In vitro methods to assess drug precipitation in the fasted small intestine – a PEARRL review

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Running title: *In vitro* methods to assess drug precipitation

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ABSTRACT

Objectives

Drug precipitation *in vivo* poses a significant challenge for the pharmaceutical industry. During the drug development process, the impact of drug supersaturation and/or precipitation on the in vivo behaviour of drug products is evaluated with *in vitro* techniques. This review focuses on the small and full scale *in vitro* methods to assess drug precipitation in the fasted small intestine.

Key Findings

Many methods have been developed in an attempt to evaluate drug precipitation in the fasted state, with varying degrees of complexity and scale. In early stages of drug development, when drug quantities are typically limited, small scale tests facilitate an early evaluation of the potential precipitation risk *in vivo* and allow rapid screening of prototype formulations. At later stages of formulation development, full scale methods are necessary to predict the behaviour of formulations at clinically relevant doses. Multicompartment models allow the evaluation of drug precipitation after transfer from stomach to the upper small intestine. Optimisation of available biopharmaceutics tools for evaluating precipitation in the fasted small intestine is crucial for accelerating the development of novel breakthrough medicines and reducing the development costs.

Conclusions

Despite the progress from compendial quality control dissolution methods, further work is required to validate the usefulness of proposed setups and to increase their biorelevance, particularly in simulating

the absorption of drug along the intestinal lumen. Coupling results from *in vitro* testing with physiologically based pharmacokinetic (PBPK) modelling holds significant promise and requires further evaluation.

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KEYWORDS

Precipitation, *in vitro* techniques, biorelevant, oral drug absorption, supersaturation

1. Introduction

Oral drug absorption is a complex process that can be affected by a range of parameters, related to the drug, the formulation and the underlying physiology of the gastrointestinal tract (GIT). Molecular size, degree of ionisation, dissolution, precipitation, gastrointestinal (GI) transit times, luminal viscosity and pH, bile salt and phospholipid concentrations, cellular permeation and intestinal drug transport and metabolism are some examples of the factors which can affect absorption of a drug and, therefore, its bioavailability.

Possible supersaturation and/or precipitation are important parameters to consider, as they can significantly affect the bioavailability of an Active Pharmaceutical Ingredient (API). Assessment of potential supersaturation and precipitation is critical, especially in cases where the API is a weak base with low aqueous solubility or a bio-enabling formulation is implemented.

Under fasting conditions, weakly basic drugs usually have higher solubility values in the acidic environment of the stomach compared to the small intestine. Due to the variability in pH values across the human GIT, weakly basic drugs have a propensity to precipitate as they move along the GIT. In particular, for weakly basic compounds, supersaturation can occur after transfer from the stomach to the small intestine. However, supersaturated states are thermodynamically unstable and the degree of supersaturation is the driving force for precipitation. Precipitation and drug absorption are competing processes in the GIT and excipient effects can be of critical importance. From 1981 until the end of 2006, 38% of the APIs approved in the U.S.A. for oral administration were basic

95 molecules[1] and as new drug entities in current pipelines tend to be somewhat larger molecules
96 (>500 Daltons) and more lipophilic, there is a need to develop reliable *in vitro* methods to simulate
97 the transfer of the drug through the GIT and accurately predict their precipitation
98 characteristics/kinetics *in vivo*.
99 The ultimate goal of bio-enabling formulations is enhanced intestinal absorption. To achieve this,
100 pharmaceutical scientists often develop formulations, which are aimed at achieving and maintaining
101 supersaturation, i.e. the so-called "spring and parachute approach".[2][3] In this way, a greater
102 amount of drug is in solution for a longer period of time in the upper small intestine and therefore,
103 available for absorption. Common methods to improve dissolution and achieve supersaturation
104 include solid phase dispersions, lipid based formulations and formulating with cyclodextrins.[4][5][6]
105 Despite the increasing interest in producing these formulations, there is still a lack of mechanistic
106 understanding about how to achieve and maintain a supersaturated state intraluminally. Therefore,
107 design of these formulations remains a challenge.[7]
108 In every case, precipitation of drug particles can result in impaired absorption of the API and reduced
109 bioavailability. Consequently, it can jeopardise both the therapeutic efficacy and safety of the drug.
110 Precipitation can further contribute to the large intra- and interindividual variability in drug exposure
111 often detected during development of new drug products[8] and can impair the chances of proving
112 efficacy in clinical trials.[9]
113 Currently, apparent supersaturation and/or precipitation of drug *in vivo* is assessed directly in the
114 human lumen or indirectly using plasma profiles (from humans or animals), *ex vivo* methods, or *in*
115 *vitro* methods. Luminal studies in humans provide the best source of information regarding the

supersaturation and/or precipitation of different compounds.[10][11][12][13][14] Despite the valuable information obtained from luminal and *in vivo* methods in humans, as well as from *ex vivo* studies,[15] they are expensive, time-consuming and can raise ethical issues. Animal pharmacokinetic studies are also a valuable source of information,[16] but differences in the GI conditions between humans and the animal model can be an important source of error when assessing supersaturation and/or precipitation. Animal studies may also raise ethical issues and are costly to conduct. Therefore, methodologies for assessing drug supersaturation and/or precipitation *in vitro* allow for understanding and predicting the behaviour of an API/formulation before it is administered to humans, and they can facilitate the development of more efficient and safe drug products for patients. Assessing the supersaturation and precipitation kinetics of a compound is important in early development stages, before first in human studies, as well as in the later stages of formulation development. In early stage of drug development, usually a small amount of the candidate-API is available and therefore, small scale techniques are necessary. On the other hand, robust full scale *in vitro* setups are needed at the stage of formulation development for the evaluation of precipitation and supersaturation after administration of clinically relevant doses, as well as understanding supersaturation and precipitation kinetics in the presence of various excipients.

The purpose of this review article is to present an up-to-date overview of the *in vitro* tools which have been proposed to predict *in vivo* precipitation, to understand their rationale and to outline strengths and weaknesses. This will highlight areas for optimisations and guide the evolution of the methodology.

2. Small scale methods to assess drug precipitation

Small-scale *in vitro* setups facilitate the use of small quantities of the API available in the early stages of drug development. They may also be useful for the evaluation of prototype formulations.[17] In addition, the use of small scale experiments allows for reducing the quantity of biorelevant media required, which helps to reduce expenses.

Smaller versions of the USP II dissolution apparatus have been developed.[18][19] The mini-paddle vessels use 250 mL, instead of at least 500 mL used in the full sized apparatus. Some of these downscaled apparatus have been shown to produce dissolution results comparable to the standard USP II apparatus.[19] However, in pharmaceutical profiling and early formulation development, an even smaller scale can be beneficial.

2.1 Single media tests

In early stage of drug development, evaluation of potential drug precipitation can be inferred by comparing solubility in simulated gastric with intestinal media. Solubility information can be obtained rapidly using high-throughput 96 well based solubility screening tests.[20,21] For example, the solubility of ketoconazole, as measured by the PASS (Partially Automated Solubility Screening) test, in Level II fasted state simulated intestinal fluid (Level II FaSSIF[22] (0.017 mg/mL) is much lower than in Level 0 simulated gastric fluid (SGF) (418.3 mg/mL)[20], indicating possible precipitation upon

153 transition from gastric to intestinal environment. Subsequent *in vivo* studies have shown
154 precipitation of ketoconazole in the upper small intestine up to 16% of the administered dose.[10]
155 Many of the high-throughput solubility tests use a solvent casting procedure, which raises concerns
156 on potential changes of drug crystallinity upon removal of the solvent in the excipient matrix.[21]
157 Another potential problem is that traces of solvent could also lead to an overestimation of solubility
158 when the medium is added. While not attempting to capture the full complexity of the *in vivo*
159 supersaturation or precipitation process, these high-throughput solubility screening tests provide
160 useful information about solubility "gaps" and thus, potential precipitation at an early stage under
161 given conditions using only microgram quantities of drug.
162 Chandran *et al.,* proposed a small scale approach using a turbidimetric spectrophotometry method
163 to quickly evaluate the precipitation potential of a drug.[23] With this method a stock solution of drug
164 was prepared using polyethylene glycol (PEG) 400 as a vehicle and precipitation inhibitors were
165 added. Drug stock solution (100µL) was added to a 96-well plate and mixed with an equal volume of
166 deionised water. This setup measured absorbance at 500 nm, which is well above the absorbance
167 range of any of the molecules tested, but provides a measure of light scattering due to the
168 precipitation of drug, leading essentially to a turbidimetric endpoint. The authors hypothesised that
169 the initial precipitation of fine particles caused a strong scattering of light, before agglomeration of
170 particles resulted. A resulting increase in effective particle size and settling allows for increased
171 transmission through the well, thus leading to decreasing absorption. The qualitative results using
172 this method correlated well with traditional UPLC methods when examining the efficacy of different
173 precipitation inhibitors, as both methods found that the 5% (w/w) d-alpha tocopheryl polyethylene

glycol 1000 succinate (TPGS) in PEG 400 formulation was the most effective at preventing camphor precipitation.[23] Benefits of using the UV spectrometer include the simple and rapid analysis of drug precipitation at multiple time points, without the requirement of extra sample preparation or sample wastage. This test could be a useful tool to rapidly assess drug precipitation and the impact of excipients in early formulation development.

2.2 Tests with medium shift (solvent shift)

Yamashita *et al.,* used a solvent shift experiment to evaluate drug precipitation.[24] In this method drug is initially dissolved in DMSO to produce a highly concentrated stock solution. The highly concentrated stock solution is diluted in Level II FaSSIF in a 96-well plate and drug precipitation is monitored by HPLC/UV analysis. This method is useful for comparing the effectiveness of different precipitation inhibitors, which can be added to FaSSIF in the setup. Yamashita *et al.,* used this test to assess the efficacy of precipitation inhibitors with itraconazole. Results were found to correlate well with the full scale paddle dissolution experiment, as both methods identified HPMC-AS (hydroxypropyl methylcellulose-acetate succinate) as the most effective precipitation inhibitor.

