2018).

1.2.1 Transfer model to characterize the supersaturation and precipitation behavior of APIs and formulations

1.2.1.1 Background and experimental set-up

The majority of marketed drugs have ionizable functional groups, and the main fraction thereof has basic properties (Manallack 2007; Manallack et al. 2013). Due to a strong pH gap between the fasted stomach and the small intestine, weak bases typically exhibit a high solubility in gastric fluids, but low solubility under intestinal conditions. Consequently, basic APIs may precipitate after entering the small intestine which, in turn, may impair oral drug absorption. However, for a certain time frame, the API often stays solubilized at concentrations considerably above the respective thermodynamic solubility (Kostewicz et al. 2004; McAllister 2010). This stage is called supersaturation and is defined as the difference between the chemical potential of the solute in solution and the chemical potential in equilibrium (Schall and Myerson 2019). Investigating the supersaturation and precipitation kinetics of weak bases provides important parameters to forecast the *in vivo* behavior, e.g., by applying the kinetic parameters generated *in vitro* to *in silico* physiologically based pharmacokinetic models (McAllister 2010; Fiolka and Dressman 2018; Berlin et al. 2014; Butler et al. 2019).

In 2004, Kostewicz et al. introduced the transfer model to investigate the precipitation behavior of weakly basic drugs. Using this set-up, the compound is dissolved in simulated gastric fluid (SGF) and pumped into simulated intestinal fluid (Kostewicz et al. 2004). Later, the disintegration of pharmaceutical formulations was incorporated into this set-up (Ruff et al. 2017). In the meantime, several modified set-ups were introduced, e.g., downscaling the amount of API and GI fluids or using the so-called dumping method to skip the use of a peristaltic pump (Fiolka and Dressman 2018). One example of a downscaled set-up is depicted in *Fig. 2*. The transfer model by Jede et al. is a 1:10 downscale of the physiological volumes and applies in-line UV analytics to assist early formulation development, e.g., by precipitation inhibitor screening (Jede et al. 2019b, 2019a; Jede et al. 2018).

1.2.1.2 Challenges in analytical method development for transfer experiments

Next to scaling of the set-ups (see previous paragraph), another difference between transfer assays can be the analytical method applied for API quantification. As the conditions in transfer assays are highly dynamic, including spontaneous precipitation and rapid changes in the concentration of dissolved drug, close monitoring of the precipitation profile is beneficial. In case (ultra-)high performance liquid chromatography ((U)HPLC) is applied, the precipitate needs to be separated from the dissolved API prior to analysis, which is often achieved by filtration or centrifugation. As this is a time-consuming step, it could hamper frequent sampling, which, in turn, is necessary to closely monitor the precipitation process. Further, precipitation may continue during sample preparation (Jede et al. 2018). In addition, undissolved API which is not completely removed during sample preparation may redissolve during dilution with the mobile phase for (U)HPLC analysis (Jede et al. 2018). Furthermore, the downscaling of the set-ups is limited by the sample volume, or vice versa the number of sampling points is limited by the total media volume available. Consequently, in-line analytics with high measurement frequency and without sample preparation are of advantage. One option is the application of derivative UV spectrophotometry (Jede et al. 2018). However, the method development for in-line UV analytics is also associated with several challenges, especially when biorelevant media are involved (see also *"1.3.5 Analytical issues for biopharmaceutical precipitation assays"*).

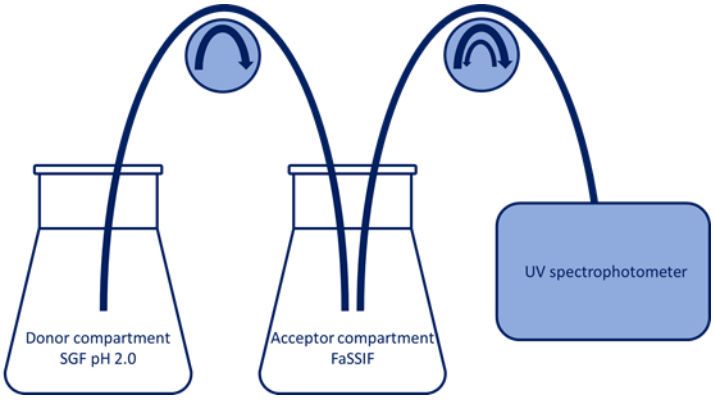

Fig. 2: Transfer model according to Jede et al. (2019b). FaSSIF; Fasted State Simulated Intestinal Fluid, SGF; Simulated Gastric Fluid.

1.3 Current state of biorelevant media for the fasted small intestine

As mentioned in the preceding chapters, biopharmaceutical *in vitro* assays typically aim to resemble the conditions in the animal or human GI tract. One essential factor for solubility and

dissolution assays is the selection of a suitable medium. For the design and selection of such media, a thorough understanding of the intestinal conditions is key.

1.3.1 Underlying physiology of human intestinal fluids

To date, numerous studies have been conducted to collect human intestinal fluids (HIF) aspirates from healthy volunteers. The review articles published by Fuchs et al. and Bergström et al. provide a holistic summary about the subject (Bergström et al. 2014b; Fuchs and Dressman 2014).

In short, the duodenal and jejunal fluids are characterized by neutral pH values ranging from 5.7 to 7.5 (Fuchs and Dressman 2014), which is an important characteristic to forecast the solubility of ionizable compounds. Of note, the strong drop in pH from the acidic gastric compartment with pH values between 1.7 and 3.3 (Bergström et al. 2014b) to more neutral values in the small intestine may cause basic APIs to precipitate. In the fasted small intestine, the predominant buffer system is represented by bicarbonate, which is a component of the pancreatic juice and the bile, but also secreted by epithelial cells into the intestinal lumen (Garbacz et al. 2014; Hall 2011). The buffer capacity was measured to range from 4-19.2 mmol/l/ΔpH in the duodenum (Litou et al. 2016; La Perez de Cruz Moreno et al. 2006; Kalantzi et al. 2006) and 2.4-4 mmol/l/ΔpH in the jejunum (Bergström et al. 2014a; Persson et al. 2005; Fadda et al. 2010). When assessing the buffer capacities and pH values reported in the literature, one should bear in mind that the buffer capacity is estimated by titrating the fluids *ex vivo*, which may lead to systematic errors. For instance, it was shown that centrifugation and freezing of aspirates from the upper GI tract significantly decreased the buffer capacity and increased the pH (Litou et al. 2020). Furthermore, the measured values are only brief snapshots, which do not represent the dynamic processes of carbon dioxide absorption and bicarbonate secretion.

With respect to API solubility and dissolution, another important characteristic of HIF is the composition of solubilizing species. The bile salts as well as phospholipids and hydrolysis products of phospholipids contained in HIF act as natural surfactants, which form mixed colloidal structures like micelles and vesicles (Fuchs and Dressman 2014). Taurocholate, glycocholate, and glycochenodeoxycholate are the three main bile salts in human intestinal fluids with mean overall bile salt concentrations of 3.3 and 3.0 mM in the duodenum and jejunum, respectively (Fuchs and Dressman 2014). Regarding the phospholipids, mainly phosphatidylcholine is present in HIF, while the majority exists as its hydrolysis product lyso-phosphatidylcholine in the small intestine (Fuchs and Dressman 2014; Bergström et al. 2014b; Kleberg et al. 2010). Furthermore, cholesterol and free fatty acids are also components of fasted HIF (Fuchs et al. 2015; Fuchs and Dressman 2014).

1.3.2 Overview of the available fasted state simulated intestinal fluids and their ability to simulate the *in vivo* conditions

Prior to the introduction of biorelevant media, plain buffer systems were the only means to determine solubility and dissolution of APIs from a biopharmaceutical perspective. Under these circumstances, mainly the pH values could be adjusted to physiological values. However, this does not reflect the influence of the solubilizing agents in HIF like bile salts and phospholipids. Therefore, biorelevant media were invented, and their compositions were constantly adapted to new scientific findings during the past years (Galia et al. 1998; Jantratid et al. 2008; Dressman et al. 1998; Dressman et al. 2007; Bou-Chacra et al. 2017; Fuchs et al. 2015). Fasted and Fed State Simulated Intestinal Fluids (FaSSIF and FeSSIF) were developed to mimic the fasted and fed state small intestinal fluids (Galia et al. 1998). Furthermore, media to simulate the gastric and colonic fluids were introduced (Vertzoni et al. 2005; Vertzoni et al. 2010). In addition to a buffer system based on phosphate or maleate salts with physiologic osmolarity, FaSSIF version-1 and -2 contain phosphatidylcholine and the bile salt sodium taurocholate (Galia et al. 1998; Jantratid et al. 2008). These two FaSSIF versions differ only slightly in terms of the phospholipid concentration, the osmolarity, and the buffer system (Galia et al. 1998; Jantratid et al. 2008). For version-3, major changes were applied to the composition of the solubilizing components (Fuchs et al. 2015). While FaSSIF version-1 and -2 do not include lysolecithin, the hydrolysis product of phosphatidylcholine, this was added to FaSSIF version-3, and so were cholesterol, sodium oleate, and the bile salt glycocholate (Fuchs and Dressman 2014; Fuchs et al. 2015; Galia et al. 1998; Jantratid et al. 2008, 2008). Furthermore, the buffer capacity and osmolarity were further adjusted (Fuchs et al. 2015).

To evaluate the ability of biorelevant media to mimic the *in vivo* solubility of various drugs, solubility data obtained in HIF aspirates from healthy volunteers were compared to solubilities measured in the biorelevant media. For example, Söderlind et al. reviewed the solubility of 24 model compounds in FaSSIF version-1 and -2 and in phosphate buffer pH 6.5 in comparison to HIF (Söderlind et al. 2010). The authors found phosphate buffer to be less predictive for ionizable compounds compared to the biorelevant media, and under-predictive for neutral substances (Söderlind et al. 2010). Augustijns et al. reviewed solubility data in fasted HIF for a large set of compounds and found a statistically significant correlation between solubilities measured in HIF and FaSSIF. However, the correlation was weaker for low solubility values compared to higher values (Augustijns et al. 2014). As reviewed by Bou-Chara et al. (2017), several studies described that dissolution testing with biorelevant media is able to mimic the *in vivo* dissolution behavior of poorly soluble drugs better than plain buffers. Consequently, solubility and dissolution testing using biorelevant media have become an important tool in the biopharmaceutical

characterization of new drugs in pharmaceutical industry and academia. Comparing the different FaSSIF versions, FaSSIF version-1 still has a high acceptance, as it is well characterized, and a broad range of solubility data is available. In contrast, version-3 is not commercially available and therefore the data generated with this medium is still rather limited. As for the predictive performance of biorelevant media, Klumpp et al. recently compared the three FaSSIF versions and found FaSSIF version-1 to yield the highest comparability to HIF data. However, differences between the media were mostly seen for acidic compounds due to the pH of the medium, and in case of neutral drugs, differences increased with higher logP values of the compounds (Klumpp et al. 2020).

Despite the advantages fasted state biorelevant media offer for the biopharmaceutical scientist, there is still room for improvement in accurately mimicking the intestinal fluids. As pointed out in the preceding paragraphs, several characteristics of HIF were covered by the FaSSIF versions, but clear differences can be pointed out for the applied buffer species and the complex composition of solubilizing agents like bile salts and phospholipids. Therefore, such biorelevant media characteristics and their impact on solubility, dissolution, and precipitation behavior of pharmaceutical drugs were investigated in the context of this thesis.

1.3.3 Solubilizing agents

As described above, the bile salt and lipid composition in HIF and biorelevant media is an important characteristic, as it determines the solubilizing capacity of the media. In HIF as well as in biorelevant media, bile salts and phospholipids can form mixed colloidal structures of different compositions and sizes. In FaSSIF version-1 for instance, several colloidal structures like globular, thread-like, and disc micelles, agglomerates of disc-micelles, and vesicles were observed (Kloefer et al. 2010; Klumpp et al. 2019). However, other studies only found micellar (Riethorst et al. 2016) or vesicular structures (Clulow et al. 2017). Comparing FaSSIF version-1 and -2, different sizes of colloidal structures were observed, although both contain the same bile salt and phospholipids (Klumpp et al. 2019). As discussed by Klumpp et al., different NaCl concentrations in the media can be an explanation due to salting effects (Klumpp et al. 2019). On the other hand, the different micelle sizes may be explained by the different bile salt to phospholipid ratios of 4/1 (FaSSIF version-1) and 15/1 (FaSSIF version-2) (Klumpp et al. 2019), because the ratio of bile salts to phospholipids can influence the structure and size of the colloidal structures (Vinarov et al. 2021; Shankland 1970). While bile salts typically form spherical micelles with low aggregation numbers and aggregates thereof (Coello et al. 1996; Kawamura et al. 1989; Glomme et al. 2007), mixed

micelles are formed in the presence of lecithin. Lecithin changes the colloidal structure due to the different contributions of bile salts and lecithin to the curvature of a micelle (Leng et al. 2003). Upon dilution of the medium, these mixed micelles can further change to vesicles (Schurtenberger et al. 1985; Sugano et al. 2007). *In vivo,* the bile salt to phospholipid ratio in the fasted state ranges from 4.5 to 39 (Kleberg et al. 2010). Regarding the colloidal structures, Elvang et al. identified up to five different size fractions in HIF and related them to small bile salt micelles, mixed phospholipid and bile salt micelles in a medium size range, and phospholipid vesicles and aggregates of larger size (Elvang et al. 2019). While these colloidal phases seemed to be consistent between the three tested volunteers, their prevalence varied between the individuals as well as between the sampling timepoints (Elvang et al. 2019). Consequently, not only the concentrations of solubilizing agents differ between subjects, but also the nature of colloidal structures. Thus, it is difficult to mimic the complex composition of the colloidal structures of HIF in *in vitro* experiments, which should be considered for the design of biorelevant media, but also for assessing the predictive power of *in vitro* data.

In addition to increasing the solubility of lipophilic drugs, bile salt micelles and mixed micelles including different bile salts and lecithin are also known to have precipitation inhibitory properties (Lu et al. 2017; Chen et al. 2015; Li et al. 2016). In this context, a recent study investigated nucleation and crystal growth of atazanavir and posaconazole in simulated intestinal media and aspirated fasted HIF (Elkhabaz et al. 2021). The researchers found the nucleation and progression of crystallization to be highly dependent on the medium. Significantly reduced crystallization kinetics were observed in the aspirated fluids, thus they recommended that media used for characterizing supersaturation should resemble the endogenous surfactants as closely as possible (Elkhabaz et al. 2021). Consequently, the composition of solubilizing agents in biorelevant media is also an important characteristic in terms of forecasting the supersaturation and precipitation of weakly basic drugs and supersaturating formulations.

1.3.4 Stability of biorelevant media

The components of FaSSIF should also be considered in terms of their chemical stability. While sodium taurocholate is characterized by a known chemical structure with a molecular weight of 537.7 g/mol, phosphatidylcholine is typically derived from natural sources with a variable composition. Soybean lecithin for instance contains a mixture of phosphatidylcholine, phosphatidylethanolamine, inositol phosphatides, and other phosphatides among triglycerides and other components (Scholfield 1981; Szuhaj 1983). Furthermore, the phosphatides like

phosphatidylcholine are composed of saturated as well as unsaturated fatty acids. The unsaturated fatty acids may be prone to lipid oxidation. Emerging oxidation products may interfere with the UV light which could cause analytical problems. In addition, hydrolysis of the phospholipids to lysolecithin may occur. Therefore, the presence of phosphatidylcholine in biorelevant media should also be considered with respect to the shelf life of the freshly prepared medium and the storage of the commercially available powder like the FaSSIF/FeSSIF/FaSSGF powder from biorelevant.com (hereafter referred to as 'SIF powder') (Kloefer et al. 2010; biorelevant.com 2021). The stability of freshly prepared FaSSIF and FeSSIF and the SIF powder, which is used to prepare the FaSSIF and FeSSIF media, was already investigated by Kloefer et al. in 2010, focusing on different properties including water and phospholipid content, and phospholipid oxidation. The researchers observed an increase in UV absorption of freshly prepared FaSSIF and FeSSIF medium over time, most likely due to phospholipid oxidation, which is why they recommended UV quantification only above 280 nm (Kloefer et al. 2010). In the same study, the SIF powder itself was considered to be stable at recommended storage conditions (2-8°C) for at least 12 months. In addition to Kloefer et al.'s study, the stability of freshly prepared FaSSIF medium over a time period of up to 120 h was recently evaluated by Klumpp et al. with respect to characteristics like buffer capacity, particle size, and turbidity (Klumpp et al. 2019). Regarding the selected stability characteristics, freshly prepared FaSSIF medium was considered to be stable for at least 96 h at room temperature (Klumpp et al. 2019). However, there is only limited knowledge about the effect of SIF powder storage on media properties like UV absorption and colloidal structures when FaSSIF and FeSSIF media are prepared from freshly opened versus older batches. Furthermore, knowledge about the impact of SIF powder age on the reproducibility of solubility, dissolution, and precipitation assays is lacking. Therefore, this point was identified as an objective of this thesis.

1.3.5 Analytical issues for biopharmaceutical precipitation assays

As described in section *"1.2.1 Transfer model to characterize the supersaturation and precipitation behavior of* APIs and formulations*"*, transfer experiments should be preferably analyzed by in-line UV spectrophotometry due to numerous advantages compared to (U)HPLC analytics. However, the same ingredients which are responsible for the instability of FaSSIF, i.e., phosphatidylcholine, are also UV active and may interfere with the UV absorption of the API. Referring to section *"1.3.4 Stability of biorelevant media"*, lipid oxidation products may also interfere with the UV signal (Kloefer et al. 2010). Furthermore, in the context of a transfer experiment, the background signal

changes dynamically due to dilution of the fluid in the intestinal compartment. Consequently, it is not sufficient to simply measure the background signal of the medium at the beginning of the experiment. Thus far, a comprehensive approach for a UV method development covering the dynamic changes in media composition and their impact on the measured UV absorption of the dissolved API was lacking. Therefore, one of the aims of this thesis was to develop a new and comprehensive approach for UV spectrophotometric method development for biorelevant transfer experiments.

1.3.6 Buffer species

Besides the solubilizing agents, the buffer species is also an important characteristic of biorelevant media. While the human intestinal fluids are mainly buffered by bicarbonate (Hogan et al. 1994), compendial media are typically prepared with non-volatile buffers, such as phosphate and maleate (Galia et al. 1998; Jantratid et al. 2008; Fuchs et al. 2015). To understand the importance of the differences between a volatile bicarbonate and a non-volatile compendial buffer, it is necessary to reflect on the unique characteristics of the bicarbonate buffer system.

In the presence of hydrogen ions, sodium bicarbonate forms carbonic acid (*Fig. 3 (A)*), which can dissociate into water and carbon dioxide (*Fig. 3 (B)*). The latter one can permeate through the mucosal membrane, enter the blood circulation, and can be exhaled through the lungs (Hall 2011). This process refers to the neutralization of acids in the intestinal liquids (Hall 2011). Carbonic acid can also dissociate into bicarbonate or carbonate and hydrogen ions (*Fig. 3 (B)*) (Hall 2011). The intrinsic pK_a of the ionization reaction of carbonic acid ($([H^+][HCO_3^-])/[H_2CO_3]$) is reported to be 3.55-3.8, while the apparent bulk pK_a is measured to be 6.04 using potentiometric titration, which refers to the equilibrium between bicarbonate and dissolved carbon dioxide ($([H^+][HCO_3^-])/[CO_2$ aq.]) (Krieg et al. 2014). In equilibrium between carbon dioxide and carbonic acid, the concentration of carbon dioxide is higher than that of carbonic acid (Al-Gousous et al. 2019). Therefore, carbon dioxide appears as the conjugate acid, and the apparent pK_a is thus also determined by the hydration and dehydration reaction between carbon dioxide and carbonic acid (Al-Gousous et al. 2019). In the diffusion layer of a dissolving drug particle, this reaction is not in equilibrium, as the diffusion rate of carbon dioxide is faster than the hydration reaction, as a consequence, the effective pK_a in the diffusion layer will be lower than the potentiometrically determined value (Krieg et al. 2014; Amaral Silva et al. 2019; Al-Gousous et al. 2019). This results in a lower effective buffer capacity in the diffusion layer of a dissolving drug particle (Amaral Silva et al. 2019).

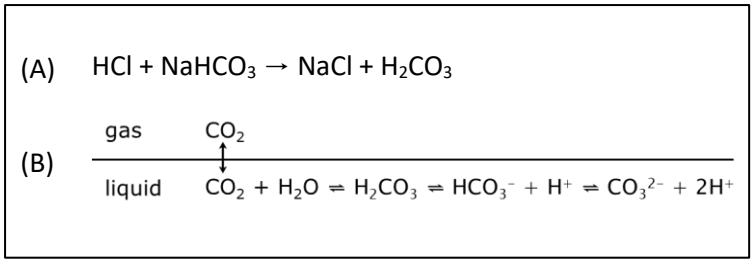

Fig. 3: Bicarbonate buffer reactions.

In addition to the unique characteristics of bicarbonate buffer in the diffusion layer of a dissolving drug particle, the bulk buffer capacity of bicarbonate buffer should be considered. It was found that phase-heterogeneous buffer systems, as present *in vivo* with dynamic bicarbonate secretion and carbon dioxide permeation through the mucosal membrane, have a higher bulk buffer capacity compared to phase-homogeneous buffer systems (Al-Gousous et al. 2018).

The impact of the buffer system and the buffer capacity on the dissolution behavior of ionizable drugs was investigated by several researchers (Krieg et al. 2014, 2015; McNamara et al. 2003b; Mooney et al. 1981; Ramtoola and Corrigan 2008; Sheng et al. 2009; Cristofoletti and Dressman 2016). For instance, McNamara et al. compared the intrinsic dissolution rate of the BCS class II drugs indomethacin and ketoprofen and found 1.5 to 3.5 times higher dissolution rates in the USP and FaSSIF phosphate buffers than in a 15 mM bicarbonate buffer (McNamara et al. 2003a). The impact of bicarbonate buffer and differences compared to non-volatile buffer species like phosphate buffer were also investigated and demonstrated with respect to the dissolution of enteric coated formulations (Amaral Silva et al. 2019; Karkossa and Klein 2017), the performance of the acidic precipitation inhibitor hydroxypropyl methylcellulose acetate succinate (HPMCAS) (Jede et al. 2019c), and the dissolution kinetics of amorphous solid dispersions including ionizable polymers (Sakamoto and Sugano 2021).

Comparing the characteristics of bicarbonate to that of standard buffers, the importance of bicarbonate buffer for dissolution testing becomes obvious. However, biorelevant media are typically prepared using non-volatile buffer systems like phosphate and maleate buffer (Fuchs et al. 2015; Jantratid et al. 2008; Galia et al. 1998). One reason for this is that bicarbonate buffer is usually not stable in *in vitro* assays due to the loss of carbon dioxide and therefore, its implementation can become challenging. For instance, a first approach described by Boni et al. to perform biorelevant dissolution testing using bicarbonate buffer came to the conclusion that bicarbonate buffer is not suitable as a medium for dissolution testing due to issues with practicability and reproducibility (Boni et al. 2007). To overcome these circumstances, different

concepts were described in the literature, like mathematical models to mimic the bicarbonate buffer properties using other buffer systems and on the other hand experimental set-ups for the use of bicarbonate buffer were developed.

Based on the properties of bicarbonate buffers, models have been developed to predict the dissolution rate of drugs in bicarbonate buffer (Krieg et al. 2014; Al-Gousous et al. 2019). Krieg et al. proposed a model to develop phosphate buffers with an equivalent buffer effect as the physiological bicarbonate buffer based on the media pH, the diffusion layer thickness, and the pK_a and intrinsic solubility of the investigated drug (Krieg et al. 2015). A recently published study by Mudie et al. provides guidance on when to use such a low buffer capacity "equivalent" phosphate buffer, which is based on easy to measure compound characteristics (Mudie et al. 2020). However, this approach requires detailed knowledge about the compound, which may be an issue in discovery and early development, profiling several drug candidates in parallel which are only roughly characterized and at a state in which physicochemical properties like the intrinsic solubility and the pK_a may exhibit certain batch-to-batch variability. Furthermore, the low phosphate buffer capacities proposed in these models may lead to an instable bulk pH (Cristofoletti and Dressman 2016). The characteristic of the phase-heterogeneous bicarbonate buffer having a higher bulk buffer capacity is also not resembled.

Besides the mathematical approaches, different concepts have been developed to maintain a stable bicarbonate buffer *in vitro*. One option is to sparge the media continuously with carbon dioxide and compressed air to achieve an equilibrated bicarbonate buffer (McNamara et al. 2003a; Krieg et al. 2014). Sparging biorelevant media with gas, however, may cause issues with foaming due to the surfactants present in the media (Boni et al. 2007). Garbacz et al. introduced the pHysio-grad® system, which is a dynamic system to maintain a bicarbonate buffer at physiologically relevant pH levels (Garbacz et al. 2013, 2014). The device consists of a pH electrode and a gas tube which are placed in the media, as well as a digital microcontroller and two proportional valves. Depending on the pH of a sodium bicarbonate solution, either carbon dioxide or compressed air/nitrogen is added to the medium to adjust the pH (Garbacz et al. 2013, 2014). These devices have been successfully used with a bicarbonate-buffered version of FaSSIF (Litou et al. 2017; Jede et al. 2019c). Due to the dynamic pH adjustment, the issues with foaming of biorelevant media are less pronounced. Furthermore, the pH can be automatically adjusted by the system in case pH changes occur during the experiment like it is the case during the addition of simulated gastric fluid in transfer experiments. A comparable dynamic system was also used by Goyanes et. al. in 2015 (2015). *Fig. 4* shows the schematic structure of a USP II dissolution vessel equipped with the pHysio-grad® device. Furthermore, bicarbonate buffers like a Krebs buffer can

be stabilized by adding a paraffin layer or a floating lid on the medium, or by using a completely sealed system (Fadda et al. 2009; Sakamoto et al. 2021).

Now that commercially available devices facilitate the application of bicarbonate buffers in *in vitro* set-ups, the questions arise as to which extent the exchange of phosphate-buffered biorelevant media with a version based on bicarbonate buffer affects the solubility, dissolution, and precipitation behavior of drugs, and under which circumstances one buffer system should be preferred over the other.

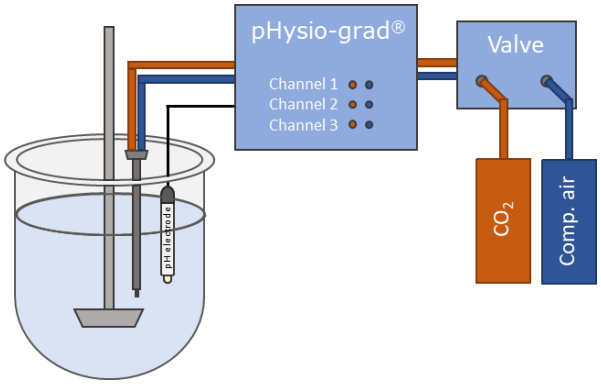

Fig. 4: USP II dissolution vessel, equipped with pHysio-grad® device.

2. Objectives

As outlined in the introduction section, biorelevant media like FaSSIF are an indispensable part of the biopharmaceutical drug characterization. However, there are still open questions and challenges associated with the use of biorelevant media for solubility, dissolution, and precipitation testing. Therefore, this work aims at addressing the following gaps:

In the preceding chapters *"1.2.1 Transfer model to characterize the supersaturation and precipitation behavior of APIs and formulations"* and *"1.3.5 Analytical issues for biopharmaceutical precipitation assays"*, it was pointed out that API quantification during transfer assays should be preferably performed by in-line UV spectrophotometry. Furthermore, it was highlighted that taking the UV absorption of biorelevant media into account during sample quantification is crucial. To address these analytical shortcomings while conducting precipitation assays, the first objective of this thesis was to identify a workflow for the development of robust, reproducible, and selective methods for API quantification for biopharmaceutical precipitation assays using in-line UV analytics.

Another shortcoming is the potential instability of SIF powder components. As summarized in the introduction section, SIF powder contains phosphatidylcholine from natural sources and is therefore prone to degradation. While some aspects of the stability of both FaSSIF and SIF powder were investigated in the past (see chapter *"1.3.4 Stability of biorelevant media"*), the impact of SIF powder aging on the outcome and reproducibility of solubility and precipitation test results has not yet been investigated. Therefore, evaluating the stability of SIF powder during storage and under stress conditions and its impact on the supersaturation and precipitation behavior of ketoconazole, as well as proposing recommendations on the use of FaSSIF to increase the reproducibility of precipitation assays, was defined as the second objective of this thesis.

Furthermore, the physiologically relevant bicarbonate buffer system was shown to impact the dissolution of APIs and their formulations compared to standard buffers. More information can be found in chapter *"1.3.6 Buffer species"*. Since the application of bicarbonate buffers became comparably straightforward during the past years, this thesis aimed at evaluating the extent to which the change of the buffer system from the non-volatile phosphate buffer to the physiological bicarbonate buffer system in fasted state simulated intestinal fluid impacts *in vitro* solubility, dissolution, and precipitation of several model compounds. Based on these results, the overarching third objective of this thesis was therefore to provide insights into the necessity of introducing bicarbonate-buffered biorelevant media as a standard tool in drug discovery and development.

3. Results and discussion

As pointed out in the introduction section in chapter *"1.2.1 Transfer model to characterize the supersaturation and precipitation behavior of* APIs and formulations*"*, precipitation assays should be preferably quantified by in-line analytics. This could be achieved by applying UV spectrophotometry, however, when the assay is performed in the presence of biorelevant media under dynamic conditions, several challenges should be considered when developing an analytical method for quantifying dissolved API: The interference of emerging precipitates with the UV light, the UV absorption of biorelevant media, the changing media composition during dilution of the intestinal compartment, and the limited API solubility in the test medium that impedes calibration measurements within the expected (supersaturated) concentration range. Some of these issues were addressed in different calibration concepts, as it will be described in more detail below. However, a comprehensive approach covering all these issues was not available. These shortcomings were addressed by designing a concept for the development of robust, accurate, and specific derivative UV spectrophotometric methods, and the results were published in the manuscript "*Increasing the robustness of biopharmaceutical precipitation assays - Part I: Derivative UV spectrophotometric method development for in-line measurements*" (Lehmann et al. 2022).

