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Early pharmaceutical profiling to predict oral drug absorption: Current status and unmet needs

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ABSTRACT

Preformulation measurements are used to estimate the fraction absorbed *in vivo* for orally administered compounds and thereby allow an early evaluation of the need for enabling formulations. As part of the Oral Biopharmaceutical Tools (OrBiTo) project, this review provides a summary of the pharmaceutical profiling methods available, with focus on *in silico* and *in vitro* models typically used to forecast active pharmaceutical ingredient's (APIs) *in vivo* performance after oral administration. An overview of the composition of human, animal and simulated gastrointestinal (GI) fluids is provided and state-of-the art methodologies to study API properties impacting on oral absorption are reviewed. Assays performed during early development, i.e. physicochemical characterization, dissolution profiles under physiological conditions, permeability assays and the impact of excipients on these properties are discussed in detail and future demands on pharmaceutical profiling are identified. It is expected that innovative computational and experimental methods that better describe molecular processes involved *in vivo* during dissolution and absorption of APIs will be developed in the OrBiTo. These methods will provide early insights into successful pathways (medicinal chemistry or formulation strategy) and are anticipated to increase the number of new APIs with good oral absorption being discovered.

1. Introduction

Large efforts are directed toward pharmaceutical profiling of active pharmaceutical ingredients (APIs) during the discovery and early development process. The aim of this profiling is to evaluate the potential of the API to display satisfactory biopharmaceutical properties. However, currently available methods are often not able to accurately predict *in vivo* API performance. The increased

Abbreviations: ADMET, absorption, distribution metabolism, elimination, toxicity; API, active pharmaceutical ingredient; BS, bilesalt; BS/PL, bile salt to phospholipid ratio; CMC, critical micellar concentration; DDI, drug–drug interaction; FaSSGF, fasted state simulated gastric fluid; FaSSIF, fasted state simulated intestinal fluid; FeSSGF, fed state simulated gastric fluid; FeSSIF, fed state simulated intestinal fluid; GI, gastrointestinal; HGF, human gastric fluid; HIF, human intestinal fluid; IVIVC, *in vitro–in vivo* correlation; IVIVR, *in vitro–in vivo* relationship; log*D*, logarithm of pH-dependent distribution of all species between octanol and water; log*P*, logarithm of partitioning between octanol and water of neutral species; lyso-PC, lyso-phosphatidylcholine; MO, mono-olein; OA, oleic acid; PBPK, physiology-based pharmacokinetics; PC, phosphatidylcholine; PEG4000, polyethyleneglycol 4000; pK_a, dissociation constant; PL, phospholipid; S, solubility; Tm, melting point.

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number of compounds profiled within the industrial research programs has led to the development of high throughput assays. In the early discovery stages when low amounts of API of limited purity is available, these provide physicochemical data allowing for categorization or binning of APIs into classes based on properties such as lipophilicity, solubility and permeability. During later discovery stages and at the phase of early development, when the compound library is significantly smaller, methods providing more accurately measured physicochemical properties are also used. In addition, the impact of factors such as components present in the gastrointestinal (GI) tract are investigated. In particular, solubilization effects obtained by colloidal lipid structures present in the GI fluid under fasted and fed conditions are explored. As part of the Oral Biopharmaceutical Tools (OrBiTo) project, this review provides a summary of the pharmaceutical profiling methods available, with focus on *in silico* and *in vitro* models typically used to forecast active pharmaceutical ingredient's (APIs) *in vivo* performance after oral administration. Here we provide a detailed review of the human and animal GI fluids under fasted and fed conditions (Section 2) and an update on the simulated intestinal fluids currently employed for *in vitro* dissolution studies (Section 3). Further, we provide a chapter on state-of-the-art methods for physicochemical and pharmaceutical profiling (Section 4), which are then set in an industrial context in Section 5. Finally, we analyse the gaps and challenges presently existing when using current *in vitro* methodologies to forecast the *in vivo* performance and the need for enabling formulations of APIs. It is expected that innovative *in silico* and *in vitro* methods that better describe molecular processes involved *in vivo* during dissolution and absorption of APIs will be developed in the framework of OrBiTo. We anticipate such models to early inform projects on successful pathways (molecular structure optimization and/or formulation strategies) to increase the number of APIs with good oral absorption being discovered.

2. Composition of GI fluids

The composition of the GI fluids has a large impact on the solubility and dissolution of poorly soluble API in the GI tract, and hence a large influence on the drug absorption. Gastric and intestinal fluids sampled from humans have been characterized in a number of studies, and this review summarizes the current knowledge with regard to pH, buffer capacity, osmolarity, surface tension and lipid concentration of GI fluids under fasted and fed conditions. It should be noted that the studies are varying in pre-dosing liquid and volume (if any), aspiration time, analytical methods and the composition of the ingested meal (for the fed state media). The focus of this review will be on data published on gastric, duodenal and jejunal composition. Although drug absorption takes place in the lower part of the gastrointestinal tract, the literature is sparse and will not be addressed further in this review (Hirtz, 1985).

The methods used for aspiration of gastric or intestinal fluids involve either intubation orally (Hernell et al., 1990; Lindahl et al., 1997; Carrière et al., 2000; Pedersen et al., 2000; Persson et al., 2005; Brouwers et al., 2006; Moreno et al., 2006; Kossena et al., 2007; Clarysse et al., 2009; AstraZeneca, data on file) or nasally (Armand et al., 1996; Kalantzi et al., 2006a, 2006b; Psachoulias et al., 2011; Vertzoni et al., 2012). After intubation, the position of the catheter is observed via fluoroscopy, or other suitable radiology methods (Dewar et al., 1982; Schindlbeck et al., 1987; Armand et al., 1996; Lindahl et al., 1997; Pedersen et al., 2000; Brouwers et al., 2006; Kalantzi et al., 2006a, 2006b; Moreno et al., 2006; Persson et al., 2006; Clarysse et al., 2009; Bevernage et al., 2011; Psachoulias et al., 2011). Intubation catheters vary in the method applied to collect GI fluid. Brouwers et al. (2006) used two double-lumen catheters to simultaneously aspirate fluid from the duodenum (Salem Sump Tube) and the proximal jejunum (Bowel Decompression Catheter), which prevented the creation of lower pressure in the intestine during aspiration. Kalantzi et al. (2006a) used a nasally intubated single lumen tube positioned in the stomach to aspirate gastric fluid and also for administration of meals prior to fed state sampling. Another more complex aspiration method utilizes a 175 cm long multichannel tube (Loc-I-Gut), which can be used to aspirate both gastric and intestinal fluid simultaneously (Hedeman et al., 1996; Lindahl et al., 1997; Pedersen et al., 2000; Holm et al., 2001a, 2001b; Nielsen et al., 2001b, 2001a; Zangenberg et al., 2001b, 2001a; Holm et al., 2002, 2003; Christensen et al., 2004; Karpf et al., 2004; Persson et al., 2005; Moreno et al., 2006; Kossena et al., 2007). The Loc-I-Gut tube has two latex balloons distally on the tube positioned 10 cm apart from each other. These balloons are inflated to prevent the tube from passing further down in the intestine after the targeted position has been reached, as determined fluoroscopically. It should be noted that introducing a catheter was found to cause duodenogastric reflux (Hoare et al., 1978). According to Nolan, there was also an increased risk of duodenogastric reflux upon rapid duodenal and jejunal intubation (Nolan, 1979). This suggested a potential problem with aspirated gastric fluid during simultaneous intestinal intubation by the Loc-I-Gut method. In addition to the different types of tubes, a Heidelberg Capsule has been administered orally to healthy volunteers to measure intestinal fluid pH (Dressman et al., 1990). The capsule is a battery-operated high frequency radio transmitter and a radio antenna signal receiver positioned around the waist of the volunteer records pH over time.