Petrusevska *et al.,* used DMSO to deliver dissolved drug in a high-throughput test.[25] McIlvaine's buffer (pH 6.8) with excipient concentrations of 0.001%, 0.01% and 0.1% (w/v) were initially dispensed into each well. The concentrated stock solution of drug in DMSO was added and the plate was shaken for five seconds to ensure adequate mixing. The plate was incubated and samples were removed at various time points up to 360 minutes. Experimental factors such as the shaking

193 frequency, incubation temperature and effect of various DMSO concentrations in the setup were
194 investigated. A DMSO concentration of ≤1% (v/v) in the assay was found to be acceptable. The
195 efficacy of 23 different excipients to prevent precipitation of two poorly soluble neutral drugs,
196 carbamazepine and fenofibrate, was examined. Distinct results were found for the two compounds,
197 highlighting the case-specific nature of precipitation inhibitor effects. The authors concluded that
198 this high-throughput test provided a reasonable starting point to select appropriate excipients to
199 help prevent precipitation of drugs.
200 Petrusevska *et al.,* carried out a follow-up study investigating the use of light scattering and turbidity
201 to evaluate drug precipitation and the efficacy of precipitation inhibitors.[26] Light scattering was
202 measured using a nephelometer, whereas turbidity was measured using a UV plate reader at 500nm.
203 Stock solutions of dipyridamole and fenofibrate in DMSO were tested using similar conditions as
204 outlined in the previous experiment. Results were compared to those obtained using standard
205 quantification methods, such as UPLC, to evaluate drug precipitation. The authors expressed a
206 preference for using the light scattering method over the turbidity as it produced less false positives
207 (4 vs. 5) and less false negatives (0 vs. 2) when examining the efficacy of different precipitation
208 inhibitors.
209 Christfort *et al.,* developed a video-microscopic tool to assess the precipitation of tadalafil and the
210 efficacy of precipitation inhibitors.[27] Using a 96-well microplate, 30 µL of a tadalafil DMSO stock
211 solution was added to FaSSIF with varying concentrations (0.0 – 5.0% w/v) of HPMC, acting as a
212 precipitation inhibitor. Micrographs were obtained using the oCelloScope system™ (Philips BioCell
213 A/S, Allerød, Denmark). The development of precipitation was monitored by both single and multi-

particle analysis. Single particle analysis determined the induction time for precipitation to occur as the time taken for the first well-defined particle to appear into focus. Using single particle analysis, the effect of varying HPMC concentrations on the induction time for crystal growth and the growth in area of a single crystal was observed over time. As single particle analysis only focuses on the growth of a single crystal, it may not be representative of the total population of crystals. In contrast, multi-particle analysis enabled the analysis of the total population of particles by examining all areas of crystal growth within the field of view. Crystal growth was quantified by determining the percentage of the area of the microscopic field of view that is covered by particles and by counting the number of particles. Results of single and multi-particle analysis correlated with each other as both found that a 0.01% (w/v) concentration of HPMC was required to observe inhibition of precipitation, with maximum inhibition occurring at a concentration of 0.1% (w/v). This visual method of assessing precipitation has significant potential to increase the understanding of the precipitation kinetics in the intestine.

The µDiss system (Pion Inc.) employs UV fibre optics to obtain real-time experimental information about drug solubility and dissolution. Information about drug supersaturation and precipitation can also be inferred using the µDiss and can be used to study dissolution from drug powder or a miniaturized disk.[28][29] Up to eight experiments can be run in parallel using volumes of media ranging from 1 mL to 10 mL. This method was employed to study dissolution for a wide variety of compounds, including poorly soluble drugs.[28] Palmelund *et al.*, developed an *in vitro* standardized supersaturation and precipitation method (SSPM) using the µDiss system.[30] High concentration stock solutions of the model drugs were prepared using DMSO, and aliquots (200 µL) were added

235 into 10 mL of Level II FaSSIF at 37°C. The model drugs tested were albendazole, aprepitant, danazol,
236 felodipine, fenofibrate, and tadalafil. After each addition of stock solution, UV absorbance was
237 measured using the *in situ* UV probes for 60 minutes or, if no precipitation was observed, for longer.
238 Precipitation was detected by a shift in the baseline UV spectrum and decrease in drug
239 concentration. Plum *et al.,* investigated the inter-lab reproducibility of the SSPM method, with
240 testing carried out at seven different sites.[31] Values obtained for three model drugs (aprepitant,
241 felodipine, fenofibrate) for apparent drug supersaturation (*aDS*) and the induction time for
242 detectable precipitation (t_{ind}) were compared across the various laboratories. While a direct
243 comparison for *aDS* and t_{ind} values between sites was not possible, it was found that 80% of the
244 partners who submitted a full data set found the same rank-ordering of drugs (aprepitant >
245 felodipine ≈ fenofibrate) when comparing β-values, which was defined as the slope of the ln(t_{ind})
246 versus ln $(aDS)^{-2}$ plot.[31]
247

248 2.3 Tests with medium and pH shifts

249 Klein *et al.,* investigated the feasibility of creating a miniaturized transfer model system to model the
250 transition from gastric to intestinal environment.[32] Two different experimental setups were tested:
251 a 96-well plate model and a mini-paddle apparatus model. In the 96-well model experiment, the
252 drug is initially dissolved in Level 0 SGF (donor phase) before 30 µL of the donor phase is pipetted
253 into the acceptor phase, consisting of 170 µL of either Level II FaSSIF or Level II FeSSIF. Drug

concentration was measured every two minutes with a UV microplate reader. In the mini-paddle setup, the drug is initially dissolved in 10 mL of Level 0 SGF and is added to 40 mL of either Level II FeSSIF or Level II FaSSIF, as shown in Figure 1. Drug concentration was determined by HPLC. Hydroxybutenyl-β-cyclodextrin complexes of both tamoxifen and itraconazole were tested using both setups and the results were consistent between platforms; tamoxifen was not found to precipitate in either setup, whereas itraconazole precipitated by approximately 90% in both methods.

The Miniaturized Intrinsic Dissolution Screening (MINDISS) setup uses minidisks of compacted drug, typically 2–5 mg, to deliver drug into a 96-well plate.[33] The minidisks are prepared in a custom-made holder, resulting in a drug surface area of 3 mm^2. Dissolution medium (0.35 mL) is added into the wells and stirred. The minidisks are added to the wells such that the drug is immersed in the dissolution media. After a set period of time, the minidisks are transferred into a new well. This transfer into new media enables a pH shift, which may help to improve the biorelevance of the test by mimicking the changing environment along the GIT.[17] Drug concentrations are determined using UPLC, while Raman spectroscopy is used to analyse the solid state characteristics of the disk. The disk intrinsic dissolution rates (DIDR) calculated from the MINDISS setup, were closely correlated (R^2 = 0.9292) to larger scale drug disk dissolution tests.

Using the MINDISS setup, the DIDR of diclofenac sodium and diclofenac potassium in SGF, pH 1.2, was found to be identical to the free acid.[33] When testing both salt forms in Level 0 SGF, a layer of free diclofenac acid was formed on the surface of the disk which controlled the DIDR. This

precipitation was thought to be due to the conversion of the salt forms of the drug to the less soluble free acid. A free base would be expected to demonstrate the converse behaviour i.e. to rapidly dissolve in acidic gastric conditions and precipitate in the more neutral intestinal environment.

2.4 Two-stage tests

The Sirius T3 instrument (Pion Inc.) is an automated titration system as shown in Figure 2.[34] Gravestock *et al.,* used it to monitor precipitation of a wide range of acidic, basic and neutral drugs.[35] It uses a fibre optic UV dip probe connected to a diode array UV spectrometer to obtain a real-time measurement of drug concentration. When examining dissolution and precipitation of drug, off-line sample analysis is susceptible to potential errors due to sample ageing. Real-time analytical technology, by contrast, avoids such errors. Drug dissolution and precipitation in 15 mL of buffered 0.15M KCl was measured at four pHs: 1.9, 3.8, 5.2 and 7.2. The pH was initially 1.9 and increased every 30 minutes. The effect of pH on the dissolution and precipitation of drugs was observed; dissolution rates of acidic compounds increased with increasing pH, whereas neutral compounds had a relatively constant dissolution rate across the four pHs. Some basic drugs, such as dipyridamole, chlorpromazine HCl and clopidogrel bisulfate, precipitated as the pH was increased. Other basic drugs, such as haloperidol, maprotiline and propranolol, did not precipitate as the pH was increased. Jakubiak *et al.* used dissolution data from the T3 to develop a dissolution and precipitation model.[9] In their studies, the dissolution testing on the T3 was carried out using two different pH values (pH 2 and pH 6.5) to simulate gastric and intestinal conditions respectively. Level

II FaSSIF was used for simulating the conditions in the upper small intestine, while a simple phosphate buffer at pH 2 was used for simulating the conditions in the stomach. After 10 minutes at pH 2, concentrated FaSSIF was added to simulate the transfer from the gastric to the intestinal environment. The drug plasma profiles estimated using their model for dipyridamole and erlotinib showed a strong correlation to the human *in vivo* plasma profile, obtained from previous clinical studies.

Mathias *et al.,* developed a micro-dissolution test to examine the effect of changing media and pH on the dissolution, supersaturation and precipitation behaviour of drugs under conditions which aim to replicate the transit through the GI tract, as shown in Figure 3.[36] Drug, either as powder or suspension, was initially added to 7 mL of Level 0 SGF. After 20 minutes, 14 mL of a 1.5 times concentrated Level II FaSSIF solution was added to simulate the changes in conditions due to transfer from the stomach to the intestine. The pH of the resulting FaSSIF solution was pH 6.5 and the drug was incubated for a further 160 minutes. The weakly basic drugs ketoconazole and erlotinib were among the evaluated drugs using this test. Ketoconazole remained supersaturated for 55 minutes upon transition from gastric to intestinal conditions, before precipitating slowly over the next 75 minutes. Erlotinib precipitated rapidly to its equilibrium crystalline solubility upon addition of FaSSIF.

2.5 Methods addressing intestinal absorption

2.5.1 Biphasic Dissolution Tests

A method to simulate the absorption step in dissolution tests is through the use of an organic layer on top of the aqueous donor layer. Drug partitioning from the aqueous to the organic layer helps to generate sink conditions in the donor layer, which can have a significant effect on drug precipitation. The disadvantage of biphasic experiments is that the organic layer is in direct contact with the aqueous layer; this can lead to effects which differ from *in vivo* drug absorption. For example, some of the organic layer may be solubilised and an emulsification could occur as a result. This issue can be especially pronounced if surface active compounds are present in the biphasic experiments, which is rather common in bio-enabling formulations.