The scattered UV signal as caused by emerging precipitates can be covered by simply measuring the UV absorption in a range of wavelengths instead of a single wavelength to allow the calculation of the derivative spectra. By applying derivative spectroscopy baseline shifts can be eliminated. This concept is not new *per se*, as derivative UV spectrophotometric methods were developed for several APIs in the past (Redasani et al. 2018). Also, Jede et al. already applied this method for in-line UV analytics in a small-scaled biopharmaceutical precipitation assay (Jede et al. 2018). By contrast, identifying a way to account for the UV active colloidal structures in biorelevant media is of higher complexity. As the composition of the medium changes dynamically during a transfer experiment (like in the model described by Jede et al. (2018)), a simple baseline measurement at the beginning of the experiment is not sufficient to exclude the background signal. Of course, it would be straightforward to simply apply wavelengths at which the medium has no UV absorption. For example, Kloefer et al. recommend using wavelengths above 280 nm due to UV absorption of the media and changes in the UV spectra of the media caused to instability of FaSSIF and FeSSIF components (Kloefer et al. 2010). However, in a review published by Redasani et al. about derivative UV spectroscopy the majority of the listed compounds were analyzed at wavelengths below 300 nm (Redasani et al. 2018), indicating that identifying suitable wavelengths may be

challenging. Furthermore, the interaction of a solubilized drug with the colloidal structures may affect the UV absorption of the drug (Werawatganone and Muangsiri 2009), which cannot be excluded even at higher wavelengths. Consequently, it should be preferred to directly calibrate in the presence of the medium to ensure that the UV signal of the medium does not interfere with the API signal. Therefore, a design of experiment (DoE) based calibration scheme was developed in the manuscript "*Increasing the robustness of biopharmaceutical precipitation assays - Part I: Derivative UV spectrophotometric method development for in-line measurements*" (Lehmann et al. 2022). The concept was intended for two stage transfer assays including biorelevant media in the acceptor compartment like the set-up depicted in *Fig. 2*. The new workflow is summarized in *Fig. 5*.

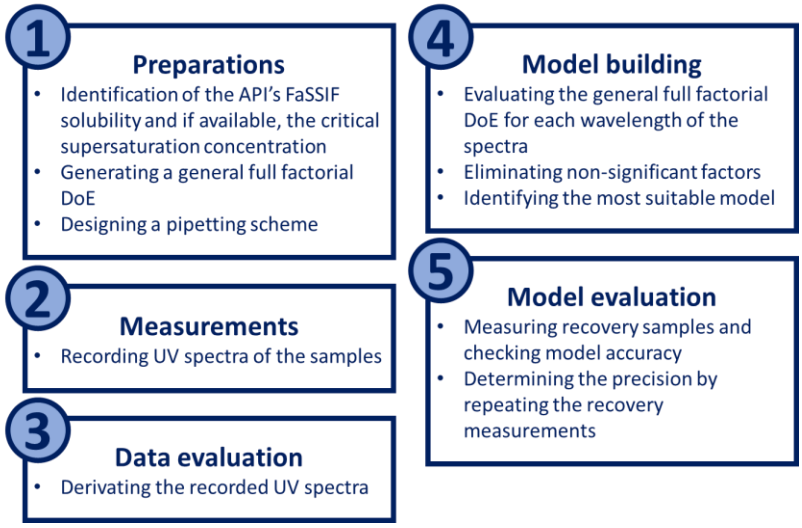

Fig. 5: Workflow for derivative UV spectrophotometric method development. API; Active Pharmaceutical Ingredient, DoE; Design of experiments, FaSSIF; Fasted State Simulated Intestinal Fluid.

The DoE concept mainly builds on the measurement of UV spectra of several API concentrations in the presences of different ratios of the donor and acceptor media (in this case SGF and FaSSIF) (Lehmann et al. 2022). For the calibration measurements, only API concentrations ranging from 0-200% of the FaSSIF solubility were applied. The slight supersaturation of the API can be induced by dissolving the compound in SGF, in which the solubility is typically high, and adding FaSSIF only directly before the measurement. However, samples with concentrations above the thermodynamic solubility should be treated with caution and monitored for potential precipitation, e.g., by measuring the samples again after a few minutes and by visual inspection

with cold light. In case precipitation is observed, lower concentrations should be used for the calibration.

As described in the manuscript "*Increasing the robustness of biopharmaceutical precipitation assays - Part I: Derivative UV spectrophotometric method development for in-line measurements*", based on the resulting derivative spectra, a linear model needs to be calculated for each wavelength including the following factors: API concentration ([API]), FaSSIF percentage ([FaSSIF]), and their interaction terms ([API^2], [FaSSIF^2], [API*FaSSIF]) (Lehmann et al. 2022). Out of these results, an optimal model, i.e., wavelength should be selected which will be used for API quantification. The selection of the wavelength is a crucial step in this workflow and should therefore be based on several checkpoints (Lehmann et al. 2022):

a. Only [API] should have a statistically significant impact on the measured UV absorption. Including factors corresponding to FaSSIF would mean that the estimated values are dependent on the actual concentration of the FaSSIF medium at each timepoint during the precipitation assay. In addition, non-linear effects should be avoided for the API (indicated by the significant factor [API^2]).
b. A high R^2 value is important to prove that the model properly predicts the measured data points.
c. The standardized effect of [API] should be high compared to the other model factors, to ensure that a change in API concentration results in a sufficient change in the UV spectrum.

Following model building and the selection of promising models, it is indispensable to validate the finally selected model. As depicted in *Fig. 5*, the accuracy of the model should be evaluated with recovery measurements, and the precision needs to be tested by repeating the recovery measurements in an independent experiment. The recovery measurements are also an important part of the workflow as they are accounting for the supersaturated concentrations during transfer experiments. As mentioned earlier, one of the challenges for UV spectrophotometric method development is the typically poor thermodynamic solubility of the API in the test medium, which is the reason why only concentrations up to 200% of the solubility of the drug in FaSSIF were proposed for model building (Lehmann et al. 2022). However, according to the authors' experience, drugs often supersaturate to higher concentrations than twice the FaSSIF solubility (Lehmann et al. 2022). Referring to the "Q2 (R1) validation of analytical procedures" from the International Council of Harmonization, it is required to evaluate a linear relationship for the range of the analytical procedure (CPMP/ICH/381/95 1995). Consequently, a method developed by the

approach presented above does not fulfill these requirements. As a compromise, higher API concentrations were included in the recovery and reproducibility measurements to verify the accuracy of the developed method at higher concentrations. Precisely, concentrations up to 900% of the FaSSIF solubility were proposed (Lehmann et al. 2022).

Another approach to overcome the limited API solubility is increasing API solubility by adding components such as organic solvents or solubilizers to the system. Unfortunately, this may alter the UV signal due to solvatochromism (Bani-Yaseen and Al-Balawi 2014) and interaction with the mixed micelles in the biorelevant media, which in turn would spoil the benefits of calibrating directly in the test media. To circumvent this shortcoming, an extended DoE model with an additional factor would be necessary, which would increase the number of experiments substantially. Changing the pH would be an additional option to increase the solubility of basic drugs for the calibration process. However, the pH may again change the UV signal of the drug due to halochromism (Welsch 2005). This behavior is for instance used in the SiriusT3 system (Pion Inc.). For the UV-metric method, extinction coefficients of the neutral and ionized forms can be determined and later used for API quantification, e.g., in dissolution experiments as depicted in the study of Gravestock et al. (2011). The authors describe that the API is typically dissolved in dimethyl sulfoxide as stock solution and then diluted in an aqueous KCl solution for pH titration. In some cases, methanol was added as a cosolvent due to poor solubility (Gravestock et al. 2011). Considering the before-mentioned points, it is important to verify that the solvents added to the system have no impact on the colloidal structures in FaSSIF, the overall UV absorption, and the extinction coefficient of the compound. In contrast, in the newly presented DoE approach (Lehmann et al. 2022), the impact of the pH on the UV absorption is automatically incorporated by the use of different FaSSIF to SGF ratios which cover nearly the complete pH range of the transfer experiment. In case the UV absorption at a specific wavelength is sensitive to the pH change, the DoE would not result in a suitable linear model for this wavelength.

The approach presented herein (see above) and published in "*Increasing the robustness of biopharmaceutical precipitation assays - Part I: Derivative UV spectrophotometric method development for in-line measurements*" was applied to the three weakly basic model compounds cinnarizine, dipyridamole, and ketoconazole (Lehmann et al. 2022). Herin, SGF pH 2.0 and FaSSIF version-1 were used as media. In each case, a derivative UV spectrophotometric method with a high R^2 value and recovery and reproducibility results within the specifications (Root mean square error ≤ 5%) was developed. Consequently, the first aim of this thesis, which was to identify a workflow for the development of robust, reproducible, and selective UV spectrophotometric methods for biorelevant precipitation assays, can be considered as fulfilled. At the same time, this

laid the foundation for a reliable determination of precipitation profiles in the following work ("*Increasing the Robustness of Biopharmaceutical Precipitation Assays - Part II: Recommendations on the use of FaSSIF*" (Krollik et al. 2022a)). As the UV method development concept, which was developed as part of this thesis, is easily adaptable also to other situations, this concept can, in the future, be applied for other types of biorelevant media like FaSSIF version-2 and FeSSIF, or for UV analytics in the presence of other media and formulation components interfering with UV light. An obstacle for the broad application of this method may be the comparably high demand of data evaluation and interpretation especially for unpracticed operators. However, to overcome this issue, a partly automated R-script for model building and interpretation was published alongside the manuscript (Lehmann et al. 2022). Furthermore, the guidance given with the points *a*) to *c*) (see above) allows for a fast and reproducible model building also for operators who are not experienced in linear model building and the underlying statistics.

Noteworthy, for none of the model compounds, a wavelength corresponding to a local minimum or maximum in the derivative spectra was identified as best calibration model (Lehmann et al. 2022). At these parts of the spectra, the FaSSIF medium often had a significant impact on the measured absorption or calculated derivative. This highlights the effect of the media on the UV spectra and the analytical method. As a consequence, the UV signal of the medium should be reproducible between different experiments. It is already known that the UV absorption of freshly prepared FaSSIF medium can alter over time (see chapter *"1.3.4 Stability of biorelevant media"* and Kloefer et al. (2010)). Selecting a wavelength at which the [FaSSIF] factor is not significant should already improve the robustness of the UV method. However, a completely altered UV signal of the medium, for instance caused by degradation products, is not covered in the method development. Hence, it is recommended to repeat the recovery measurements in case the FaSSIF medium preparation deviates from the calibration measurements, e.g., storage time of the medium or change of the SIF powder batch.

As depicted in the preceding paragraphs, the impact of the UV absorption of biorelevant media on the UV method development was concluded. However, it was outlined in the introduction section (see *"1.3.4 Stability of biorelevant media"*) that there is only rare knowledge about the effect of SIF powder storage on media properties like UV absorption and colloidal structures when FaSSIF and FeSSIF media are prepared from fresh versus older batches. Therefore, these questions were addressed in the manuscript "*Increasing the Robustness of Biopharmaceutical Precipitation Assays - Part II: Recommendations on the use of FaSSIF*" (Krollik et al. 2022a). As it can be derived from the title of the publication, not only the stability of SIF powder, but also its impact on the

reproducibility of biopharmaceutical precipitation assays was investigated. The key findings of the manuscript are summarized in the following paragraphs (Krollik et al. 2022a).

Long-term storage of SIF powder under recommended storage conditions resulted in constantly increasing UV absorption of the freshly prepared FaSSIF medium, exhibiting a maximum between 230 and 245 nm (*Fig. 6A*). Furthermore, using the medium in a miniaturized precipitation assay with UV analytics (as described by Jede et al. (2018), see *Fig. 2*), the supersaturation and precipitation behavior of the model drug ketoconazole appeared to change from a two-phasic precipitation to an increased supersaturation and a single precipitation onset in FaSSIF medium prepared from longer stored SIF powder (*Fig. 6C*). At this point, however, it was not possible to judge if the precipitation kinetics of ketoconazole changed or if the measured transfer profiles were just the result of incorrect UV analytics. *Fig. 6B* depicts that the derivatives of the medium UV signal changed with increasing age of the SIF powder. The UV method development for API quantification was performed based on the DoE approach presented above, which means that FaSSIF medium was included in the calibration process. However, calibration measurements were performed with a freshly opened SIF powder batch. It was concluded from the previously discussed manuscript that the UV absorption of the FaSSIF medium plays a crucial role in UV method development (Lehmann et al. 2022). Consequently, the increasing UV signal of older SIF powder samples (see *Fig. 6A and B*) invalidated the applied analytical method. The observations that the transfer profiles measured in older FaSSIF samples exceeded the theoretical profile and that the equilibrium concentration appeared to increase (*Fig. 6C*), was also correlated with the invalid UV method. To gain deeper insights into the aging process of SIF powder and the impact thereof on the supersaturation and precipitation behavior of ketoconazole, SIF powder was stored under defined stress conditions (light, humidity, and temperature) and used for UV spectrophotometric method development as well as for transfer experiments. To not overestimate differences based on the inherent variability of precipitation processes, the experiments were performed as three independent experimental runs with all storage conditions in parallel. For the SIF powder samples stored at elevated temperature and humidity (for detailed storage conditions see caption of *Fig. 7*), comparable to the long-term storage, an increase in UV absorption and changes in the ketoconazole transfer behavior were observed (*Fig. 7A* and *C*). Thus, it was concluded that SIF powder aging impacted the supersaturation and precipitation behavior of the model drug ketoconazole. Using the individually stressed SIF powder samples for the UV spectrophotometric method development resulted in different models at different wavelengths for all samples. This once again highlights the impact of the applied biorelevant

media on UV method development and underlines the recommendations made in the previous paragraphs.

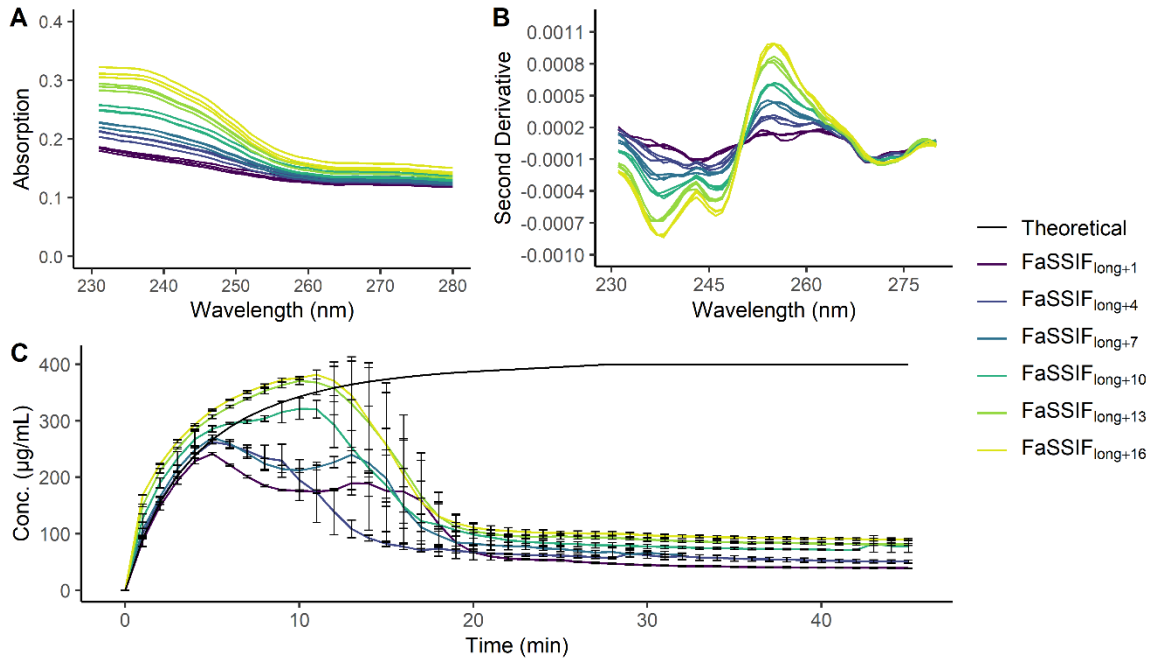

Fig. 6: Impact of long-term storage on SIF powder. A, UV absorption measured during placebo transfer experiments in equilibrium between SGF and FaSSIF (1:1). B, Second derivatives calculated from the absorption profiles displayed in A. C, Mean transfer profiles of ketoconazole, error bars indicate the standard deviation (n = 3). For the theoretical profile, no precipitation is assumed. FaSSIF-long+X; Fasted State Simulated Intestinal Fluid prepared from SIF powder which was stored for X months under recommended storage conditions.

In a next step, liquid chromatography coupled with mass spectrometry (LC-MS analytics) was conducted, to gain deeper insights into the differences between freshly opened and aged SIF powder samples. To the author's knowledge, it was the first time that such data for SIF powder had been published (Krollik et al. 2022a). Based on the composition of SIF powder, especially lipid degradation caused by oxidation and hydrolysis can be expected (see chapter *"1.3.4 Stability of biorelevant media"* for more details). This was confirmed by the results of the LC-MS analytics in which decreasing concentrations of several phosphatidylcholine molecules were observed with increasing age and applied stress conditions (Krollik et al. 2022a). Additional structures, which most likely refer to peroxidation and hydrolysis products, were also found in some samples. These findings can be correlated to the increase in UV absorption, which might be caused by peroxidation products (Kloefer et al. 2010). Comparing the different samples, the effect of storage at enhanced humidity and temperature was more pronounced than that of light stress and long-

term storage (Krollik et al. 2022a). Moreover, it needs to be emphasized that testing the same SIF powder batch in the same experiment but at different ages was not possible, so differences found may not be only related to aging, but also to batch-to-batch variability. Of note, the applied analytics facilitated elucidation of some of the phospholipids on a molecular level, however, the method did not focus on other lipid components and the included bile salt sodium taurocholate.

Moreover, for the FaSSIF medium prepared with SIF powder samples that were stored at increased temperature and humidity, a decreased size of the colloidal structures in the media and an increase in polydispersity were observed by dynamic light scattering (DLS) (*Fig. 7B*) (Krollik et al. 2022a). As pointed out in the introduction (chapter *"1.3.3 Solubilizing agents"*), the content and composition of the bile salts and lipids can impact the formed colloidal structures. Consequently, at least for the SIF powder stored at enhanced temperature and humidity the storage conditions seemed to impact the formed colloidal structures. The decreasing concentrations of phospholipids in the stressed SIF powder samples may have shifted the colloidal structures from vesicles with phospholipids to smaller bile salt micelles. Furthermore, lysolecithin, the concentration of which may have increased during storage at high humidity, is known to form micelles by itself while phosphatidylcholine forms vesicles (Kleberg et al. 2010; Carey and Small 1970).

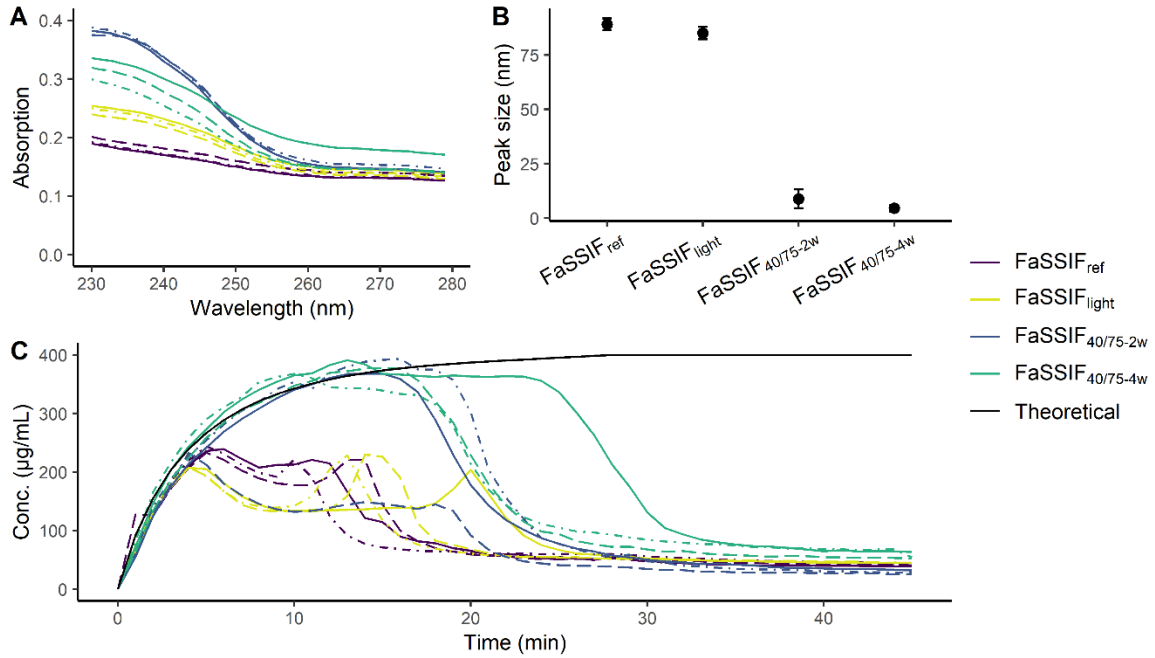

Fig. 7: Impact of stress conditions on SIF powder. A, UV absorption measured during placebo transfer experiments in equilibrium between SGF and FaSSIF (1:1). B, Size of colloidal structures in FaSSIF prepared with differently stored SIF powder measured by dynamic light scattering, error bars indicate the standard deviation between the samples (n = 3). C, Transfer profiles of ketoconazole. For the theoretical profile, no precipitation is assumed. The different line types indicate independent repetitions of the transfer experiments. FaSSIF; Fasted State Simulated Intestinal Fluid, ref; Reference, Light; Exposed to visible and UV light according to ICH Q1B, 40/75-2w and 40/75-4w; Storage at 40 °C and 75% relative humidity for 2 and 4 weeks.

The results of this work (Krollik et al. 2022a) provide additional data and insights to the overall picture of the FaSSIF stability described in the previous work from Kloefer et al. (2010) and Klumpp et al. (2019). While in the current thesis the focus was on the SIF powder stability, Klumpp et al. investigated the stability of different versions of biorelevant media after preparation of the medium itself (Klumpp et al. 2019). With respect to the buffer and the colloidal structures, the authors concluded that FaSSIF version-1 is stable at room temperature for at least 96 h (Klumpp et al. 2019). However, they did not test the UV absorption nor the molecular composition of the solubilizing agents during storage. Kloefer et al. investigated UV absorption of FaSSIF and FeSSIF and observed an increasing UV signal of the freshly prepared media and thus, already brought awareness to this topic in the context of UV analytics (Kloefer et al. 2010). Taking these results together with the results of the current thesis, not only the age of the SIF powder *per se*, but also the age of FaSSIF medium prepared from the SIF powder needs to be considered for UV method development, because both the age of the SIF powder and the age of the prepared medium influence the UV signal. The robustness of the analytical method towards storage time of the prepared FaSSIF medium can be assessed by the reproducibility experiments conducted during

method development (Krollik et al. 2022a). Kloefer et al. further investigated the lipid composition and lipid oxidation of SIF powder after one year of storage and found only low levels of lipid oxidation and no relevant changes in the composition of the SIF powder (Kloefer et al. 2010). This somehow contrasts to the results of the current study, in which a substantial increase in UV absorption after long-term storage of SIF powder was observed which may be related to lipid oxidation (Krollik et al. 2022a). However, the absolute concentrations of lipid oxidation products were not determined in the current study (Krollik et al. 2022a), and Kloefer et al. did not show UV spectra of the corresponding FaSSIF medium. Consequently, the impact of the low content of oxidated lipids on UV analytics cannot be assessed by the data published in the study by Kloefer and colleagues (Kloefer et al. 2010). Next to the lipid composition, Kloefer et al. tested the dissolution of three compounds in FaSSIF media containing either smaller or larger colloidal structures and observed no impact of the size on the dissolution results (Kloefer et al. 2010). This is in line with the similar solubilities of the model compound ketoconazole observed in freshly opened versus stressed SIF powder batches (Krollik et al. 2022a).

As for elucidating the impact of SIF powder age on the transfer behavior of ketoconazole, an impact on the precipitation kinetics of ketoconazole was concluded (Krollik et al. 2022a). Based on solubility experiments conducted with SIF powder of different age, effects of SIF powder age on the thermodynamic solubility of ketoconazole were excluded (Krollik et al. 2022a). However, at this point, the peculiarity of ketoconazole having a two-phasic precipitation profile in FaSSIF prepared from freshly opened SIF powder bottles should be mentioned. Similar observations of ketoconazole transfer profiles were also made by Jede et al. (2019b) and Zygouropoulou et al. (Zygouropoulou et al.). Theories discussed by the authors included precipitation of different polymorphic forms and precipitation faster than the transfer rate. However, it was not possible to finally explain the two-phasic precipitation behavior. Regarding the results of the current study, a certain sensitivity of ketoconazole towards the change in FaSSIF concentration during dilution with SGF may also be reasonable. Alternatively, this observation may be correlated to a first precipitation process of ketoconazole taking place in or at the surface of the micellar and/or vesicular structures, limiting the particle growth (Krollik et al. 2022a). For a deeper understanding of these processes follow-up experiments like in-line solid state analytics such as Raman or experiments elucidating the interaction of ketoconazole with the colloidal structures may be helpful. Considering the unique precipitation behavior of ketoconazole, other compounds may not be as sensitive towards the FaSSIF age. Nonetheless, these experiments highlighted to which extent SIF powder age may impede the reproducibility of transfer experiments (Krollik et al. 2022a). This is of particular relevance, as biopharmaceutical precipitation assays are often

associated with a high variability. For instance, high variability was observed for indinavir precipitation profiles in an interlaboratory ring-study (Berben et al. 2019). The results depicted in the manuscript "*Increasing the Robustness of Biopharmaceutical Precipitation Assays - Part II: Recommendations on the use of FaSSIF*" clearly emphasized the importance of the storage conditions and the age of SIF powder on the outcome and reproducibility of transfer experiments and UV method development (Krollik et al. 2022a). Hence, one of the goals of the current thesis was to provide guidance on how to handle biorelevant media to increase the reproducibility of precipitation assays, which was also covered in the manuscript (Krollik et al. 2022a). Next to adhering to the appropriate storage conditions of SIF powder, intra-laboratory quality control tests (by measurement of UV absorption and size of colloidal structures) and the incorporation of the medium to the DoE-based method development were recommended (Krollik et al. 2022a).

Furthermore, in the same manuscript, the combined effect of SIF powder age and concentration on the ketoconazole transfer behavior was investigated (Krollik et al. 2022a). It was found that higher SIF powder concentrations (150% and 200% compared to normal FaSSIF) led to smaller colloidal structures than FaSSIF with the standard concentration. Further, the transfer experiments with higher SIF powder concentrations resulted in greater areas under the curve (AUC) and higher maximum concentrations (c_{max}) compared to standard FaSSIF (see *Fig. 8*). More importantly, the differences between stressed and reference SIF powder were diminished for the 200% SIF powder concentration (Krollik et al. 2022a). Consequently, the reproducibility is likely to be increased using double-concentrated SIF powder. In addition to the benefits with respect to the reproducibility, this approach can be justified for transfer set-ups with 1:1 dilution based on human physiology. FaSSIF version-1 was designed to resemble the fasted human intestinal fluids (Galia et al. 1998). However, during the transfer, the medium is diluted, resulting in a decreased bile salt and phospholipid concentration. Fiolka and Dressman already recommended to adjust the medium composition with respect to dilution in two-stage experiments (Fiolka and Dressman 2018).

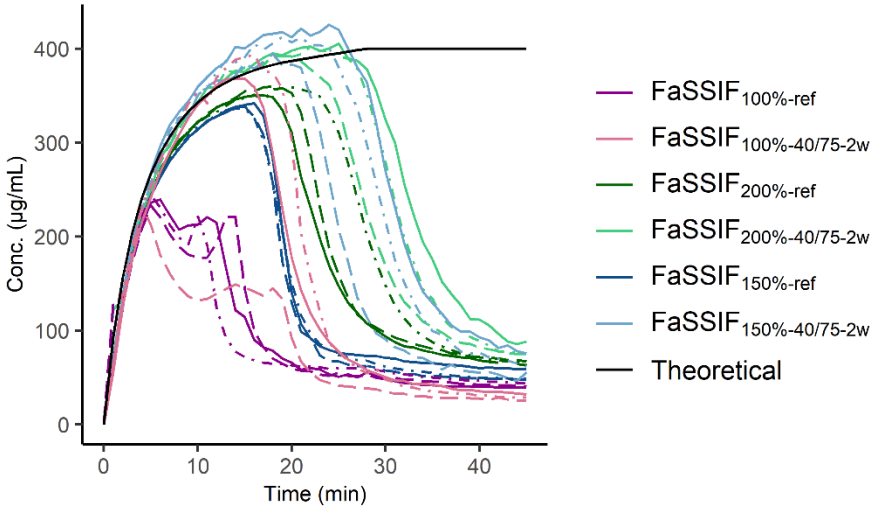

Fig. 8: Transfer profiles of ketoconazole performed with FaSSIF$_{ref}$ and FaSSIF$_{40/75\text{-}2w}$ (100% SIF powder, see Fig. 7) and FaSSIF$_{150\%}$ and FaSSIF$_{200\%}$ prepared with differently stored SIF powder. For the theoretical profile no precipitation is assumed. The different line types indicate independent repetitions of the transfer experiments. FaSSIF; Fasted State Simulated Intestinal Fluid, ref; Reference, 40/75-2w; Storage at 40 °C and 75% relative humidity for 2 weeks.