The protocols for aspiration in the fasted state conditions vary; some investigators administered liquid to the volunteers (Dewar et al., 1982; Persson et al., 2005; Brouwers et al., 2006; Kalantzi et al., 2006a, 2006b; Moreno et al., 2006; Clarysse et al., 2009; Bevernage et al., 2011, 2012b; Psachoulias et al., 2011), whereas others did not (Piper et al., 1965; Finholt and Solvang, 1968; Lindahl et al., 1997; Moreno et al., 2006; Persson et al., 2006; Pedersen et al., 2013). In some studies a solution of a non-absorbable marker (PEG4000) was administered to enable corrections for water absorption and/or secretion (Kalantzi et al., 2006a, 2006b; Persson et al., 2006). This can result in lower concentrations of salt and/or lipids measured in the fasted volunteers due to dilution by the marker. The emptying water from the stomach has been found to follow an exponential curve with a half-time of 8–15 min (Brener et al., 1983; Dressman, 1986; Armand et al., 1994), and hence, the effect of the dilution will be dependent on the sampling time-point post fluid administration. Another study examined gastric emptying with relation to the three different phases of the interdigestive migrating myoelectric complex (IMMC), where subjects received either 50 mL and 200 mL of water (Oberle et al., 1990). Overall the observed emptying half-life was fastest in phase I, and slowest in phase III. The emptying half-life was faster in all three phases for subjects receiving 200 mL of water. When 50 mL of water was administered the emptying half-life ranged from 9.0 ± 4.9 min (Phase I) up to 60.6 ± 21.0 (Phase III). For patients receiving 200 mL of water the emptying half-life ranged from 4.9 ± 2.1 to 22.8 ± 17.8 min.

Studies of GI fluid characteristics in the fed state vary with regard to the composition of the administered meal prior to sampling. Nutritional supplements such as Ensure Plus® (Kalantzi et al., 2006a, 2006b; Clarysse et al., 2009), Scandishake Mix (Clarysse et al., 2009), Shak Iso (Carriere et al., 1993) and Biosorbin MCT® (Schindlbeck et al., 1987) contain the same nutrients as a meal and are often used. However, in some studies homogenized meals were administered (Dewar et al., 1982; Hernell et al., 1990;

Armand et al., 1996; Kossena et al., 2007; Vertzoni et al., 2012) and a typical composition was 70 g olive oil, 1 whole egg, 1 egg white and 70 g of sugar as described by Armand et al. (1996) Often the meal or nutritional drink is infused directly into the stomach through the intubated catheter. Similarly to fasted state studies, PEG4000 has also been used to allow corrections for water absorption/secretion known to occur in the fed state (Kalantzi et al., 2006a, 2006b; Persson et al., 2006).

During characterization of the fluids; pH, buffer capacity, osmolarity, surface tension, bile salt (BS) and phospholipid (PL) concentration is measured in both the fasted and the fed state. While some properties mainly rely on one methodology for determination, e.g. a standard pH electrode is used to determine pH and freezing point depression is used to measure osmolarity, other properties have been measured utilizing different techniques in different studies. Surface tension has been measured using the Du Noey ring method (Kalantzi et al., 2006a, 2006b), the Du Noey–Paday method (Clarysse et al., 2009) or the Wilhelmy plate method (Persson et al., 2005). These methods are described in Sections 4.7 and 4.8.1.1.1. BS concentration has been quantified using commercially available enzyme kits (Dewar et al., 1982; Hernell et al., 1990; Armand et al., 1996; Persson et al., 2005, 2006; Kalantzi et al., 2006a, 2006b; Kossena et al., 2007), GC-Flame ionization detector (Schindlbeck et al., 1987), gel permeation chromatography (Mansbach et al., 1975), HPLC-ELS (Persson et al., 2006), GC–MS (Clarysse et al., 2009) and HPLC charged aerosol detector (Vertzoni et al., 2008). PL concentration has also been determined using commercially available enzymatic kits (Kossena et al., 2007; Clarysse et al., 2009). Further, degradation of PL thereby freeing phosphate to be determined by a colorimetric method (Mansbach et al., 1975; Dewar et al., 1982; Hernell et al., 1990), HPLC-evaporative light scattering detector (Persson et al., 2005, 2006; Kalantzi et al., 2006b) or HPLC-charged aerosol detector (Vertzoni et al., 2012) have been used. It is likely that all the different analytical techniques will contribute to the variability in the surface tension, BS and PL concentrations observed.

2.1. Human intestinal fluid in the fasted state

2.1.1. pH

All samples discussed here have been analyzed with a standard pH electrode, with the exception of the study performed by Dressman et al. (1990) who used the Heidelberg capsule. Variations in measured pH may be a result of bicarbonate evaporating during sample handling, and/or administration of water to the test subjects. The reported pH values of fasted gastric, duodenal and jejunal fluids are shown in Fig. 1. In the stomach, pH values of 1.7 up to 3.3 (median of 2.5) have been reported (Dressman et al., 1990; Lindahl et al., 1997; Efentakis and Dressman, 1998; Pedersen et al., 2000; Kalantzi et al., 2006a; Pedersen et al., 2013; AstraZeneca, data on file), whereas pH of the duodenum have been found to be 5.6–7.0 (median of 6.3) (Brouwers et al., 2006; Kalantzi et al., 2006a, 2006b; Moreno et al., 2006; Kossena et al., 2007; Clarysse et al., 2009; Psachoulias et al., 2011). It should be noted that the four lowest duodenal pH values were measured in the same study and consisted of four experiments with the same test subjects over two separate days (Psachoulias et al., 2011). Interestingly, these four results showed little variation between the sampling days. In the jejunum, the pH has been measured in a large number of different studies reporting pH values of 6.5–7.8 (median of 6.9) (AstraZeneca, data on file). From these studies the general trend of the increase in pH from stomach to jejunum were observed.