The miBIdi-pH (miniscale biphasic dissolution model with pH-shift) is a small-scale biphasic dissolution test which incorporates a pH shift to evaluate drug release and precipitation, as shown in Figure 4.[37][38] The organic lipid layer acts as an absorptive sink as drug partitions from the aqueous phase into the organic phase. The system consists of 50 mL of aqueous media covered by a 15 mL octanol layer, which acts an absorptive sink, in a miniaturised USP dissolution apparatus II. Drug concentration is determined by online UV-spectrometry. Frank *et al.,* investigated the utility of this system to predict the *in vivo* dissolution processes of two weakly basic drugs: dipyridamole and BIXX.[37] Precipitation was observed for both drugs upon shift of the pH from an acidic gastric environment to the neutral intestinal environment. The correlation to *in vivo* data for both drugs

was greatly improved using the biphasic dissolution model compared to single phase dissolution experiments**.** A level A IVIVC (*In-Vitro In-Vivo* Correlation) was established (R^2 = 0.95) between the fraction absorbed *in vivo* and the fraction dissolved in octanol for the BIXX formulations tested, whereas the single phase dissolution tests were not found to be predictive of *in vivo* performance. The inForm (Pion Inc.) has also been proposed for biphasic dissolution experiments to study precipitation. The inForm setup employs a fibre optic UV dip probe to measure drug concentration in real time, and uses a potentiometric pH probe to monitor pH of the media in real time to facilitate *in situ* pH control. Biphasic experiments have been carried out using the inForm on a range of acidic, basic and neutral compounds using a solvent shift process.[39] Drugs were initially dissolved using DMSO to prepare a concentrated stock solution and samples were added using an automatic liquid handling needle into the aqueous layer. The aqueous layer consisted of 40 mL of an acetate-phosphate buffer at pH 6.5, while the organic layer consisted of 30 mL of decanol. All the neutral and basic drugs were found to precipitate when injected into the aqueous layer at a dose level of 10 mg. Fenofibrate, a neutral compound, was added at two dose levels: 5 mg and 10 mg. Precipitation was observed at both dosing levels and the quantity of drug which partitioned into the lipid layer, was the same after one hour. This indicated that in both cases fenofibrate rapidly precipitated to its equilibrium solubility in the aqueous layer and only dissolved fenofibrate was able to partition across from the aqueous into the lipid layer. To date, published data with respect to biphasic dissolution experiments using the inForm setup with a pH shift is very limited.[40]

352 *2.5.2 Compartmental methods using non-cellular biomimetic membranes*

353 Recently, a two chamber system has been introduced called the µFlux (Pion Inc).[41] Drug
354 concentrations in the both the donor and acceptor chambers can be measured by fibre optic UV
355 probes. A membrane separates the two chambers and a biomimetic membrane coated with lipids,
356 which is a scaled-up version of the parallel artificial membrane permeability assay (PAMPA)
357 membrane, is typically used. Uptake through the membrane into the acceptor chamber aims to
358 represent drug absorption *in vivo*. Incorporation of an absorption step helps to improve the
359 biorelevance compared to single chamber systems, as drug absorption can generate sink conditions
360 in the donor chamber, which is beneficial when assessing drug precipitation. Zhu *et al.*, used the
361 µFlux apparatus to study the effect of an increased gastric pH on the kinetic profiles of many drugs,
362 including ketoconazole and nilotinib, as shown in Figure 5.[42] Initially 400µL of drug suspension was
363 added to 7 mL of gastric fluid in the donor chamber. The pH of gastric fluid was either at pH 2 or pH
364 6, simulating typical gastric pH and acid suppression respectively. The acceptor chamber was filled
365 with 21 mL of an acceptor sink buffer (ASB). After twenty minutes, 14 mL of 1.5 times concentrated
366 Level II FaSSIF solution was added to the donor chamber and the concentrations in both chambers
367 were monitored for 160 minutes. The resulting FaSSIF solution in the donor chamber had a pH of
368 6.5. In the experiment simulating normal gastric pH, ketoconazole maintained a supersaturated state
369 for at least twenty minutes after addition of the concentrated FaSSIF and readily partitioned across
370 the membrane into the acceptor compartment. In contrast, nilotinib was only transiently

371 supersaturated after the addition of the FaSSIF solution in the experiment simulating normal gastric
372 pH and appeared to precipitate quickly. The smaller surface area of the biomimetic membrane
373 compared to the human intestine hampers the transfer of drug from the donor into the acceptor
374 chamber. Therefore, precipitation may be overestimated in the donor chamber. This limitation must
375 be considered when mimicking the relationship between absorption and precipitation using the
376 µFlux.

377 Sironi *et al.*, investigated a dissolution/ permeation system using an Ussing chamber with a
378 Permeapad® acting as an intestinal barrier between the acceptor and donor side.[43] Permeapad®
379 consists of thin layer of soy phosphatidylcholine on a hydrophilic support sheet. A good correlation
380 has been found between the permeability coefficients found using Permeapad® with those found
381 using Caco-2 cells ($R^2 = 0.75$)[44] and the PAMPA membrane ($R^2 = 0.76$). The volume of media in donor
382 and acceptor compartments was 7 mL and 6 mL respectively. Phosphate-buffered saline (pH 7.35 -
383 7.45) was used as both acceptor and donor media. Hydrocortisone (BCS class II) suspension and
384 hydrocortisone methanolate tablets were tested using this setup. For the suspension, a constant rate
385 of permeation into the acceptor chamber was observed. This constant flux indicated that permeation
386 through the membrane was the rate limiting step. In contrast, the tablets had a variable rate of
387 permeation through the membrane for the initial three hours of the experiment. As the
388 concentration plateaued in the donor chamber approaching equilibrium solubility after three hours, a
389 linear increase of drug was subsequently observed in the acceptor chamber. The area to volume ratio

(0.25 cm^2/mL) in this experiment was a limiting factor when trying to achieve a substantial decrease in donor chamber drug concentrations within a reasonable period of time. The authors calculated that it would take an area to volume ratio of 5.9 cm^2/mL to achieve a 90% permeation of hydrocortisone into the acceptor chamber within four hours. The inter-laboratory variability of these biomimetic membranes needs to be further investigated. The compatibility of the Permeapad® membrane with surfactants, co-solvents and biorelevant media,[45] and ability to be used over a long duration, up to 94.5 hours in the experiment, are advantages compared to cellular membranes, such as Caco-2. To evaluate this setup's usefulness in assessing drug precipitation in the upper fasted small intestine, further studies must be carried out incorporating a pH shift from gastric to intestinal media.

2.5.3 Compartmental methods using cellular membranes

Kobayashi *et al.*,[46] proposed a system for predicting drug absorption using Caco-2 cells, which also accounted for the pH change from the stomach to the intestine. The drug was dissolved in a vessel that simulates the stomach (pH 1.0, volume of medium 3 mL) and a pump transferred the dissolved drug to a vessel (pH 6.0, volume of medium 3mL) for pH adjustment. The solution with the adjusted pH was then transferred to the compartment containing the Caco-2 monolayer. The same setup was also used by Sugawara *et al*.,[47] where additionally the effect of pH change in the "gastric vessel" (i.e. simulating achlorhydria or patients administered with proton pump Inhibitors or H2-receptor antagonists) was evaluated. Significant differences were found in the cumulative permeation of two albendazole formulations at raised and normal gastric pH in this experiment. These results qualitatively agreed with a previous rabbit study carried out using the same albendazole formulations.[48] However, the culturing

time required for the Caco-2 cells limits the throughput capacity of this method. Issues concerning the poor reproducibility of results, and incompatibility with some solubilising excipients (e.g. surfactants) and media (e.g. FeSSIF) also further limit the use of Caco-2 cell monolayers as intestinal barriers in studies examining intestinal precipitation.[45]

3. Full scale methods to assess drug precipitation

In late stages of formulation development, where larger amounts of the API are available, full scale methods and setups are required, in order to accurately characterise and predict the behaviour of the formulation, after administration of clinically relevant doses. These full scale techniques aim to evaluate the supersaturation and/ or precipitation of the drug product and to help understand the effect of different excipients on its kinetics. The main goal is to link the bioavailability of the drug product to the amount of drug which is in solution in the upper small intestine, where absorption mainly takes place.

3.1 Compendial Apparatus and Methods

3.1.1 USP I and USP II dissolution apparatus and methods

The basket (USP I) and paddle (USP II) apparatus were first introduced into the United States Pharmacopeia in the 1970's for evaluating the dissolution characteristics of oral drug products.[49] They have primarily been used to fulfil a QC function for testing a variety of oral dosage forms[50] and provide a large volume of media for a dosage form to dissolve in a well stirred environment.[51]

Dissolution testing using either the USP I or USP II apparatus is conducted under various parameters and conditions, including variations in hydrodynamics, type and volume of dissolution medium.[52] Typically the volumes used in the basket/paddle apparatus range from 500-1000 mL and these large volumes are often useful to generate sink conditions required to achieve complete dissolution. However, they are far in excess of volumes in the human stomach and intestine, which do not typically exceed 250 mL in the fasted stomach and 30-100 mL in the fasted upper small intestine.[53][54]

The simple aqueous buffers typically used in the USP methods fail to reflect the composition of the GI contents. This can lead to a misinterpretation of the *in vivo* dissolution profile, where supersaturation, precipitation and re-dissolution might occur. Apart from the pharmacopoeial buffers, different levels of biorelevant media can be used for simulating the composition of the GI fluids. Biorelevant media have demonstrated advantages over compendial media when assessing drug performance *in vivo*.[22][55][56] Wagner *et al.,* carried out an experiment comparing the use of compendial and biorelevant media with the USP II apparatus for Compound A, a basic BCS class IV drug.[56] It was found to have a much greater solubility and dissolution rate constant (z value) in biorelevant media representing the upper fasting intestine, compared to simple media at the same pH. The STELLA® software was used to model the predicted drug plasma profiles from the dissolution data and a stronger correlation to the human *in vivo* data was observed from the profiles predicted from the dissolution experiments using biorelevant media.

The transfer process from the stomach through different parts of the intestine is not taken into consideration when using the compendial USP I and USP II dissolution methods. This process is

important for IR formulations of weak bases, as the drug might precipitate as it enters the small intestine, and MR formulations, which are commonly designed to deliver the drug to distal, as well as proximal sites of the GI tract.

3.2 The "Dumping Test"

Kambayashi *et al*.,[57] proposed a simple pH-shift test, the so called "dumping test", in which 50 mL solutions of two weak bases, dipyridamole and ketoconazole, in 0.02 N HCl at various concentrations were "dumped" into 450 mL of FaSSIF-V2. In this case, FaSSIF-V2 had higher concentration of sodium taurocholate and lecithin, so after "dumping" of the drug solutions, the final concentrations of sodium taurocholate and lecithin in the dissolution vessel corresponded to the composition of FaSSIF-V2. The results from this *in vitro* setup were successfully coupled with Stella® software and a predictive model for the total and dissolved concentration in small intestine for both drugs, after oral administration in the fasted state was established. The advantage of this simple approach is that it could be used as an early assessment and pre-screening tool for drug precipitation during early stages in drug product development to facilitate the design and development of new drug products. The performance of this method as a screening tool and its possible preference over the more complicated transfer methods should be further investigated.

3.3 Compartment methods not addressing intestinal absorption

3.3.1 Closed Systems

It was Kostewicz *et al.*,[58] who first introduced the so called "transfer model", which simulates the transfer of drug from the stomach to the upper small intestine. This setup is a two compartment compendial dissolution method where contents of the vessel, in which dosage form's performance under simulated gastric conditions (donor compartment) is evaluated, are transferred with a pump into another vessel, where the conditions in the small intestine are simulated (acceptor compartment) (Figure 6). In that study, the donor compartment containing the dissolved drug in 125 mL SGFfast was transferred at a constant rate between 0.5-9.0 mL/min (values within the observed physiological range) into the acceptor compartment 500mL Level II FaSSIF. The results indicated that this setup is useful in predicting supersaturation and precipitation of all weakly basic compounds tested, under fasting conditions. Furthermore, it was clear that gastric emptying rates may play an important role on the precipitation kinetics. Such effects of the transfer rate can be considered by mathematical modelling as it has been proposed for the *in vitro* transfer test by Arnold *et al*.[59] The classical transfer test was here used together with an on-line particle analyser and in-line Raman spectroscopy to study the kinetics of drug precipitation. A nucleation and growth model was used at two transfer rates (4 and 9 mL/min) and experimental results for dipyridamole were in good agreement with the model.