Investigations regarding the power of fresh vs. aged SIF powder for resembling the luminal conditions were not part of this thesis. For the robustness of the results, either fresh or aged SIF powder batches can be used as long as the process is standardized. However, as the supersaturation and precipitation behavior of drugs are important characteristics to draw conclusions on further development of drug candidates or to serve as input parameters for *in silico* modelling, the *in vitro* conditions should be selected carefully. In the current manuscript, different precipitation kinetics were observed in differently aged and stored SIF powder samples albeit applying the same overall SIF powder concentration. Furthermore, based on the results from the stability study under stress conditions and testing of increased SIF powder concentrations, ketoconazole precipitation was assumed to be sensitive towards the colloidal structures of FaSSIF (Krollik et al. 2022a). As the molecular composition as well as the colloidal structures of FaSSIF are different to that of HIF, precipitation of ketoconazole in intestinal fluids may be different compared to biorelevant media (see *"1.3.1 Underlying physiology of human intestinal fluids"* and *"1.3.3 Solubilizing agents"*). Of note, *in vivo* also other conditions like the pH, transit time, and absorption through the mucosal membrane can impact drug precipitation. In a human aspiration study with healthy volunteers, the degree of ketoconazole precipitation was found to be low (Psachoulias et al. 2011). As the stressed FaSSIF samples (enhanced temperature and humidity) resulted in considerably higher supersaturation and probably slower nucleation (*Fig. 7C*) (Krollik et al. 2022a), this data may fit better to the limited precipitation assumed *in vivo*. However, the

available data is not sufficient to make a clear statement on the predictive power of fresh and aged FaSSIF. With regards to differences in drug precipitation between biorelevant media and HIF, Elkhabaz et al. showed that atazanavir and posaconazole crystallization kinetics were significantly reduced in the aspirated fluids compared to simulated intestinal media (Elkhabaz et al. 2021). In another study, mixed micelles obtained from a mixture of several bile salts and lecithin were shown to inhibit the precipitation of supersaturated telaprevir solutions, while no effect was observed for sodium taurocholate and lecithin (FaSSIF) compared to buffer (Lu et al. 2017). This indicates that there is still the necessity to improve the composition of biorelevant media to reflect the *in vivo* precipitation inhibitory properties of HIF. In the future it may be helpful to judge the predictive power of biorelevant media not only based on solubility and dissolution, but also based on the supersaturation and precipitation behavior of compounds. In addition to basic compounds, this might also be beneficial for supersaturating drug delivery systems.

As depicted in the objectives section, also the buffer system applied to FaSSIF was a focus of this thesis. In chapter *"1.3.6 Buffer species",* different approaches of implementing the bicarbonate buffer characteristics to *in vitro* testing are summarized. On the one hand, there are mathematical approaches to adjust a phosphate buffer to achieve equivalent API dissolution compared to a bicarbonate buffer ((Krieg et al. 2015), see also *"1.3.6 Buffer species"*). However, as described earlier, this approach is associated with some challenges. Especially in early discovery and development, a "one-fits-all" medium would be more suitable. On the other hand, there are experimental set-ups available to incorporate bicarbonate buffer. Therefore, in the manuscript "*The effect of buffer species on biorelevant dissolution and precipitation assays - Comparison of phosphate and bicarbonate buffer*", phosphate-buffered FaSSIF ($FaSSIF_{phosphate}$) was compared to FaSSIF prepared with bicarbonate buffer ($FaSSIF_{bicarbonate}$) in the context of solubility, dissolution, and precipitation testing for a broad range of model compounds (Krollik et al. 2022b).

With respect to solubility, the buffer species turned out to have only a minor impact. Observed differences were either deemed not relevant (< 25% difference between solubility values) or associated with different bulk pH values and salt concentrations (Krollik et al. 2022b). In contrast, a higher impact of the buffer system was found for the rate at which the solubility was reached. To evaluate differences between the buffer species, the observed dissolution profiles were fitted using a linearized Weibull equation and based on the resulting fits, an index (τ_D) describing the speed of the dissolution process was calculated (Langenbucher 1972; Weibull 1951; Costa and Sousa Lobo 2001). Differences between the buffer systems were assessed by statistically significant differences between the fitting parameters (Krollik et al. 2022b). In addition, a relevant difference was defined as two-fold distinction of the τ_D values in order not to over-emphasize

differences and to account for the higher degree of variability which is associated with fitting a large amount of data. Based on these criteria, for eleven out of 20 evaluable compounds, differences in rate of dissolution were observed (*Fig. 9*) (Krollik et al. 2022b).

As described above (see section "*1.3.6 Buffer species*"), bicarbonate buffer is known to have a lower effective buffer capacity in the diffusion layer of dissolving drug particles, which can lead to differences in the dissolution behavior of ionizable compounds compared to non-volatile buffer systems such as a phosphate buffer (Amaral Silva et al. 2019). As discussed in the manuscript "*The effect of buffer species on biorelevant dissolution and precipitation assays - Comparison of phosphate and bicarbonate buffer*", the dissolution behavior of several compounds was in accordance with the knowledge about the unique buffering properties of bicarbonate buffer, like the faster dissolution of flufenamic acid and probenecid in phosphate buffer, and the observation that free bases of prazosin and compound A exhibited no substantial differences in the rate of dissolution (Krollik et al. 2022b). However, parts of the results were not explainable solely by the compounds' ability to impact the pH on the surface of the dissolving drug particle (based on their pK_a and intrinsic solubility). Examples include aprepitant, ketoconazole, and the neutral compounds danazol, felodipine, and fenofibrate, which all dissolved significantly faster in $FaSSIF_{bicarbonate}$. This led to the conclusion that next to the surface pH, also other factors like common ion effect, salt solubility, and wettability affect the dissolution behavior in phosphate- and bicarbonate-buffered biorelevant media during solubility testing under non-sink conditions. Consequently, based on the available data, a prediction of the effect of changing the buffer species from phosphate to bicarbonate buffer on the dissolution was not possible in all cases. Furthermore, it is noteworthy that a pronounced effect of the buffer species on drug dissolution was observed for the tested salts and the cocrystal (Krollik et al. 2022b): Prazosin-HCl was found to reach its solubility faster in $FaSSIF_{bicarbonate}$, while the dissolution of the free base, prazosin, was not affected. For glybenclamide-K, a pronounced supersaturation was observed in bicarbonate buffer at the first measured time point (15 min), while the solubility was already in equilibrium in the phosphate-buffered medium. For the compound A cocrystal, the fitted τ_D values were not affected by the buffer species, but a three times higher supersaturation (29.9 µg/mL vs. 9.2 µg/mL, 4 h values) was observed after four hours in $FaSSIF_{bicarbonate}$ compared to $FaSSIF_{phosphate}$. By contrast, the parent was not affected. Comparing salt and parent forms of basic drugs, a higher dissolution rate is typically expected for the salts due to the ability of the corresponding acid to lower the surface pH and therefore enhancing the dissolution rate. In bicarbonate buffer, the lower effective buffer capacity in the diffusion layer may have resulted in a stronger alteration of the surface pH compared to phosphate buffer. This would be in line with the results from a study

of Uekusa et al. who found decreasing supersaturation of the pioglitacone-HCl salt with increasing phosphate and maleate buffer capacities (Uekusa et al. 2020). Another salt that was investigated as part of this work was amiodarone-HCl, for which an overall lower solubility in bicarbonate buffer was observed (Krollik et al. 2022b). As salting effects were previously documented for amiodarone-HCl in the literature (Ravin et al. 1969; Ravin et al. 1975), this effect may be attributed to the slightly higher chloride concentration in the bicarbonate-buffered FaSSIF compared to standard $FaSSIF_{phophate}$ (Krollik et al. 2022b).

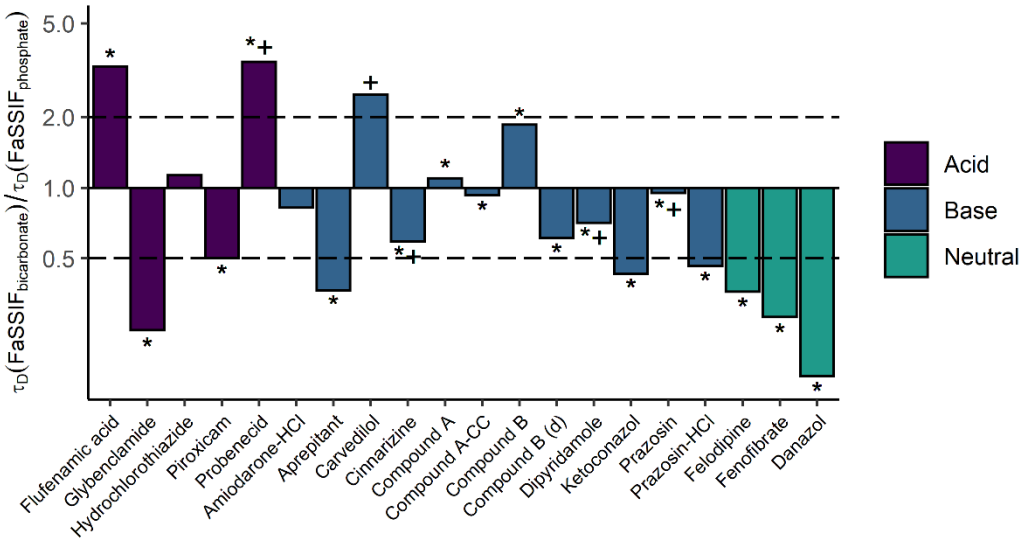

Fig. 9: Relative differences of the calculated τ_D -values between $FaSSIF_{phosphate}$ and $FaSSIF_{bicarbonate}$ (and/or + = regression parameters loga and/or b from linear regression are statistically significant different between the buffer systems, for details regarding the regression parameters see Krollik et al. (2022b)). FaSSIF; Fasted State Simulated Intestinal Fluid, CC; cocrystal; (d); disordered material.*

Overall, the observed differences in rate of dissolution between bicarbonate- and phosphate-buffered biorelevant media under non-sink conditions turned out to be multifactorial and it was not possible to make a quantitative assessment. In this context, it must be noted that in the assessment of the τ_D values, it was not feasible to account for concurrent precipitation processes during dissolution due to supersaturation in the calculations (Krollik et al. 2022b). However, applying this technique allowed for a statistical comparison of the dissolution profiles. In addition, in the current study, the dissolution behavior was investigated in the context of solubility measurements under non-sink conditions, and consequently, no conclusions can be drawn on the intrinsic dissolution and precipitation rates of the compounds. Therefore, further investigations would be needed in the context of intrinsic dissolution as well as supersaturation and precipitation

rates to gain a deeper understanding of the factors influencing solubility and dissolution behavior in phosphate- vs. bicarbonate-buffered biorelevant media. The results of such experiments should be seen in relation to previous literature regarding surface pH and effective buffer capacity in bicarbonate buffer (Krieg et al. 2014, 2015). Furthermore, a higher number of compounds may help to assess the relevance of the observed differences or to elucidate further reasons for them.

Next to statistical descriptors, also the predictive power of the media for the *in vivo* situation is of high importance. The manuscript "*The effect of buffer species on biorelevant dissolution and precipitation assays - Comparison of phosphate and bicarbonate buffer*" aimed at comparing solubility data of phosphate- and bicarbonate-buffered FaSSIF with solubilities measured in HIF, to draw conclusions on the *in vivo* predictive power of the media (Krollik et al. 2022b). Following the minor differences in thermodynamic solubility between the buffer systems, neither bicarbonate- nor phosphate-buffered FaSSIF turned out to be superior in predicting solubility in HIF.

While the predictive power of biorelevant media is often evaluated by comparing thermodynamic solubilities of pharmaceutical compounds in the media of interest to solubility data measured in HIF aspirates *ex vivo*, also the limitations of this procedure need to be acknowledged. *In vivo,* the pH and buffer capacity are adjusted dynamically by absorption and secretion processes, which is not resembled in static solubility measurements. This can result in a shift in pH during the experiment (Söderlind et al. 2010). Furthermore, the pH and buffer capacity of HIF samples can be altered by the applied freeze-thaw cycles and sample handling like centrifugation (Litou et al. 2020). One option to gain more insightful data could be solubility testing in HIF aspirates with continuous pH (or, more specifically, carbon dioxide) adjustment to simulate the dynamic buffer system in the small intestine. In addition, data on the dissolution speed are needed to further assess the quality and the need of *in vitro* bicarbonate buffers to predict *in vivo* dissolution. Therefore, solubility testing in HIF, using a more biorelevant timeframe, such as three instead of 24 hours (La Cruz-Moreno et al. 2017) to account for physiological transit times, could be applied. Furthermore, the unique property of the *in vivo* bicarbonate buffer being a phase-heterogeneous buffer system with enhanced bulk buffer capacity (Al-Gousous et al. 2018) is not represented in static solubility measurements in HIF samples. This particularity of bicarbonate buffer is also not reflected when phosphate buffers with low buffer capacities are applied to resemble the reduced bicarbonate buffer capacity in the diffusion layer (see section "*1.3.6 Buffer species*"). Consequently, the bulk pH may change during the experiment and thus, the additional adjustment of the pH could become necessary. For instance, Cristofoletti and Dressman matched phosphate and maleate buffers to achieve equivalent surface pH values to bicarbonate buffer for weak acid

drugs, but pointed out that the trade-off between a medium with low buffer capacity and the necessity to titrate the buffer to keep the bulk buffer pH stable needs to be taken into consideration (Cristofoletti and Dressman 2016).

Another important point to consider for the design of buffer systems for dissolution testing and the rating against *in vivo* data is the high variety of buffer capacities measured *in vivo* (see "*1.3.1 Underlying physiology of human intestinal fluids*"). As noted before, *ex vivo* measurements of the buffer capacity can be affected by the experimental procedure (Litou et al. 2020), making it difficult to assess the actual buffer capacity of the intestinal fluids. In the manuscript "*The effect of buffer species on biorelevant dissolution and precipitation assays - Comparison of phosphate and bicarbonate buffer*", the buffer capacities were not varied (Krollik et al. 2022b). However, in cases where the buffer capacity has a relevant impact on the dissolution of drugs and excipients, it may be of added value to test a variety of physiological buffer strengths as described by Sakamoto and Sugano for a nifedipine formulation to characterize the effect of bicarbonate buffer on ionizable polymers (Sakamoto and Sugano 2021). Furthermore, Litou et al. recently evaluated data suggesting that the *in vivo* buffering system of the fasted upper GI tract is not only dependent on bicarbonate but also on proteins (Litou et al. 2020), which should be taken into consideration for the design of *in vivo* predictive buffers.

As described above, the effect of exchanging phosphate by bicarbonate buffer in FaSSIF medium on the rate of dissolution of several APIs turned out to be multifactorial. Considering the study results (Krollik et al. 2022b) together with the discussion in the preceding paragraphs regarding the unique properties of bicarbonate buffer (see also chapter "*1.3.6 Buffer species*"), this strongly highlights the special role of bicarbonate buffer for simulating the conditions in the human intestinal fluids and clearly points out the importance of carefully selecting the buffer system for the design of *in vitro* experiments. Although it was not possible to rate phosphate- and bicarbonate-buffered biorelevant media in terms of their predictive power for *in vivo* dissolution, the additional use of physiological bicarbonate buffers already in early discovery and development is recommended for simulating dissolution processes. Regarding thermodynamic solubility, the buffer species (phosphate vs. bicarbonate) was demonstrated to be of minor importance. Furthermore, in a stage at which a compound's behavior is well characterized like in late-stage development and quality control, surrogate buffers like phosphate buffers with an adjusted buffer strength may be sufficient and beneficial for robust and simple experimental set-ups.

In addition to solubility and dissolution, the impact of the buffer species on the supersaturation and precipitation behavior of four basic model drugs was tested (Krollik et al. 2022b) in a transfer

model similar to the one developed by Kostewicz et al. (Kostewicz et al. 2004). As depicted in *Fig. 10*, the transfer results of the tested model compounds indicate that the supersaturation and precipitation behavior of the neat API is not markedly influenced by the buffer species (Krollik et al. 2022b). These results were to some extent in contrast with study results from Jede et al. who investigated the effect of the buffer species on the transfer behavior of weakly basic drugs in combination with a precipitation inhibitor (Jede et al. 2019c). One of their key findings was that bicarbonate-buffered FaSSIF better predicted the precipitation inhibitory effect of the acidic polymer HPMCAS on the precipitation of several weakly basic APIs (which was confirmed in a rat pharmacokinetic study), but they also tested the neat APIs in both buffer systems. While also no differences were found for ketoconazole, they observed a slight increase in AUC of lapatinib-ditosylate monohydrate and a pronounced increase in AUC and c_{max} for pazopanib-HCl in bicarbonate- compared to phosphate-buffered FaSSIF. With respect to the results published in the manuscript "*The effect of buffer species on biorelevant dissolution and precipitation assays - Comparison of phosphate and bicarbonate buffer*", these findings may be correlated with the observed effect of the buffer system on salts and cocrystals (Krollik et al. 2022b), with potentially higher (re)dissolution of the salts in bicarbonate buffer during the transfer of the suspended APIs from the gastric to the intestinal compartment (Jede et al. 2019c). However, in the current study, no impact of the buffer system on the transfer behavior of the suspended salt amiodarone-HCl could be detected (Krollik et al. 2022b). As mentioned earlier, the solubility of amiodarone-HCl is sensitive to the chloride concentration and therefore may have also been determined by this (Ravin et al. 1969). Consequently, not enough data is available to draw a clear conclusion on the effect of the buffer species on the transfer behavior of neat APIs. Based on the available data from literature and this work, the buffer system seems to be of minor importance when dissolved APIs are transferred from an artificial stomach to an artificial intestinal compartment, whereas suspended APIs may be partly affected. Hence, additional testing in bicarbonate-buffered media should be considered for such compounds. Also for the characterization of formulations containing ionizable excipients, the selection of a suitable buffer system becomes crucial (Jede et al. 2019c; Sakamoto and Sugano 2021).

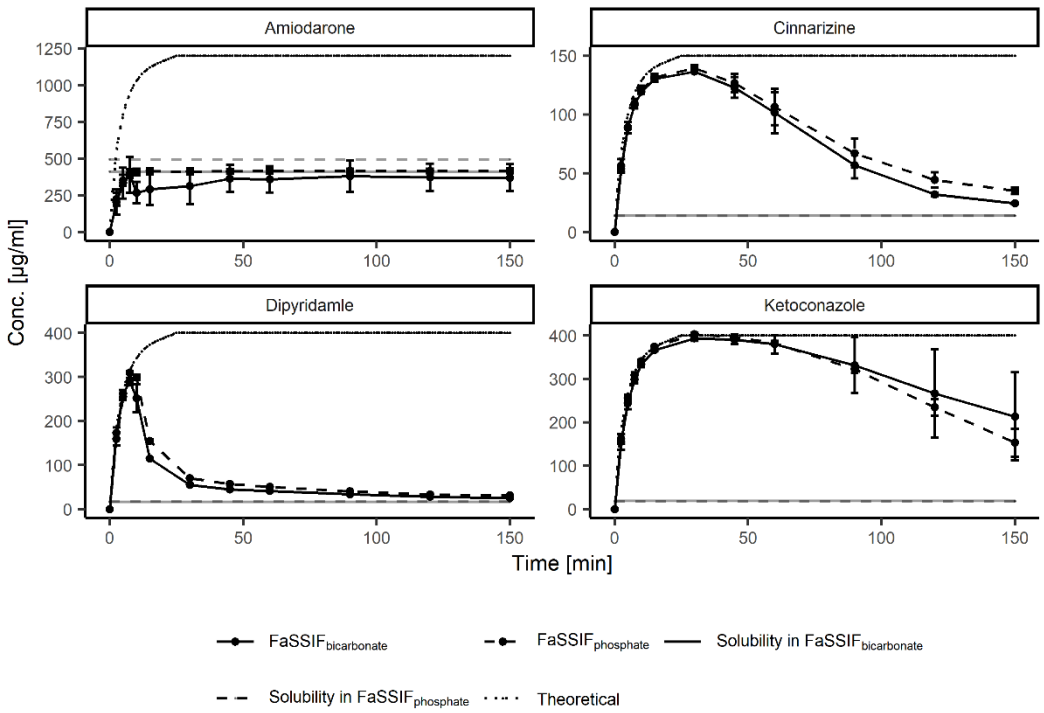

Fig. 10: Transfer profiles in phosphate- and bicarbonate-buffered FaSSIF (transfer media) and their respective solubilities in the media, error bars indicate the standard deviation, the dotted line indicates the theoretical concentrations in case no precipitation occurs. FaSSIF; Fasted State Simulated Intestinal Fluid.

4. Summary and outlook

As outlined in the introduction section, *in vitro* assays play a crucial role in the biopharmaceutical assessment of APIs. During the past two decades, biorelevant media became an indispensable tool to forecast the *in vivo* solubility and dissolution of pharmaceutical drug candidates, and to assess absorption risks like low solubility or API precipitation. Nevertheless, *in vitro* set-ups are still a simplification of the conditions in the human GI tract. This thesis aimed to shed light on some of the remaining open questions, aiming at providing a better understanding of the effects of biorelevant media on solubility, dissolution, and precipitation processes, and providing guidance for a more streamlined usage in the future. The results of this work can be outlined in brief as follows:

- First, a new DoE-based method development was introduced which increased the robustness and accuracy of derivative UV spectrophotometric methods for API quantification in biorelevant precipitation assays.
- Second, based on this new approach, the impact of SIF powder aging on the supersaturation and precipitation behavior of the model drug ketoconazole was investigated. Recommendations on the use of biorelevant media for precipitation assays were developed to further improve the reproducibility of transfer experiments and to enhance data reliability.
- Third, it was investigated under which circumstances the physiological bicarbonate buffer should be applied to FaSSIF medium for *in vitro* solubility, dissolution, and precipitation testing to resemble the *in vivo* conditions.

As pointed out in the discussion, some questions regarding the predictive power of the media for *in vivo* still remain open. These topics should clearly be addressed in future studies. To conclude about the predictive power of differently buffered biorelevant media for *in vivo* dissolution processes, a starting point could be solubility and dissolution testing in HIF aspirates under dynamic pH and carbon dioxide adjustment to better represent the dynamic *in vivo* conditions in the GI tract and thus to gain a deeper understanding of the *in viv*o dissolution processes as compared to static thermodynamic solubility testing in HIF. In terms of aged versus fresh SIF powder, a more mechanistic understanding regarding the impact of colloidal structures on the precipitation kinetics of APIs in biorelevant media versus HIF is necessary. Clarifying the impact of different colloidal structures, their compositions, and concentrations could help to guide the design and selection of *in vivo* predictive biorelevant media for precipitation testing.

The translation of the generated *in vitro* data to actual *in vivo* absorption predictions using *in silico* tools (see *Fig. 1*) was not covered in this thesis. On the one hand, *in silico* models may be a supporting tool to guide the selection of the most *in vivo* predictive experimental set-up, for instance, by comparing absorption predictions based on differently generated *in vitro* data, like precipitation kinetics from freshly opened vs. aged SIF powder batches. On the other hand, a thorough understanding of the *in vitro* processes and their limitations is required when input parameters from *in vitro* experiments are used for *in silico* modelling. Likewise, the specific impact of the *in vitro* parameters on the *in silico* prediction should be well understood.

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6. Research paper

6.1 Increasing the robustness of biopharmaceutical precipitation assays - Part I: Derivative UV spectrophotometric method development for in-line measurements

Published in the Journal of Pharmaceutical Sciences, 2022, 111 (1), pp. 146-154, DOI: 10.1016/j.xphs.2021.08.025

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Journal of Pharmaceutical Sciences 111 (2022) 146–154

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmaceutics, Drug Delivery and Pharmaceutical Technology

Increasing the Robustness of Biopharmaceutical Precipitation Assays – Part I: Derivative UV Spectrophotometric Method Development for in-line Measurements

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ARTICLE INFO

Article history:
Received 4 May 2021
Revised 25 August 2021
Accepted 25 August 2021
Available online 28 August 2021

Keywords:
Precipitation
FaSSIF
UV spectrophotometry
Method development
In vitro
Dissolution

ABSTRACT

In vitro precipitation assays are often applied to support drug and formulation development. Current methods applied to quantify the amount of dissolved drug, in particular (U)HPLC, require time-consuming sample preparation. Furthermore, small precipitates formed during the nucleation phase may not be removed quantitatively by filtration or centrifugation of the sample. Given the drawbacks of standard (U)HPLC analyses during the application in transfer experiments, it was the aim of this work to develop a robust and simple to implement in-line UV spectrophotometric method which accurately reflects the precipitation profile obtained from *in vitro* transfer assays. Based on the three model compounds cinnarizine, dipyridamole, and ketoconazole, the manuscript describes the development of a design of experiments (DoE) based approach to develop derivative UV spectrophotometric methods accounting for the change in media composition over time due to the dilution of simulated intestinal with simulated gastric fluid. An R script was developed which automatically identifies suitable wavelengths for in-line measurements. As an outcome of this study, a fast, robust, accurate, and specific derivative UV spectrophotometric methodology for measuring the concentration of dissolved drugs in *in vitro* transfer experiments was successfully developed. This method can flexibly be applied to multi-compartmental precipitation assays.

Introduction

Weakly basic drugs typically show high solubility in the acidic, fasted stomach, while their solubility is significantly lower at the rather neutral pH under intestinal conditions. Therefore, these drugs may be prone to precipitation when entering the small intestine, which may impact the rate and/or extent of drug absorption.[1–3] To estimate the precipitation behavior of orally administered weak bases and de-risk formulation development, biopharmaceutical *in vitro* precipitation assays are typically applied.[4, 5] The first *in vitro* transfer model was proposed in 2004 by Kostewicz et al.[5] This model comprises a gastric and a small intestinal compartment, and the drug solution or suspension is transferred from the gastric into the intestinal compartment. To date, various *in vitro* set-ups have been developed, which also account for simultaneous drug absorption during the transfer.[6–8]

Besides the selection of a suitable *in vitro* transfer set-up, a robust analytical method is key for the reliable quantification of the precipitation kinetics of the drug of interest. For this purpose, analytical methods such as high- and ultra-high-performance liquid chromatography (HPLC; UHPLC) are frequently used, because these methods are typically accessible in biopharmaceutic laboratories and thus convenient to apply. However, conducting UV spectrophotometry compared to (U)HPLC analyses may lead to different results. Also, the two methods differ in their sample preparation.[9, 10] For example, when conducting (U)HPLC analyses, the operator is required to draw samples from the intestinal compartment to measure the amount of drug remaining in solution after

Abbreviations: API, Active Pharmaceutical Ingredient; DMSO, Dimethyl sulfoxide; DoE, Design of Experiments; FaSSIF, Fasted State Simulated Intestinal Fluid; HPLC, High-Performance Liquid Chromatography; LC, Liquid Chromatography; QQ plot, Quantile-Quantile plot; RMSE, Root-Mean-Square Error; SDS, Sodium Dodecyl Sulfate; SGF, Simulated Gastric Fluid; SGFsp, Simulated Gastric Fluid sine pancreatin; UHPLC, Ultra-High-Performance Liquid Chromatography.

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https://doi.org/10.1016/j.xphs.2021.08.025
0022-3549/

precipitation has started. Subsequent sample preparation includes filtration or centrifugation to separate dissolved from undissolved drug. Given the often very small particle size of the precipitate, it may be challenging to remove all undissolved particles from the sample. Therefore, (U)HPLC analyses may lead to an overprediction of the amount of drug dissolved and, in turn, an underprediction of the precipitated fraction.[10, 9] Furthermore, the sampling frequency is limited by the time needed to withdraw and prepare the samples, and by the cumulative volume removed from the intestinal compartment.

The application of UV in-line analytics offers significant benefits compared to liquid chromatography (LC) methods such as HPLC and UHPLC. First, the number of sampling points that can be measured over time is markedly higher compared to LC methods, because the sampling frequency is not limited by the time needed to withdraw and prepare the samples. Second, UV spectrophotometry does not require the removal of samples from the set-up, and it thus does not lead to a reduction of the liquid volume available in the intestinal compartment.

On the downside, the method development of an in-line UV spectrophotometric method is comparably complex due to various challenges:

Interference of precipitates with UV light

During the precipitation assay, light scattering effects occur due to the formation of precipitate, leading to a baseline shift which impedes a straightforward evaluation of the UV absorption values.[7] One strategy to overcome this challenge is to measure a range of wavelengths rather than applying just one specific wavelength. Furthermore, derivative UV spectrophotometry can be applied, which eliminates baseline offsets. To date, derivative UV spectrophotometric methods have been developed and published for a number of APIs.[11]

UV absorption of biorelevant media

One key element of biopharmaceutical precipitation assays is the utilization of simulated intestinal fluids like Fasted State Simulated Intestinal Fluid (FaSSIF) to mimic intestinal conditions more closely.[12] Of note, the bile salts and phospholipids contained in biorelevant media themselves are UV-active. During the conduct of a precipitation assay, the concentration of bile salts and phospholipids in the intestinal compartment gradually changes over time due to the dilution with simulated gastric fluid (SGF). As a result, the UV absorption of the medium itself is not constant over time. Ideally, analytical methods which are not impacted by factors other than the UV absorption of the API should be preferred.

Impact of limited API solubility on calibration curves

The ICH guideline "Q2 (R1) validation of analytical procedures" describes methodological requirements to obtain an accurate, specific, precise, and robust method. One of them is to "evaluate a linear relationship across the range of the analytical procedure".[13] This requirement for the calibration curve interferes with the nature of a transfer experiment, where concentrations above the thermodynamic solubility of the drug (i.e., supersaturation) frequently occur.[6] To circumvent this problem, the calibration curve could be constructed by replacing the biorelevant medium used to prepare the analytical standard with a medium in which the API yields sufficient solubility (e.g., organic solvents). This is, however, not recommended, because the UV spectrum of the drug is often influenced by the pH and polarity of the medium and may thus shift (solvatochromism). This, in turn, could lead to wrong analytical results.[14, 15]

In general, precipitation assays are prone to high variances, as seen in a large ORBITO ring study from 2019. For example, the precipitation profiles of indinavir during a two-stage dissolution test varied significantly between the various laboratories. One reason for the differences discussed in the ORBITO study was the utilization of a semi-automated UV system.[16]

To overcome the challenges associated with LC analyses and to satisfy the ICH requirements for the validation of analytical procedures, the aim of this work was to set up a new DoE-based workflow for an in-line UV spectrophotometric method development intended to quantify the amount of dissolved drug in the course of *in vitro* precipitation assays. We aimed to investigate the practicability and method development performance using three model substances, namely ketoconazole, dipyridamole, and cinnarizine. In the second part of this two-part publication,[17] the presented approach is applied to investigate the influence of ageing FaSSIF powder on the precipitation behavior of ketoconazole.