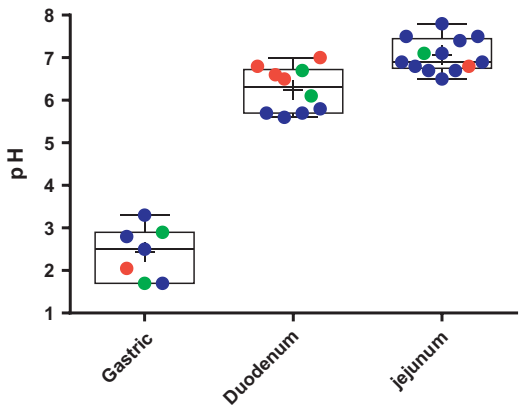

Fig. 1. pH measured in fasted gastric, duodenal and jejunal fluids. The Box–whisker plots show minimum and maximum values, and 25, 50 and 75 percentile. The cross indicates the mean value. Each data point represents a group of participants (n = 1–10 colored red; n = 11–20 colored blue; n > 20 colored green) reported in one publication. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.1.2. Buffer capacity and osmolarity

The buffer capacity of GI fluids is presented in Fig. 2a. Buffer capacity of gastric fluid has been determined in three studies to be 13.3–19.0 mM/ΔpH (median 14.3) (Kalantzi et al., 2006a; Pedersen et al., 2013; AstraZeneca, data on file), whereas in duodenum 5.6 and 8.5 mM/ΔpH have been reported (Kalantzi et al., 2006a; Moreno et al., 2006). In contrast to the rather sparse data on buffer capacity of gastric and duodenal fluids, nine studies have determined the buffer capacity in the jejunum (AstraZeneca, data on file). These investigations resulted in a median value of 4 mM/ΔpH. Hence, the jejunal buffer capacity is in general lower than the buffer capacity of the duodenum, which in turn is significantly lower than that of the gastric fluid.

Gastric fluid osmolarity has been reported to be between 119 and 221 mOsm (median of 202 mOsm; Fig. 2b) (Lindahl et al., 1997; Pedersen et al., 2000; Kalantzi et al., 2006a; Pedersen et al., 2013; AstraZeneca, data on file). Duodenal fluid osmolarity has been determined in three studies with values between 137 and 224 mOsm being reported (median 197 mOsm), (Kalantzi et al., 2006a; Moreno et al., 2006; Clarysse et al., 2009). The osmolarity in the jejunum has been reported to be 200–300 mOsm (median 280 mOsm) (Moreno et al., 2006; AstraZeneca, data on file), with values tightly grouped near the median. The only study finding a significantly lower osmolarity (200 mOsm) did not use the Loc-I-Gut perfusion tube for aspiration, which may provide a methodological explanation of the result (Moreno et al., 2006). Osmolarity in jejunal fluid is clearly raised as compared to gastric and duodenal fluids (Fig. 2b). Interestingly, the lowest osmolarity values measured in duodenal and jejunal fluids were not a result of co-administration of water, indicating the influence of other factors such as sampling methods and inter-individual physiological variability.

2.1.3. Surface tension, bile salt and phospholipid composition

The surface tension in gastric juice has been reported to be 31–45 mN/m (median of 36.8 mN/m) (Fig. 3) (Efentakis and Dressman, 1998; Kalantzi et al., 2006a; Pedersen et al., 2013; AstraZeneca, data on file). This is significantly lower than the surface tension of water (72 mN/m), and is primarily due to the presence of pepsin, but also a result of refluxed BS from the duodenum. BS levels in fasted gastric fluids have been found to be between 0.0 and 0.8 mM (median of 0.28 mM) (Lindahl et al., 1997; Pedersen et al., 2000, 2013; Kalantzi et al., 2006a; AstraZeneca, data on file), which indicate that reflux from the duodenum does not occur in all individuals (Fig. 4a). It has been argued that duodenal reflux can be

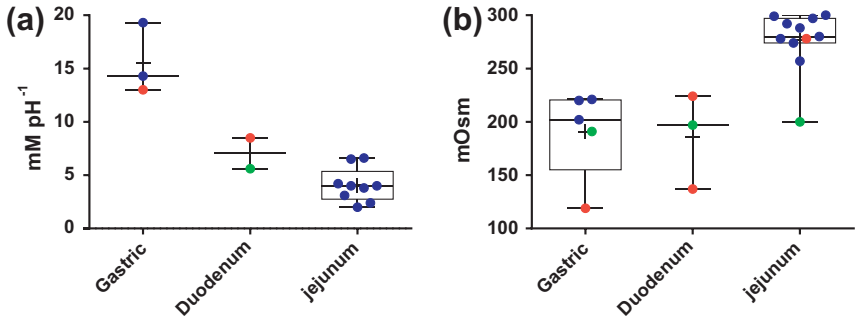

Fig. 2. (a) Buffer capacity and (b) osmolarity of fasted state gastric, duodenal and jejunal fluids. The Box–whisker plots show minimum and maximum values, as well as 25, 50 and 75 percentile. The cross indicates the mean value. Each data point on the graph represents a group of participants (n = 1–10 colored red; n = 11–20 colored blue; n > 20 colored green) reported in one publication. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

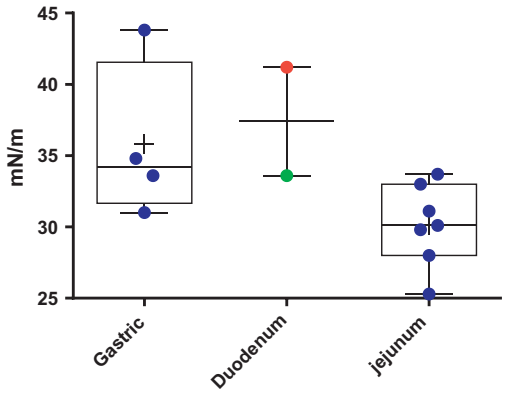

Fig. 3. Surface tension of fasted gastric, duodenal and jejunal fluids. Box–whisker plots show minimum and maximum values, as well as 25, 50 and 75 percentile. The cross indicates the mean value. Each data point represents a group of participants (n = 1–10 colored red, n = 11–20 colored blue and n > 20 colored green) reported in one publication. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

induced by the cannula used to sample the fluids and therefore is not physiologically relevant (Hoare et al., 1978). This is still under debate and more studies are warranted to clarify the importance of duodenal reflux to the stomach. In the duodenum, BS will be present in micelles with PL. However, only Dewar and co-workers have measured the concentration of lyso-phospholipid in gastric fluids and they found the concentration to be 0.029 mM (Dewar et al., 1982).