Due to the shortcomings of the initial transfer model, such as the zero order rate of drug pumping from the donor compartment to the acceptor compartment, Ruff *et al.*,[60] attempted to optimise

the experimental conditions of the originally proposed transfer model, using ketoconazole as model compound. In this study, the "average" physiological GI conditions were taken into consideration, while the impact of extreme conditions was also evaluated. To reflect fasting gastric emptying behaviour *in vivo,* a first order transfer rate with half-life of 9 minutes was used. Generally, the optimised transfer model by Ruff *et al.* was successful in simulating the *in vivo* dosage form performance. Nonetheless, one disadvantage of this model is that it fails to take the absorption process into consideration, which might be crucial to whether precipitation occurs or not, and thus also in determining drug plasma concentrations. It was concluded that this *in vitro* model over-predicted the precipitation behaviour of ketoconazole. The authors also mention that for BCS Class II compounds, which have high or moderate permeability values, *in vivo* precipitation may be reduced due to the continuous *in vivo* absorption of the drug through the intestinal mucosa. This may not apply to BCS Class IV drugs with low permeability characteristics, where possible precipitation seriously affects the amount of drug available for absorption. To circumnavigate the lack of absorption in the *in vitro* model, the authors coupled the results obtained with the transfer model to a PBPK model, where absorption was taken into account. With this approach not only was precipitation shown not to occur in the intestinal compartment, but the plasma profile was accurately simulated in humans.

3.3.2 Open systems

The Artificial Stomach Duodenal (ASD) model has two chambers representing the stomach and the

duodenum. In the standard setup, the gastric and duodenal chambers have a maximum capacity of 400mL and 50mL respectively,[61] with fluid transfer controlled by a series of five pumps, accounting for stomach and duodenal secretions and chamber emptying. The initial starting volumes in the chambers, the flow rate of fresh media into the chambers and the empting rate from the chambers can all be adjusted to fit the experimental requirements (e.g. *in vitro* modelling of fasted/fed state, human or dog model).[61][62][63][64] Dilute HCl and FaSSIF are typically used as gastric and duodenal fluid respectively. Dissolution is the primary process which occurs in the ASD's gastric and duodenal chambers. However, concurrent precipitation can also occur in these chambers. The ASD model has been used to examine the relative bioavailability of various drugs.[61][62][65] When assessing the performance of the weakly basic drug galunisertib, the ASD showed that the formulations maintained supersaturation upon transition into the duodenal chamber and that no significant precipitation occurred throughout the experiment (150 min).[63] In order to account for the information obtained from the ASD model in the absorption modelling, a precipitation time of 11 hours was estimated by the GastroPlus® software. This estimate exceeds the usual small intestine transit times which are observed *in vivo* and confirms that galunisertib could maintain supersaturation in the small intestine for a longer period than 15 min, which is the default value used in GastroPlus® when no experimental data are available. Combining the ASD data and other biopharmaceutical results (e.g. permeability) as inputs for GastroPlus®, the simulated plasma concentrated profiles for the three tablet formulations were found to have AUCs of between 90-105% of the observed human clinical data. The model was able to successfully rank the *in vivo* bioavailability of the different formulations of galunisertib used in the clinical trials. The ASD model

was also used to check the effect of gastric pH on LY2157299, a weakly basic BCS class II drug, which had showed variability of absorption in early studies carried out in dogs.[64] Compared to humans, dogs have a larger variability of basal gastric pHs, which can be a source of error when assessing the *in vivo* performance of drugs with a pH dependent solubility in dogs. To model the variability in dog gastric pH, experiments were carried out in the ASD using gastric fluid at pH 2 and pH 4.5, using 10^{-2} N HCl or 10^{-4} N HCl respectively. While the ASD model was able to qualitatively predict the effect of variability of stomach pH on the bioavailability of LY2157299, it overestimated the influence of the raised gastric pH on the absorption of LY2157299.

Takeuchi *et al.*,[66] evaluated the performance of a three compartment setup (Gastrointestinal Simulator-GIS) for predicting *in vivo* dissolution and precipitation. The three compartments of the GIS represent the stomach, the duodenum and the jejunum, where different buffer species, volumes and pH values were used to mimic the *in vivo* conditions. The fluid transfer rate from the gastric to the duodenal compartment was set at a first order rate with a half-life between 5-10 minutes. In this particular setup, paddles were adjusted to give a high-speed burst at certain intervals to simulate the contractions in the stomach and the duodenum. Gastroplus® software was used to determine the *in vitro* gastric emptying time, which provided the best fit to *in vivo* data for two BCS Class I drugs, propranolol and metoprolol. Overall, the GIS was able to predict the *in vivo* performance of the investigated compounds. The GIS setup was also used by Matsui *et al*.,[67] to investigate the impact of elevated gastric pH. When coupled with *in silico* modelling GIS could be useful for assessing *in vivo* precipitation of BCS Class II weakly basic compounds, but incorporation of an absorptive site, to mimic the continuous drug removal from the intestine, might be beneficial for enhancing its *in vivo*

predictability.

A slightly modified form of the GIS (mGIS), was used by Tsume *et al*., [68] to investigate the absorption kinetics of the weakly basic drug dasatinib. In this study, the *in vitro* results from the dissolution experiments performed in the USP apparatus II and mGIS, were coupled with Gastroplus® in order to predict plasma concentrations. The predicted plasma profiles were compared with clinical data. The dissolution profiles of dasatinib acquired with the USP apparatus II did not indicate precipitation and resulted in absorption profiles, which did not match the human data. On the other hand, the dissolution profiles acquired with the mGIS exhibited supersaturation and precipitation of dasatinib and, when coupled with Gastroplus®, resulted in better plasma concentration predictions. Despite the fact that the PBPK model underestimated the overall Cmax and AUC, something that could be partially attributed to underestimated permeability values, the study clearly demonstrated the benefit of assessing drug supersaturation and/ or precipitation with a more complex setup. Tsume *et al*., have used also the GIS to assess the supersaturation/ precipitation kinetics of the two weakly basic compounds; dipyridamole and ketoconazole.[69] For both compounds, and in accordance to previous studies,[70][10][58][60] the precipitation rates observed in the intestinal compartments of GIS were overestimated, most likely due to lack of an absorptive compartment. This study highlighted once more the importance of accounting also for the absorption process when assessing precipitation with various setups *in vitro*.

3.4 Compartment methods which attempt to account of absorption

Although models which do not account for the intestinal absorption process can be useful in predicting *in vivo* drug supersaturation and/ or precipitation, the *in vivo* performance of a drug product is highly dependent not only on the GI transfer, but also on other important parameters, such as the intestinal permeability. As mentioned previously, for drugs with high or moderate permeability values, *in vitro* setups can overpredict *in vivo* precipitation as the sink conditions created by continuous removal of the drug through the gut wall are not simulated *in vitro*. In order to account for drug absorption in the *in vitro* experiment, a number of models have been setup.

3.4.1 Using appropriate flow rates to take into account both absorption and transit process

These methods have been proposed primarily for evaluating products of highly permeable APIs. Psachoulias *et al*.,[70] introduced a three-compartment setup for the prediction of intraluminal precipitation of ketoconazole and dipyridamole. This setup consisted of a gastric, a duodenal and a reservoir compartment. The reservoir compartment contained concentrated Level II biorelevant medium with the purpose of keeping pH values, lecithin and bile salt concentrations constant in the duodenal compartment, thereby compensating for the dilution that occurs when the simulating

585 gastric fluid is pumped into the duodenal compartment. During each experiment the volume of the
586 medium in the duodenal compartment was kept constant at 60 mL. The flow rates between the
587 compartments were regulated by a multi-channel peristaltic pump and a first order gastric emptying
588 rate of 15 minutes was used. The contents of the duodenal compartment were completely renewed
589 with fresh medium every 15 minutes. Using this experimental setup the measured *in vitro* duodenal
590 compartment concentrations were in line with the luminal concentrations measured in healthy
591 volunteers in a previously performed clinical studies.[10][71] Dose-dependent *in vitro* precipitation was
592 observed for ketoconazole. However, XRPD studies indicated differences in the solid state
593 characteristics of the precipitates; *in vitro* the precipitate of ketoconazole was crystalline, but *in vivo*
594 it was amorphous. Despite the good results presented with this methodology, the equipment is not
595 commercially available, thus restricting its application in the pharmaceutical industry.
596 Recently, Kourentas *et al.*,[72] introduced a new setup (Biorelevant Gatrointestinal Transfer system-
597 BioGIT) for simulating GI transfer and assessing duodenal concentrations, drug supersaturation
598 and/or precipitation of highly permeable APIs, by using commercially available equipment. This
599 setup also consists of three compartments: gastric, duodenal and reservoir compartment (Figure 7).
600 The reservoir compartment is used for maintaining the composition of the medium in the duodenal
601 compartment constant. Gastric emptying half-life is 15 minutes. The volume of the dissolution
602 medium in the gastric compartment is 250mL (10 mL resting volume, plus 240 mL to account for
603 administration with a glass of water) and the volume of the duodenal compartment is set at 40 mL.
604 Fluid from the duodenal compartment is moved away with a constant flow rate of 11.6 mL/min, so

that the volume in the duodenal compartment is kept constant throughout the experiment. These flow rate and volumetric values were estimated from luminal data previously collected from healthy adults. In this study, the ability of the BioGIT model to predict intraluminal concentrations of dipyridamole, ketoconazole and posaconazole was evaluated. With the BioGIT setup the precipitated fraction *in vivo* was successfully predicted in every case.[73][74] Recently, BioGIT data were successfully used for informing PBPK modelling software and predicting the plasma profile of a moderately precipitating salt of weak base.[75] The method was shown to be useful for providing information on the impact of GI transfer on intraluminal concentrations of drugs, which are given as fast disintegrating tablets and capsules, dispersions or solutions. However, one should note here, that BioGIT has been designed to simulate intraluminal concentrations of highly permeable drugs, after administration in the fasted state. Therefore, flow rates might need to be adjusted to simulate concentrations of drugs with different permeability characteristics.[76] Evaluation of intra- and inter-laboratory reproducibility of BioGIT data is currently in process.[77]