Methods and materials

The DoE-based derivative UV spectroscopy method development and its validation comprises five steps, namely preparations, measurements, data evaluation, model building, and method validation (Fig. 1). The model is built based on API concentrations which are stable for at least the time of analysis (see Measurements section), since it is not possible to measure concentrations above the critical supersaturation concentration without changing the media (see Introduction above). Later, recovery measurements are conducted at concentrations which are close to the critical precipitation concentration (the maximum observed concentration before precipitation occurred; see Validation section). As during the precipitation assay the proportion of simulated intestinal fluid in the acceptor compartment changes over time due to dilution with SGF, this factor is included in the linear model. Subsequently, a model is identified for which the changing concentrations of bile salts and phospholipids (reflected by the percent of FaSSIF powder used to prepare FaSSIF) is not significant (see model building).

Preparations

For each method development, 100 mL simulated gastric fluid sine pancreatin (SGFsp) pH 2.0 were prepared by dissolving 200 mg sodium chloride in 100 ml Milli-Q water. The pH was adjusted to 2.0 with 1 M hydrochloric acid. As simulated intestinal fluid, double-concentrated FaSSIF was prepared.[16] For this, 84 mg sodium hydroxide, 790 mg monobasic sodium phosphate monohydrate, 619 mg sodium chloride, and 448 mg FaSSIF/FeSSIF/FaSSGF powder (biorelevant.com Ltd, United Kingdom) were dissolved in 100 ml purified water. The pH was adjusted to 6.5 with 1 M hydrochloric acid or 1 M sodium hydroxide solution.

Stock solutions with concentrations of 300 μg/ml (cinnarizine), 600 μg/ml (dipyridamole), and 800 μg/ml (ketoconazole) were prepared using SGFsp. All chemicals except for FaSSIF/FeSSIF/FaSGF powder were obtained from Merck KGaA, Darmstadt, Germany, or its subsidiaries.

Based on the FaSSIF solubility of the model compounds (dipyridamole: 30 μg/ml; ketoconazole: 40 μg/ml; cinnarizine: 15 μg/ml; all values rounded [18–21]), a general full factorial design and a respective pipetting scheme were developed. The concentration of the API is the first factor ([API]) with five levels, ranging from 0 μg/ml to a concentration twice as high as the thermodynamic solubility of the drug in FaSSIF (see Discussion). During a typical precipitation assay, the concentration of the simulated intestinal fluid is diluted by 50 % (one part SGF, one part FaSSIF).[9, 5] As a consequence, a second factor with

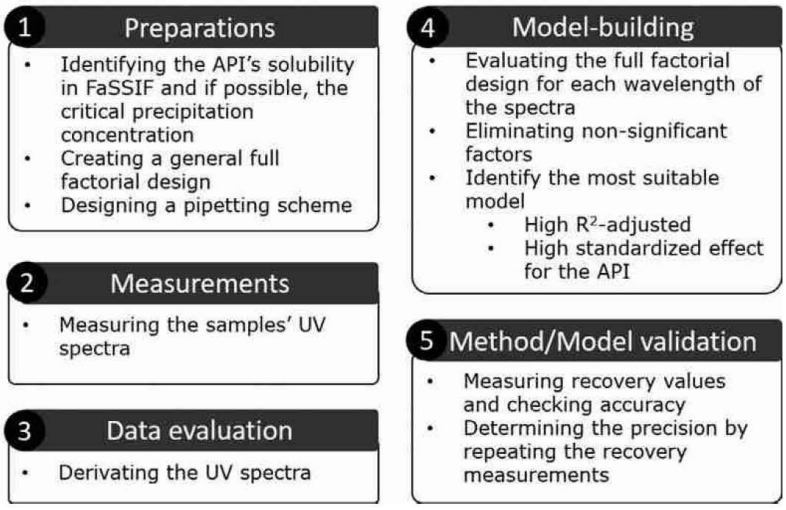

Fig. 1. Workflow overview.

four levels for the FaSSIF concentration was implemented into the model ([FaSSIF]), with the FaSSIF concentration being between 50 % (lower limit) and 90 % (upper limit). FaSSIF concentrations above 90 % are not feasible, since the API stock solution added contains SGF to ensure complete dissolution. Not considering values greater than 90 % during method building is justified, because the FaSSIF concentration is above 90 % only at the beginning of the experiment and decreases in the course of the experiment. Table 1 presents a generic pipetting scheme based on the DoE.

Measurements

The samples were transferred into a 1 mm flow-through cuvette (Flow Cell Quartz 1 mm 113 μL, Agilent Technologies) and measured using a Cary 3500 UV spectrophotometer (Agilent Technologies, Waldbronn, Germany). The scan range was set from 400 nm to 200 nm with an average time of 0.02 s, a data interval of 0.5 nm, a spectral bandwidth of 1.0 nm, and a scan rate of 1500 nm/min. The samples were freshly prepared directly before the measurements according to the pipetting scheme depicted in Table 1.

Table 1
Pipetting scheme based on the full factorial model (sample 1-20) and for the recovery measurements (R1-R4).

Sample	Concentration API [μg/ml]	Concentration FaSSIF [%]	FaSSIF [μl]	SGF pH 2.0 [μl]	stock solution [μl]
1	0 % FaSSIF solubility	50	**2500**	**2500**	**0**
2	50 % FaSSIF solubility	50	**2500**	**2375**	**125**
3	100 % FaSSIF solubility	50	**2500**	**2250**	**250**
4	150 % FaSSIF solubility	50	**2500**	**2125**	**375**
5	200 % FaSSIF solubility	50	**2500**	**2000**	**500**
6	0 % FaSSIF solubility	63.3	**3165**	**1835**	**0**
7	50 % FaSSIF solubility	63.3	**3165**	**1710**	**125**
8	100 % FaSSIF solubility	63.3	**3165**	**1585**	**250**
9	150 % FaSSIF solubility	63.3	**3165**	**1460**	**375**
10	200 % FaSSIF solubility	63.3	**3165**	**1335**	**500**
11	0 % FaSSIF solubility	76.6	**3830**	**1170**	**0**
12	50 % FaSSIF solubility	76.6	**3830**	**1045**	**125**
13	100 % FaSSIF solubility	76.6	**3830**	**920**	**250**
14	150 % FaSSIF solubility	76.6	**3830**	**795**	**375**
15	200 % FaSSIF solubility	76.6	**3830**	**670**	**500**
16	0 % FaSSIF solubility	90	**4500**	**500**	**0**
17	50 % FaSSIF solubility	90	**4500**	**375**	**125**
18	100 % FaSSIF solubility	90	**4500**	**250**	**250**
19	150 % FaSSIF solubility	90	**4500**	**125**	**375**
20	200 % FaSSIF solubility	90	**4500**	**0**	**500**
For the recovery/reproducibility measurements:					
R1	80 % FaSSIF solubility	96	**4800**	**0**	**200**
R2	248 % FaSSIF solubility	70	**3500**	**880**	**620**
R3	500 % FaSSIF solubility	60	**3000**	**750**	**1250**
R4	900 % FaSSIF solubility	50	**2500**	**250**	**2250**

After the measurements, each sample was visually inspected for the absence of precipitates.

Data evaluation

For all UV spectra, the second derivatives were calculated initially by applying the savgol filter of the python package scipy.signal (version 1.6.0, http://www.scipy.org) with a window length of 19, a poly-order of 5, and "interp" as mode.[22] The respective values for each measured wavelength derived from this procedure were used as response factor in a linear model. Utilizing a self-written R script (see supplemental material 1) created with R (version 4.0.2, The R Foundation), a model with the factors [API], [FaSSIF], [API*FaSSIF], [API*API], and [FaSSIF*FaSSIF] was developed and evaluated for each wavelength. For further evaluation, only those models were considered where the coefficient of determination (R^2) was above 0.99 and the [API] was the only significant factor in the model. The models were ranked according to their standardized effect of the API concentration. Beginning with the highest ranked model, it was evaluated whether the residuals were distributed normally. This evaluation was conducted graphically using a QQ plot and by plotting the fitted values against the measured ones. If no suitable wavelength was identified, the procedure was repeated with the first derivatives and, if necessary, with the third derivatives.

Method/model validation

Four recovery measurements were conducted to validate the model. For this purpose, samples with API and FaSSIF concentrations within and outside the range of concentrations used during model building were measured (see Table 1). As some of the API concentrations were nine times higher than the thermodynamic FaSSIF solubility, these measurements were carried out directly after preparation of the recovery standard. The formation of precipitates during the preparation of the recovery standards and the subsequent measurement was excluded by visual inspection.

Subsequently, the root-mean-square error (RMSE) between the recovery measurements and the real concentrations was calculated in the R script (see supplemental material 1). A method was deemed acceptable if the RMSE was below 5 %. The recovery measurements were repeated on another day with freshly prepared samples to check the reproducibility. The same 5 % limit for RMSE was applied.

Results

Figs. 2–4 summarize the models identified as part of this work. For all three model compounds, a suitable wavelength with an accurate, precise, specific, linear, and robust analytical model was determined. A suitable model for ketoconazole was found using the second derivative (243 nm). For dipyridamole and cinnarizine, suitable methods were identified using the first derivative (264.5 nm and 259.5 nm, respectively) (panel A in Figs. 2–4).

As indicated by the Pareto charts, the FaSSIF concentration is a non-significant factor at each model compound's wavelength. Furthermore, no quadratic effects, neither for the API, nor for the FaSSIF concentration, were observed (panel B in Figs. 2–4). These two observations are an important prerequisite for a highly accurate and robust model. The high R^2 values (0.9998 for dipyridamole, 0.9945 for ketoconazole, and 0.9977 for cinnarizine) indicate that the models properly predict the measured data points. The QQ plots in combination with the residual-versus-fitted-values plots indicate that the residuals are normally distributed, with minor outliers for ketoconazole and cinnarizine (panels C and D in Figs. 2–4), for which no relevant influence on the accuracy is expected. The RMSEs obtained for all recovery as well as reproducibility measurements were less than 5 %, underlining the precision of the models (panels E and F in Figs. 2–4). A comparison of the recovery and reproducibility measurements suggests that the deviations are randomly distributed.

Discussion

Many examples of how derivative UV spectroscopy is applied have been previously described in the literature.[11, 23] However, the implementation of UV spectrophotometric methods in biorelevant precipitation assays, as described by Jede et al. in 2019, is comparably new.[9] One of the advantages of derivative UV spectroscopy is that interfering signals caused by precipitates, which may lead to baseline shifts and altered UV spectra, can be avoided. Baseline shifts are eliminated and the absorption bands are sharpened by applying a derivative filter on the spectra.[24] The decision which derivative order should be selected depends on the original band width and how bands are overlapping.[11] However, since the approach presented herein is a screening tool to select the most suitable wavelength for UV spectroscopy analyses, we started with the second derivative per default, because it represents a compromise between a strong elimination of interference on the one hand and sufficient signal strength on the other hand. If no suitable wavelength was identified, the first derivative was applied, followed by the third derivative. With every derivation, the bands decrease in size, and the signal-to-noise ratio increases.[23] As a consequence, the probability to find a suitable wavelength or model in higher order derivations is limited. The influence of the derivation order is exemplified in Fig. 5, which illustrates that the application of a higher degree derivative (in this case, the second derivative) can cause higher variance and differences in the precipitation profiles.

While the application of the analytical method described above is rather simple, the method development itself is associated with various challenges. As described in the Introduction section, the changing concentration of simulated intestinal fluid (e.g., FaSSIF) over time is one factor which needs to be taken into account during UV spectrophotometry method development, because the bile salts and lecithin contained in FaSSIF are UV-active and thus influence the measured UV spectra. To overcome this hurdle, the concentration of simulated intestinal fluid can be accounted for during method development. A full factorial DoE, as described in the Method section, provides the basis to evaluate not only singular effects of API and FaSSIF on the signal, but also interaction terms between the API and FaSSIF. For example, interactions can occur when drug solubilization in micelles affects the UV absorption behavior of the API.[25] When the FaSSIF and API UV spectra are analyzed separately, these interaction terms, along with quadratic factors of the API or FaSSIF concentration, may be overlooked. By applying the approach for UV spectroscopy method development described above, it was possible to identify a suitable wavelength for each of the three model APIs. At this wavelength, only the concentration of API is a significant factor, and interactions or non-linear effects were excluded. Surprisingly, none of the wavelengths identified for the three model APIs was a local UV or derivative maximum or minimum, which is usually used for UV spectroscopy. In the case of cinnarizine, dipyridamole, and ketoconazole, the local extremes could not be used due to overlay and interaction effects with FaSSIF (see supplemental material 2). The use of a local extreme for construction of the calibration curve can have a significant impact on the calculated precipitation curves, as shown in Fig. 6. Although calculated based on the exact same measured UV data, the shape of the precipitation profile, the maximum concentration, and the equilibrium solubility at the end of the experiment differ markedly, depending on the wavelength used during calibration and calculation of the measured concentrations. It should be emphasized that the measurement points 20 minutes after the start of the experiment show the lowest variance for the 244 nm model, which is

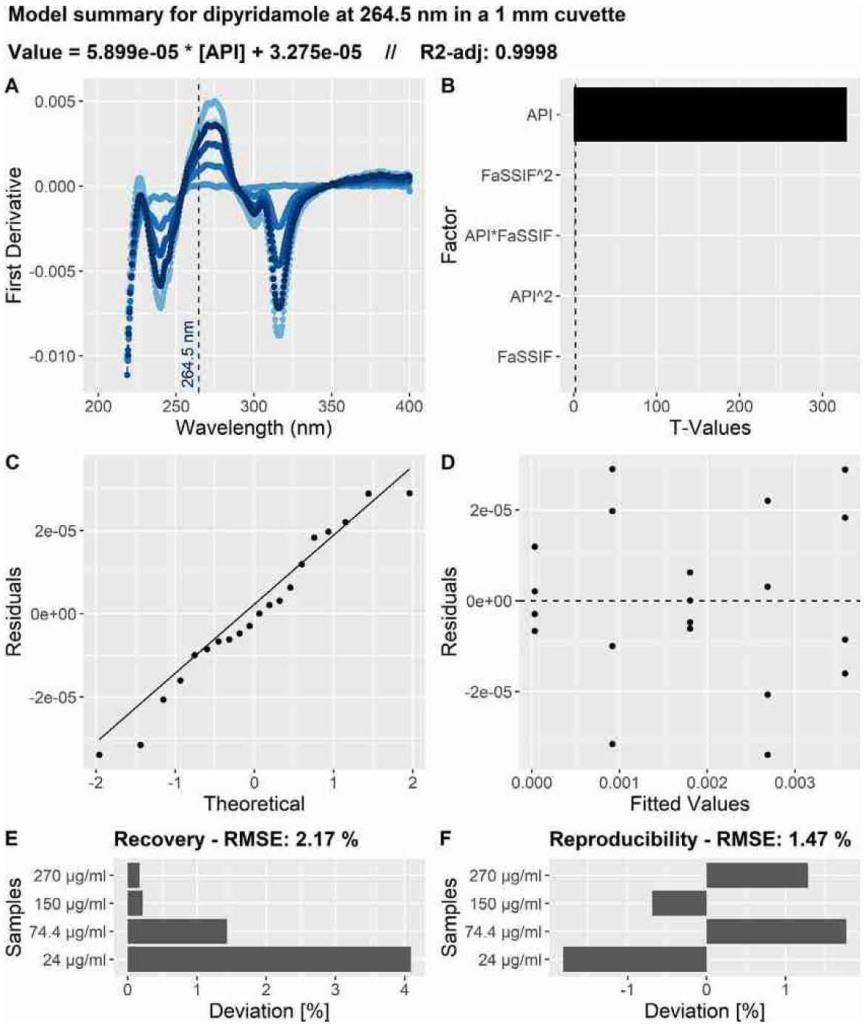

Fig. 2. Model summary for dipyridamole at 264.5 nm. A) First derivatives of the measurements. B) Pareto chart of the standardized effects for the model factors and their (quadratic) products. C) QQ plot of the residuals. D) Residuals versus fits plot. E) Recovery measurements and the observed deviations [%]. F) Reproducibility measurements and observed deviations [%].

another advantage of this method development. Of note, ketoconazole is an example where the impact of the wavelength on the resulting profile is rather high. For cinnarizine and dipyridamole, the impact was less pronounced.

The second major challenge during UV spectroscopy method development is the attempt to fulfill the ICH Q2 (R1) requirement, i.e., that the calibration curve covers all concentrations of interest.[13] It is in the nature of the precipitation assays that the measured concentrations exceed the thermodynamic solubility of the drug (supersaturation). However, the state of supersaturation is metastable and can thus not be used for constructing calibration curves. To circumvent this challenge, the method development described herein follows a compromise. The full factorial model is built using supersaturated API concentrations. However, the concentrations are below the critical precipitation concentration, i.e., they are stable for a certain period of time. Only during the recovery and reproducibility measurements, higher concentrations are used for various reasons. First, drug precipitation in the standards used for constructing the calibration curve would yield incorrect results. Second, as precipitation can occur very fast, the operator should have enough time for conducting the measurements. The absence of precipitation can be confirmed by visual inspection of the samples, or by repeating the UV-measurements after 1 or 2 minutes. If neither the signal, nor the spectrum changes in height and shape, the absence of precipitates

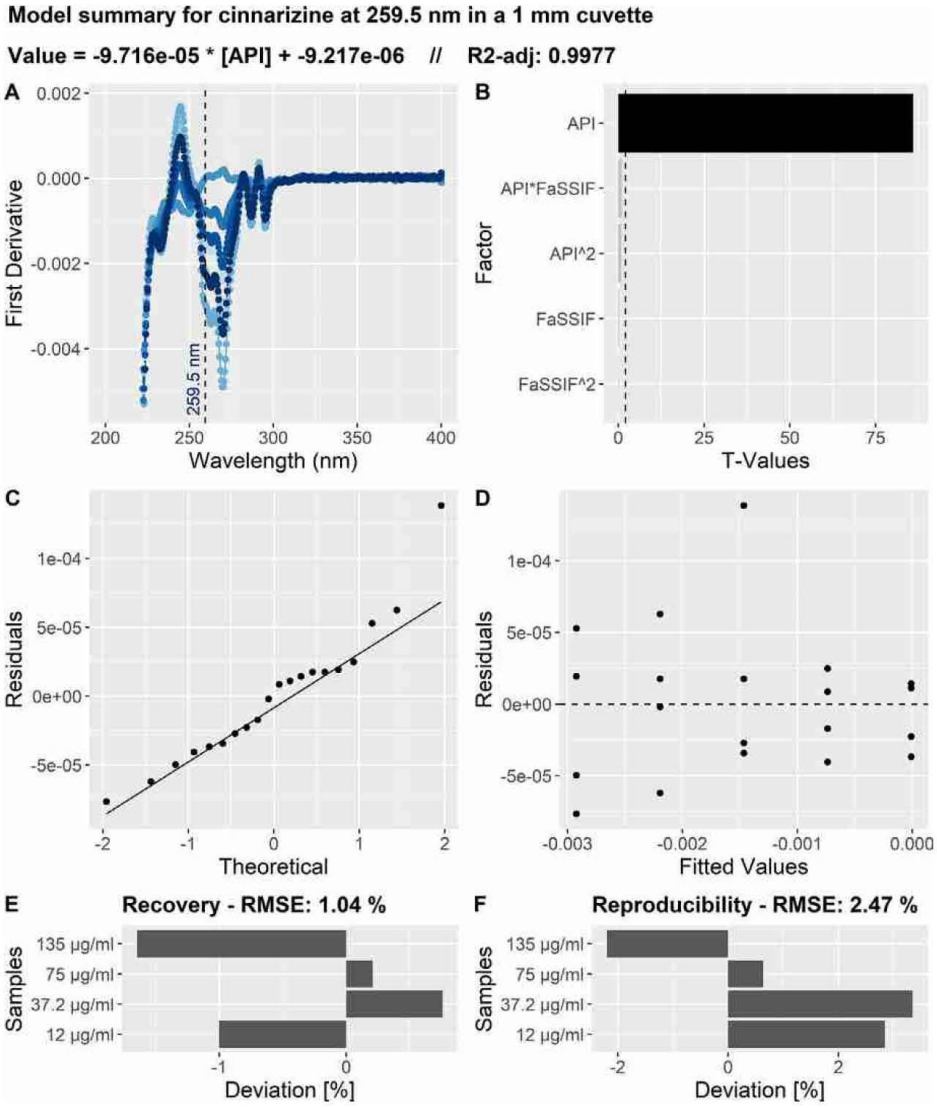

Fig. 3. Model summary for cinnarizine at 259.5 nm. A) First derivatives of the measurements. B) Pareto chart of the standardized effects for the model factors and their (quadratic) products. C) QQ plot of the residuals. D) Residuals versus fits plot. E) Recovery measurements and the observed deviations [%]. F) Reproducibility measurements and observed deviations [%].

can be assumed. If precipitation occurs, lower API concentrations should be used for the recovery measurements.

When constructing the calibration curve, another approach to account for concentrations above the thermodynamic drug solubility in the SGF/FaSSIF mixture would be spiking the medium with an organic solvent (e.g., DMSO), or the addition of a surfactant (e.g., sodium dodecyl sulfate (SDS)).[20] However, according to the authors' experience, even small changes in the composition of the medium may have a significant impact on the resulting UV spectra. This is caused by solvatochromism and altered solubilization properties of the micelles. Organic solvents can impact the hydrogen bonding capacity and the dielectric constant of the solvent, which, in turn, can affect the electronic state of the API.[14] Similarly, the addition of acids or bases leads to pH shifts in the medium, allowing protonation or deprotonation of the API, which results in alterations of the UV spectra (halochromism).[26] By applying a solubilization enhancer (e.g.,

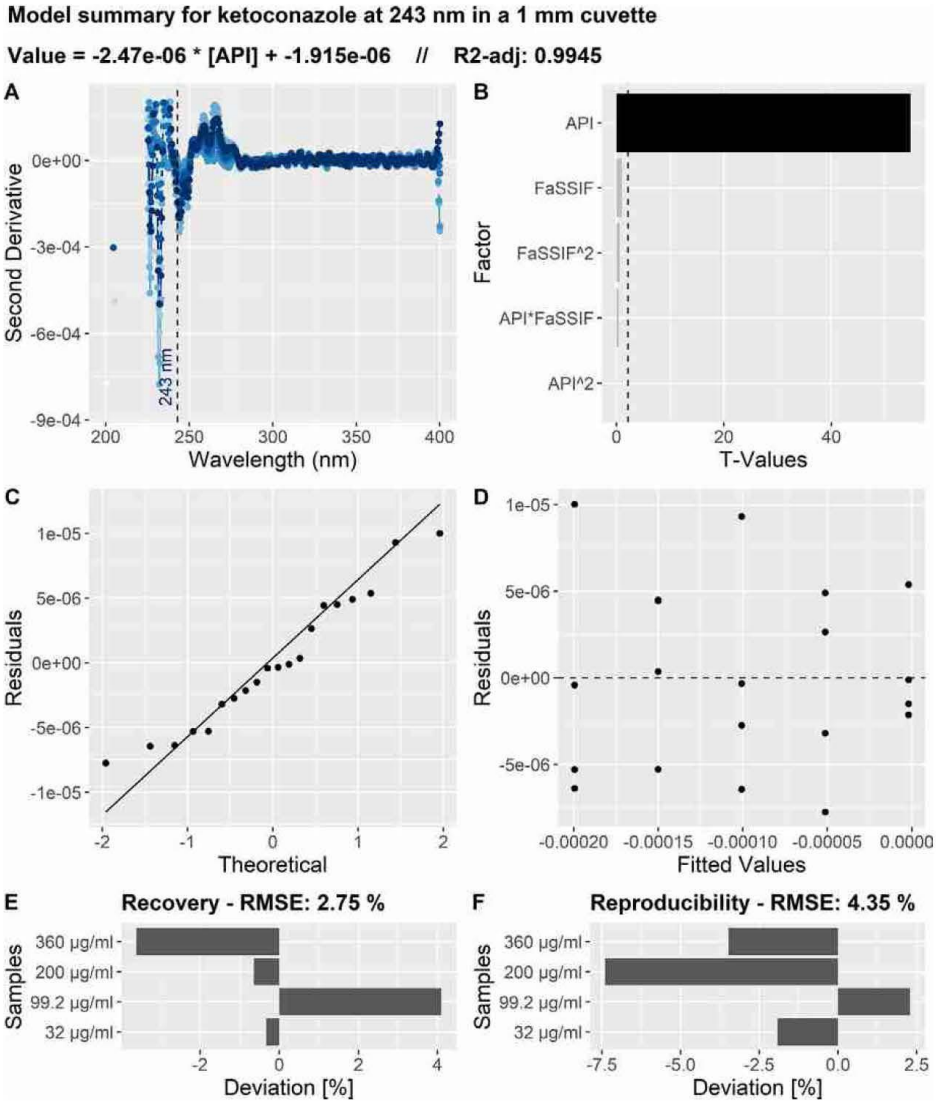

Fig. 4. Model summary for ketoconazole at 243 nm. A) First derivatives of the measurements. B) Pareto chart of the standardized effects for the model factors and their (quadratic) products. C) QQ plot of the residuals. D) Residuals versus fits plot. E) Recovery measurements and the observed deviations [%]. F) Reproducibility measurements and observed deviations [%].

SDS), an additional covariate is added to the model. Solubilizers interact not only with the API, but also with the micelles contained in FaSSIF.[27] As a consequence, this factor would have to be considered in the full factorial model, which would markedly increase the number of experiments (for example, 60 additional measurements if the same number of levels (i.e. 4) would be defined as for FaSSIF). Furthermore, the higher API consumption may be a drawback, e.g., in drug discovery.

Two inherent challenges of the proposed method development are the time-consuming model building and validation steps. For each wavelength, an individual model needs to be generated and verified with respect to its suitability and robustness. Using commercial software tools (e.g., Minitab®), this process may take several hours if model building and factor elimination is conducted manually. As stated above, the medium has a strong influence on the absorption spectrum of an API due to solvatochromism and halochromism

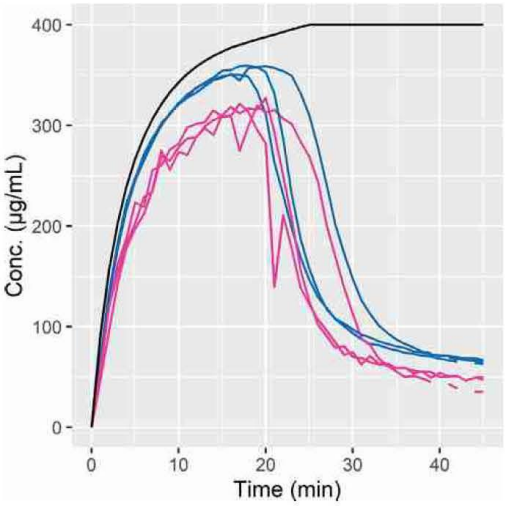

Fig. 5. Results of a ketoconazole ($n = 3$) precipitation assay according to Jede et al.[9] The ketoconazole concentration in the donor compartment was 800 μg/ml, and the drug was dissolved in SGFsp pH 2.0. In the acceptor compartment, double-concentrated FaSSIF was applied. The blue lines were calculated by applying the first derivative of the measured UV spectrum, while the pink lines were calculated by applying the second derivative. The black line represents the theoretical curve. In both cases, the wavelength was 257 nm. The plot is based on data published in an accompanying manuscript ("Increasing the robustness of biopharmaceutical precipitation assays – Part II: Recommendations on the use of FaSSIF")[17].

effects. Consequently, an individual method development would be necessary for each medium investigated, which would markedly increase the time burden. To save time, an R script was developed which semi-automatically builds and evaluates the models. Furthermore, the R script automatically generates the graphical abstracts of the model (e.g., see Figs. 2–4). Only minimum input from the operator is needed, which increases model robustness. Utilizing this script, a suitable method can be identified in a few minutes. The R script is provided in supplement 1. Please note that the R script is only applicable if a wavelength can be identified for which solely the API concentration is significant. Sometimes, as seen for example in the second part of this series of publications,[17] it may not be possible to identify a wavelength at which FaSSIF does not interfere. However, since the FaSSIF concentration at a specific time point can be calculated easily, this problem can be solved by implementing [FaSSIF] into the model. For this purpose, the R script needs to be adjusted minimally.

Finally, the question arises whether LC analyses such as (U)HPLC, which are usually applied on a standard basis, would be more suitable compared to the approach presented herein.[16] Compared to an in-line UV spectrophotometric analysis, (U)HPLC analyses have three drawbacks: First, sampling and sample preparation are time-consuming. As a consequence, the measurement intervals are significantly longer compared to in-line UV analysis, which, in turn, limits the number of samples that can be taken during the precipitation assay. Second, every sample analyzed by (U)HPLC reduces the fluid volume in the intestinal compartment, which further limits the number of samples that can be drawn from the compartment. Additionally, down-scaling of precipitation assays as described by Jede et al. is limited.[9] Third, filtration or centrifugation of the samples is required prior to (U)HPLC analysis. During the nucleation phase of drug precipitation, small particles form that may not be quantitively

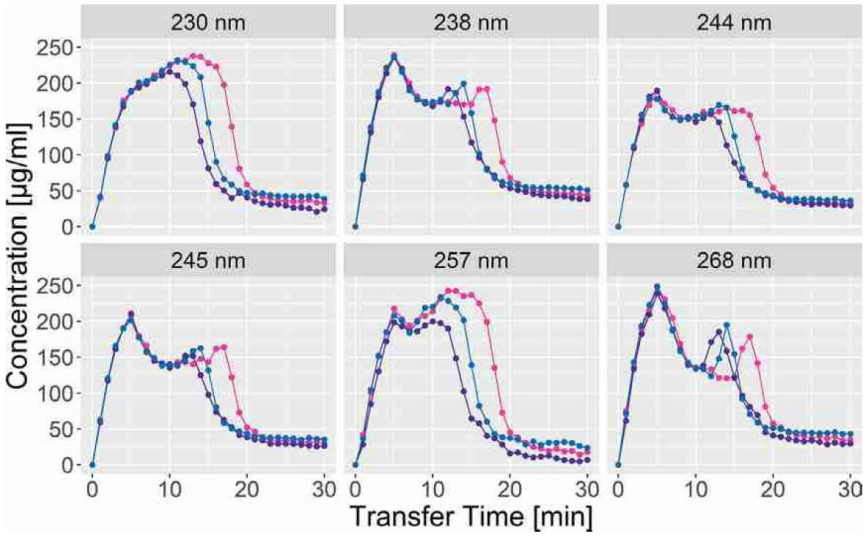

Fig. 6. Results of a ketoconazole ($n = 3$) precipitation assay according to Jede et al. The ketoconazole concentration in the donor compartment was 800 μg/ml, and the drug was dissolved in SGFsp pH 2.0. In the acceptor compartment, normal-concentrated FaSSIF (double buffered) was applied.[9] All six precipitation profiles were calculated using the exact same measured data. However, only the 244 nm profile was evaluated with a built model according to this manuscript. The precipitation curves were calculated with models of the local extremes (230 nm – R^2: 93.8, 238 nm – R^2: 99.7, 245 nm – R^2: 98.8, 257 nm – R^2: 97.3, and 268 nm – R^2: 97.5) without considering 'FaSSIF' as factor. The plot is based on unpublished data. For details about the models please see supplement 2.

separated from the bulk liquid when drawing samples. If these precipitates are not separated prior to the LC analysis, they may dissolve and lead to false results.[9] The application of an automated in-line derivative UV spectrophotometric method enables frequent measurements (up to every 10 seconds), enabling an accurate precipitation profile obtained from the precipitation assay.