The surface tension of duodenal fluid is in the same range as that found for gastric fluids. Only two studies have measured the surface tension and these analysed fluids sampled at two different sites in the duodenum (Kalantzi et al., 2006a; Clarysse et al., 2009). As seen in Fig. 3, there is a tendency of decreased surface tension in the jejunum compared to the stomach and duodenum (AstraZeneca, data on file). This may be a result of pancreatic secretion, in particular the secretion of bile salts from the gall bladder. The BS concentration in the duodenum has been determined to 2.5–5.9 mM (median of 3.25 mM) (Armand et al., 1996; Brouwers et al., 2006; Kalantzi et al., 2006a; Moreno et al., 2006; Kossena et al., 2007; Clarysse et al., 2009; Psachoulias et al., 2011) which is slightly higher than that of the jejunum which range from 1.4 mM to 5.5 mM (median of 2.52 mM) (Lindahl et al., 1997; Pedersen et al., 2000; Persson et al., 2005; Moreno et al., 2006; AstraZeneca, data on file), as shown in Fig. 4a. The concentration of PL in duodenal (Brouwers et al., 2006; Kossena et al., 2007; Clarysse et al., 2009; Psachoulias et al., 2011) and jejunal (Persson et al., 2005; AstraZeneca, data on file) fluids follows the pattern of the bile (Fig. 4b).

Human bile compositions reported in three duodenal studies (Persson et al., 2005; Brouwers et al., 2006; Moreno et al., 2006) and eleven jejunal studies (Moreno et al., 2006 and ten unpublished studies by AstraZeneca (data on file)) are shown in Fig. 5. Based on these studies it is evident that conjugated bile acids constitute the main component of human bile. The single most occuring bile acid is cholic acid, conjugated to either taurine or glycine, followed by chenodeoxycholic acid and deoxycholic acid. The PL levels are higher in the duodenum (median of 0.26 mM) than in the jejunum (median of 0.19 mM) (Fig. 4b). The most common PL present in the bile is phosphatidylcholine (PC), which is hydrolysed to lyso-phosphatidylcholine (lyso-PC) in the lumen of the small

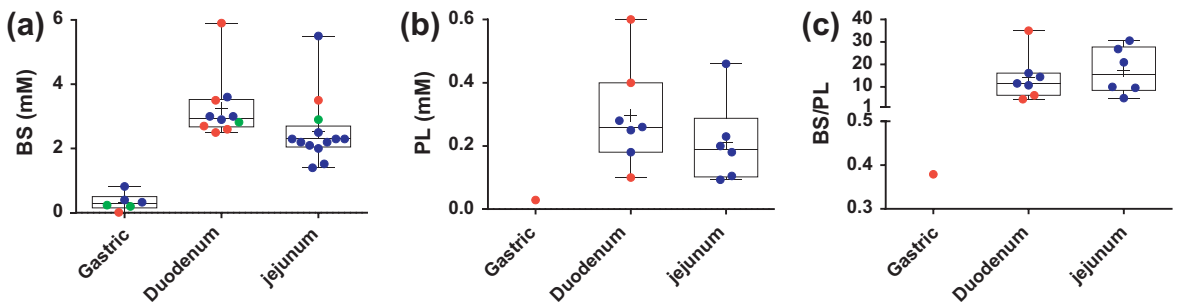

Fig. 4. (a) Bile salt (BS) concentration, (b) phospholipid (PL) concentration, and (c) BS/PL ratio in fasted gastric, duodenal and jejunal fluids. Box–whisker plots show minimum and maximum values, as well as 25, 50 and 75 percentile. The cross indicates the mean value. Each data point represents a group of participants (n = 1–10 colored red, n = 11–20 colored blue and n > 20 colored green) reported in one publication. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

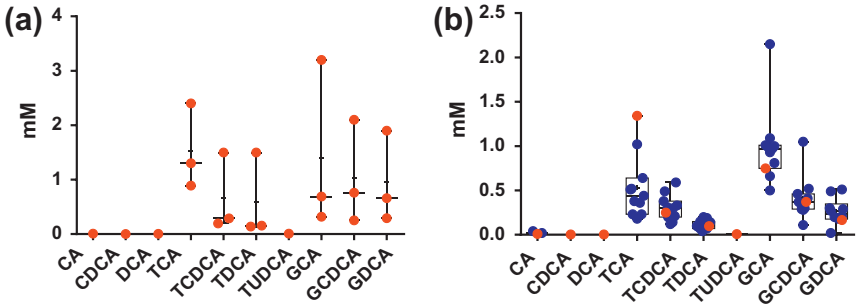

Fig. 5. Bile salt composition in human (a) duodenum and (b) jejunum. CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; TCA: taurocholic acid; TCDCA: taurochenodeoxycholic acid; TDCA: taurodeoxycholic acid; TUDCA: Tauroursodeoxycholic acid; GCA: glycocholic acid; GCDCA: glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid. Box–whisker plots show minimum and maximum values, as well as 25, 50 and 75 percentile. The cross indicates the mean value. Each data point represents a group of participants (n = 1–10 colored red, n = 11–20 colored blue) reported in one publication. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

intestine. At the concentrations of PC and lyso-PC present in the aqueous fluids of the small intestine, lyso-PC forms micelles, whereas PC forms vesicles. PC will, however, form micelles together with the bile acids (Carey and Small, 1970; Kleberg et al., 2010b). Based on this literature survey, it becomes clear that the levels of BS and PL in fasted duodenal and jejunal fluids have large variability. Whether this reflects actual individual differences or is a result of different sampling techniques and analytical methods used in the published investigations remains to be shown.

An analysis of the ratio of BS to the PL concentration (BS/PL) in the fasted state shows that the median of the BS concentration is 11.5 times higher than the median of the PL concentration in the duodenum. The corresponding ratio in the jejunum is 15.5 (Fig. 4c).

2.2. Human intestinal fluid in the fed state

Compared to the literature on fasted state GI fluids published characterization of fed state GI fluids is rather sparse. Furthermore, the investigations have used different meals, as well as different aspiration procedures making comparisons and analysis of results difficult.

2.2.1. pH

The variations in pH between gastric fasted and fed state will largely be governed by the administered meal itself. For instance the commonly used Ensure Plus® with 10 mg/mL PEG4000 has a pH of 6.6 (Kalantzi et al., 2006a), which is reflected in an increased gastric pH to 4.5 and 6.7 (Dressman et al., 1990; Kalantzi et al., 2006a). Because of the pretreatment in the stomach, the pH of the intestinal fluids is not affected to the same extent as gastric fluids (Fig. 6). In the duodenum, the fed state fluid has been reported to be between 5.4 and 6.5 (median of 6.0) (Mansbach et al., 1975; Dressman et al., 1990; Armand et al., 1996; Kalantzi et al., 2006a, 2006b; Clarysse et al., 2009; Vertzoni et al., 2012), which is overlapping the pH in the fasted duodenum (5.6–7.0 (median of 6.3) see above). In jejunal fluids, only one study has recorded the pH and found it to be 6.1 (Persson et al., 2005).