Utilising a similar approach, based on the compendial dissolution apparatus II, Gu *et al.*,[78] described a multi-compartmental model with 4 compartments, comprising of a gastric, intestinal, absorption and a reservoir compartment, to maintain the composition in the intestinal compartment (Figure 8). The novelty of this setup was the addition of the “absorption compartment”, to simulate the uptake of drug across the intestinal membrane. All compartments were placed in a water bath at 37°C temperature and the pH in each vessel was maintained at a constant value. The drug was transferred with different flow rates between the compartments, the volumes of which were kept constant and

controlled by a peristaltic pump. Vessel 1 contained 250 mL of dissolution medium in order to simulate the available volume of gastric fluids in the stomach in the fasted state. Vessel 2 contained 250 mL of dissolution medium, simulating the composition of the upper small intestine, and after the inflow from vessel 1 for one hour the volume in vessel 2 increased to 500 mL. Vessel 3 contained 600mL of ethanol and 100 mL of 0.1 N HCl solution in order to maintain drug concentrations below their solubility values throughout the experiment. In this study, the precipitation kinetics of two weak bases, cinnarizine and dipyridamole was investigated. It was concluded that this method could successfully predict drug precipitation in the lumen, and the results from this multi-compartmental system correlated better with the *in vivo* data compared with the conventional dissolution methods. Cinnarizine and dipyridamole were found to have significantly different precipitation characteristics, despite both being fully dissolved at gastric pH. Approximately 40% of the cinnarizine was found to precipitate in the intestinal vessel compared to <10% of the dipyridamole dose. The setup from Gu *et al.,* simulates the absorption process in a simple dissolution apparatus and no complex additions/methods are needed. Furthermore, the flow rates between the intestinal and absorption compartments can be adjusted to reflect different permeability values, thus facilitating its use in the investigation of precipitation kinetics for APIs with different permeability properties. However, the remaining challenge of this setup is that it is difficult to adjust the flow rates to the absorption compartment so that they would correlate with *in vivo* permeability values.

Mitra *et al.*,[8] proposed yet another setup to simulate the dynamic environment of the GIT; the "simulated stomach duodenum" model (SSD) (Figure 9). The SSD model was modelled after the system described by Carino *et al*.,[62] (section 3.3.2) and it is a four compartment model, where the

removal of drug from the duodenum is also taken into account. The study explored the ability of the SSD to predict the supersaturation of different dose strengths of dipyridamole under fasted conditions, as well as to investigate the impact of different surfactants, which are commonly used in oral preparations. In the SSD model, basal volumes in both gastric and duodenal compartment were used, based on mean fluid volumes previously reported in clinical studies in fasted adults. The basal gastric volume was set at 50 mL and the duodenal at 30 mL. The gastric emptying was simulated by a first order pattern with a half-life of 15 minutes, until the basal gastric volume was restored. Afterwards, the gastric emptying was kept constant at 1.7 mL/min. The setup was able to predict the supersaturation kinetics of dipyridamole, when compared to *in vivo* data. Furthermore, the effect of different surfactants commonly used in oral preparations, as well as the effect of different gastric emptying patterns on dipyridamole supersaturation was investigated. The SSD model provided good correlation of the amount of drug in solution in the duodenal compartment of the SSD to the bioavailability of different dosage strengths of dipyridamole *in vivo*. However, again in this setup does not take into account the application of different flow rates to adjust for low permeability values. Furthermore, the SSD model is not based on a commercially available setup, such as the USP II dissolution apparatus, and agitation is performed using magnetic stirrers at 200 rpm, which entails hydrodynamics that are less reproducible and not physiologically relevant. In order to investigate its usefulness in predicting drug precipitation, more studies with different compounds are needed.

Another multicompartment method incorporating a chamber simulating the systemic circulation was

665 proposed by Selen *et al*.[79] The FloVitro™ (Dow Chemicals) is a three compartment system with
666 chambers simulating the gastric, intestinal and systemic compartments (cells) and flow rates between
667 cells. The fluid volume in the cells varies depending on the compound tested; typically 40 mL in the
668 gastric cell, 200 mL in the intestinal cell and 1 - 2 L in the systemic cell are used. The primary use of the
669 FloVitro™ has been to predict the *in vivo* plasma profile based on the profile which is achieved in the
670 systemic cell by using a variety of drugs, including: ibuprofen, furosemide, paracetamol and doxycycline
671 hydrochloride.[79][80] The effect of fed or fasted state media has also been examined on the dissolution
672 profiles of danazol and furosemide.[81] However, there have not been any publications to date
673 illustrating its application to precipitation of poorly soluble weak bases and further studies will need to
674 be undertaken.
675
676 *3.4.2 Simulating the intestinal epithelial barrier*

677 Ginski *et al*., [82] proposed a "two-step" dissolution/Caco-2 system with the aim of simulating
678 simultaneous dissolution and absorption in the GIT. This would enable prediction of the dissolution-
679 absorption relationship for different compounds and allow a comparison with results from clinical
680 studies. This continuous system consisted of a dissolution apparatus and a side-by-side diffusion cell.
681 The drug is dissolved in the dissolution compartment and, after a filtration with a 10 µm filter, is
682 transferred with a pump to a donor compartment, containing the Caco-2 monolayer. In this
683 particular study, fast and slow dissolving formulations of piroxicam, metoprolol tartrate and
684 ranitidine hydrochloride were tested. Generally, this two-step setup was able to reflect the

685 qualitative dissolution-absorption relationships. This setup can be considered as a first attempt to
686 couple dissolution with permeation through Caco-2 cell lines, although it is obvious that several more
687 factors would need to be considered. For example the lack of appropriate first order gastric
688 emptying, GI transfer and the level of biorelevance of the media need to be taken into account to
689 better simulate the *in vivo* drug absorption and accurately predict plasma concentrations.
690 In the same logic of assessing simultaneously dissolution and permeation through cell monolayers,
691 Kataoka *et al.*,[83] introduced a dissolution/permeation system (D/P), which consisted of an apical
692 and basolateral compartment mounted with a Caco-2 monolayer. The volume of apical and
693 basolateral compartments was 8 mL and 5.5 mL respectively. Magnetic stirrers were placed in both
694 compartments and Hanks balanced salt solution issued in both sides as a transport medium. Kataoka
695 *et al.*,[84] utilised the same technique, but improved its biorelevance by using a modified Hank's
696 balanced salt solution containing sodium taurocholate (3 mM) and lecithin (0.75 mM) as a transfer
697 medium. Overall, the D/P system was proposed to be a useful tool for formulation selection during
698 drug development.[85] Nonetheless, it is mainly used for powders or suspensions and despite the use
699 of a more biorelevant transfer medium, the D/P system is far from properly mimicking *in vivo*
700 conditions, such as hydrodynamics, fluid volumes, GI transfer etc. Furthermore, to the best of our
701 knowledge it has not been used to study precipitation and/or supersaturation kinetics.
702 In a bid to overcome these deficiencies, Motz *et al.*,[86] introduced a new system which consists of
703 the flow through dissolution apparatus (USP apparatus IV) and a flow through permeation module.
704 The latter includes an open apical and a closed basolateral compartment with a Caco-2 monolayer
705 between them. The flow rates of the dissolution medium which was transferred from the USP IV to

706 the permeation module had to be carefully adjusted, to assure the viability and integrity of the Caco-
707 2 cell membrane. Indeed, the authors suggested the use of a stream splitter, which successfully
708 allowed the combination of compendial and commercially available USP apparatus IV with a
709 permeation/Caco-2 compartment. Krebs buffer was chosen as the dissolution and permeation buffer
710 for the installation of this apparatus. While the use of biorelevant dissolution media could be more
711 physiologically relevant and perhaps produce better results, the authors acknowledged that a first
712 evaluation of this new setup was the initial scope of the study. Despite the fact that, the Caco-2 cell
713 monolayers is a useful *in vitro* technique to assess drug permeation and allows good predictions of
714 *in vivo* drug permeability,[87][88][89] there are many limitations in their use, as mentioned previously in
715 section 2.5.3, that need to be considered. These limitations include: different cultures of Caco-2 cells
716 resulting in data variability, difficulty in predicting paracellular transport as a result of tighter
717 junctions between the colonic cells and long period of time required for cell culture.[90] The lower
718 surface area for the Caco-2 cell line compared to the intestinal membrane is also disadvantageous
719 when examining the relationship between precipitation and absorption.
720
721 As mentioned previously, an alternative to the absorptive cell monolayers for simulating drug
722 absorption is through the use of biphasic media.[91][92][93] In this case, the drug is dissolved in the
723 aqueous phase and partitions in the organic phase. The drug concentration profile that is acquired
724 from the organic phase could be an effective surrogate of the amount of drug available for
725 absorption. Vagani *et al*.,[94] developed a system by combining biphasic media and a flow through
726 (USP IV) technique. In particular, the USP apparatus IV is combined with a USP II apparatus. The cells

from the USP IV apparatus are used to hold the formulations and the dissolution vessels from the USP II apparatus contain the biphasic dissolution media, maintained at 37 °C. This system has also been used by Shi *et al*.[95] Overall, this system established good IVIVC with the drug concentrations obtained in the organic phase and the biopharmaceutical performance of the different formulations was well discriminated. Tsume *et al*.,[96] combined the Gastrointestinal Simulator (GIS) introduced by Takeuchi *et al*., with biphasic media in order to investigate whether the addition of the organic layer would lead to better predictions of the plasma concentrations of two poorly soluble, weakly basic compounds, raloxifene and ketoconazole. Indeed, the results of ketoconazole showed slower decline of drug concentration in the small intestinal compartments with the presence of the organic phase, than those without the addition of the biphasic system, thus providing another calculated precipitation rate constant. Incorporation of these data to physiologically based pharmacokinetic (PBPK) models and simulation with Gastroplus® suggested a slight improvement in the *in vivo* predictions of ketoconazole. The combination of GIS with an organic layer provided also information about the partitioning characteristics of these two drugs to the organic phase.