To summarize, the combination of derivative UV spectroscopy with a DoE-based method development to select the most suitable wavelength for UV measurements offers a robust method especially for automated in-line measurements applied to precipitation assays. In contrast to conventional UV spectroscopy methods, the selection of an appropriate wavelength takes the changes in FaSSIF concentrations during the conduct of the precipitation assay into account. With the methodology presented in this manuscript, challenges inherent to the sample preparation required prior to (U)HPLC analyses can be circumvented, which saves time and resources and increases accuracy, reproducibility, and robustness.

Conclusion

With the approach presented in this work, robust, accurate, and specific derivative UV spectrophotometric methods for the quantification of drug concentrations in precipitation assays can be identified in a straightforward manner. The DoE-based method development provides a simple solution which takes the impact of the changes in media composition during the transfer into account. By applying semi-automated R scripts, suitable models can be identified within a short time frame and without being biased by the operator. The UV spectrophotometric method presented herein is superior to LC analysis with respect to temporal resolution, detection of small precipitates, loss of sample material, and sample preparation efforts. Moreover, the presented methodology can be applied in all assays with biorelevant media or interfering components where UV spectroscopy is to be used and precipitation complicates method development.

Supplementary materials

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.xphs.2021.08.025.

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6.2 Increasing the robustness of biopharmaceutical precipitation assays - Part II: Recommendations on the use of FaSSIF

Published in the Journal of Pharmaceutical Sciences, 2022, 111 (1), pp. 155-163, DOI: 10.1016/j.xphs.2021.08.026

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Journal of Pharmaceutical Sciences 111 (2022) 155–163

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmaceutics, Drug Delivery and Pharmaceutical Technology

Increasing the Robustness of Biopharmaceutical Precipitation Assays – Part II: Recommendations on the use of FaSSIF

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ARTICLE INFO

Article history:
Received 5 May 2021
Revised 25 August 2021
Accepted 25 August 2021
Available online 27 August 2021

Keywords:
Supersaturation and precipitation
Biorelevant media
Stability testing
Transfer model
Two-stage dissolution
Fasted state simulated intestinal fluid
FaSSIF

ABSTRACT

Biopharmaceutical precipitation assays are an important *in vitro* tool to characterize the precipitation behavior of weakly basic drugs during their transit from the stomach into the small intestine. To mimic the intestinal fluids more closely, biorelevant media like Fasted State Simulated Intestinal Fluid (FaSSIF) and versions thereof are often applied. When applying UV analytics to measure the drug concentration during the transfer experiments, changes in the UV spectrum of the medium have been observed when FaSSIF was stored over a longer period of time or under accelerated conditions. Therefore, this study aimed at evaluating the stability of FaSSIF under various storage conditions. Furthermore, the impact of stressed FaSSIF on the supersaturation and precipitation behavior of ketoconazole was investigated. As a result of this study, it was demonstrated that the FaSSIF powder composition changes during storage, which, in turn, impacts the supersaturation and precipitation behavior of ketoconazole in *in vitro* transfer studies. Based on the results of this study, we provide recommendations on the application of FaSSIF in biopharmaceutical precipitation assays with the aim to increase reproducibility and enhance data reliability for those compounds where changing FaSSIF composition may impact the supersaturation and precipitation behavior.

Introduction

Over the last two decades, *in vitro* precipitation testing became an important tool to characterize the supersaturation and precipitation behavior of weakly basic drugs during their transit from the acidic stomach (donor compartment) to the small intestine (acceptor compartment). Due to their pH-dependent solubility behavior, basic drugs typically exhibit high solubility in the gastric fluid, but low solubility under intestinal conditions. This may lead to precipitation of the drug after oral intake and may subsequently decrease its absorption. To date, several *in vitro* transfer set-ups were published, and these assays differ in terms of the media volumes and composition, the transfer rate and order, and the applied analytical method. While in many cases (U)HPLC ((Ultra) High Performance Liquid Chromatography) analytics are applied to measure the amount of dissolved drug during the precipitation experiment, the advantages of in-line UV spectrophotometry were demonstrated recently.[1,2] In the first part of this two-part publication, we highlight the benefits of using UV spectrophotometry, but also the importance to account for the use of biorelevant media for analytical method development.[2] As a consequence, we introduced a new procedure on how to perform UV spectrophotometric method development for biorelevant transfer assays using a design of experiment (DoE) based approach.

Biorelevant media like Fasted State Simulated Intestinal Fluid (FaSSIF) are typically applied to resemble the *in vivo* conditions found in the intestine more closely in *in vitro* set-ups[3]. As FaSSIF is designed to mimic human intestinal fluids (HIF) in the upper intestinal tract, it is prepared with a buffer comprising physiological pH and osmolarity. Bile salts and phospholipids are added to mimic the solubilizing capacity of HIF.[4] In case of FaSSIF-V1, the medium can be prepared with a commercially available powder consisting of sodium taurocholate and lecithin (FaSSIF/FeSSIF/FaSSGF powder[5]) As lecithin is a product from natural sources and contains unsaturated fatty acids, problems may occur with respect to the stability of the components.

Abbreviations: ANOVA, analysis of variance; AUC, area under the curve; API, active pharmaceutical ingredient; DLS, dynamic light scattering; DoE, design of experiment; FaSSIF, fasted state simulated intestinal fluids; FeSSIF, fed state simulated intestinal fluids; HIF, human intestinal fluids; LC, liquid chromatography; MS, mass spectrometry; RH, relative humidity; SGF_{sp}, simulated gastric fluid *sine pepsin*; SIF powder, FaSSIF/FeSSIF/FaSSGF powde; UHPLC, Ultra High Performance Liquid Chromatography.

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https://doi.org/10.1016/j.xphs.2021.08.026
0022-3549/

Although the powder is considered stable under recommended storage conditions, oxidation of unsaturated phospholipids was observed to a small extent in FaSSIF and FeSSIF, resulting in an increasing UV signal.[6] Consequently, Kloefer et al. recommend wavelengths above 280 nm for UV-spectrophotometric compound quantification.[6]

Although the powder was stored as recommended, we observed alterations in the UV spectrum of FaSSIF samples during storage, using in-line UV spectrophotometry. Therefore, the aims of this study were threefold: First, to evaluate the stability of FaSSIF powder during storage and under stress conditions. Second, to investigate the impact of FaSSIF stability on the supersaturation and precipitation behavior of ketoconazole. Third, to propose recommendations on the use of FaSSIF to increase the reproducibility of precipitation assays.

Materials and Methods

Materials

Sodium dihydrogen phosphate monohydrate, sodium hydroxide, sodium chloride, and hydrochloric acid 1 M were purchased from Merck Millipore (Darmstadt, Germany). For the transfer experiments, ketoconazole was purchased from Caesar & Lorentz GmbH (Hilden, Germany), and for the solubility measurements, ketoconazole was purchased from Sigma-Aldrich (Darmstadt, Germany). FaSSIF/FeSSIF/FaSSGF-powder was purchased from biorelevant.com Ltd (London, United Kingdom). LC-MS-grade isopropanol, methanol, trifluoroacetic acid and acetonitrile were obtained from Merck KGaA (Darmstadt, Germany), and formic acid from VWR International LLC (Radnor, PA, USA). A phosphatidylcholine soybean standard as European pharmacopoeia reference standard was purchased from Sigma-Aldrich (Darmstadt, Germany).

Media for Transfer and Solubility Experiments

For the solubility experiments, FaSSIF-V1 was prepared with FaSSIF/FeSSIF/FaSSGF-powder (SIF powder) according to the instructions from biorelevant.com. In case of the transfer experiments, the phosphate buffer concentration was doubled to prevent a decrease in pH during the transfer, as suggested by Jede et al.[7] Further transfer experiments were performed with FaSSIF which was prepared with higher SIF powder concentrations (150 and 200%) to account for the dilution during the transfer with simulated gastric media. For FaSSIF preparation, different SIF powder batches were used (see Table 1) and for some experiments, stressed SIF powder samples were used for media preparation (see section 2.3). As noted in Table 1, batches FFF-0120-A and FFF-1020-B were used at different time points after first opening of the SIF powder containers. However, batches were used at least 11 months before their stated expiry date. Samples of batch FFF-0119-A were tested over a longer period of time until the expiry date was reached.

For the gastric compartment in the transfer experiments, simulated gastric fluid *sine pepsin* (SGF_{sp}) was applied. The pH was adjusted to 2.0 to keep the pH in the acceptor compartment at a physiological level, as formerly proposed by Jede et al.[7]

Stability Studies

While FaSSIF was always prepared freshly upon use, for some experiments the SIF powder used for media preparation was stored under conditions deviating from the recommended storage conditions to accelerate the degradation process. Therefore, SIF powder was weighed into a volumetric flask and (1) exposed to light stress by applying visible and UV light according to ICH guideline Q1B requirements using the SUNTEST CPS+ (Atlas Material Testing Technology, Mount Prospect, IL, USA), (2) stored in an open volumetric flask for 2 and 4 weeks in a Binder KBF 240 climate chamber (Binder GmbH, Tuttlingen, Germany) at 40°C and 75% relative humidity (RH). Stressed SIF powder samples were not used for every experiment, see Table 1 for more details.

Solubility Experiments

Solubility of ketoconazole was tested in $FaSSIF_{sol\text{-}ref}$, $FaSSIF_{sol\text{-}ref\text{-}8m}$, $FaSSIF_{sol\text{-}40/75\text{-}2w}$ (see Table 1 for details). 10 ml FaSSIF, preheated to 37°C, was added to 1.2 mg ketoconazole, and samples were stirred with 150 rpm and heated at 37°C in an incubator for 25 h. Additional sampling points were collected to ensure that the equilibrium was already reached after 25 h. Samples of 0.5 ml were collected and directly centrifuged with 21382 g at 37°C for 2 min. The supernatant was diluted with extraction medium, followed by UHPLC analysis with a gradient starting from 95% mobile phase A (water, 0.1% trifluoroacetic acid) changing to 95% mobile phase B (acetonitrile) within 2.5 min, and a total run time of 4 min using a Waters ACQUITY™ UPLC™ H-Class PLUS system (Waters Corporation, Milford, MA, USA) and a Waters AQUITY UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm, 130Å, Waters Corporation).

The experiments were performed as n = 1 in each tested medium on three individual days, resulting in a total number of n = 3.

Transfer Experiments

Transfer experiments were performed using the small-scale transfer model developed by Jede et al., which is a 1:10 down-scale of the physiological fluid volumes [1]. In detail, two 50 ml Erlenmeyer flasks were connected with a peristaltic pump (Reglo ICC, Ismatec, Cole-Parmer GmbH, Wertheim, Germany) and transfer tubes (Tygon-LMT-55, Ismatec). 24 mg ketoconazole were dissolved in 30 ml SGF_{sp} (donor compartment) and stirred with a magnetic stirrer at 150 rpm, resulting in a clear solution. Afterwards, 25 ml of the solution (containing 10% of the human dose) were transferred into 25 ml FaSSIF (acceptor compartment). To mimic gastric emptying in fasted humans as closely as possible first order kinetics with a half-life of 5 min were selected.[7] The transfer rate of the peristaltic pump was controlled by a self-programmed software (Python Software Foundation, version 3.7[8]). The 25 ml donor solution were transferred within 28 min. In parallel, samples were withdrawn automatically every minute through a stainless steel inlet filter (2 μm) using a second peristaltic pump, and UV spectra were recorded in a 1 mm quartz flow cell with the Cary 60 UV-VIS spectrophotometer (Agilent Technologies, Santa Clara, CA, United States). Afterwards, samples were recycled into the acceptor compartment. The experiment was carried out in an incubator at 37°C.

The transfer experiments were performed with $FaSSIF_{long}$, $FaSSIF_{ref}$, $FaSSIF_{light}$, $FaSSIF_{40/75\text{-}2w}$, $FaSSIF_{40/75\text{-}4w}$, $FaSSIF_{150\%\text{-}ref}$, $FaSSIF_{200\%\text{-}ref}$, $FaSSIF_{150\%\text{-}40/75\text{-}2w}$, and $FaSSIF_{200\%\text{-}40/75\text{-}2w}$ (see Table 1 for more details).

For $FaSSIF_{long}$, $FaSSIF_{ref}$, $FaSSIF_{light}$, $FaSSIF_{40/75\text{-}2w}$, and $FaSSIF_{40/75\text{-}4w}$, additional placebo transfer experiments (without ketoconazole) were performed to record the changing background signal deriving from the altering media composition.

All transfer experiments were performed in triplicate. To account for the high variability which is often associated with transfer experiments, and to improve data reliability, the transfer experiments performed with $FaSSIF_{ref}$, $FaSSIF_{light}$, $FaSSIF_{40/75\text{-}2w}$, and $FaSSIF_{40/75\text{-}4w}$ were performed as n = 1 in parallel in three individual experiments, resulting in n = 3. The same approach was applied for the comparison between $FaSSIF_{150\%\text{-}ref}$, $FaSSIF_{200\%\text{-}ref}$, $FaSSIF_{150\%\text{-}40/75\text{-}2w}$, and $FaSSIF_{200\%\text{-}40/75\text{-}2w}$.

Table 1
Media Preparation With Differently Stored SIF Powder for Transfer, Solubility, and LC-MS Experiments, and UV Spectrophotometric Method Development.

Experiment	Storage	Medium Abbreviation	Batches used	Note
Transfer experiments during long-term storage at recommended conditions	As recommended[a]	$FaSSIF_{long}$ + X months opened	FFF-0119-A	Transfer experiments were conducted every three months until expiry (15 months)
Transfer experiments and UV spectrophotometric method development with higher SIF powder concentrations	As recommended[a], open for 3 months	$FaSSIF_{200\%\text{-}ref}$	FFF-1020-B	150% and 200% SIF powder concentration compared to standard FaSSIF
		$FaSSIF_{150\%\text{-}ref}$		
	2 weeks 40°C, 75% RH	$FaSSIF_{200\%\text{-}40/75\text{-}2w}$		
		$FaSSIF_{150\%\text{-}40/75\text{-}4w}$		
Transfer experiments and UV spectrophotometric method development with reference and stressed SIF powder, DLS measurements	As recommended[a]	$FaSSIF_{ref}$	FFF-0120-A	
	Light stress[b]	$FaSSIF_{light}$		
	2 weeks 40°C, 75% RH	$FaSSIF_{40/75\text{-}2w}$		
	4 weeks 40°C, 75% RH	$FaSSIF_{40/75\text{-}4w}$		
Solubility experiments	As recommended[a], open for 3 months	$FaSSIF_{sol\text{-}ref}$	FFF-1020-B	Normal concentrated phosphate buffer was used to prepare the media
	As recommended[a], open for 8 months	$FaSSIF_{sol\text{-}ref\text{-}8m}$	FFF-0120-A	
	2 weeks 40°C, 75% RH	$FaSSIF_{sol\text{-}40/75\text{-}2w}$	FFF-1020-B	
LC-MS experiments	As recommended[a], open for 1 month	SIF-ref1m	FFF-1020-B	SIF powder was dissolved in methanol instead of a phosphate buffer
	As recommended[a], open for 6 months	SIF-ref6m	FFF-0120-A	
	As recommended[a], open for 8 months	SIF-ref8m	FFF-0120-A	
	As recommended[a], open for 15 months	SIF-ref15m	FFF-0119-A	
	Light stress[b]	SIF-light	FFF-0120-A	
	2 weeks 40°C, 75% RH	SIF-40/75-2w		
	4 weeks 40°C, 75% RH	SIF-40/75-4w		

FaSSIF, Fasted State Simulated Intestinal Fluid; SIF powder, FaSSIF/FeSSIF/FaSSGF-powder; RH, relative humidity.
[a] Stored in the original closed container at 5°C.
[b] SIF powder was exposed to visible and UV light according to ICH Q1B.

Quantitative Determination of Ketoconazole

For the determination of ketoconazole concentrations, a newly developed UV-based method was applied. The underlying science of this method is described in detail elsewhere.[2]

Ketoconazole concentrations were determined in the transfer experiments in $FaSSIF_{long}$, $FaSSIF_{ref}$, and the stressed samples using the Cary 60 UV-spectrophotometer equipped with 1 mm quartz flow cells. Calibration measurements were conducted between 210 and 290 nm. Five different concentration levels from 0 to 100 μg/ml ketoconazole and three different FaSSIF concentrations from 50 to 90% were used for the DoE-based method development.

For the transfer experiments with $FaSSIF_{150\%}$ and $FaSSIF_{200\%}$, measurements were performed with the Cary 60 in 1 mm quartz flow cells and between 200 and 400 nm. Five different concentration levels from 0 to 100 μg/ml ketoconazole and four different FaSSIF concentrations from 75 to 180% were used for the DoE based method.

Details on the applied media are given in Table 1.

DLS Measurements

Dynamic light scattering experiments (DLS) were performed to determine the size of colloidal structures in $FaSSIF_{ref}$, $FaSSIF_{light}$, $FaSSIF_{40/75\text{-}2w}$, $FaSSIF_{40/75\text{-}4w}$, $FaSSIF_{150\%\text{-}ref}$, $FaSSIF_{150\%\text{-}40/75\text{-}2w}$, $FaSSIF_{200\%\text{-}ref}$, and $FaSSIF_{200\%\text{-}40/75\text{-}2w}$. Experiments were performed using a Malvern Nano ZS Zetasizer (Malvern Panalytical Ltd, Malvern, United Kingdom) with 173° backscatter at 25°C. The samples were measured with automated measurement position and attenuator in disposable semi-micro cuvettes.

LC-MS Experiments

To investigate changes in SIF powder composition during storage, LC-MS experiments were conducted. As SIF powder is composed of sodium taurocholate and soybean lecithin, an HPLC method was selected separating soy phosphatidylcholine, which is one of the main phospholipid fractions in soy lecithin.[9,10] The chosen analytical method uses an isocratic mobile phase with isopropanol, deionized water, and methanol (70:22:8), 0.5 ml flow rate and a Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 5 Micron, Agilent Technologies).[11] Chromatograms were recorded at 205 nm. In the current study, experiments were performed using an Agilent 1200 HPLC system with a DAD detector. For subsequent mass spectrometry (MS) and MS^n experiments, the HPLC system was coupled with a Bruker amaZon SL mass spectrometer (Bruker, Billerica, MA, United States), comprising electron-spray-ionization and an ion-trap detector. For fragmentation helium gas was applied to the samples. The neutral mobile phase was not compatible with the MS analysis (due to decreasing signal during sequence). Hence, 0.5% formic acid was added to the mobile phase, which resulted in consistent mass signals. Due to the UV cut-off of formic acid, the UV chromatograms were recorded with the neutral mobile phase. To prove linearity of the UV analytical method, a calibration curve was prepared with a soybean phosphatidylcholine standard between 10 and 1000 μg/ml. Chromatograms were analyzed using the Bruker Compass DataAnalysis 4.4 SR1 software, and for fragment characterization the software Mass Frontier™ 1.0 (High Chem. Ltd., Bratislava, Slovakia) was applied.

Data Evaluation and Statistical Analysis

To evaluate the differences between the observed transfer profiles using $FaSSIF_{ref}$, $FaSSIF_{light}$, $FaSSIF_{40/75\text{-}2w}$, and $FaSSIF_{40/75\text{-}4w}$, the $AUC_{0\text{-}40min}$ (area under the curve) was calculated from the transfer profiles applying the trapezoidal rule. Additionally, c_{max},

Table 2
Derivative UV Methods for in-line Analytics in 1 mm Quartz Flow Cells.

Medium	Derivative	Wavelength [nm]	Significant model factors for regression equation	R^2-adj. [%]	RMSE (Maximum deviation), recovery [%]
$FaSSIF_{ref}$	First	253	API, FaSSIF	1.0	1.7 (< 3%)
$FaSSIF_{light}$	First	259	API, FaSSIF, FaSSIF*FaSSIF	0.997	6.0 (< 9%)
$FaSSIF_{40/75\text{-}2w}$	First	261	API, FaSSIF	0.989	3.0 (< 4%)
$FaSSIF_{40/75\text{-}4w}$	Second	238	API	0.836	5.6 (< 10%)
$FaSSIF_{200\%\text{-}ref}$	First	257	API, FaSSIF	0.999	1.8 (3%)
$FaSSIF_{200\%\text{-}40/75\text{-}2w}$	Second	270	API, FaSSIF, API*FaSSIF	0.984	6.5 (< 10%)

FaSSIF, Fasted State Simulated Intestinal Fluid, for further details see Table 1; RSME, root-mean-square-error; API, active pharmaceutical ingredient.

corresponding t_{max}, and the time at which the first precipitation processes started (t_p) were derived from the graph. With these values, a one-way analysis of variance (ANOVA) was performed. Dunnett's method was selected as post-hoc test with $FaSSIF_{ref}$ as control group. The significance level was set to 0.05. The same approach was applied for the DLS measurements and to compare the solubility of ketoconazole in $FaSSIF_{sol\text{-}ref}$, $FaSSIF_{sol\text{-}ref\text{-}8m}$, and $FaSSIF_{sol\text{-}40/75\text{-}2w}$.

Unpaired t-tests were applied with a significance level of 0.05 to compare the $AUC_{0\text{-}40min}$, c_{max}, and t_{max} from the transfer experiments using $FaSSIF_{150\%\text{-}ref}$ and $FaSSIF_{150\%\text{-}40/75\text{-}2w}$, and $FaSSIF_{200\%\text{-}ref}$ and $FaSSIF_{200\%\text{-}40/75\text{-}2w}$. Equal variances were assumed, and a Benjamini-Hochberg correction was applied to control the false discovery rate.[12]

Results

Method Development

Derivative UV methods were developed for ketoconazole in different FaSSIF samples, using the DoE approach described above and elsewhere in detail.[2] Primarily, it was intended to develop a model which can quantify API (active pharmaceutical ingredient) concentrations in fresh as well as in stressed FaSSIF samples. Unfortunately, the influence of aging FaSSIF was a significant factor in model building over the complete measured wavelength range. Therefore, an individual method was developed for each FaSSIF species. Details can be found in Table 2. It should be noted that, with increasing FaSSIF UV absorption (see Figs. 2A and 3A), the determination of a suitable wavelength became more complex, which is also the reason that deviations higher than 5% were accepted for the recovery measurements as well as R^2-adj.-values below 0.99 and the implementation of interaction terms.

Transfer Experiments

Impact of SIF Powder Stability on UV Absorption and Transfer Behavior of Ketoconazole

Placebo transfer experiments were performed using the same batch of SIF powder over the course of 15 months, starting approximately one month after opening the SIF powder container. During this time, the SIF powder was stored as recommended in a closed container at 5°C. However, changing UV spectra were observed in the placebo transfers. As displayed in Fig. 1A, UV absorption is

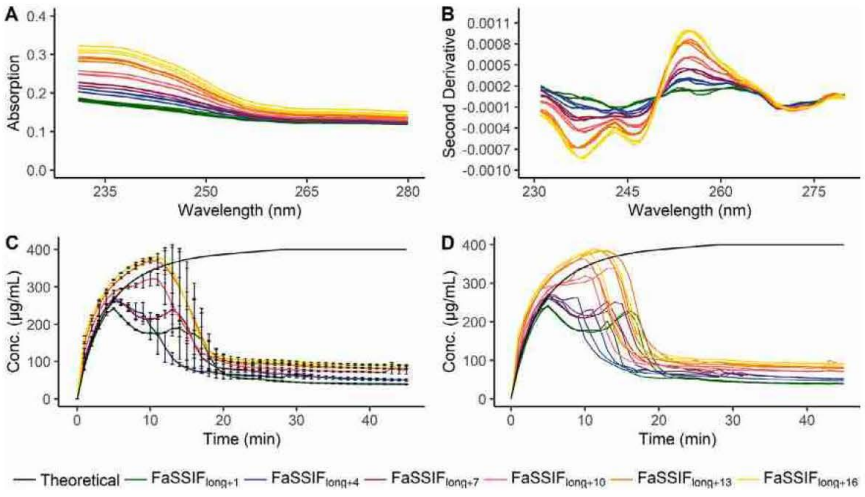

Figure 1. Impact of long-term storage on the UV spectrum of SIF powder and ketoconazole precipitation behavior. A, UV absorption measured during placebo transfer experiments in equilibrium between SGF and FaSSIF (1:1). B, Second derivatives calculated from the absorption profiles displayed in A. C, Mean transfer profiles of ketoconazole, error bars indicate the standard deviation (n = 3). D, Single transfer profiles for n = 3. For the theoretical profile, no precipitation is assumed.
FaSSIF: Fasted State Simulated Intestinal Fluid, for further details see Table 1.

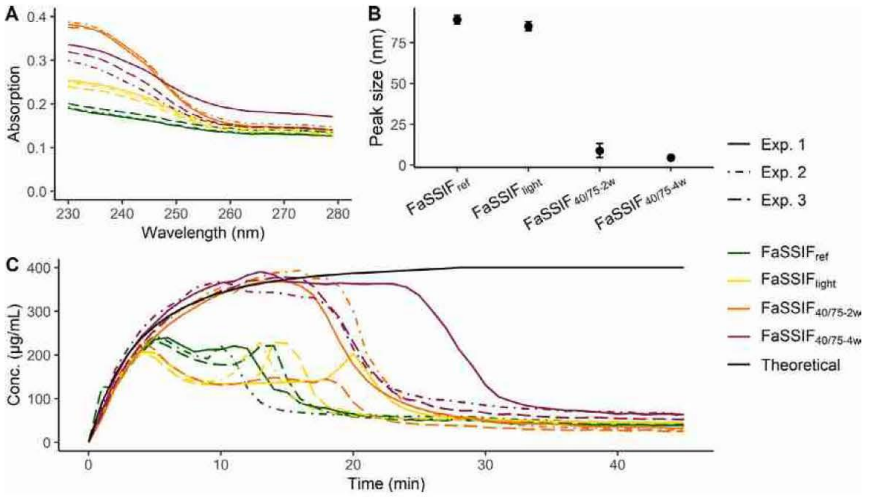

Figure 2. Impact of stress conditions on SIF powder. A, UV absorption measured during placebo transfer experiments in equilibrium between SGF and FaSSIF (1:1). B, Size of colloidal structures measured in FaSSIF prepared with differently stored SIF powder, error bars indicate the standard deviation between the samples (n = 3). C, Transfer profiles of ketoconazole. For the theoretical profile, no precipitation is assumed.

FaSSIF: Fasted State Simulated Intestinal Fluid, for further details see Table 1; Exp.: experiment

continuously increasing during storage, with a maximum between 230 and 245 nm.

According to the changes in UV spectra, differences in supersaturation and precipitation behavior of ketoconazole between the experiments were observed (Fig. 1C). Using FaSSIF from the freshly opened container, ketoconazole exhibits a characteristic transfer profile with initial precipitation in the first minutes and a second precipitation phase after around 15 minutes before equilibrium is reached. By contrast, transfer experiments with SIF powder which was stored at 5°C and tightly sealed once opened for ten months and longer show increased supersaturation and only one single precipitation onset. The changes in UV absorption of FaSSIF have an impact on the calculated derivative spectra (Fig. 1B) which were utilized for API quantification. The analytical method development was performed with $FaSSIF_{ref}$ which is a freshly opened SIF powder with low UV absorption. As the UV absorption increases with storage time, the calibration curve cannot be used for FaSSIF samples stored for a longer period of time. Therefore, no valid conclusions can be drawn on the absolute differences between the transfer profiles using fresh and older SIF powder samples. To address this shortcoming, SIF powder was actively aged using stress factors like humidity, enhanced temperature, and UV/VIS light. By exposing SIF powder to these stress factors, the measured UV absorption during the placebo transfers increased, with the highest increase after storage for two weeks at 40°C and 75% RH (Fig. 2A). Differences were also found in the transfer profiles of ketoconazole (Fig. 2C). Statistical analyses of the observed transfer profiles proved that the media had a significant impact on AUC, c_{max}, and t_p, with p-values of 0.007, 0.007, and 0.039 (ANOVA), respectively. Applying Dunnett's post-hoc test, the $FaSSIF_{40/75-4w}$ differed significantly from $FaSSIF_{ref}$ in terms of $AUC_{0\text{-}40min}$ and c_{max}, with 2.1- and 1.6-fold higher values for $FaSSIF_{40/75-4w}$ (p-values 0.006 and 0.012). For transfer experiments with $FaSSIF_{40/75-2w}$ no statistically significant differenence was found compared to $FaSSIF_{ref}$ due to an outlier (Exp. 3, see Fig. 3A). This may be the result of spontaneous precipitation caused by, e.g., particle impurities. However, the influence of stressed media on the supersaturation and precipitation behavior of ketoconazole is obvious (Fig. 2C). Like the results obtained from the long-term storage tests, AUC, c_{max}, and the profile shape changed along with increasing duration and intensity of the applied stress factors.