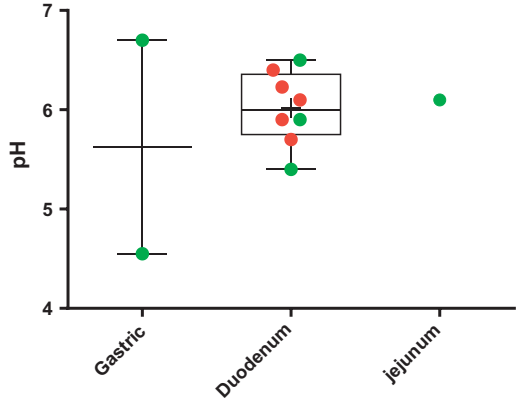

Fig. 6. pH in fed gastric, duodenal and jejunal fluids. Box–whisker plots show minimum and maximum values, as well as 25, 50 and 75 percentile. The cross indicates the mean value. Each data point represents a group of participants (n = 1–10 colored red and n > 20 colored green) reported in one publication. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.2.2. Buffer capacity

The buffer capacity is raised for gastric, duodenal and jejunal fluids in the fed state compared to fasted state. The buffer capacity in the fed state studies has been analyzed by one-way titration of sodium hydroxide or hydrochloric acid (Kalantzi et al., 2006b; Vertzoni et al., 2012), or two-way titration by a first titration with sodium hydroxide then followed by hydrochloric acid titration (Persson et al., 2005; Kalantzi et al., 2006a). However, the current literature is rather sparse (Fig. 7a). One study measured the gastric buffer capacity to 19.5 mM pH^{-1} (Kalantzi et al., 2006a). The duodenal buffer capacity has been measured to be between 24 and 30 mM pH^{-1} (Kalantzi et al., 2006a, 2006b; Vertzoni et al., 2012), whereas the only study performed on jejunal fluid obtained 13.9 mM pH^{-1} (Persson et al., 2005). Similar to buffer capacity, osmolarity data are also rather scarce (Fig. 7b). The fed gastric juice has been determined to have an osmolarity of 388 mOsm (Kalantzi et al., 2006a), whereas the fed state duodenal fluid ranged from 276 to 416 mOsm (Fig. 7b) (Kalantzi et al., 2006a, 2006b; Clarysse et al., 2009). No studies of osmolarity of fed jejunal fluids have been reported so far. Overall, the buffer capacity and the osmolarity are higher in the fed state as compared to the fasted (Fig. 2a and b).

2.2.3. Surface tension, bile salt and phospholipid

Similar surface tension (~30 mN/m) values are observed in all GI compartments in the fed state. The surface tension of fed gastric fluids (30.5 mN/m; Fig. 8) (Kalantzi et al., 2006a) is lower than the lowest fasted state gastric surface tension (Fig. 3). Fed state duodenal fluids have surface tension values of 27.8–35.4 mN/m (median of 31.3 mN/m), which is also lower as compared to the fasted state (Kalantzi et al., 2006a, 2006b; Clarysse et al., 2009). Fed state jejunal fluids has a surface tension of 30.0 mN/m (Persson et al., 2005) and hence, although results

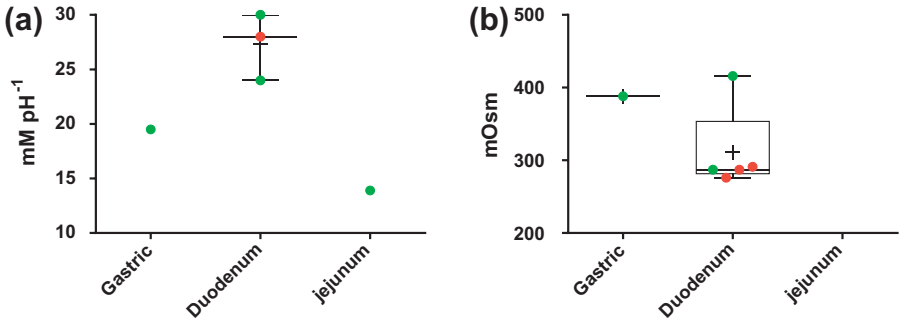

Fig. 7. (a) Buffer capacity and (b) osmolarity in fed gastric, duodenenal and jejunal fluids. Box–whisker plots show minimum and maximum values, as well as 25, 50 and 75 percentile. The cross indicates the mean value. Each data point represents a group of participants (n = 1–10 colored red and n > 20 colored green) reported in one publication. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

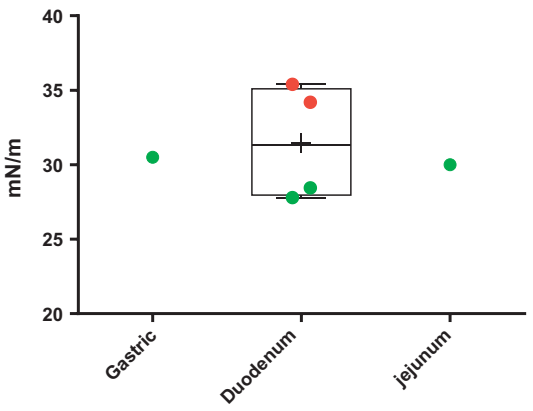

Fig. 8. Surface tension in fed gastric, duodenal and jejunal fluids. Box–whisker plots show minimum and maximum values, as well as 25, 50 and 75 percentile. The cross indicates the mean value. Each data point represents a group of participants (n = 1–10 colored red and n > 20 colored green) reported in one publication. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

are sparse, the literature suggests the surface tension to be similar in fasted and fed jejunal fluids.

BS concentration, PL concentration and the BS/PL in the fed state are shown in Fig. 9. BS concentration is lower in the fed state gastric fluids (0.051 and 0.31 mM reported by Dewar et al. (1982) and Schindlbeck et al. (1987), respectively), which is a result of the dilution of the stomach content caused by the food. In the duodenal fluids the BS concentration has been reported to vary between 3.6 and 24.0 mM (median of 11.8 mM) which points to the increased bile secretion into the duodenum during the fed state (Mansbach et al., 1975; Hernell et al., 1990; Armand et al., 1996; Kalantzi et al., 2006a, 2006b; Kossena et al., 2007; Clarysse et al., 2009; Vertzoni et al., 2012). Jejunal fluids have been sampled in two studies and BS was determined to 4.5 and 8.0 mM (Persson et al., 2005; Persson et al., 2006), respectively, which is an increase when compared to the fasted state (median of 2.52 mM).

The PL concentration in fed gastric fluid has been measured to 0.022 mM (Dewar et al., 1982). Since phospholipids are secreted with the bile, their level is significantly increased in the intestine, compared to the fasted state. Duodenal fluid has been sampled in eight studies and PL concentration was determined to be in a range from 1.2 to 6.0 mM (median of 2.15 mM) (Mansbach et al., 1975; Hernell et al., 1990; Armand et al., 1996; Kalantzi et al., 2006b; Kossena et al., 2007; Clarysse et al., 2009; Vertzoni et al., 2012). The PL concentration in fed jejunal fluid has been reported to be 2.0–3.0 mM in two studies (Persson et al., 2005, 2006). The amount of phospholipids present in the administered meal will play a significant role. For example, the presence of raw egg will increase the level of phospholipids.