To account for the absorption process, Hate *et al.*, developed an apparatus coupled with a high surface membrane area.[97] The apparatus consisted of a donor compartment, where drug dissolution takes place, a buffer reservoir and a receiver chamber, to collect drug after diffusion through the membrane. A hollow fibre membrane was used to simulate intestinal absorption, due to its high surface area per unit volume of fluid, thus facilitating higher mass transfer. A pump is used to control the transfer of

746 fluid (Figure 10). In particular, fluid from the donor compartment is transferred to the inner side of the
747 hollow membrane, where at the same time fluid from the reservoir compartment is transferred to the
748 outer side of the hollow membrane. The drug diffused through the membrane into the fresh buffer,
749 which was collected into the receiver chamber. The donor fluid emerging from the membrane module
750 was then recycled back into the donor chamber. Three different formulations of the weakly basic drug
751 nevirapine were tested using this apparatus. Initially, the media in the donor chamber was 0.1M HCL.
752 After 30 minutes, the pH was adjusted to pH 6.5 using 0.17 M Na_2HPO_4 and the absorption system was
753 connected. When assessing the performance of nevirapine tablets and powder, rapid precipitation of
754 nevirapine down to its equilibrium solubility was observed in the donor compartment, upon transition
755 to pH 6.5. When a precipitation inhibitor (HPMC-AS) was used, there was no precipitation observed in
756 the donor chamber for up to 160 minutes, while an increase in drug concentration was observed in the
757 receiver chamber. However, no significant differences were observed in the dissolution profiles, when
758 the performance of nevirapine tablet, with or without the addition of the absorption membrane was
759 assessed. Overall the apparatus could be a useful tool for formulation screening and for assessing drug
760 precipitation and/or supersaturation. However, more data is needed to support its further application.
761
762 The artificial membrane insert system (AMI-system) was proposed by Berben *et al.,*[98] as a method to
763 simulate the passive absorption of drug in the intestine without the use of cells based systems, such
764 as Caco-2. The AMI-system consists of a regenerated cellulose membrane mounted between two
765 plastic rings, as shown in Figure 11,[98] and has shown comparable results to Caco-2 cells when

766 assessing the permeability coefficients of poorly soluble drugs. To study the interplay between
767 supersaturation, precipitation and absorption, Berben *et al.,* carried out an experiment to examine
768 the potential of the AMI system using loviride, posaconazole, itraconazole and fenofibrate.[99] Initially,
769 samples were added to Level II fasted state simulated gastric fluid (FaSSGF) with constant stirring at
770 300rpm, with magnetic stirrers. After 15 minutes, the acidic medium was added to concentrated
771 Level II FaSSIF. Following another 15 minute period of stirring, a sample of the intestinal fluid (665µL)
772 was added to the donor side of the AMI system. In the case of loviride, the meta-stabilised
773 supersaturated state resulted in higher concentrations of drug in the acceptor compartment,
774 whereas lower concentrations of drug were found in the acceptor chamber when precipitation was
775 induced. An enhanced permeation into the acceptor compartment was also observed for
776 posaconazole when it was administered as an acidified suspension compared to a neutral suspension
777 using the AMI-system (concentration at 120 minutes: acidified suspension: 1.12 ± 0.01 nmol and
778 neutral suspension: 0.44 ± 0.01 nmol). This trend corresponded to an *in vivo* study carried out by
779 Hens *et al.,*[12] which found a twofold increase in $AUC_{0\text{-}8hr}$ following administration of the acidified
780 suspension versus the neutral suspension. When evaluating the performance of the AMI system
781 using three different bioenabling formulations: Sporanox® solution (itraconazole), Lipanthyl®
782 capsules and Lipanthylnano® tablets (fenofibrate), the drug concentrations achieved in the acceptor
783 compartment of the AMI-system were qualitatively well correlated with the respective intraluminal
784 drug concentrations. However, further validation of the setup is required with other compounds.
785 Overall, the AMI-system when coupled with the two-stage dissolution test proved to be a useful early

786 screening tool in assessing the possible *in vivo* precipitation of APIs as well as the performance of
787 formulated drug-products.

788 Finally, the TNO TIM-1 is a computer controlled multi-compartmental model of the human GIT. It
789 was developed by the TNO Nutrition and Food Research centre based on data from *in vivo* human
790 studies[100] and simulates the dynamic digestive and physiological processes in the stomach and small
791 intestine.[101] The TIM-1 system models the absorption of small molecules through their uptake from
792 filtration membrane systems or dialysis in the ileal and jejunal sections of the system. In this way the
793 amount of drug in solution which is available for absorption (expressed as "bioaccessibility") can be
794 measured. However, some active processes such as active absorption, efflux and intestinal
795 metabolism are not modelled by this system. It has been suggested that the TNO TIM-1 system can
796 be coupled with other intestinal absorption systems to facilitate modelling of these processes, thus
797 enabling an estimation of oral bioavailability of compounds.[102] The majority of studies carried out
798 with the TIM-1 have focused on the absorption of nutritional compounds and there are only a limited
799 number of published studies focusing on the uptake of pharmaceutical compounds.[103][104][105][106]
800 The biorelevance of the TIM-1 system would indicate significant potential to model precipitation of
801 drug in the GIT. However, the complexity of the system, time required for set up and the limited
802 throughput are significant limitations when considering its potential use as a tool to predict *in vivo*
803 precipitation.
804 Recently, Van Den Abeele *et al*., have used an updated version of TIM-1 (TIMagc) with the aim of
805 investigating the intraluminal behaviour of diclofenac in the fasted and fed state.[107] The results

obtained from the *in vitro* setup were compared with intraluminal and systemic data collected from healthy volunteers after the administration of diclofenac tablets along with 240 mL of water. The data obtained with this method can suggest slow dissolution and/or precipitation of diclofenac in the stomach, but it was not possible to mechanistically discern between these two mechanisms. The potential of TIM-1 to assess precipitation in the upper small intestine must be further investigated using weakly basic drugs and bio-enabling formulations.

4. Coupling full scale *in vitro* testing with Physiologically Based Pharmacokinetic (PBPK) modelling

PBPK modelling has been widely used and rapidly developed in the last years with many applications in academia and in the pharmaceutical industry. Furthermore PBPK modelling has gained acceptance at various regulatory agencies as part of the submission package. In 2016 the Committee for Medicinal Products for Human Use (CHMP) of EMA and FDA published a draft guidance regarding the qualification of PBPK modelling, regarding its use to support marketing authorisation.[108][109] This guidance aims to provide general information on which details should be included in a PBPK modelling report and which elements are needed in order for a PBPK platform to be qualified and evaluated for an intended purpose. Generally, PBPK modelling can be used for the prediction of human PK profiles from preclinical data and it is a useful tool for evaluating and optimising a clinical trial design. It can also be utilised for extrapolating the drug's pharmacokinetic behaviour in healthy

824 volunteers to patient populations, where clinical studies are difficult to be conducted. In order for a
825 successful drug model to be built by using the "bottom-up" approach, the quality of the input data
826 is of high importance.[110] Coupling *in vitro* data with *in silico* methods can be very important in
827 optimising and validating both *in vitro* and *in silico* models. Commercially available software, such as
828 Simcyp® Simulator, Gastroplus® and PK-Sim®, or open source and in-house modelling platforms can
829 incorporate *in vitro* data of supersaturation and precipitation kinetics. This can then lead to a better
830 understanding of the importance of supersaturation and/ or precipitation *in vivo*. In many cases,
831 combination of the *in silico* and *in vitro* methods to assess supersaturation and precipitation has
832 proven to be successful.[111][112][57][113] Gastroplus® software handles precipitation by incorporating a
833 mean precipitation time. This parameter is the mean time for particles to precipitate from solution,
834 when the local concentration exceeds the drug solubility and it is a function of luminal pH. Default
835 precipitation time is 900 sec, which was determined from exponential fit to the dipyridamole
836 transfer data, published by Kostewicz *et al*.[58] Certara® recently introduced the "Simcyp *In Vitro* Data
837 Analysis Toolkit- SIVA" which is designed to model *in vitro* experiments and estimate parameters for
838 input to *in vivo* simulations with the mechanistic PBPK Simcyp® Simulator. For modelling
839 supersaturation and precipitation, SIVA and Simcyp® Simulator use an empirical approach based on
840 critical supersaturation concentration and precipitation rate constant obtained in *in vitro*
841 experiments.[111] One issue with the available software is that they rely mostly on precipitation
842 kinetics which at best have been estimated with non-validated *in vitro* setups. Validation of *in vitro*
843 methodologies should ideally be based on intraluminal data in humans. It should also be noted that
844 it is difficult to build a successful, validated model which can predict the behaviour of a drug *in vivo*,

especially for BCS II, III and IV.[114] Nonetheless, coupling *in vitro* data with *in silico* models can help also to optimise the *in vitro* techniques which are used until today and understand which are the critical parameters for drug supersaturation and precipitation.

5. Conclusions

The increasing prevalence of poorly soluble drugs and use of bio-enabling formulations to achieve supersaturated states *in vivo* has triggered great interest in *in vitro* precipitation modelling. Overall, much progress has been made from the standard equipment used in QC testing and various *in vitro* models have been developed. Small scale tests are beneficial, especially in early stage of drug development, as drug quantities are often limited. Employing a small scale approach also facilitates the rapid parallel screening of multiple prospective formulations. Multicompartment models have proven useful to evaluate precipitation of drug upon transfer from the gastric to the intestinal environment. However, it would be reasonable to state that no single *in vitro* test is suitable for modelling precipitation in all circumstances. Further progress is still to be made to improve the predictive capabilities of such models, especially in terms of simulating the absorption of drug along the intestinal lumen. Coupling the results of *in vitro* tests with PBPK modelling has significant potential and must be investigated further. Improving the biopharmaceutics tools to predict *in vivo* precipitation will be a key step to improving the efficacy and reducing the development costs of

863 medicines.

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Acknowledgements

This work was supported by the European Union's Horizon 2020 Research and Innovation Programme under grant agreement No 674909 (PEARRL)

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1192 **LIST OF ABBREVIATIONS**

1193

Abbreviation	**Meaning**
aDS	Apparent Drug Supersaturation
AMI	Artificial Membrane Insert system
API	Active Pharmaceutical Ingredient
ASB	Acceptor Sink Buffer
ASD	Artificial Stomach Duodenal
BCS	Biopharmaceutics Classification System
BioGIT	Biorelevant Gastrointestinal Transfer system
CHMP	Committee for Medicinal Products for Human Use
D/P	Dissolution/Permeation
DIDR	Disk Intrinsic Dissolution Rate
DMSO	Dimethyl sulfoxide
EMA	European Medicines Agency
FaSSGF	Fasted State Simulated Gastric Fluid
FaSSIF	Fasted State Simulated Intestinal Fluid
FDA	Food and Drug Administration
FeSSIF	Fed State Simulated Intestinal Fluid
GIS	Gastrointestinal Simulator
HPLC	High-Performance Liquid Chromatography

HPMC	Hydroxypropyl Methylcellulose
IR	Immediate Release
IVIVC	*In-Vitro In-Vivo* Correlation
MDCK	Madin-Darby Canine Kidney
miBldi-pH	Miniscale Biphasic Dissolution Model with pH-Shift
MINDISS	Miniaturized Intrinsic Dissolution Screening
MR	Modified Release
PAMPA	Parallel Artificial Membrane Permeability Assay
PASS	Partially Automated Solubility Screening
PBPK	Physiologically Based Pharmacokinetic modelling
P_{eff}	Intestinal Membrane Permeability
PEG	Polyethylene Glycol
PK	Pharmacokinetic
QC	Quality Control
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SSD	Simulated Stomach Duodenum
SSPM	Standardized Supersaturation and Precipitation Method
t_{ind}	Induction time for Detectable Precipitation
UPLC	Ultra-Performance Liquid Chromatography
USP	United States Pharmacopeia
UV	Ultraviolet
XRPD	X-Ray Powder Diffraction