Impact of SIF Powder Concentration on Transfer Behavior of Ketoconazole

To investigate the impact of different SIF powder concentrations on the shape of the transfer profile of ketoconazole, transfer experiments were performed with higher SIF powder concentrations, using a fresh reference batch and stressed SIF powder samples (Fig. 3B). During the second experiment with $FaSSIF_{150\%\text{-}40/75\text{-}2w}$, air bubbles formed in the flow cell interfering with the UV signal. For this reason, minutes 5 to 19 had to be excluded from the graph, and the sample was not used for AUC calculations. Comparing $FaSSIF_{150\%\text{-}ref}$ and $FaSSIF_{150\%\text{-}40/75\text{-}2w}$, AUC and c_{max} differed statistically significant with 1.6- (p-value 0.002) and 1.2-fold (p-value 0.008) higher values in $FaSSIF_{150\%\text{-}40/75\text{-}2w}$, respectively. In case of the double-concentrated FaSSIF, a statistically significant difference was found only for c_{max}, with a p-value of 0.001 and 1.1-fold higher values in $FaSSIF_{200\%\text{-}40/75\text{-}2w}$.

Size of Colloidal Structures in FaSSIF Samples

During the transfer experiments with $FaSSIF_{ref}$, $FaSSIF_{light}$, $FaSSIF_{40/75-2w}$, and $FaSSIF_{40/75-4w}$, the latter two samples did not show opalescence, although opalescence is a characteristic of FaSSIF-V1. Therefore, the size of colloidal structures such as micelles and vesicles was measured in the four samples. For all FaSSIF samples prepared with stressed SIF powder, PDI (polydispersity index) values above 0.1 were calculated. For $FaSSIF_{40/75-2w}$ and $FaSSIF_{40/75-4w}$, this resulted in a high variability of the average particle size, with partially high values above the measurable range. However, only one clear peak was detected in the size range between 0.3 nm and 10 μm. This implied that a second fraction of relatively large colloidal

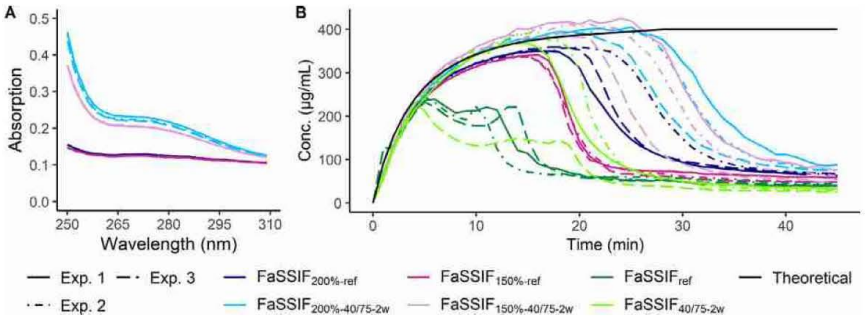

Figure 3. A, UV absorption of FaSSIF samples used for transfer experiments with 150 and 200% SIF powder concentration, displayed in B. B, Transfer profiles of ketoconazole performed with $FaSSIF_{ref}$ and $FaSSIF_{40/75\text{-}2w}$ (100 % SIF powder) and $FaSSIF_{150\%}$ and $FaSSIF_{200\%}$ prepared with differently stored SIF powder. For the theoretical profile no precipitation is assumed.

FaSSIF: Fasted State Simulated Intestinal Fluid, for further details see Table 1; Exp.: experiment

structures was also present in these samples. To compare the size of the colloidal structures in the measured size frame, the size of the detected peak was compared. Results are displayed in Fig. 2B. For the media, an overall statistical significance was found with a p-value < 0.001. $FaSSIF_{40/75\text{-}2w}$, and $FaSSIF_{40/75\text{-}4w}$ were found to differ significantly from the reference (p-value < 0.001), as shown by a post-hoc test.

In case of the higher concentrated FaSSIF samples ($FaSSIF_{200\%}$ and $FaSSIF_{150\%}$), neither the reference nor the stressed samples appeared opalescent, indicating smaller colloidal structures compared to $FaSSIF_{ref}$. DLS measurements clearly displayed a fraction of colloidal structures below 10 nm, but measurements resulted in high PDI values. This is in line with the results obtained from the $FaSSIF_{40/75\text{-}2w}$ and $FaSSIF_{40/75\text{-}4w}$ samples. The high PDI values may be attributed to the presence of a second fraction of colloidal structures which was not clearly detected by the instrument.

Solubility Experiments

The thermodynamic solubility of ketoconazole was found to be comparable between $FaSSIF_{sol\text{-}ref}$, $FaSSIF_{sol\text{-}ref\text{-}8m}$, and $FaSSIF_{sol\text{-}40/75\text{-}2w}$ with values of 25.3, 25.4 and 27.2 μg/ml, respectively (p-value 0.474, ANOVA).

LC-MS Characterization

LC-MS analysis with focus on phosphatidylcholine was carried out to further characterize differences between the differently stored and aged SIF powder samples to a fresh reference. During calibration measurements with a purified phosphatidylcholine standard from soybean, linearity was proven for the main peaks (retention time 13-18 min, marked in Fig. 4 with corresponding molecular masses) between 10 and 1000 μg/ml with R^2 values above 0.9997. Equivalent peaks were also detected in the SIF powder samples. The peak area was found to decrease compared to the reference (6 months open at time point of measurement, SIF-ref6M) with increasing age and stress factor, showing the highest effect after storage of the SIF powder at 40°C and 75% relative humidity for four weeks (SIF-40/75-4w) (Table 3). Additionally, a freshly opened reference batch (1 month) was compared to SIF-ref8M in a sperate experimental run (see Table 3).

Subsequent MS^n analysis identified corresponding structures to the obtained phosphatidylcholine peaks in the UV chromatograms. The structure of phosphatidylcholines include a phosphocholine head group and various fatty acids which are esterified in position sn-1 and -2.[11] As soybean phospholipids are known to be mainly composed of C-16 and C-18 saturated and unsaturated fatty acids, namely palmitic, palmitoleic, stearic, oleic, linoleic, and linolenic

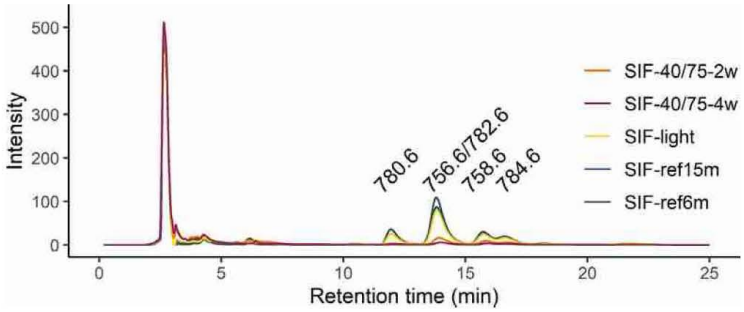

Figure 4. UV chromatograms (205 nm) obtained from the LC-MS analysis of differently stored SIF powder samples. Peaks are marked with corresponding masses from the MS analysis (m/z, $[M+H]^+$).

SIF: FaSSIF/FeSSIF/FaSSGF powder, for further details see Table 1

Table 3
Decrease in Peak Area Obtained During LC-MS Analysis of Several SIF Powder Samples Compared to a Reference.

m/z: $[M+H]^+$	Difference in peak area compared to SIF-ref6m [%]				Difference in peak area compared to SIF-ref1m [%]
	SIF-ref15m	SIF-light	SIF-40/75-2w	SIF-40/75-4w	SIF-ref8m
780.6	-27.6	-29.6	-90.1	-96.1	-6.2
756.6/782.6	-19.2	-26.3	-84.2	-94.3	-11.3
758.6	-17.7	-20.5	-67.9	-86.6	-9.2
784.6	-16.0	-21.2	-67.9	-87.0	-10.6

SIF, FaSSIF/FeSSIF/FaSSGF powder, for abbreviations see Table 1.

acid,[9] the analysis was performed with focus on these fatty acids. The molecular mass m/z 780.6 ($[M+H]^+$) may refer to one linoleic and one linolenic acid residue, while m/z 756.6 ($[M+H]^+$) most likely refers to one saturated C-16 chain (palmitic acid) and linolenic acid. The m/z of 782.6 ($[M+H]^+$) may refer to two linoleic acid chains, however, also one stearic and one linolenic acid residue would be in accordance with the MS^n results. In case of m/z 758.6 ($[M+H]^+$), fatty acid residues most likely refer to palmitic and linoleic acid. The m/z of 782.6 ($[M+H]^+$) again comprises two fatty acids with 18 carbon atoms, which may be oleic and linoleic acid.

During storage, additional peaks were detected in the mass spectra, with retention times between 6.4 and 8.2 min, indicating higher hydrophilicity compared to the phospholipids contained in FaSSIF prepared from fresh SIF powder. Subsequent MS^n analysis of m/z 790.6 and 814.6 ($[M+H]^+$) indicated that these may be peroxidation products of the phosphatidylcholines. However, it was not possible to clarify the complete reaction mechanism. Additionally, in all samples, the mass m/z 520.4 ($[M+H]^+$) was found at retention times between 3.8 and 4.6 min, which may represent a hydrolysis product of a phospholipid, as the presence of lysolecithin is common in lecithin. While fewer degradation products were observed during long term storage, the impact of temperature and humidity stress seemed to have a higher impact on the variety of the masses detected. For instance, in the SIF-40/75-4w sample, m/z 496.4 and 524.4 ($[M+H]^+$) with short retention times were found in addition.

Discussion

The placebo transfer experiments performed as part of this work clearly demonstrate the impact of storage time and conditions of SIF powder on the UV absorption of FaSSIF. As displayed in Fig. 1A and 2A, UV absorption particularly increased between 230 and 245 nm during storage. For the stress conditions, the highest increase in UV absorption was observed after two weeks storage at 40°C and 75% RH.

With respect to the precipitation behavior of ketoconazole, changes in the transfer profile were observed during long term-storage of the SIF powder. While AUC and c_{max} increased over time, the shape of the profile also changed (Fig. 1C). However, based on only these results, it remains questionable whether the changes in the precipitation profile of ketoconazole were evoked by the aging of the SIF powder used to prepare FaSSIF, or if the fact that the increased UV absorption at later sampling points is not properly compensated also contributes to this observation. Therefore, transfer experiments with stressed SIF powder samples were conducted. During these experiments, the increasing UV absorption of FaSSIF during storage was compensated by performing an individual UV method development for each stressed SIF powder sample. Furthermore, these transfer experiments were conducted in parallel for every stress condition as well as the reference to exclude inter-day variability and to reduce external confounding factors such as temperature and humidity. The results of the transfer experiments (Fig. 2C) clearly confirm that the supersaturation and precipitation behavior of ketoconazole is affected by aging of FaSSIF, which resulted in higher c_{max} and AUC values when the SIF powder was stored at increased temperature and humidity. In addition, aging SIF powder was found to have a significant impact on the outcome and accuracy of the analytical method. Consequently, the applied SIF powder needs to be considered during the UV method development as described in the first part of this two-part publication.[2] Although not quantifiable, some of the variation observed in the transfer experiments may be attributed to the process of media stressing which was performed individually for every experiment.

In addition to the impact of aged or stressed SIF powder on the precipitation behavior of ketoconazole, differences were also found in the size of colloidal structures in the media. While FaSSIF is known to be slightly opalescent if colloidal structures in the medium are larger than approximately 50 nm,[6] $FaSSIF_{40/75\text{-}2w}$ and $FaSSIF_{40/75\text{-}4w}$ appeared clear, indicating a decrease in the size of colloidal structures. This was also confirmed by DLS measurements (Fig. 2B). However, DLS measurements also showed high polydispersity in the media containing smaller colloidal structures, which indicates that not only smaller, but also larger structures are present. The measured size values in $FaSSIF_{ref}$ and $FaSSIF_{light}$ are in line with DLS data published in literature for FaSSIF-V1.[13] The size of colloidal structures typically increases during the first two hours after preparation. However, this was not the case for the media prepared with stressed SIF powder, in which the micelles remained small at approximately 5 nm.

In the literature, the presence of several colloidal structures in FaSSIF-V1 is documented, including thread-like, globular, and disc-like micelles, vesicles, and aggregates of different sizes.[6,13] In another study, no vesicles were observed in simulated intestinal fluids.[14] Bile salts typically form small, spherical micelles with low aggregation numbers. As discussed in the literature, these micelles can form larger aggregates (secondary micelles)[15–18] With the addition of lecithin, mixed micellar structures are formed, which change the colloidal structure due to different contribution of lecithin and bile salts on the curvature.[19] As described in the literature, the obtained micellar structures can change to vesicles during dilution, meaning that the shape and size of the colloidal structures are concentration-dependent[20, 21] and further the size of mixed micelles depends on the ratio between bile salts and phospholipids.[22] With respect to the LC-MS results, demonstrating decreasing concentrations of phospholipids during storage at 40°C and 75% RH, it might be reasonable that the smaller colloidal structures measured in $FaSSIF_{40/75\text{-}2w}$ and $FaSSIF_{40/75\text{-}4w}$ are caused by micelles which mainly contain bile salts and are therefore significantly smaller compared to the colloidal structures in $FaSSIF_{ref}$. Nevertheless, it was not possible to clarify the colloidal structure in detail with the applied analytical methods.

However, in the current study, the resulting effect of the colloidal structure on solubility and supersaturation of ketoconazole is of higher interest. Mixed micelles obtained from a mixture of several bile salts and lecithin were shown to inhibit the precipitation of supersaturated telaprevir solutions.[23] By contrast, no effect was observed for sodium taurocholate and lecithin (FaSSIF) compared to

buffer.[23] With respect to the solubilizing capacity, the chain length and saturation number of phospholipids were found to be related to their drug solubilizing capacity.[24] In the current study, the thermodynamic solubility of ketoconazole was not affected by aged FaSSIF, which is in line with the thermodynamic equilibrium after the transfer experiments. According to our data, $FaSSIF_{sol\text{-}ref}$ and $FaSSIF_{sol\text{-}8m}$ also had no relevant impact on the thermodynamic solubility of cinnarizine and dipyridamole (data on file). These results are in accordance with published dissolution experiments that were performed with FaSSIF with different sizes of colloidal structures and preparation methods which resulted in comparable dissolution profiles.[6,25] This implies that the media particularly impact the precipitation kinetics. Using freshly opened SIF powder batches (e.g. $FaSSIF_{ref}$), ketoconazole exhibits a unique transfer profile with an initial precipitation phase within the first minutes and a second precipitation phase after 10 to 15 minutes. The equilibrium solubility was reached at the end of the second precipitation phase. This phenomenon was already observed by Jede et. al. as well as by Zygouropoulou et. al.[7,26] While it was not possible to fully explain this behavior, the precipitation of different polymorphic forms and the transfer being faster than the precipitation rates were discussed as potential reasons in these studies. With respect to the findings of the current study, a significant impact of the colloidal structures in FaSSIF on the precipitation kinetics of ketoconazole may also be reasonable. On the one hand, the change in FaSSIF concentration due to dilution with SGF may be a possible explanation for the two different precipitation processes. On the other hand, this observation may be correlated to a first precipitation process of ketoconazole taking place in or at the surface of the micellar and/or vesicular structures, limiting the particle growth.

Biorelevant precipitation assays are often associated with high variability. This was, for instance, documented in an interlaboratory ring-study, which resulted in low intra-laboratory but high inter-laboratory variability for the supersaturation and precipitation behavior of indinavir.[27] The authors discussed the sample dilution and heterogeneous nucleation due to vessel cleanliness as potential reasons.[27] As the study was performed with different SIF powder samples provided by each partner, an impact of different SIF powder age may also be reasonable.

The differences observed in the transfer behavior can be correlated with a changing SIF powder composition during storage. LC-MS analytics demonstrated decreasing contents of the main phosphatidylcholine components (Table 3). While smaller differences between the tested SIF powder batches (SIF-ref1m, SIF-ref6m and SIF-ref15m) might be explained by the different compositions of the original batches, the stressed samples clearly illustrate the impact of temperature and humidity on the phosphatidylcholine concentrations. With respect to LC-MS analysis, degradation most likely takes place in the form of oxidation of the unsaturated fatty acids and hydrolysis of phospholipids to lysolecithin, while the impact of the stress conditions applied to the samples was higher compared to long-term storage. The presence of peroxidation products in aged and stressed SIF powder samples can be correlated with the increasing UV absorption during storage (Fig. 2A), which was already described in the literature as a result of phospholipid oxidation for freshly dissolved FaSSIF and Fed State Simulating Intestinal Fluid (FeSSIF) over 24 h at room temperature and to a small extent after one year storage of the SIF powder.[6] As displayed in Fig. 2A, UV absorption of $FaSSIF_{40/75\text{-}4w}$ is lower compared to $FaSSIF_{40/75\text{-}2w}$. This observation may be explained with a reduced concentration of the peroxidation products in the four weeks sample due to further reactions of these degradation products. The LC-MS analytical data support this hypothesis as the mass intensity of the mentioned degradation products (m/z 790.6 and 814.6 ($[M+H]^+$) as well as the corresponding UV absorption at 235 nm is reduced in $FaSSIF_{40/75\text{-}4w}$ compared to $FaSSIF_{40/75\text{-}2w}$.

Of note, the analytical focus of this study was primarily set on the composition and molecular characterization of phosphatidylcholine and its degradation products, as it is the main component of soy lecithin.[9] Accordingly, no conclusions can be drawn on other phospholipid fractions like phosphatidylethanolamine, phosphatidylinositol, or phosphatidylserine. Sodium taurocholate, which is also a main component of SIF powder, eluted directly with the injection peak due to its higher hydrophilicity. The respective mass spectra did not indicate any differences in the taurocholate composition. However, a slight decrease in sodium taurocholate content has been reported during storage at enhanced temperature.[6] Therefore, a contribution of the taurocholic acid to the enhanced supersaturation of ketoconazole in stressed and aged FaSSIF cannot be excluded, while the changing lipid fraction most likely impacts the micellar structure and characteristics.

While the structure, composition, and number of micelles seem to be the main driver for increasing the supersaturation of ketoconazole, the question arises if an elevated initial SIF powder concentration can improve ketoconazole supersaturation and diminish differences between stressed and reference SIF powder, because double-concentrated FaSSIF shows a reduced colloidal structure size as observed in aged FaSSIF. Therefore, FaSSIF was prepared with 150% and 200% of the SIF powder needed to prepare standard FaSSIF ($FaSSIF_{150\%}$ and $FaSSIF_{200\%}$). As displayed in Fig. 3B, all transfers resulted in higher supersaturation compared to $FaSSIF_{ref}$. Additionally, the differences between fresh and stressed media (2 weeks 40°C, 75% RH) were reduced especially by using $FaSSIF_{200\%}$. Therefore, the use of $FaSSIF_{200\%}$ may decrease the variability caused by different lipid and micellar compositions. Furthermore, FaSSIF was designed to resemble the fluid in the fasted human intestine based on the composition of HIF aspirates.[4] However, the medium is constantly diluted with gastric medium during the transfer experiment, resulting in lower bile salt and phospholipid concentrations than intended for the human small intestine. Therefore, the advantages of the use of a concentrated medium should be taken into account when selecting the intestinal medium for precipitation assays.[3] Consequently, we recommend the use of double-concentrated FaSSIF for *in vitro* transfer model set-ups with equal volumes of gastric and intestinal media, as this may improve the biorelevance as well as the reproducibility of the assay.

This study aimed at investigating the stability of FaSSIF during storage and to evaluate its impact on the supersaturation and precipitation behavior of ketoconazole. While the impact of aged FaSSIF as well as different FaSSIF concentrations on the transfer behavior of ketoconazole were demonstrated, the impact on other basic compounds was not investigated. It is important to note that ketoconazole shows a unique precipitation behavior in transfer experiments (biphasic precipitation). Hence, it is not clear to which extent the results are applicable also to other compounds. However, the results of the current study point out the influence of biorelevant media in the method development when using UV analytics as well as the importance of using FaSSIF with equivalent absorption spectra, that is, FaSSIF of comparable age.

Based on the results of this study, we provide the following recommendations on the use of FaSSIF to increase the reproducibility and data reliability when conducting transfer experiments:

- The applied biorelevant media should be implemented in the UV method development (see also part I of this two part publication[2])
- For experiments in which comparability and reproducibility of the results need to be ensured, we strongly recommend using SIF powder of consistent age for media preparation. Based on the results of the current study, we propose to use SIF powder from the same bottle within six months after first opening (see Fig. 1).

Whenever possible, newly produced SIF powder batches should be used to keep the amount of degradation products as small as possible. Furthermore, we recommend an intra-laboratory quality control test procedure that includes UV absorption and the size of colloidal structures (see below).

- An intra-laboratory quality control test should be implemented to ensure the correctness of the analytical method and comparability of FaSSIF batches and their UV profiles. Therefore, UV spectra should be measured during UV spectrophotometric method development and later compared to UV absorption of FaSSIF samples used for transfer experiments.
- As the micellar size and structure turned out to be an important characteristic of the media influencing ketoconazole's transfer behavior, DLS measurements of the media may serve as an additional tool for quality control of the media.
- As the experiments performed herein clearly demonstrated the impact of incorrect storage of SIF powder, we recommend to store SIF powder as recommended by the supplier, that is, protected from humidity, and keeping the powder refrigerated.
- We recommend the use of double-concentrated SIF powder, because the concentration of bile salts is closer to the physiological levels in particular after completion of the transfer. Furthermore, the results of this study indicate that the use of double-concentrated SIF powder diminishes the difference in precipitation behavior between fresh and aged SIF powder.

Conclusion

This study shows that the composition of SIF powder used to prepare FaSSIF media changes during storage, which impacts the supersaturation and precipitation behavior of ketoconazole. By contrast, the solubility of ketoconazole was not affected. Based on the study results, recommendations on the application of FaSSIF in biopharmaceutical precipitation assays were developed with the aim to increase reproducibility and enhance data reliability.

Declaration of Competing Interest

None.

Acknowledgements

The authors would like to thank Kristian Beran for his assistance in the lab by performing preliminary stability experiments.

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6.3 The effect of buffer species on biorelevant dissolution and precipitation assays - Comparison of phosphate and bicarbonate buffer

Published in the European Journal of Pharmaceutics and Biopharmaceutics, 2022, 171, pp. 90-101, DOI: 10.1016/j.ejpb.2021.09.009

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Study conception and design; literature research; data acquisition; data evaluation, interpretation, and discussion; drafting of the manuscript

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Collaboration in study conception and design; data evaluation, interpretation, and discussion; revision of the manuscript

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European Journal of Pharmaceutics and Biopharmaceutics 171 (2022) 90–101

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

The effect of buffer species on biorelevant dissolution and precipitation assays – Comparison of phosphate and bicarbonate buffer

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ARTICLE INFO

Keywords:
Biorelevant media
Solubility testing
Dissolution
Bicarbonate buffer
Precipitation

ABSTRACT

Biorelevant solubility and dissolution testing is an important tool during pharmaceutical development, however, solubility experiments performed using biorelevant media often do not properly match the solubility data observed in human intestinal fluids. Even though the bicarbonate buffer is the predominant buffer system in the small intestine, *in vitro* assays are commonly performed using non-volatile buffer systems like phosphate and maleate. In the current study, bicarbonate- and phosphate-buffered biorelevant media were applied to solubility, dissolution, and precipitation testing for a broad range of model compounds. It was found that the medium affects primarily the dissolution kinetics. However, with the knowledge of the unique buffering properties of bicarbonate buffer in the diffusion layer, it was not always possible to predict the effect of buffer species on solubility and dissolution when changing from phosphate to bicarbonate buffer. This once again highlights the special role of bicarbonate buffer for simulating the conditions in the human intestinal fluids. Moreover, it is necessary to further investigate the factors which may cause the differences in solubility and dissolution behavior when using phosphate- vs. bicarbonate-buffered biorelevant media.

1. Introduction

In vitro solubility and dissolution testing are important determinants during the development of new drug candidates, because they are key for candidate selection and bioperformance prediction throughout pharmaceutical development. Although solubility *per se* is simply defined as the amount of a drug that can dissolve molecularly in a specific volume, solubility measurements can become complex, because various determination methods may lead to different results [1]. As *in vitro* dissolution and solubility assays should be as biopredictive as possible, the test conditions need to be selected carefully. One aspect is the selection of the test medium, because its composition, pH, and solubilizing capacity are important factors which determine the outcome of the test.

Thus far, biorelevant media, such as FaSSGF (Fasted State Simulated Gastric Fluid), FaSSIF, and FeSSIF (Fasted/Fed State Simulated Intestinal Fluid) [2–5], are considered to be the gold standard for biorelevant and biopredictive solubility and dissolution testing. The composition of biorelevant media is based on HIF (human intestinal fluids) aspirates, and the media were designed to mimic gastrointestinal pH, bile salt and phospholipid concentrations, and osmolarity as accurately as possible. An overview about important characteristics of HIF and FaSSIF-V1 is displayed in Table 1.

To date, several versions of FaSSIF have been described in literature, and these versions differ in their pH, osmolarity, and nature and composition of bile salts and phospholipids [2–4]. With the newer versions, however, the performance in predicting the HIF solubility of various model compounds has not been improved markedly [4,20,21]. For this reason and to ensure comparability, FaSSIF-V1 still has a high acceptance.

Recently, not only the composition of biorelevant media with respect to their pH, bile salt concentrations, and osmolarity was recognized to be a key determinant for the outcome of a solubility and dissolution study, but also the buffer species and buffer capacity. Of note, current versions of biorelevant media simulating the small intestine employ non-volatile, convenient to apply buffers, such as phosphate and maleate, while the

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https://doi.org/10.1016/j.ejpb.2021.09.009
Received 20 May 2021; Received in revised form 7 September 2021; Accepted 22 September 2021
Available online 27 September 2021

Table 1
Composition and characteristics of fasted HIF and FaSSIF-V1. Depending on the available data, either the mean value, a range, or both are given in the table.

	HIF		FaSSIF-V1
	Duodenum – mean (range)	Jejunum – mean (range)	
pH	6.5 (5.7 7.5) [6]	6.8 (6.1 7.5) [6]	6.5 [2]
Buffer species	Mainly bicarbonate-buffered		Phosphate [2]
Buffer capacity (mmol/l/ΔpH)	(4 19) [7 9]	(2.4 4) [10 12]	(10 12) [13,14]
Osmolarity (mosmol/kg)	197 (137 224) [6,10]	264 (200 300) [6,10]	270 [13]
Bile salts (mM)	3.3 [6]	3 [6]	3 (sodium taurocholate) [2]
Phospholipids (mM)	0.32 (0.003 2.7) [6]		0.75 (lecithin) [2]
Bicarbonate (mM)	(17 20) [15]	(6 20) [15 17]	/
Sodium (mM)	/	142 [16,18]	148
Chloride (mM)	/	(126 135) [16,18]	106
Potassium (mM)	/	(4.8 5.4) [16,18]	/
Calcium (mM)	/	0.5 [18]	/
Surface tension (mN/m)	33.8 (28 46) [6]		54 [19]

FaSSIF-V1, Fasted State Simulated Intestinal Fluid version 1; *HIF*, Human intestinal fluid(s).

main buffer species in HIF is bicarbonate [22].

Regarding the buffer capacity, it is difficult to make reliable statements which buffer capacity should be used in biorelevant media, since the buffer capacity is determined *ex vivo* by titration of aspirates (Table 1) which does not reflect the dynamic, local situation in the intestine.

A bicarbonate buffer differs from other buffer systems through its unique buffering capacity. In the presence of hydrogen ions, sodium bicarbonate, secreted by the exocrine pancreas, forms carbonic acid (Eq. (1)). While the intrinsic pK_a of the ionization reaction of carbonic acid ($([H^+][HCO_3^-]/[H_2CO_3]$, Eq. (2)) is reported to be 3.55–3.8, the apparent bulk pK_a, which refers to the equilibrium between bicarbonate and dissolved carbon dioxide ($[H^+][HCO_3^-]/[CO_2$ aq.]), is measured to be 6.04 [23] using potentiometric titration. As the equilibrium between carbon dioxide and carbonic acid is shifted towards the side of carbon dioxide, carbon dioxide appears as the corresponding acid and therefore, the apparent pK_a is also determined by the hydration and dehydration reaction between carbon dioxide and carbonic acid [24]. However, as reported by Krieg et al. [23], the effective pK_a in the diffusion layer of a dissolving drug particle is lower than 6.04, because the diffusivity of carbon dioxide is much higher than the hydration reaction. This leads to a lower effective buffer capacity in the diffusion layer [23]. Due to the physiological conditions including carbon dioxide permeation through the mucosal membrane on the one hand and bicarbonate secretion on the other hand, *in vivo* bicarbonate buffer is a phase-heterogeneous buffer system with the particularity of having a higher bulk buffer capacity compared to phase-homogeneous systems like phosphate and maleate buffer [25].

$$HCl + NaHCO_3 \rightarrow NaCl + H_2CO_3 \quad (1)$$

gas CO_2 ↕

liquid $CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \rightleftharpoons CO_3^{2-} + 2H^+$ (2)

It has been shown previously that the buffer system and the buffer capacity can have a pronounced impact on the dissolution behavior of ionizable drugs [23,26–30]. One example how a bicarbonate buffer effects dissolution of ionizable compounds was published by Krieg et al. [26]. The authors state that, by using mathematical models, the prediction of the *in vitro* dissolution rate of a specific drug in bicarbonate buffer is possible. Subsequently, the authors matched phosphate and bicarbonate buffer concentrations to reach equal dissolution rates in both buffer systems. Thus, phosphate buffers may be generated, having the same effective buffer capacity in the diffusion layer of a dissolving drug particle like bicarbonate buffer [24,26,31]. According to the published theory, the equivalent buffer concentration of a phosphate buffer depends on the media pH, the pK_a, and the intrinsic solubility of the investigated drug, as well as the diffusion layer thickness [26]. For weakly basic drugs, Krieg et al. proposed to use very low phosphate buffer concentrations (below 2 mM) to match a physiological (approximately 10 mM) bicarbonate buffer [26]. A recently published study by Mudie et al. provides guidance on when to use such a low buffer capacity "equivalent" phosphate buffer, which is based on easy to measure compound characteristics [32]. Unfortunately, this approach is difficult to implement in early drug discovery, because, in this stage of development, these measured physicochemical values can vary from batch to batch. In addition, a huge number of compounds needs to be characterized, so that a "one-fits-all" medium would be more suitable.