In a single study the BS/PL ratio in fed gastric fluid was found to be 2.3 (Dewar et al., 1982). From seven studies of the fed duodenal fluid the BS/PL ratio ranged from 2 to 16 (median of 3.4) (Mansbach et al., 1975; Hernell et al., 1990; Armand et al., 1996; Kalantzi et al., 2006b; Kossena et al., 2007; Clarysse et al., 2009; Vertzoni et al., 2012), and from two studies, the fed jejunal fluid was found to be 2.3 and 2.7 (Persson et al., 2005, 2006). Hence, the BS/PL ratio is significantly lowered in the fed state (Fig. 9c), as compared to the fasted state (Fig. 4c). This can either be due to increased PL content as a result of the administered meal itself or be related to absorption of PL in the fasted state.

2.2.4. Monoglycerides and free fatty acids

Monoglycerides and free fatty acids are surface-active molecules that are formed during digestion of triglycerides.

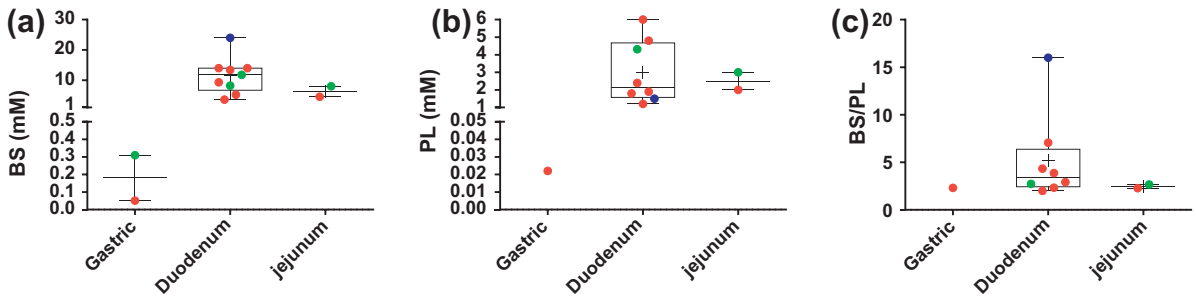

Fig. 9. (a) Bile salt (BS) concentration, (b) phospholipid (PL) concentration, and (c) BS/PL ratio in fed gastric, duodenal and jejunal fluids. Box–whisker plots show minimum and maximum values, as well as 25, 50 and 75 percentile. The cross indicates the mean value. Each data point represents a group of participants (n = 1–10 colored red; n = 11–20 colored blue and n > 20 colored green) reported in one publication. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Quantification is generally performed by HPLC with evaporative light scattering (Persson et al., 2005; Kalantzi et al., 2006b; Vertzoni et al., 2012). To date, the concentration of these components in the fed gastric fluid has not been reported. In the duodenum, monoglyceride concentration has been reported in two studies to be 5.9 and 8.1 mM and the free fatty acid concentration to be 39.4 and 52.0 mM (Fig. 10) (Kalantzi et al., 2006b; Vertzoni et al., 2012). In fed jejunal fluid the concentration of monoglycerides and free fatty acids have been determined to be 2.2 and 13.2 mM, respectively (Persson et al., 2005). The concentration of monoglycerides and free fatty acids will be related to the fat content of the administered meal. The lower concentration of monoglycerides and free fatty acids in the jejunum when compared to the duodenum indicates absorption of these components.

2.3. Animal gastrointestinal fluid

The physiology of animal models are discussed in detail elsewhere (see e.g. Sjögren et al., submitted for publication). Here a brief overview of the GI fluids of commonly used laboratory animals is provided and linked to dissolution, solubility and absorption profiles as the project moves from preclinical (animal) to clinical (human) studies.

The rat stomach consists of 2 compartments with different pHs; in the fasted state pH has been found to be 7 in the anterior region and pH 5 in the glandular region of the stomach (Smith, 1965), whereas a more recent study by McConnell and coworkers measured the pH in the content of the fasted rat stomach to be 3.2 (McConnell et al., 2008). In the fed state the pH in the anterior portion of the stomach is 4.3, but drop in the posterior stomach to pH 3.1, and hence the gastric compartment becomes significantly more acidic when food is ingested (Ward and Coates, 1987). In contrast, a McConnell and coworkers found the pH of the content of the fed rat stomach to be 3.9. (McConnell et al., 2008). A pH change in the fed state will, however, be dependent on the meal type given. In the fasted small intestine of rat the pH increases from 6.5 to approximately 7.1 from the proximal to distal parts (Smith, 1965). This is slightly higher compared to findings by McConnell et al., who found pH to be 5.9 in the duodenum and 6.1 in the jejunum. In the fed state pH was 5.0 in the duodenum and 5.1 in jejunum. (McConnell et al., 2008).

Canine gastric pH varies along the length of the stomach. The anterior gastric pH is 5.5 and drops to 3.4 in the posterior stomach (Smith, 1965). The pH increases in the small intestine from pH 6.2 in the proximal part to pH 7.5 in the distal (Kararli, 1995) and the pH of canine intestinal fluid has been confirmed by Kalantzi et al., who reported a fasted pH of 7.1 (Kalantzi et al., 2006b).

In a study by Smith and colleagues the anterior stomach pH in pigs was found to be 4.3 and decreased towards pH 2.2 towards the posterior part of the stomach (Smith, 1965). The pH of the pig small intestine was 6.0 increasing to pH 7.5 along its length (Smith, 1965).

In comparison to human GI fluids, the gastric juice is less acidic in animal models often used for absorption studies, here exemplified with rat, dog and pig. In contrast, the pH range of the small intestine in these species is similar to that observed in man. Hence, the different gastrointestinal pH-profile between animals and human may particularly impact on dissolution, solubility and absorption profiles when solid oral dosage forms with a residence time in the gastric compartment are studied.

2.3.1. Buffer capacity, bile salts and phospholipids

In fasted dogs a buffer capacity of 1.4–4.2 mM/pH and osmolarity of 69–207 mOsm have been reported (Kalantzi et al., 2006b). These values varied over the sampling time investigated and the last sampling point had higher buffer capacity and osmolarity than earlier time points. In the small intestine of the fasted rat, Staggers and colleagues reported bile salt concentration in the range of 33.5–61.3 mM (Staggers et al., 1982), whereas a significantly lower concentration (17–18 mM) was reported by Kararli (1995). In comparison, much lower concentrations of bile salt (2.4–9.4 mM) were measured in the small intestine of dogs (Kalantzi et al., 2006b). The phospholipid concentration in fasted rat small intestine was measured to 6.2–6.5 mM (Staggers et al., 1982), whereas up to 8.12 mM was determined in dogs (Kalantzi et al., 2006b). Similar to man, the most abundant bile salt was taurocholic acid. The main phospholipid components of the canine bile were phosphatidylcholine (94.5%) and phosphatidylethanolamine (5.5%) and phosphatidylcholine has also been identified as the main phospholipid in porcine gallbladder bile (Alvaro et al., 1986). For a more detailed description of the composition of animal bile, the reader is referred to a recent review by Holm et al. (2013).