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FIGURES:

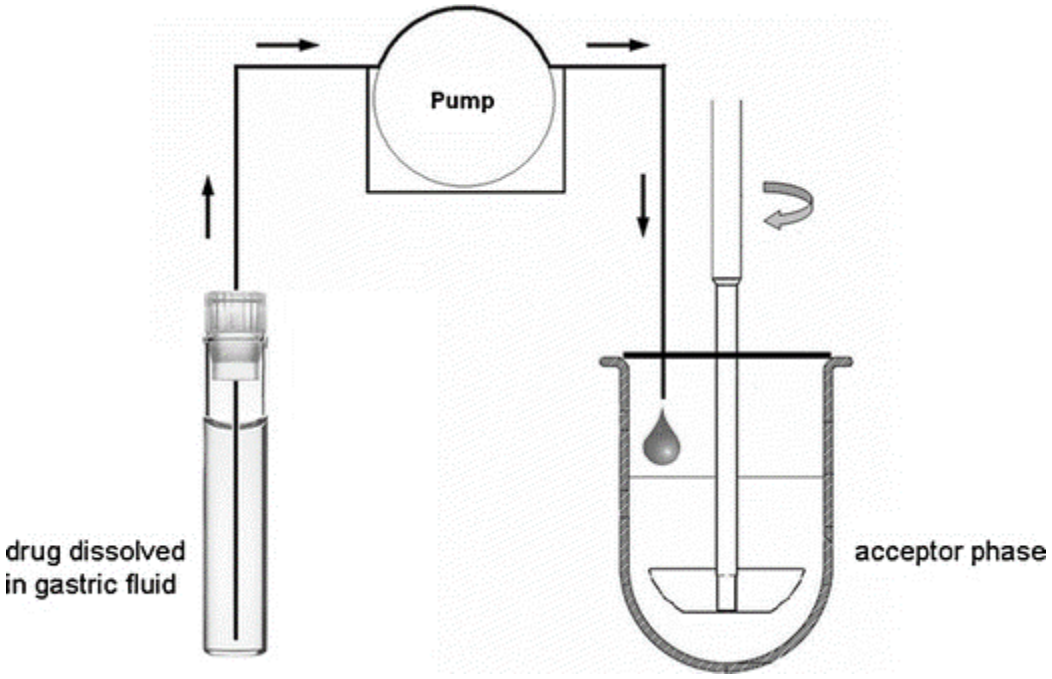


Figure 1: Schematic of miniaturized transfer model system proposed by Klein et al.[32] *Reproduced with permission from Springer.*

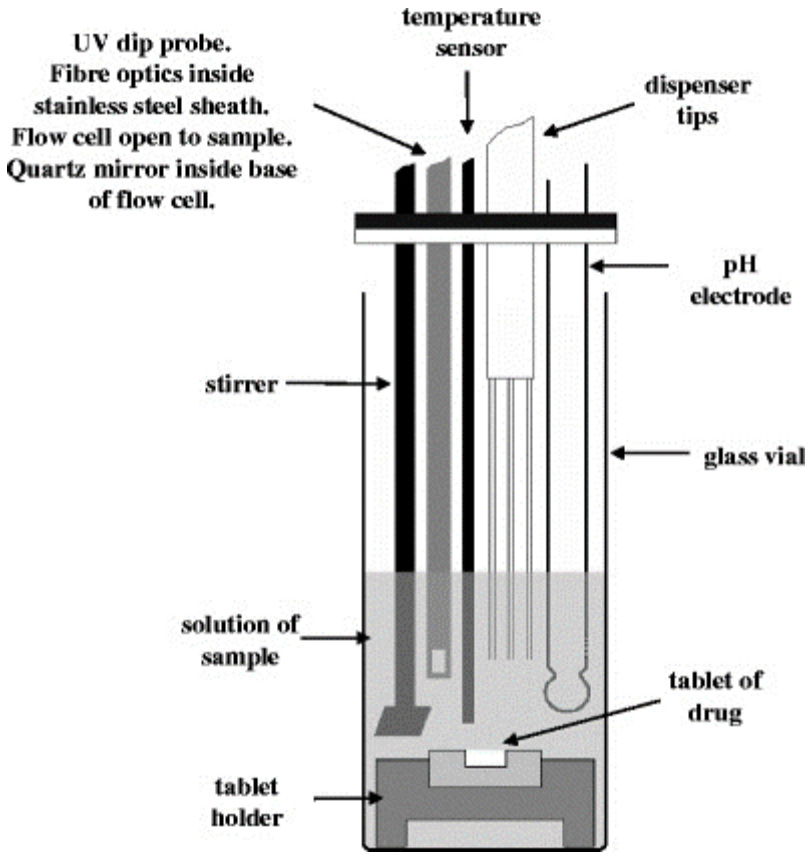


Figure 2: Schematic of the Sirius T3 instrument.[34] *Reproduced with permission from Springer.*

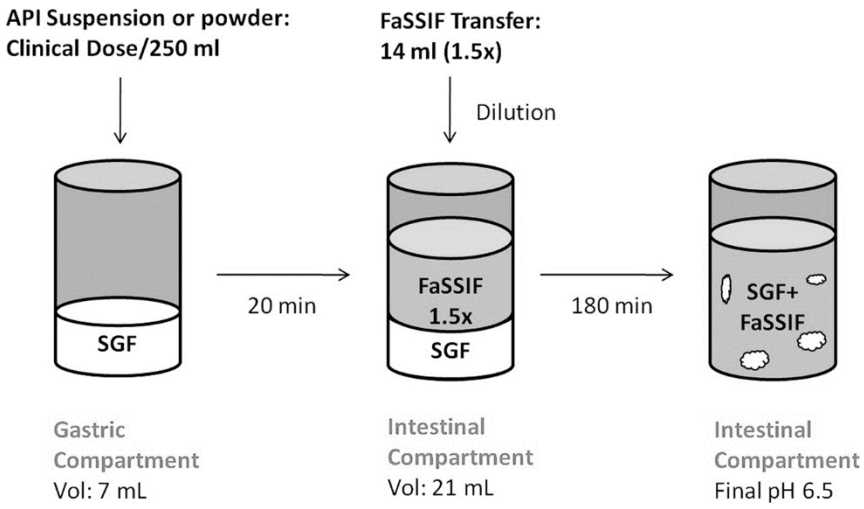


Figure 3: Schematic of the experiment carried out by Mathias et al.[36] *Reprinted (adapted) with permission from Mathias et al. Copyright 2013 American Chemical Society*

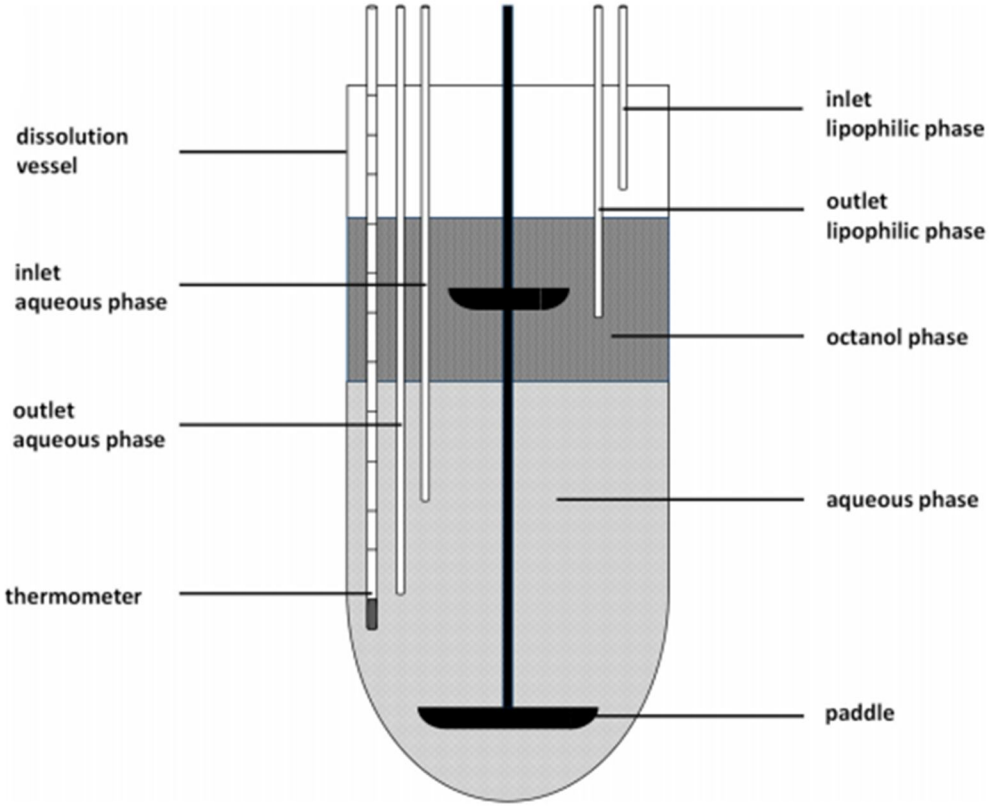


Figure 4: Schematic of the miBldi-pH apparatus.[38] *Reproduced with permission from Elsevier.*

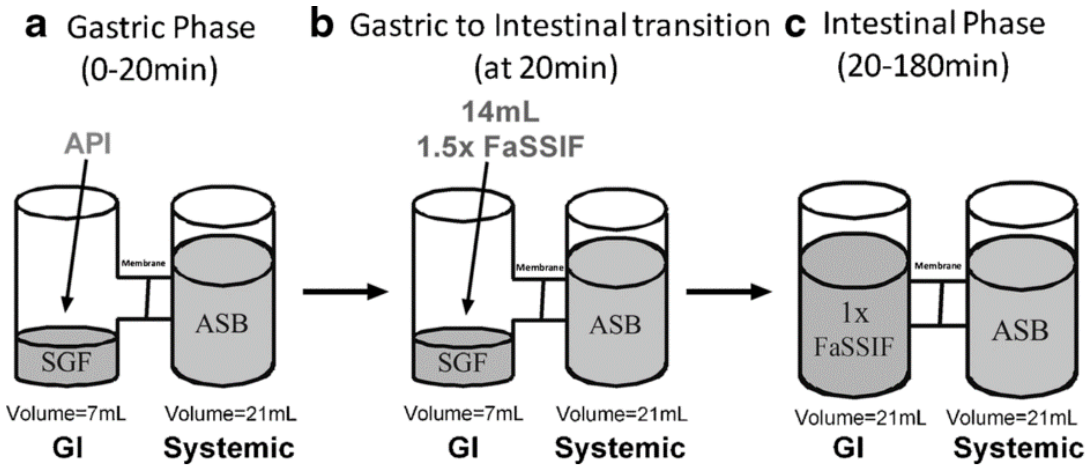
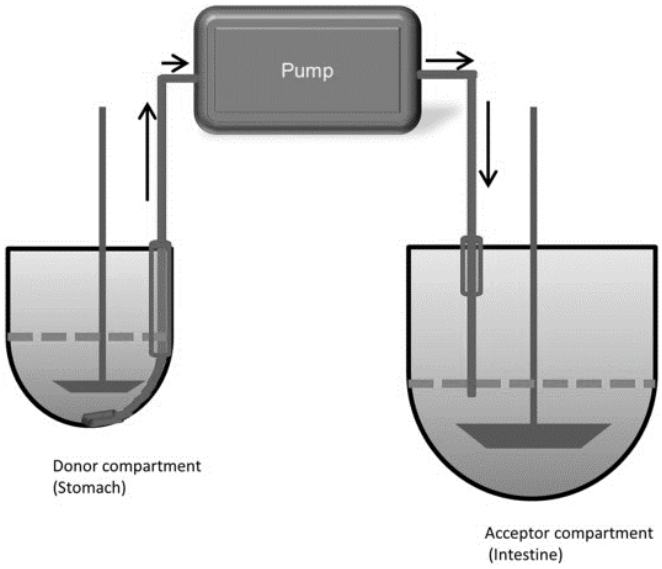