The buffer species does not only affect solubility and dissolution of the neat API (active pharmaceutical ingredient), but is also known to impact dissolution of enteric coated formulations [33] and the effect of acidic precipitation inhibitors, such as hydroxypropyl methylcellulose acetate succinate [34]. Consequently, the application of the bicarbonate buffer system to *in vitro* solubility and dissolution methodology needs to be considered. Challenges associated with the handling of the volatile bicarbonate buffer have been overcome by the implementation of dynamic systems to maintain a phase-heterogeneous bicarbonate buffer at physiologically relevant pH levels, such as the pHysio-grad® device (see Fig. A.1, supplementary material) [35,36].

To summarize, bicarbonate buffers differ from non-volatile buffer systems such as phosphate and maleate, particularly in terms of the effective buffer capacity in the diffusion layer and the increased bulk buffer capacity. Limited amount of data and compound, especially during early discovery and development phases, make it difficult to develop equivalent phosphate buffers with the help of published models [24,26]. Furthermore, the use of low concentrated equivalent phosphate buffers is reported to lead to difficulties by maintaining the bulk pH during solubility experiments. At the same time, commercially available devices [36,37] facilitate the application of bicarbonate buffers in *in vitro* set-ups. Thus, this work strives to answer the questions as to how a bicarbonate buffer, applied to biorelevant media such as FaSSIF, affects the solubility, dissolution, and precipitation behavior of drugs compared to "standard" (phosphate-buffered) FaSSIF, and if one of these buffer systems is superior over the other regarding accurately predicting *in vivo* dissolution and solubility.

To answer these questions, FaSSIF-V1 ($FaSSIF_{phosphate}$) and a modified version, applying a bicarbonate instead of a phosphate buffer ($FaSSIF_{bicarbonate}$), were used to investigate the influence of the buffer system on the solubility and dissolution behavior of a broad range of model compounds, including eight acidic, thirteen basic, and five neutral compounds. Additionally, four basic APIs were selected to determine the impact of the buffer system on their supersaturation and precipitation behavior.

2. Materials and methods

2.1. Materials

Sodium bicarbonate, sodium dihydrogen phosphate monohydrate, sodium hydroxide, hydrochloric acid 1 M, sodium chloride, trifluoroacetic acid and gradient grade acetonitrile were purchased from Merck KGaA (Darmstadt, Germany). Ketoconazole was purchased from Caesar & Lorentz GmbH (Hilden, Germany), carvedilol from abcr GmbH (Karlsruhe, Germany), indomethacin from Euro OTC Pharma GmbH (Bönen, Germany), probenecid and glybenclamide-K from Cayman

Chemical Company (Ann Arbor, MI, USA), aprepitant from Advanced ChemBlocks Inc (Burlingame, CA, USA), felodipine from Combi-Blocks Inc. (San Diego, CA, USA), prazosin-HCl, flufenamic acid, amiodarone-HCl (for the solubility experiments) from Alfa Aescar Thermo Fisher (Kandel, Germany), and prazosin (free base) from VWR International GmbH (Darmstadt, Germany). Hydrochlorothiazide, danazol, piroxicam, glybenclamide, griseofulvin, amiodarone-HCl (for the transfer experiments), glipizide, quinidine, dipyridamole, and cinnarizine were purchased from Sigma Aldrich (Darmstadt, Germany). Carbamazepine was purchased from Fagron GmbH and Co. KG (Glinde, Germany). Fenofibrate, compound A, compound A hydroxybenzoic acid-cocrystal (compound A-CC), crystalline compound B, and compound B disordered material (d) were available in-house. Important physicochemical characteristics of the model compounds can be found in Table 3. FaSSIF/FeSSIF/FaSSGF-powder was purchased from biorelevant.com Ltd (London, United Kingdom).

2.2. Media composition

$FaSSIF_{phosphate}$ was prepared from FaSSIF/FeSSIF/FaSSGF-powder according to the instructions for FaSSIF-V1 from biorelevant.com. $FaSSIF_{bicarbonate}$ contained the same amount of FaSSIF/FeSSIF/FaSSGF-powder compared to $FaSSIF_{phosphate}$, however, a bicarbonate instead of a phosphate buffer was used for media preparation. The blank bicarbonate buffer was developed yielding the same bulk buffer capacity (10 to 12 mmol/l/ΔpH [13,14]) and osmolarity (270 mosmol/kg [13]) as $FaSSIF_{phosphate}$. For this purpose, sodium bicarbonate and sodium chloride were dissolved in water, and the appropriate amount of carbon dioxide necessary for a buffer with a pH of 6.5 was automatically passed into the solution by the pHysio-grad® device. The concentration of sodium bicarbonate was adapted from recently published bicarbonate-buffered biorelevant media to achieve the desired buffer capacity [34,38], which was additionally verified experimentally (see supplementary material).

For the transfer experiments in $FaSSIF_{phosphate}$ and $FaSSIF_{bicarbonate}$, an increased buffer capacity of 20 mmol/l/ΔpH was used to prevent a strong decrease in pH during the transfer experiments (see below). The media were prepared according to the instructions described by Jede et al. [34], except for the FaSSIF/FeSSIF/FaSSGF-powder, the concentration of which was doubled. Due to the 1:1 dilution during the transfer, the higher concentration of FaSSIF/FeSSIF/FaSSGF-powder ensures that the final bile and lecithin concentration in the acceptor compartment after the transfer corresponds to that of original FaSSIF. To diminish the decrease in pH during the transfer experiment, SGFsp (Simulated Gastric Fluid *sine pepsin*) was prepared according to Jede et al. [34], i.e., yielding a pH of 2.0 instead of 1.2. Media compositions can be found in Table 2.

2.3. Solubility testing

Solubility experiments were performed to determine the thermodynamic solubility as well as the rate at which this value is reached (later referred to as "rate of dissolving"). The solubility of the 26 compounds was initially measured up to 22 h in $FaSSIF_{phosphate}$ and $FaSSIF_{bicarbonate}$. Experiments were performed in a USP II dissolution apparatus (DT 726, Erweka GmbH, Langen, Germany) equipped with mini vessels. Stirrer speed was set to 75 rpm and the medium temperature to 37 °C. For the experiments using $FaSSIF_{bicarbonate}$, the dissolution apparatus was equipped with the pHysio-grad® device (Physiolution GmbH, Greifswald, Germany). An excess amount of API (around 3-fold of respective maximal solubility found in literature, but the same amount for each vessel within the single experiments) was added to 250 mL of the medium. Experiments were performed in triplicate. For glybenclamide and indomethacin, 1 mL samples were taken after 0.25, 0.5, 0.75, 1, 4, and 22 h. For the other 24 compounds, samples were additionally taken after 2 h. In case the concentrations were still strongly increasing after 22 h, indicating that the equilibrium was not reached yet, the measurements were continued up to 92 h (amiodarone-HCl, felodipine, fenofibrate). Upon withdrawal of the sample from the vessel, the samples were immediately centrifuged for 2 min with 15000 rpm (21,382 g). After centrifugation, the supernatant was appropriately diluted with the respective extraction medium, followed by HPLC analysis (for analytical methods see supplementary material). To exclude inter-day variability, the experiments for every compound were performed on the same day with the same drug substance.

2.4. Transfer experiments

The transfer experiments with ketoconazole, cinnarizine, dipyridamole, and amiodarone-HCl were carried out in a USP II dissolution apparatus (Erweka DT 726) equipped with mini vessels. A 1:1.25 downscale of the physiological set-up (250 mL gastric and 250 mL intestinal compartment) was used for practical reasons. For this purpose, 200 mL SGFsp pH 2.0 were transferred into 200 mL $FaSSIF_{bicarbonate}$ or $FaSSIF_{phosphate}$ (transfer media). The media temperature was set to 37 °C, and the stirrer speed to 75 rpm. For the experiments using $FaSSIF_{bicarbonate}$, the acceptor vessels were additionally equipped with the pHysio-grad® device. The amount of API was downscaled based on the physiological human dose. For ketoconazole and dipyridamole, 160 mg (1:1.25 downscale of a human dose of 200 mg), for cinnarizine 60 mg (equivalent to 75 mg human dose), and for amiodarone-HCl 480 mg (equivalent to 600 mg human dose) were introduced into the donor vessel and stirred for 30 min. Subsequently, the mixture was transferred into the acceptor compartment using a 1st order kinetic with a 5 min half-life [34].

Samples were taken after 2.5, 5, 7.5, 20, 15, 30, 45, 60, 90, 120, and 150 min and filtered immediately using a 0.45 μm PTFE filter (Whatman, GE Healthcare, Amersham, United Kingdom). The first 2 mL were returned into the acceptor vessel. As ketoconazole exhibited high filter adsorption on the PTFE filter, a 0.2 μm regenerated cellulose filter (Schleicher & Schuell, Dassel, Germany) was used, and only 0.5 mL were returned. Samples were appropriately diluted with the respective extraction medium, followed by HPLC analysis (for analytical methods, see supplementary material). For every compound, the transfer

Table 2
Composition of $FaSSIF_{phosphate}$ and $FaSSIF_{bicarbonate}$ for solubility and transfer experiments.

	$FaSSiF_{phosphate}$ - solubility	$FaSSiF_{bicarbonate}$ - solubility	$FaSSiF_{phosphate}$ - transfer [34]	$FaSSiF_{bicarbonate}$ - transfer [34]	SGF_{sp} pH 2.0 [34]
FaSSIF/FeSSIF/FaSSGF- powder (g/l)	2.240	2.240	4.480	4.480	
NaH_2PO_4 (mM)	29		56.8		
NaOH (mM)	q.s.		q.s.		
$NaHCO_3$ (mM)		13.5		22.5	
CO_2 (mM)		q. s.		q. s.	
NaCl (mM)	106	130	77.5	111.8	34.2
pH	6.5	6.5	6.5	6.5	2

FaSSIF/FeSSIF, Fasted/Fed State Simulated Intestinal Fluid; *FaSSGF,* Fasted State Simulated Gastric Fluid; SGF_{sp}, Simulated Gastric Fluid Sine Pepsin.

Table 3
Physicochemical characteristics, HIF solubilities, solubility values determined in $FaSSIF_{phosphate}$ and $FaSSIF_{bicarbonate}$, and statistical parameters and calculated τ_D values from the linear Weibull regression. A statistically significant difference between the buffer systems is highlighted in bold.

Compound	Ionization behavior (pK_a)[a]	HIF solubility (mean) (mg/ml)	Buffer used for FaSSIF preparation	Conc. (22 h) ± SD (mg/ml)	p-value solubility	logα	p-value for logα	b	p-value for b	τ_D (min)	R^2 linear model
Amiodarone-HCl[a]	Base (8.4)	0.376 [21]	Bicarbonate	0.41 ± 0.03	0.023[b]	0.0909	0.544	0.3678	0.342	106.0	88.7
			Phosphate	0.494 ± 0.007		0.1107		0.3331		129.0	
Aprepitant	Base (3.1)	0.013 [21]	Bicarbonate	0.0218 ± 0.0019	0.771	0.4201	**<0.001**	1.080	0.294	146.9	62.9
			Phosphate	0.023 ± 0.003		1.357		1.644		401.4	
Carbamazepine	Neutral	0.283 0.336 (0.308) [21,43 45]	Bicarbonate[f]	0.2636 ± 0.0026	**0.001**			N/A			
			Phosphate[f]	0.2598 ± 0.0026							
Carvedilol	Base (8.8)	0.036 [21]	Bicarbonate	0.074 ± 0.006	0.458	−0.3654	0.054	0.5668	**0.011**	13.60	91.0
			Phosphate	0.067 ± 0.009		−0.4087		0.3931		5.476	
Cinnarizine	Base (8.4)	0.0118 [45]	Bicarbonate	0.0141 ± 0.0010	0.525	0.5371	**<0.001**	0.9945	**<0.001**	208.1	95.5
			Phosphate	0.0137 ± 0.0002		1.125		1.461		353.3	
Compound A			Bicarbonate	0.0034 ± 0.0003	0.138	−0.1543	**0.001**	0.9416	0.064	87.50	98.7
			Phosphate	0.00358 ± 0.00003		−0.2055		0.8714		79.67	
Compound A-CC	Base (3)	N/A	Bicarbonate[f]	0.00487 ± 0.00009	**0.003**	−0.0756	**0.008**	1.0614	0.548	70.67	99.4
			Phosphate[f]	0.0020 ± 0.0008		−0.10730		1.0413		76.07	
Compound B			Bicarbonate	0.00363 ± 0.00008	0.716	−0.4006	**0.003**	0.3698	0.206	4.953	79.11
			Phosphate	0.00380 ± 0.00016		−0.3112		0.2302		2.669	
Compound B (d)	Base (1.8)	N/A	Bicarbonate	0.0124 ± 0.0005	0.646	0.3261	**<0.001**	0.7141	0.515	171.7	96.04
			Phosphate	0.0122 ± 0.0007		0.5031		0.7511		280.5	
Danazol	Neutral	0.002 0.0132 (0.0085) [21,43 47]	Bicarbonate	0.00887 ± 0.00009	0.011[b]	−0.09146	**<0.001**	0.8193	0.545	46.40	91.6
			Phosphate	0.00915 ± 0.00009		0.5105		0.7407		293.3	
Dipyridamole	Base (6.6)	0.029 [21]	Bicarbonate	0.0165 ± 0.0003	0.764	−0.4357	**<0.001**	0.6331	**0.002**	12.30	96.3
			Phosphate	0.01707 ± 0.00017		−0.2589		0.4805		17.35	
Felodipine[c]	Neutral	0.014 [21]	Bicarbonate	0.049 ± 0.005	0.239	0.8851	**0.010**	0.8923	0.079	589.0	90.9
			Phosphate	0.0453 ± 0.0009		1.060		0.7387		1634	
Fenofibrate[d]	Neutral	0.0197 [45]	Bicarbonate	0.0107 ± 0.0020	0.977	1.411	**0.006**	0.9486	0.849	1842	90.3
			Phosphate	0.0108 ± 0.0019		1.884		0.925		6530	
Flufenamic acid	Acid (3.8)	0.428 [21]	Bicarbonate	0.66 ± 0.05	0.764	0.5480	**<0.001**	0.7454	0.414	326.1	98.8
			Phosphate	0.671 ± 0.007		0.1699		0.7762		99.32	
Glipizide	Acid (4.3)	0.042 0.2889 (0.172) [21,43 45]	Bicarbonate	0.0423 ± 0.0028	0.369			N/A			
			Phosphate	0.0447 ± 0.0019							
Glybenclamide		0.0092 0.0154 (0.0123) [43 45]	Bicarbonate	0.00254 ± 0.00015	0.183	0.3223	**<0.001**	0.9604	0.414	129.9	89.8
			Phosphate	0.00230 ± 0.00003		0.7906		0.840		524.1	
Glybenclamide-K	Acid (4.3)		Bicarbonate[f]	0.0058 ± 0.0011	0.679			N/A			
			Phosphate[f]	0.0063 ± 0.0014							
Griseofulvin	Neutral	0.017 0.0246 (0.0208) [21,43,44]	Bicarbonate	0.0161 ± 0.0003	0.268			N/A			
			Phosphate	0.0158 ± 0.0001							
Hydrochloro-thiazide	Acid (9.4)	1.060 [45]	Bicarbonate	0.926 ± 0.009	0.091	−0.7589	0.566	0.4996	0.797	1.816	75.0
			Phosphate	0.917 ± 0.009		−0.7219		0.4593		1.608	
Indomethacin	Acid (3.8)	0.7699 0.8477 (814) [43 46]	Bicarbonate	0.48 ± 0.04	0.74			N/A			
			Phosphate	0.489 ± 0.005							

(continued on next page)

Table 3 (*continued*)

Compound	Ionization behavior (pK_a)[e]	HIF solubility (mean) (mg/ml)	Buffer used for FaSSIF preparation	Conc. (22 h) ± SD (mg/ml)	p-value solubility	log*a*	p-value for log*a*	*b*	p-value for *b*	τ_D (min)	R^2 linear model
Ketoconazole	Base (7.2)	0.056–0.3264 (0.149) [21,43,45,46]	Bicarbonate	0.0189 ± 0.0008	0.158	0.4446	**<0.001**	0.8079	0.426	213.0	93.6
			Phosphate	0.0171 ± 0.0012		0.6839		0.7447		497.2	
Piroxicam	Acid (6.4)	0.397 [21]	Bicarbonate[f]	0.45 ± 0.06	0.985	−0.4104	**<0.001**	0.9932	0.097	23.17	79.3
			Phosphate	0.45 ± 0.06		−0.07417		0.6512		46.16	
Prazosin		0.100 [48]	Bicarbonate	0.045 ± 0.007	0.685	−0.1884	**<0.001**	0.4096	**<0.001**	20.81	97.1
			Phosphate	0.0435 ± 0.0004		−0.2659		0.6073		21.89	
Prazosin-HCl	Base (7.2)	N/A	Bicarbonate	0.0700 ± 0.0020	0.052	0.08923	**<0.001**	0.7694	0.421	78.37	95.9
			Phosphate	0.0667 ± 0.0008		0.3239		0.7216		168.7	
Probenecid	Acid (3.6)	0.738 [21]	Bicarbonate	2.933 ± 0.029	**<0.001**	0.1884	**<0.001**	0.9502	**0.001**	94.72	91.1
			Phosphate	2.128 ± 0.023		−0.1936		0.5747		27.62	
Quinidine	Base (9)	0.6414 [45]	Bicarbonate	1.360 ± 0.014	**<0.001**	−0.4631	N/A	0.4308	N/A	5.047	N/A
			Phosphate	1.137 ± 0.011		N/A		N/A		<15 min	

N/A: no data available or calculation not feasible, *HIF*: human intestinal fluids; *FaSSIF*: Fasted State Simulated Intestinal Fluid.

[a] Solubility testing was prolonged to 50 h.

[b] No statistical significance with Benjamini-Hochberg correction of the p-values.

[c] Solubility testing was prolonged to 48 h.

[d] Solubility testing was prolonged to 92 h.

[e] Predicted values from literature [44].

[f] Dissolution profiles indicate simultaneous dissolution and precipitation processes in the buffer.

experiments were performed in triplicate.

2.5. Statistical analysis and data evaluation

Prior to all evaluations, outliers were identified with the Grubbs test and excluded from further calculations.

2.5.1. Thermodynamic solubility

For each compound, the measured concentration at the last sampling-point was compared between $FaSSIF_{phosphate}$ and $FaSSIF_{bicarbonate}$ using an unpaired *t*-test ($\alpha = 0.05$). Equal variances were presumed. Additionally, since many t-tests were performed, the Benjamini-Hochberg correction was applied for all measurements to control the FDR (false-discovery-rate) [39]. The limit for the FDR was set to 5% [39]. To assess the relevance of the differences found, bioequivalence criteria (80–125%) were applied, so that only a relative difference between the solubility values higher than 20% or 25% was considered as a relevant difference.

To address the biorelevance of the obtained solubilities, the ratio between the respective solubility of a drug in $FaSSIF_{phosphate}$ or $FaSSIF_{bicarbonate}$ and the mean HIF solubility (Table 3) was calculated for each compound.

2.5.2. Rate of dissolving

To investigate the differences regarding the shapes of the dissolution profiles, fitted Weibull functions of the measured dissolution profiles (see 2.3) were compared for $FaSSIF_{phosphate}$ and $FaSSIF_{bicarbonate}$. Additionally, the regression parameters obtained from fitting the measured data to a Weibull function were used to determine τ_D (time at which 63.2 % of the compound is dissolved) [40]. This value was used to interpret the relevance of statistical differences between the dissolution curves. Since the fitting of a large amount of data is associated with higher degree of variability and in order to not over-emphasize differences between the dissolution profiles, a relevant difference was defined as 2-fold distinction.

The dissolution data was fitted using the linearized Langenbucher adaption (Eqs. (3) and(4)) [40] of the original Weibull equation [41].

$$m = 1 - \exp\left[\frac{-(t - T_i)^b}{a}\right] \quad (3)$$

Eq. (3): Langenbucher adaption of the Weibull equation. For dissolution processes, m is the fraction of drug in solution at time t, T_i is the lag time until the onset of the dissolution process, and a is a time constant and b describes the shape of the curve [41,42].

$$\log[-ln(1-m)] = blog(t - T_i) - \log a \quad (4)$$

Eq. (4): Log-log plot of $-ln(1-m)$ versus t, representing the linearized version of the Langenbucher adaption [40,42].

To harmonize the curves, the fraction dissolved, *m*, was calculated by dividing each concentration per timepoint *t* by the terminal concentration . If this value was an outlier (identified as per Grubbs test), the mean value was used. To exclude the equilibrium part of the profile from the analysis and to focus on the initial dissolution process, the regression analysis was performed up to the first time point with $m \geq 0.95$, but at least with three timepoints.

The parameters *a* and *b* were estimated with the linear regression tool using the Minitab® 19.2 software (Minitab, LLC, State College, PA, USA). As T_i represents the time until the onset of the dissolution process (lag time), this is only relevant for, e.g., disintegration time of tablets and capsules, or coatings. Since the neat API was used the lag time was excluded from the regression equation.

To compare the different buffers, a linear model with buffer as categorical variable and an interaction term (time*buffer) was used to compare the regression parameters *a* and *b*. The significance level was set to 0.05. The quality of the linear model was assessed by the R^2 values.

The Benjamini-Hochberg correction was applied to control FDR (with 0.05).

2.5.3. Transfer experiments

The $AUC_{0\text{-}150min}$ and c_{max} values were compared, using unpaired t-tests, to address differences in the supersaturation and precipitation behavior. The $AUC_{0\text{-}150min}$ was calculated from the single profiles using the trapezoidal rule.

3. Results

3.1. Media characterization

To ensure that the only difference between the media applied in this study is the buffer system, $FaSSIF_{phosphate}$ and $FaSSIF_{bicarbonate}$ were compared in terms of their key physio-chemical parameters. More details about the experimental methods can be found in the supplementary materials. The characterization proved that both media are comparable regarding osmolarity (259.4 ± 0.8 mosmol/kg for $FaSSIF_{phosphate}$ versus 270.1 ± 1.7 mosmol/kg for $FaSSIF_{bicarbonate}$), buffer capacity (10.4 ± 0.3 mmol/l/ΔpH for $FaSSIF_{bicarbonate}$ versus 10–12 mmol/l/ΔpH for $FaSSIF_{phosphate}$ (literature value [13,14])) and surface tension (51 ± 3 mN/m for $FaSSIF_{phosphate}$ versus 54.7 ± 0.8 mN/m for $FaSSIF_{bicarbonate}$). The values correspond to the published data of FaSSIF-V1 in previous studies (Table 1). To reach equal osmolarities in both media, it was necessary to add more sodium chloride to $FaSSIF_{bicarbonate}$ than it is the case for $FaSSIF_{phosphate}$, resulting in different chloride concentrations (106 mmol/l for $FaSSIF_{phosphate}$ versus 130 mmol/l for $FaSSIF_{bicarbonate}$). The micelle size of $FaSSIF_{bicarbonate}$ was found to be reproducibly smaller compared to $FaSSIF_{phosphate}$ (n = 3, separate preparation using two different FaSSIF/FeSSIF/FaSSGF-powder batches). Even though the difference is statistically significant, it is only 3 nm (74 ± 5 nm for $FaSSIF_{phosphate}$ versus 71 ± 5 nm for $FaSSIF_{bicarbonate}$, paired *t*-test: mean difference 3 nm, 95% CI 1.208; 5.143, p-value 0.02). As for the relevance of this difference, please see additional considerations in the discussion section.

3.2. Solubility measurements

3.2.1. Thermodynamic solubility

Regarding the solubility at the last measured time point, four out of 26 analyzed compounds showed a statistically significant difference between $FaSSIF_{phosphate}$ and $FaSSIF_{bicarbonate}$. Two out of the four compounds were bases (quinidine, compound A-CC). However, also for probenecid as acidic substance as well as carbamazepine as a neutral compound, statistically significant differences were observed (Table 3). Only for probenecid and compound A-CC, the difference was higher than 25%, while for the other two compounds, it was below 25% (Fig. 1). In case of quinidine and probenecid, the pH values measured after the solubility test in $FaSSIF_{phosphate}$ were 7.3 and 5.9, respectively. The pH in $FaSSIF_{bicarbonate}$ remained at pH 6.5 for quinidine and decreased during probenecid dissolution to 6.3, which could explain the observed differences in thermodynamic solubility (Fig. 1).

Comparing the measured solubility values to the solubility of the same compounds in HIF, the biorelevant solubilities are within 2-fold for 13 out of 20 compounds (Fig. 2A). Of note, there is no trend for systematic over- or underprediction for acids, bases, and neutral compounds. The quality and relevance of the available HIF data for this purpose are discussed below.

3.2.2. Rate of dissolving

To assess the rate of dissolving, the dissolution profiles were fitted using a linearized Weibull function (Eq. (4)). In total, most models resulted in R^2 values above 90% (Table 3). However, some fits yielded in a relatively low R^2 value. In the current study, our focus was the statistical comparison of regression parameters (*a* and *b* from linear Weibull regression). Therefore, the linearized Langenbucher model was used as standard procedure. Of note, a nonlinear regression model with additional regression parameters like a biphasic model may lead to better individual fits in some cases. On the downside, the application of such a model would impede the statistical comparability.

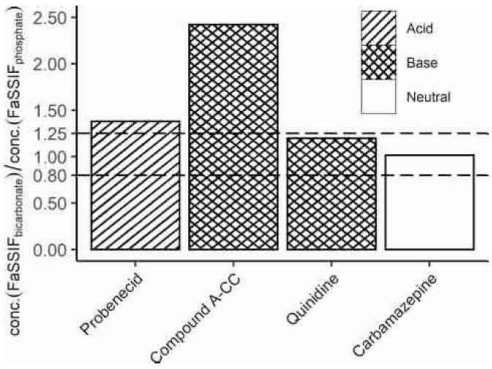

Fig. 1. Ratio between the mean solubility values in $FaSSIF_{phosphate}$ and $FaSSIF_{bicarbonate}$ of those compounds where a statistically significant difference in the solubility was observed after 22 h.

Regarding the regression parameters (Eq. (3) and Eq. (4)), significant differences were found for the time scale parameter *a* (13 compounds), the shape parameter *b* (one compound), or both parameters (four compounds) (Table 3). τ_D differs more than 2-fold for eleven out of the evaluable compounds (Fig. 2B). The corresponding dissolution profiles are displayed in Fig. 4 and Fig. A.2 (supplementary materials).

For 3 out of 26 compounds (griseofulvin, indomethacin, glipizide), the equilibrium solubility was reached very fast, and the Weibull function could not be modeled. These compounds were subsequently excluded from further calculations. The same applies to quinidine, but only for $FaSSIF_{phosphate}$. Carbamazepine was also excluded from the analysis because it showed initial supersaturation followed by slow precipitation to equilibrium in both media, and the resulting shape of the solubility profile could not be represented by a Weibull distribution. As piroxicam and compound A-CC showed a supersaturation, the regression was only performed up to 4 h to address the initial dissolution. Applying this approach, simultaneous drug precipitation is not included in the calculation. However, the Weibull equation can still be applied to fit the dissolution profiles up to the measured maximum concentration and τ_D is used to compare the dissolution speed until this maximum is reached. τ_D should not be treated as an intrinsic dissolution rate, but as a time parameter describing simultaneous drug dissolution and precipitation processes.

3.3. Dissolution and precipitation behavior

The transfer profiles of ketoconazole, cinnarizine, and amiodarone-HCl are comparable between $FaSSIF_{phosphate}$ and $FaSSIF_{bicarbonate}$ (Fig. 3). Only for dipyridamole, transfer profiles differed significantly in terms of $AUC_{0\text{-}150min}$ and c_{max}, with a difference of 16% (p-value < 0.001) and 7% (p-value 0.019), respectively. In case of the cinnarizine transfer using $FaSSIF_{bicarbonate}$, only two vessels were used for calculation due to one clogged tube.

4. Discussion

Solubility testing of 26 model compounds led to the conclusion that the rate of dissolving is highly affected by the buffer system. Although 18 out of 26 compounds showed a statistically significant difference in

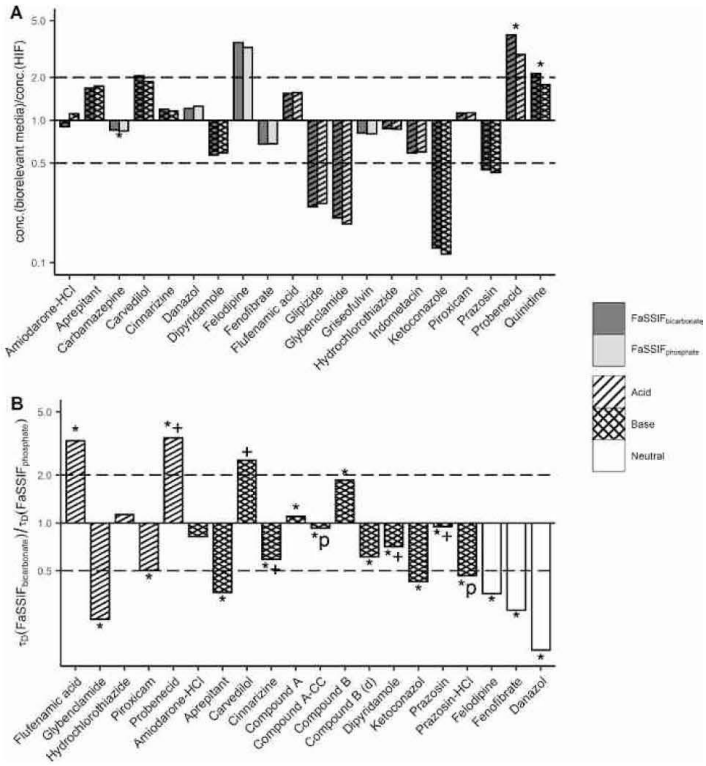

Fig. 2. A) Ratios of $FaSSIF_{phosphate}$ and $FaSSIF_{bicarbonate}$ solubilities to solubilities obtained in HIF (* Statistically significant difference between measured biorelevant solubilities). B) Relative differences of the calculated τ_D -values between $FaSSIF_{phosphate}$ and $FaSSIF_{bicarbonate}$ (* and/or + log*a* and/or*b* from linear regression are statistically significant different between the buffer systems, p dissolution profiles indicate simultaneous dissolution and precipitation).

terms of the regression parameters (Table 3), it is important to consider the relevance of these differences. In this study, we propose to calculate a time value which corresponds to a specific fraction of dissolved compound (e.g. τ_D, see section 2.5.2). Eleven out of the 20 evaluable compounds showed more than a 2-fold difference in τ_D between $FaSSIF_{phosphate}$ and $FaSSIF_{bicarbonate}$ (Fig. 2B). While this is a comparably simple way to compare the rate of the dissolution processes, it should be noted that similar τ_D values do not necessarily indicate that the dissolution behavior is equal, as also the shape of the dissolution profile and the end point need to be considered.