In comparison to human small intestinal fluid in the fasted state, the buffer capacity and osmolarity determined in dogs are similar. Both bile salt and phospholipids, and the ratio thereof, are present at higher levels in rats than in humans, whereas the levels measured in dogs are similar. In addition the phospholipid composition is similar in intestinal fluids of these animals and in human intestinal fluids (Alvaro et al., 1986). The higher levels of solubilizing colloidal structures in the rat small intestine, as compared to dog and human, may result in an overestimation of the solubility and hence, absorption in this model for lipophilic compounds. In particular, precipitation of lipophilic weak bases in the small intestine may be difficult to predict with this animal model, since neither the pH of the gastric compartment nor the amount of solubilizing micelles in the small intestine are reflecting those observed in human GI fluids.

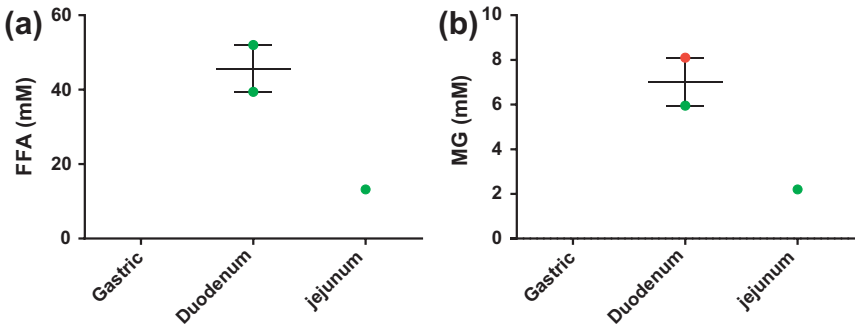

Fig. 10. (a) Free fatty acid (FFA) and (b) monoglyceride (MG) concentration in fed duodenal and jejunal fluids. Box–whisker plots show minimum and maximum values, as well as 25, 50 and 75 percentile. The cross indicates the mean value. Each data point represents a group of participants ($n = 1$–10 colored red and $n > 20$ colored green) reported in one publication. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Simulated GI fluids

With the purpose to simulate the solubility and dissolution of API in the GI tract several media simulating gastric and intestinal fluids have been developed. In the following section, the most commonly used biorelevant media simulating GI fluids will be described.

3.1. Simulated gastric fluids

Vertzoni et al. first published a fasted state simulated gastric fluid (FaSSGF) in 2005 (Table 1) (Vertzoni et al., 2005). It was later updated by increasing the osmolarity to better comply with physiologically measured values (Vertzoni et al., 2007; Erceg et al., 2012). Several attempts to simulate the gastric conditions during the fed state have been made, however, the characteristics of gastric fluids in the fed state will be highly dependent on the composition of the ingested meal. The pH in fed state simulated gastric fluid (FeSSGF) is raised from 1.6 to 5.0 and the buffer capacity is 25 mM/pH, which is close to that reported for fed state human gastric fluid (HGF) (Jantratid et al., 2008). Long life milk has been applied to simulate intragastric conditions during intake of a meal in the stomach. Pepsin and hydrochloric acid is added to imitate the digestion of milk proteins (Macheras et al., 1986; Fotaki et al., 2005). The osmolarity is raised to 400 mOsm, which correlates with HGF data, reviewed in the previous section. As expected, FeSSGF does not contain bile salts or phospholipids (Jantratid et al., 2008) since these components are found only at low concentrations in the fed stomach.

3.2. Simulated intestinal fluids

3.2.1. Bile salts and phospholipids

Of the most abundant bile salts, sodium taurocholate is the preferred BS employed in simulated intestinal fluids since it is fully ionized at intestinal pH. Taurocholate has a pK_a of 2 (O'Maille and Richards, 1977; Bortolini et al., 2011) compared to a pK_a of 3.9 for glycocholate (Fini and Roda, 1987). Despite chemical differences in the BS in man and animals porcine and ox extract have been used to better resemble physiological bile composition, which contains a mixture of different BS. The exact batch composition needs to be determined because of the large batch-to batch variability (Vertzoni et al., 2004; Kleberg et al., 2010b; Reppas and Vertzoni, 2012). Vertzoni et al. (2004) reported problems with the production of fed state simulated intestinal fluid (FeSSIF) with consistent quality due to batch variability in crude sodium taurocholate from ox extract (Vertzoni et al., 2004), which pinpoints the analytical need. The chemical differences between the BS were by Söderlind and co-workers concluded to have minimal impact on drug solubility at concentrations of 2–6 mM. They compared felodipine, budesonide, danazol and retinol in aqueous solutions of various concentrations of BS and PL with a BS/PL ratio of 4. The BS used were glycholic acid, taurocholic acid, glychodeoxycholic acid, taurodeoxycholic acid and glychochenodeoxycholic acid (Söderlind et al., 2010).

The most commonly used PL in biorelevant media is phosphatidylcholine (PC) because of its high abundance in bile secretion (Kleberg et al., 2010b). Egg or soy are the two most common sources of PC used in biorelevant media, with egg PC having a higher amount of saturated fatty acids compared to soy PC. A study by Vertzoni et al. suggests that the fatty acid composition of PC is only likely to have significant effects on solubilization of highly lipophilic drugs (Vertzoni et al., 2004). The critical micellar concentration (CMC) of sodium taurocholate together with PC was found to be 0.2 mM. At a BS/PL ratio less than 1, vesicles are formed, between 1 and 2, mixed micelles are the dominant form, whereas for a BS/PL ratio greater than 2, mixed micelles or micelles comprised purely of BS are formed (Holm et al., 2013).

Table 1
Simulated fasted and fed state gastric media.

	FaSSGF[a]	FaSSGF-V2[b]	FeSSGF[c]
pH	1.6	1.6	5.0
Buffer capacity (mM/pH)	–	–	25
Buffer type	HCl	HCl	Acetate
Long life milk buffer ratio	–	–	1:1
Osmolarity (mOsm)	120.7	186.9	400
BS (μM)	80	80	–
PL (μM)	20	20	–
BS/PL	4	4	
Pepsin (mg/mL)	0.1	0.1	
Surface tension (mN/m)	42.6	42.6	

FaSSGF, Fasted state simulated gastric fluid; FaSSGF-V2, Fasted state simulated gastric fluid version 2; FeSSGF, Fed state simulated gastric fluid; BS, Bile salt; PL, phospholipid; HCl, hydrochloric acid.
[a] Vertzoni et al. (2005).
[b] (Vertzoni et al., 2007).
[c] Jantratid et al. (2008).