Figure 5: Schematic of dissolution-permeation experimental setup (µFlux apparatus) used by Zhu et al. ASB = Acceptor Sink Buffer.[42] *Reproduced with permission from Springer.*



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1215 *Figure 6: The "transfer model" proposed by Ruff et al.*[60] *Reproduced with permission from Elsevier.*

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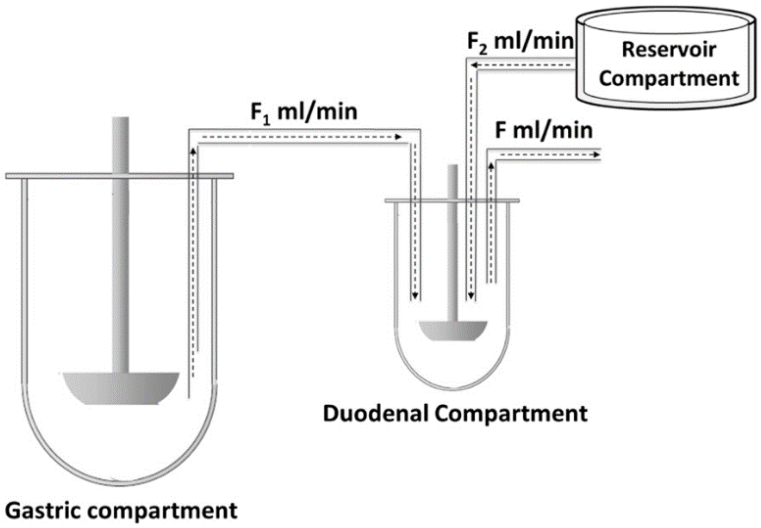

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1218 *Figure 7: The BioGIT system proposed by Kourentas et al.*[72] *F_1 and F_2 are the incoming flow rates and*

1219 *F is the outgoing flow rate ($F=F_1+F_2$). Reproduced with permission from Elsevier.*

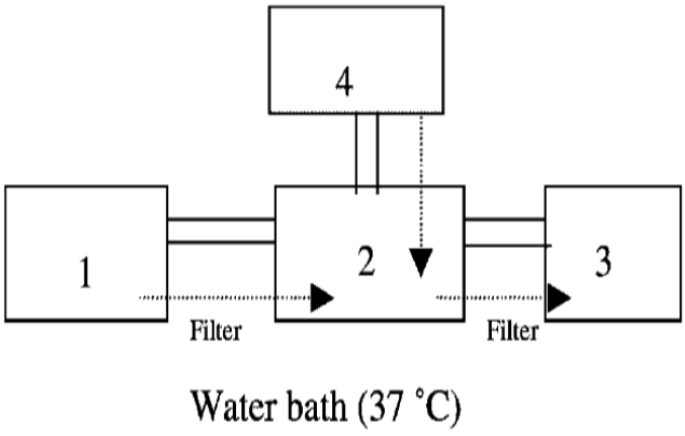
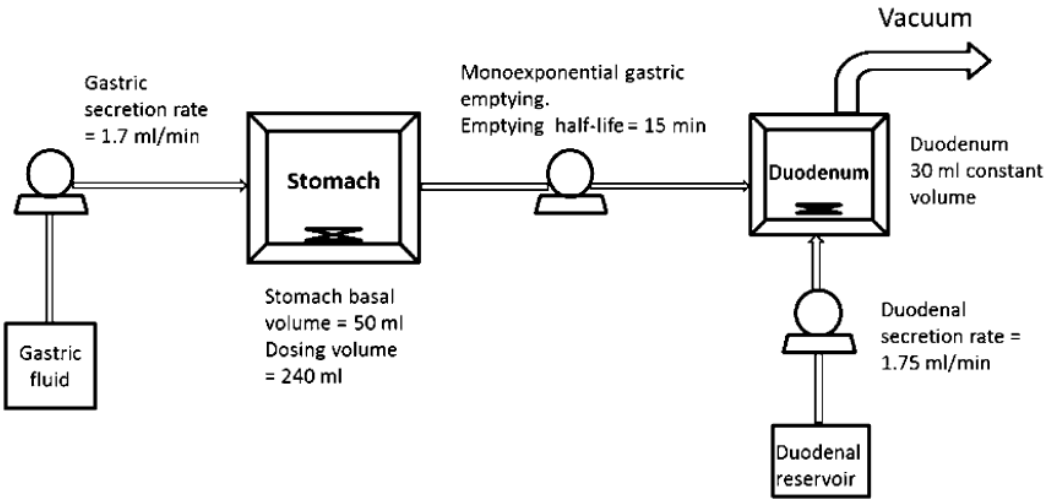


Figure 8: Multicompartment dissolution system by Gu et al.[78] *Vessel 1 "gastric" compartment simulating the stomach conditions; Vessel 2: "intestinal" compartment simulating the intestinal conditions; Vessel 3: "absorption" compartment simulating absorption; Vessel 4: reservoir vessel containing the dissolution medium identical to that in Vessel 2. Reproduced with permission from Elsevier.*



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1227 *Figure 9: Schematic diagram of the simulated stomach duodenum model (SSD) Reprinted (adapted)*

1228 *with permission from Mitra et al.*[8] *Copyright 2014 American Chemical Society.*

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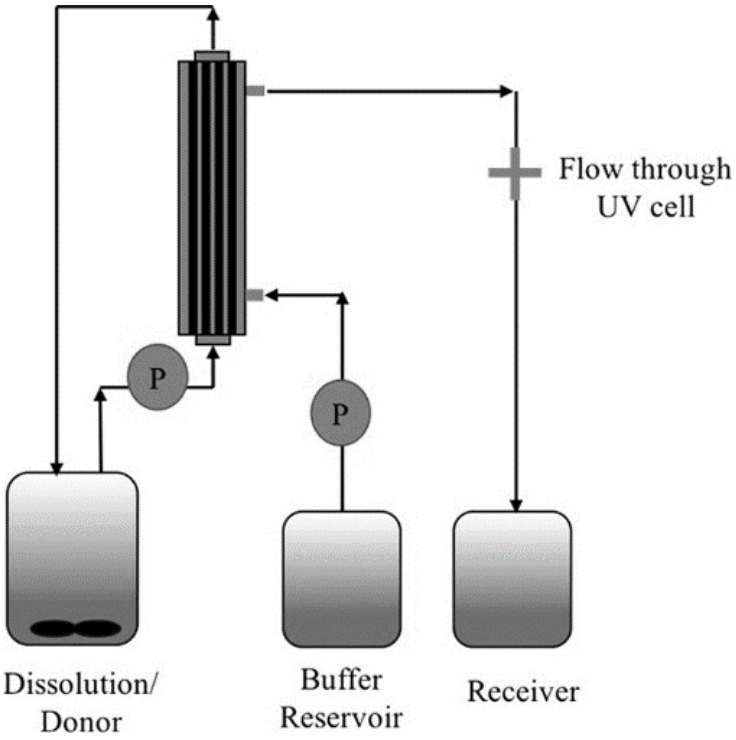

Figure 10: Schematic of the apparatus used by Hate et al.[97] *The hollow fibre membrane is represented by the grey and black tube. Reprinted (adapted) with permission from Hate et al. Copyright 2017 American Chemical Society.*

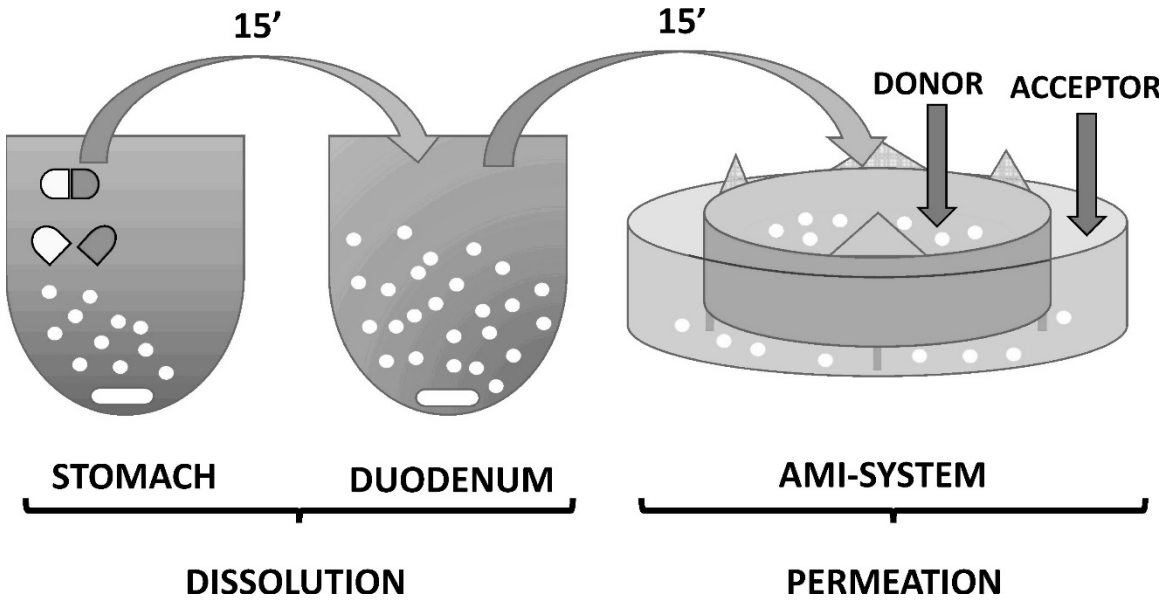

Figure 11: Schematic of the AMI-system proposed by Berben et al.[99] *Reproduced with permission from Elsevier*