Regarding the thermodynamic solubility, only a few compounds showed statistically significant as well as relevant differences (difference higher than 20 or 25%, Fig. 1).

The data obtained in this study indicate that the observed differences in solubility as well as in the rate of dissolution are caused not only by one but by several reasons, namely buffer capacity and surface pH, salt solubility, ion effects, and wettability.

4.1. Buffer capacity and rate of dissolving

As described above, bicarbonate buffer is known to have a lower effective buffer capacity in the diffusion layer, which can lead to differences in the dissolution behavior of ionizable compounds compared to non-volatile buffer systems such as a phosphate buffer. During dissolution of acidic compounds, this attribute results in a lower pH in the diffusion layer, followed by a reduced dissolution rate as described, for instance, for ibuprofen and indomethacin under sink conditions [23,24,26,27,30]. In case of basic compounds, the increasing surface pH results in a lower buffer capacity due to the higher difference between buffer pK_a and pH, causing slower dissolution compared to phosphate buffer, as previously demonstrated for haloperidol [26]. Since the extent of the buffer effect on the dissolution rate depends on the physicochemical parameters of the drug, several mathematical models are published to calculate phosphate buffer molarities that match physiological bicarbonate buffer performance [24,26]. For example, Mudie et al. provided recommendations on when to use such an adjusted phosphate buffer, based on the pK_a and intrinsic solubility of the compound [32]. Following this approach for ionizable compounds, in general either a faster dissolution, or no difference is expected in phosphate buffer.

These observations are in line with the findings of this study for flufenamic acid, probenecid, quinidine, and carvedilol, which exhibit markedly faster dissolution in phosphate buffer, as well as compound A (free base), compound B/ -d, hydrochlorothiazide, prazosin, amiodarone-HCl, and dipyridamole with no substantial difference between both buffer systems. Results for dipyridamole and flufenamic acid are additionally in line with results published by Mudie et al., who compared intrinsic dissolution rates in a low-concentrated phosphate buffer (0.2 and 0.4 mM) to a 50 mM phosphate buffer [32]. However, glybenclamide, prazosin-HCl, aprepitant, and ketoconazole

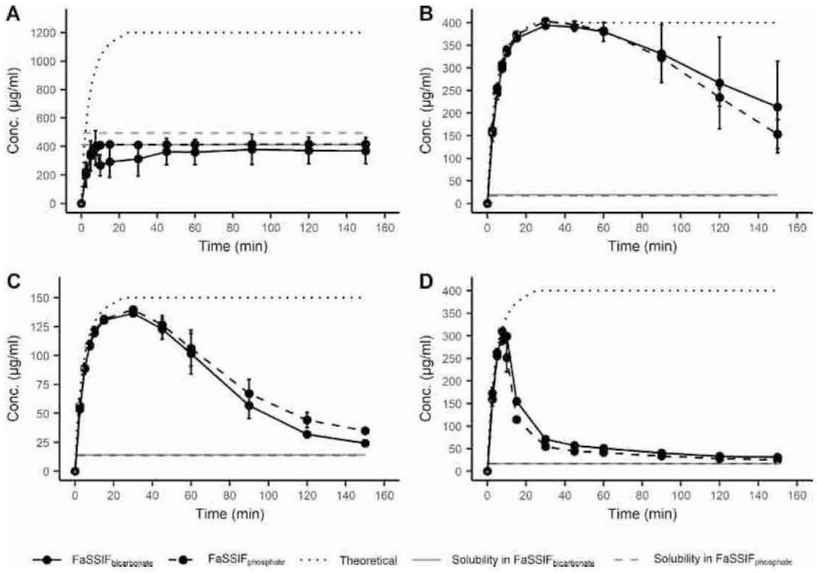

Fig. 3. Comparison of the dissolution and precipitation behavior of amiodarone-HCl (A), ketoconazole (B), cinnarizine (C), and dipyridamole (D) (error bars standard deviation) in FaSSIF$_{phosphate}$ and FaSSIF$_{bicarbonate}$ (transfer media). The theoretical profile represents the concentration time profile which results from the first order transfer in case the respective API is completely dissolved during the experiment.

unexpectedly dissolved faster in FaSSIF$_{bicarbonate}$. Additionally, compound A-CC, piroxicam and glybenclamide-K exhibited a higher or more stable supersaturation in FaSSIF$_{bicarbonate}$, and the neutral compounds danazol, felodipine, and fenofibrate showed significantly faster dissolution in FaSSIF$_{bicarbonate}$.

This leads to the conclusion that different dissolution rates in phosphate- and bicarbonate-buffered biorelevant media during solubility testing under non-sink conditions cannot only be explained by the pK_a value and the intrinsic solubility of the tested compounds.

4.2. (Common) ion effect

According to the solubility product, the solubility of a compound can significantly decrease in the presence of counterions, either coming from the media or as part of the formulation. Important examples for relevant counterions are chloride and sodium ions, but also bicarbonate and phosphate ions can form poorly soluble complexes with positively charged molecules. The formation of phosphate precipitates and aggregates during solubility measurements in phosphate buffer was discussed by Avdeef in 2014 for several APIs [49].

Amiodarone-HCl is an example for a compound of which solubility is known to be affected by counterions. In the current study, a particularly high variability was observed in FaSSIF$_{bicarbonate}$, and therefore, no statistical significance was observed for thermodynamic solubility (p-value = 0.023) and rate of dissolving. However, the absolute mean values were found to be consistently higher in FaSSIF$_{phosphate}$ (e.g., 20% at 15 min and 16% after 50 h, see also Fig. 4A). Moreover, the partly linear increase in solubility leads to poor Weibull fits. During the solubility experiment, the consistency of the undissolved residue changed to highly viscous, gel-like material in both tested buffer systems, which is in line with the findings published in literature that amiodarone can be present in several metastable states such as transparent sols or gels [50] and has the ability to form micelles [51]. Accordingly, it can be assumed that the chloride salt transformed at least partly to the free base during dissolution in FaSSIF$_{phosphate}$ and FaSSIF$_{bicarbonate}$. In addition, Ravin et al. observed an effect of several anions on the thermodynamic solubility of amiodarone-HCl, which was associated with an effect of the anions on the CMC (critical micelle concentration) of amiodarone-HCl [51,52]. Furthermore, Korth-Bradley and Ludwig observed precipitation of amiodarone-HCl when combined with a sodium bicarbonate solution for injection. The authors discussed a potential pH change as a reason for the precipitation; on the other hand, the results from Ravin et al. (see above) may also be an explanation for the findings of Korth-Bradley and Ludwig [53]. Comparing FaSSIF$_{phosphate}$ and FaSSIF$_{bicarbonate}$ in the current study, a combination of the common-ion effect due to higher chloride concentration in FaSSIF$_{bicarbonate}$ (see section 3.1) and an influence of chloride and bicarbonate ions on the micellar structure of amiodarone are likely. However, it is difficult to differentiate between micellar and dissolved compound. In summary, it is hardly feasible to distinguish between buffering and salting effects in this special case. However, this clearly illustrates the relevance to consider physiological amounts of counterions, not only to reach comparable osmolarities and ionic strengths, but to additionally account for the risk of a potential common ion effect. In this context, FaSSIF prepared with bicarbonate buffer is superior over media comprising unphysiological ion species such as phosphate and maleate.

For prazosin-HCl, the common ion effect was also documented for chloride ions at a pH of 1 [54]. However, in the current study, the solubility of prazosin-HCl was not found to be affected by the higher chloride concentration in FaSSIF$_{bicarbonate}$. As the magnitude of the common ion effect depends on the solubility product of the resulting salts as well as the pH (pH_{max}) [55], it is reasonable that prazosin-HCl solubility is not affected at pH 6.5 by the applied chloride concentrations.

4.3. Solubility of salts and surface pH

Compared to the parent form, a higher dissolution rate is expected

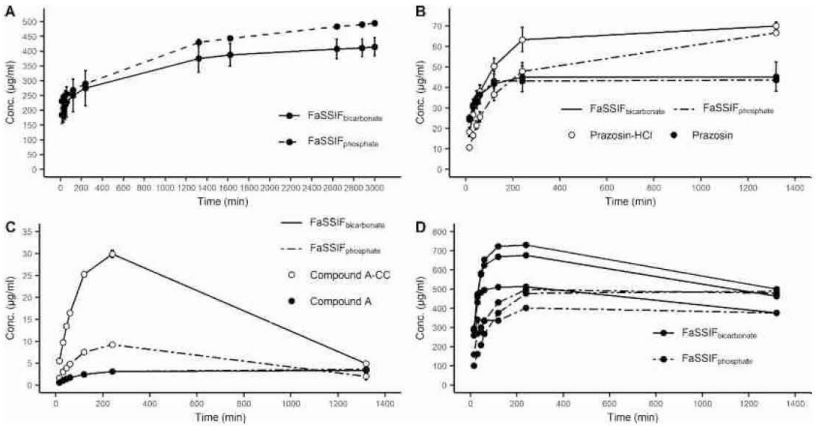

Fig. 4. Dissolution profiles in FaSSIF$_{phosphate}$ and FaSSIF$_{bicarbonate}$ of amiodarone-HCl (A), prazosin free base and prazosin-HCl (B), compound A free base and compound A-CC (C), and piroxicam (N 3) (D) (error bars standard deviation).

for salts, because the corresponding acid of a dissolving basic drug salt alters the surface pH to lower levels and consequently enhances its dissolution rate. In this study, dissolution of prazosin free base was not affected by the buffer species, as expected due to its weak ability to alter the surface pH. In contrast, the hydrochloride salt of prazosin dissolved faster in FaSSIF$_{bicarbonate}$ than in FaSSIF$_{phosphate}$ (Fig. 4B). To explain this observation, it is assumed that the lower effective buffer capacity of the bicarbonate buffer results in a lower surface pH of the dissolving salt in bicarbonate compared to phosphate buffer, which yields a higher dissolution rate. Correspondingly, Uekusa et al. observed lower supersaturation of pioglitazone-HCl salt with increasing phosphate and maleate buffer capacities [56].

For the potassium salt of glybenclamide, a pronounced supersaturation was observed after 15 min in FaSSIF$_{bicarbonate}$, while at this timepoint, the equilibrium was already reached in FaSSIF$_{phosphate}$. As no sampling points below 15 min are available, a higher supersaturation in the phosphate buffered medium cannot be excluded. However, the supersaturation was more stable in FaSSIF$_{bicarbonate}$, which is in line with the considerations for prazosin-HCl.

In case of compound A (free base), supersaturation was observed in the first four hours, followed by precipitation to a level which corresponds to the thermodynamic solubility of the free base (Fig. 4C). Cocrystals often convert to the less soluble free form which is associated with crystallization [57]. The extent of supersaturation is much greater in FaSSIF$_{bicarbonate}$ compared to FaSSIF$_{phosphate}$, with solubility values of 29.9 µg/ml and 9.2 µg/ml (4 h values), respectively. Regarding the dissolution profile, it is not certain that the maximum solubility was already reached after four hours, as no samples between 4 h and 22 h were analyzed. For the calculation of τ_D, the 4 h values were used as an endpoint. Comparing the calculated $\tau_D$values, no difference in the rate of dissolving can be observed, however, the absolute solubility values differ remarkably (29.9 µg/ml and 9.2 µg/ml, 4 h values). It has to be further noted that the τ_D value for the 4 h maximum, does not distinguish between simultaneous dissolution and precipitation processes in the supersaturated media. While the solubility and dissolution behavior of the free base is not affected by the buffer system, the observations for the cocrystal could be again related to the weaker buffering capacity of bicarbonate buffer in the diffusion layer, resulting in an even lower surface pH compared to the phosphate buffered medium, and thus enhancing the dissolution rate of basic drugs. Moreover, the thermodynamic solubility of the cocrystal seems to be 2.4-fold higher in FaSSIF$_{bicarbonate}$ (the equilibrium after 22 h was ensured with additional sampling points after 24 h (data not shown)), which is in line with the difference found after four hours (approximately 3-fold difference), while the absolute values correspond to the solubility of the free base.

In summary, the dissolution of the salts and the cocrystal tested herein seems to be highly affected by the buffer system, exhibiting higher rates of dissolving and supersaturation in bicarbonate buffer. Therefore, the implementation of bicarbonate buffer to dissolution testing should be considered in particular for this type of compounds.

4.4. Wettability

In addition to counterion and surface pH effects, the wettability of powder particles is also an important factor influencing their dissolution rate. Although bile salt and phospholipid concentrations are the same in FaSSIF$_{phosphate}$ and FaSSIF$_{bicarbonate}$, differences in wetting of the solid API were observed for some compounds during solubility experiments. Especially for the neutral compounds fenofibrate, felodipine, and danazol, the wetting in the bicarbonate-buffered media was better compared to FaSSIF$_{phosphate}$ (as determined by visual observation). Also, the faster dissolution of fenofibrate, felodipine, and danazol in FaSSIF-$_{bicarbonate}$ cannot be explained by salting effects or differences regarding the surface pH.

The surface tension measurements did not indicate a significant difference between the buffer systems. However, this does not exclude differences in the wetting and solubilizing capacities of the media used. According to the Young equation, the contact angle also depends on the interfacial energy of the solid–vapor and solid–liquid interfaces [58] and the latter one can be influenced by the different buffer salts. For example, the interfacial energy between solid and liquid interface could be affected differently between the buffers. Accordingly, Luner observed a smaller contact angle on polymethylmethacrylate blocks for a TDC (taurodeoxycholic acid) solution in SGF$_{sp}$ at pH 1.2 compared to a solution in simulated intestinal fluid with a pH of 7.4. As discussed by Luner, the differing wetting behavior may be attributed to different fractions of ionized TDC due to the pH of the buffers used or due to the influence of counterions on the aggregation number of TDC [58]. In the current study, FaSSIF-V1 was used, which contains sodium taurocholate and phospholipid mixed micelles. Comparing FaSSIF$_{phosphate}$ and FaSSIF$_{bicarbonate}$, the micelles were found to be significantly smaller in FaSSIF$_{bicarbonate}$ (see 3.1). This may be related to the higher chloride concentration in FaSSIF$_{bicarbonate}$. Sodium chloride can lead to a smaller micelle size and a higher CMC due to salting-in effects [59]. In addition,

the different pH values during measurement of the micellar size may lead to different micellar sizes due to different surface pH. Of note, the pH was not adjusted to 6.5 for the DLS (dynamic light scattering) measurements due to instability of bicarbonate buffer (see supplementary material for details). However, the difference between the micelle sizes is only 3 nm and is thus deemed to be of minor importance for the overall performance of the media. Furthermore, the interaction of the micellar structures with the API during the dissolution experiments is of higher importance. It is reasonable that the compounds interact differently with the micellar structures due to different surface pH values in the buffer systems.

Despite the micelle size, sodium chloride can also affect the water structure. While ions are typically surrounded by a tightly bound layer of water molecules, the residence time of water is shorter on water structure breakers like chloride and potassium ions than the residence time at another water molecule in pure water [60]. Regarding $FaSSIF_{phosphate}$ and $FaSSIF_{bicarbonate}$, the higher chloride concentration may improve the wettability in the bicarbonate-buffered medium. While more recent investigations came to the result that the combined effects of cations and anions in solution need to be considered for the water structure [61] and subsequently the wettability, the extent of this effect on the dissolution rate is difficult to assess.

Less effective wetting of the powder particles in $FaSSIF_{phosphate}$ was also observed for the acidic compound piroxicam. During dissolution, piroxicam showed supersaturation up to four hours only in $FaSSIF_{bicarbonate}$. Following subsequent precipitation, the equilibrium solubility was comparable to that of piroxicam in $FaSSIF_{phosphate}$ (Fig. 4D). Similar supersaturating behavior of piroxicam was already observed by Jinno et al. at different pH levels in phosphate-citrate-buffer. This was associated with a crystal form conversion, as the white anhydrous form converts to the yellow monohydrated form [62] over time. In the current study, the anhydrous (white) form was used for solubility testing, however, the residual powder changed from white to yellow during the solubility experiment in both buffers, indicating a conversion into the monohydrate (yellow) form. Due to the different wetting behavior of piroxicam in the buffer systems, the initial dissolution of the anhydrous form may have been greater in bicarbonate buffer, while the anhydrous piroxicam may not have dissolved substantially prior to the conversion to the monohydrate form in $FaSSIF_{phosphate}$.

In addition to the buffer salts, the solubility experiments also slightly differed in terms of the experimental set-up. To maintain a constant pH in $FaSSIF_{bicarbonate}$, the dissolution vessels needed to be equipped with a gas tube and a pH electrode. As the pHysio-grad® device is a dynamic system which passes only small amounts of gas to the medium depending on the current pH, the formation of gas bubbles during the experiments is of minor relevance compared to bicarbonate buffers which are continuously sparged with carbon dioxide. However, the additional equipment compared to a standard dissolution vessel could result in different hydrodynamics which may have an influence on the amount of floating drug particles on the surface of the medium.

Finally, it can be concluded that the dissolution behavior in both buffer systems is affected by multiple factors and is therefore challenging to predict for a broad range of compounds. However, for a further differentiation between the two media, intrinsic dissolution experiments of neutral compounds instead of powder dissolution could be helpful.

4.5. A critical view on HIF solubilities

As shown in Fig. 2A, $FaSSIF_{phosphate}$ and $FaSSIF_{bicarbonate}$ are able to mimic the solubility of twelve compounds tested in HIF within a two-fold range of the solubility found in HIF, but at the same time, the solubilities of eight compounds were remarkably over- or underestimated, which is in line with observations described in literature [21,44]. Neither bicarbonate-, nor phosphate-buffered FaSSIF was found to be superior in predicting drug solubility in HIF aspirates.

The predictive power of biorelevant media is often evaluated by comparing results against solubility data generated in HIF [11,21,44,45], however, it is important to also acknowledge limitations of this approach. For example, various values for the buffer capacity of HIF are published in the literature, ranging from 5.6 to 19.7 mmol/l/ΔpH in the duodenum to 2.4 to 4 mmol/l/ΔpH in the jejunum (table 1). Litou et al. recently demonstrated that the sample handling of HIF aspirates can have an impact on the buffer capacity and pH estimated *ex vivo* [63], which is in line with the observation by Kalantzi et al. who discovered that the pH of duodenal aspirates increased by up to 6% after 20 min at room temperature [9]. It is important to note that the buffer capacity measured in HIF is actually a brief snapshot, because *in vivo* the pH of the intestinal fluids is adjusted dynamically, including the secretion of bicarbonate and absorption of carbon dioxide. This implies that the buffer capacity measured with the current protocols may underestimate the *in vivo* buffer capacity in the small intestine.

As for solubility testing in HIF, it is important to note that experiments are usually performed using HIF samples that were frozen after sampling and thawed prior to solubility testing. Consequently, the instable bicarbonate buffer can lose carbon dioxide, resulting in a decreasing buffer capacity and changes in pH. Since pH and buffer capacity of the medium are crucial factors for the dissolution and solubility of ionizable compounds, it is important to account for these factors when conducting solubility tests in HIF. Moreover, it is important to consider the enhanced buffer capacity of a phase-heterogeneous bicarbonate buffer, which is not resembled by *ex vivo* solubility experiments using HIF. The impact of phase-heterogeneous buffer systems on solubility measurements was also observed in this study during dissolution of probenecid and quinidine, where the pH was less stable in the phase-homogeneous phosphate buffer. Similar observations regarding the pH were made by Söderlind et al., who measured a substantially lower pH after an *in vitro* solubility experiment of probenecid in HIF [21].

While biorelevant media are used to simulate the solubility and dissolution behavior of orally administered drugs in the human intestinal lumen, its predictability should not be rated exclusively by the solubility measured in aspirates *ex vivo*. Additionally, the biorelevance of thermodynamic solubility may be questioned due to the unphysiological time frame of *in vitro* solubility tests. One approach to tackle challenges associated to the current HIF protocol may be solubility testing in HIF aspirates with a continuous pH or more specifically carbon dioxide adjustment to simulate the dynamic buffer system in the small intestine. Additionally, solubility testing in HIF, using a more biorelevant timeframe, such as three instead of 24 h [64], could be applied.

4.6. Dissolution and precipitation behavior

Basic drugs typically show high solubility and dissolution in the fasted stomach, whereas solubility tends to be lower in the small intestine. Therefore, precipitation testing is of particular importance especially for weak bases. Recently, Jede et al. demonstrated the superiority of bicarbonate buffer to predict the effect of acidic polymers on the precipitation behavior of weakly basic drugs [34]. The transfer results of the neat APIs used in the study of Jede et al., demonstrated that the buffer system also affects the supersaturation behavior of pazopanib-HCl and lapatinib-ditosylate in the absence of polymeric excipients, resulting in a higher AUC in bicarbonate buffer [34]. Therefore, the buffer effect on the neat API is of special interest. In the current study, no relevant difference in the precipitation behavior was observed for the four selected model compounds. To further investigate the relevance of the buffer system in precipitation assays, it would be necessary to test a higher number of compounds. However, at this point, the buffer choice seems to be more relevant when characterizing drug formulations.

5. Conclusion

The media applied in this study yielded only small differences in

thermodynamic solubility of the model compounds, which are mainly associated with the enhanced bulk buffer capacity of the heterogeneous bicarbonate buffer system. With respect to the dissolution kinetics, differences were found for several compounds. However, it is not possible to decide about the superiority of one buffer system over the other in terms of their ability to mimic *in vivo* solubility. The differences found cannot be fully explained with the current knowledge about the unique bicarbonate buffer characteristics and its impact on dissolution processes. Due to the multitude of factors influencing the solubility of the model compounds in $FaSSIF_{phosphate}$ and $FaSSIF_{bicarbonate}$, it was not possible to make either a quantitative assessment of the influencing factors, or to make predictions regarding the impact of the different factors like surface pH, salting effects or wettability on drug solubility. Consequently, it is necessary to further investigate the factors which may cause the differences in solubility and dissolution behavior when using phosphate- vs. bicarbonate-buffered biorelevant media. In the current study, the dissolution behavior of the various model compounds was characterized as part of thermodynamic solubility measurements. Consequently, no conclusions can be drawn on compound specific differences regarding intrinsic dissolution and precipitation rates. To further investigate the impact on dissolution in biorelevant media, the supersaturation and precipitation rates could be characterized in focused experiments. Furthermore, results should be related to previous literature regarding surface pH and effective buffer capacity in bicarbonate buffer [23,26]. In the meantime, we suggest to additionally test the solubility of drug candidates in bicarbonate-buffered FaSSIF, since bicarbonate is the physiologically relevant buffer species.

To evaluate the biopredictive power of the phosphate- and bicarbonate-buffered media, data on the physiological dissolution process in the intestinal fluids with respect to dynamic buffer, bile, and pH adjustment in the small intestine would be necessary. In contrast to static solubility measurements in HIF, dissolution studies in aspirates under dynamic pH and carbon dioxide adjustment may have the potential to provide a more accurate data set that better represents actual *in vivo* conditions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank Anja Victor, Armin Schüler, and Christina Habermehl for their helpful discussions regarding the statistical evaluation of dissolution data.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2021.09.009.

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7. List of publications

7.1 Research paper – part of the dissertation

Lehmann, Andreas; Krollik, Katharina; Beran, Kristian; Hirtreiter, Carina; Kubas, Holger; Wagner, Christian (2022): Increasing the Robustness of Biopharmaceutical Precipitation Assays - Part I: Derivative UV Spectrophotometric Method Development for in-line Measurements. In Journal of pharmaceutical sciences 111 (1), pp. 146–154. DOI: 10.1016/j.xphs.2021.08.025.

Krollik, Katharina; Lehmann, Andreas; Wagner, Christian; Kaidas, Jonathan; Bülhoff, Janina; Kubas, Holger; Weitschies, Werner (2022): Increasing the Robustness of Biopharmaceutical Precipitation Assays - Part II: Recommendations on the use of FaSSIF. In Journal of pharmaceutical sciences 111 (1), pp. 155–163. DOI: 10.1016/j.xphs.2021.08.026.

Krollik, Katharina; Lehmann, Andreas; Wagner, Christian; Kaidas, Jonathan; Kubas, Holger; Weitschies, Werner (2022): The effect of buffer species on biorelevant dissolution and precipitation assays - Comparison of phosphate and bicarbonate buffer. In European Journal of Pharmaceutics and Biopharmaceutics 171, pp. 90–101. DOI: 10.1016/j.ejpb.2021.09.009.

7.2 Research paper – not included in the dissertation

Jung, Fabian; Thurn, Manuela; Krollik, Katharina; Gao, Ge Fiona; Hering, Indra; Eilebrecht, Elke et al. (2021): Predicting the environmental emissions arising from conventional and nanotechnology-related pharmaceutical drug products. In Environmental research 192, p. 110219. DOI: 10.1016/j.envres.2020.110219.

Jung, Fabian; Thurn, Manuela; Krollik, Katharina; Li, David; Dressman, Jennifer; Alig, Edith et al. (2021): Sustained-release hot melt extrudates of the weak acid TMP-001: A case study using PBB modelling. In European Journal of Pharmaceutics and Biopharmaceutics 160, pp. 23–34. DOI: 10.1016/j.ejpb.2021.01.007.

7.3 Poster presentations

Krollik, Katharina; Lehmann, Andreas; Wagner, Christian; Kubas, Holger; Weitschies, Werner (2020): Investigation of buffer species for biorelevant dissolution and precipitation assays, UNGAP Spring Meeting 2020, Ljubljana, Slovenia.

Krollik, Katharina; Lehmann, Andreas; Wagner, Christian; Kubas, Holger; Weitschies, Werner (2021): Biopharmaceutical Precipitation Assays – Investigating the Impact of FaSSIF on the Supersaturation and Precipitation Behavior of Ketoconazole, AAPS PharmSci360 2021, Philadelphia, United States.

Krollik, Katharina; Lehmann, Andreas; Wagner, Christian; Kubas, Holger; Weitschies, Werner (2022): Challenges in predicting the micellar solubilization of three weakly basic compounds in Fasted State Simulated Intestinal Fluid, PBP World Meeting 2022, Rotterdam, Netherlands.

8. Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

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Date and Place	Katharina Krollik

9. Acknowledgements

Mein besonderer Dank gilt Prof. Dr. Werner Weitschies für seine überaus wertvolle Betreuung. Den spannenden wissenschaftlichen Austausch, die konstruktive und offene Kommunikation, sowie das mir entgegengebrachte Vertrauen habe ich stets sehr geschätzt.

Darüber hinaus möchte ich Dr. Christian Wagner meinen großen Dank aussprechen. Mit seiner fachlichen Expertise und Erfahrung, dem stetigen Austausch und der kontinuierlichen Unterstützung hat Christian Wagner einen unverzichtbaren Anteil zu dieser Arbeit beigetragen.

Dr. Andreas Lehmann möchte ich neben der hervorragenden fachlichen Betreuung dafür danken, dass er mir zwischen Labor und Schreibtisch stets mit Rat und Tat zur Seite gestanden hat und dabei immer ein offenes Ohr für mich hatte. Darüber hinaus hat er mich maßgeblich bei meiner persönlichen Weiterentwicklung unterstützt hat und konnte mich mit seiner inspirierenden Art oft für neue Themengebiete begeistern.

Nicht zuletzt möchte ich mich auch bei Dr. Holger Kubas für den stetigen wissenschaftlichen Austausch bedanken, bei dem ich sehr von seiner langjährigen Erfahrung in der pharmazeutischen Entwicklung profitieren konnte.

Dr. Markus Weigandt danke ich für die Möglichkeit in der industriellen Forschung und Entwicklung zu promovieren und die Ermöglichung der Teilnahme an internationalen Kongressen.

Ein weiterer Dank gilt Janina Engemann, Victoria Jede, Donnia Robins, Melinda Kern, Jonathan Kaidas und Dr. Simone Hansmann. Ihre Unterstützung und Beistand auf zahlreichen Wegen (im Labor, beim Review, Yoga, gemeinsame Mittagspausen, Mario Kart, …) haben einen unerlässlichen Anteil an meiner Arbeit.

Danken möchte ich außerdem Kristian Beran und Chiara Hirtreiter für ihre Unterstützung im Labor und Dr. Anja Victor, Armin Schüler und Dr. Christina Habermehl für die hilfreichen Diskussionen zum Thema Statistik. Prof. Dr. Dressman möchte ich für die spannende Diskussion zum Thema FaSSIF danken.

Ein besonderer Dank gilt außerdem meinen geschätzten Kollegen in Research Pharmaceutics. Die zahlreichen Team Events, sei es der Biopharm Döner oder online Spieleabend, sollen an dieser Stelle nicht unerwähnt bleiben, da sie ein Zeichen für das hervorragende Arbeitsklima sind. Darüber hinaus möchte ich mich bei allen Kollegen in PharmTech und AD bedanken, die mich während meiner Doktorarbeit fachlich unterstützt haben.

Zusätzlich möchte ich der Doktoranden Gruppe bei Merck einen großen Dank aussprechen. Ich werde unsere Dok-Breakfasts und -Dinner sowie unsere fachlichen und privaten Gespräche in sehr positiver Erinnerung behalten.

Mein Dank gilt außerdem den Arbeitskreisen der Biopharmazie und Pharmazeutischen Technologie der Universität Greifswald für die herzliche Aufnahme während meiner Aufenthalte in Greifswald und den spannenden Austausch. Ein besonderer Dank gilt Dr. Ulrike Hanke, für die Organisation der Lehrveranstaltungen und dafür, dass sie stets ein wertvoller und verlässlicher Ansprechpartner war.

Meiner Familie, insbesondere meiner Mama und meinen Bruder, danke ich für ihre vielfältige und fortwährende Unterstützung auf meinem bisherigen Lebensweg. Ein ganz besonderer Dank gilt außerdem Maxim für seine bedingungslose Unterstützung, sein Verständnis und seinen unverzichtbaren Rückhalt.