3.2.2. Composition of media

Recently published fasted and fed biorelevant intestinal media are shown in Table 2. Dressman and colleagues introduced fasted state simulated intestinal fluid (FaSSIF) in 1998, and this was updated to FaSSIF-V2 in 2008 (Dressman et al., 1998; Galia et al., 1998; Jantratid et al., 2008). The buffer was changed to maleic acid in order to comply with the pH of the fasted and fed state (see below) and physiological osmolarity. In addition, this resulted in retarding the rancidity of fat and oil. Further, the FaSSIF-V2 has a lower PL concentration resulting in an increased BS/PL ratio from 4 to 15 (Jantratid et al., 2008). Importantly, physical stability of FaSSIF-V2 is better; it is stable for at least 7 days, while FaSSIF shows signs of phase separation after 24 h. The increased stability has been accredited to the lowered PL concentration (Söderlind et al., 2010). Sheng and co-workers compared phosphate buffer with physiologically more relevant bicarbonate buffer and found that phosphate buffer had a higher intrinsic dissolution rate compared to bicarbonate buffer for drugs with pK_a values below 5.5 (Sheng et al., 2009).

Fed state intestinal media primarily differ from fasted media by the addition of higher levels of BS and PL. Further, the addition of lipid digestion products, such as free fatty acids and monoglycerides have been employed (Grove et al., 2005; Nielsen and Müllertz, 2005; Sunesen et al., 2005), which has been shown to be important in order to achieve *in vitro–in vivo* relationships (IVIVR) (Sunesen et al., 2005). As described in Section 2, the pH is often lower in the small intestine in the fed state, as compared to the fasted. Fed state simulated intestinal fluid (FeSSIF) was first introduced in 1998 by Dressman and co-workers and later updated to FeSSIF-V2 (Dressman et al., 1998; Galia et al., 1998; Jantratid et al., 2008). A reduction of buffer capacity, osmolarity, amount of BS and PL was implemented in FeSSIF-V2 and the BS/PL ratio was increased from 4 to 5. In addition, mono-olein (MO) and oleic acid (OA) were added to simulate the lipolysis of triglycerides by gastric and pancreatic lipase (Jantratid et al., 2008). In contrast, in the Copenhagen fasted and fed media (Grove et al., 2005; Nielsen and Müllertz, 2005; Kleberg et al., 2010a) the pH is kept at 6.5, BS/PL at 4 and maleate is used as the buffer component. In the fasted state medium the osmolarity is kept at 270 mOsm, whereas it may vary in the fed state media. Additionally, lipolysis products (OA and MO) were added to the fed state media in different concentrations and studied at a ratio of 2 and 6. The OA/MO ratio did not affect the surface tension instead the total surfactant concentration had an influence (i.e. BS + PL + OA + MO). Varying the OA/MO ratio, however, did produce different colloidal struc-

Table 2
Simulated intestinal media.

	FaSSIF[a]	FaSSIF-V2[b]	Copenhagen Fasted[c]	FeSSIF[a]	FeSSIF-V2[b]	Copenhagen Fed[c]
pH	6.5	6.5	6.5	5	5.8	6.5
Buffer Capacity (mM/pH)	10	10	–	75	25	–
Buffer type	KH_2PO_4	Maleic Acid	Trizma Maleate	Acetate	Maleic Acid	Trizma Maleate
Osmolarity (mOsm)	270	180	270	635	390	Varies
Surface tension (mN/m)	45.5	–	–	46.3	40.45	
Particle size	–	–	–	–	–	–
BS (mM)	3	3	2.5	15	10	5–20
PL (mM)	0.75	0.2	0.625	3.75	2	1.25–5
BS/PL	4	15	4	4	5	4
MO (mM)	–	–	–	–	5	0–10
OA (mM)	–	–	–	–	0.8	0–45

Abbreviations used: FaSSIF, fasted state simulated intestinal fluid; FeSSIF, fed state simulated intestinal fluid; BS, bile salt; PL, phospholipid; MO, mono-olein; OA, oleic acid.
[a] Galia et al. (1998).
[b] Jantratid et al. (2008).
[c] Kleberg et al. (2010b).

tures which may impact on the solubilization capacity (Kleberg et al., 2010a). Although large efforts have been directed toward the establishment of improved simulated media to better mimic dissolution in fasted and fed HGF and human intestinal fluid (HIF), few studies have found a good correlation between the simulated media and human fluids (Kleberg et al., 2010a).

4. Physicochemical characterization methods of API

Physicochemical properties of the API such as lipophilicity, solubility and the solid state play a crucial role in GI absorption. These properties will significantly influence the biopharmaceutical performance of the API because of their impact on dissolution, precipitation, permeability and food interaction in the stomach and intestine. Computational models able to accurately predict these properties are highly sought after as they can inform on structural features that are fundamental for the resulting physicochemical, biopharmaceutical and/or pharmaceutical property. Furthermore, miniaturized experimental methods preferably with high throughput that accurately predict *in vivo* absorption are also warranted. In the following sections, the current status of *in silico* predictions of physicochemical parameters, state-of-the art experimental methods to measure these properties and the impact of the physiological conditions that the APIs are exposed to during the dissolution and absorption *in vivo* will be discussed.

4.1. Molecular descriptors and in silico predictions of physicochemical parameters

During the 1990s the interest and efforts to link calculated molecular properties to *in vivo* API performance increased. The relationship between lipophilicity and permeability had long been known, however, during this time an increased use of calculated lipophilicity in the form of the logarithm of the partition coefficient between octanol and water (log*P*), was used. Although this property seem to be fairly straight forward to calculate either from fragment-based/group contribution or more statistically advanced models (Pliska et al., 1996; Eros et al., 2002; Mannhold et al., 2009), evaluation of their applicability to specific compound series obtained in dedicated projects have often proven otherwise (Tetko and Poda, 2004; Mannhold et al., 2009). Tools that accurately predict this property are highly important since lipophilicity is related to both solubility and permeability, and hence will greatly influence the absorption. In addition, lipophilicity has been identified to drive pharmacological potency, (Kubinyi, 1979; Andrews et al., 1984) often through nonspecific binding, which exacerbated the risk for toxicological effects to occur (Leeson and Springthorpe, 2007; Hughes et al., 2008). In addition to these physiologically relevant aspects, the lipophilicity can be combined with melting point and/or entropy of fusion to determine which molecular property is of most importance for the dissolution and solvation (Yalkowsky and Valvani, 1980; Jain and Yalkowsky, 2001; Wassvik et al., 2006) and thereby give an early indication of the type of enabling formulation that may be needed.

The seminal analysis performed by Lipinski and co-workers linked simple physicochemical properties (i.e. molecular weight, log*P*, hydrogen bond acceptor and donors) to absorption through the 'rule of five' (Lipinski et al., 2001). Molecular weight, hydrogen bond donor and acceptor properties are easily calculated and more than 30 different computational methods to predict log*P* have been developed (Mannhold et al., 2009). A similar system to Lipinski's rule-of-five is the 'Oral PhysChem Score' (Lobell et al., 2006), which uses a 'traffic light' approach where API values of MW, calculated log*P*, number of rotatable bonds, calculated solubility, and topological polar surface area (TPSA) are each assigned a corresponding traffic light colour (green, 0; yellow, 1; red, 2, see Table 3). The values are summed; and a lower score indicates better biopharmaceutical properties of the API.

Ritchie and co-workers recently published a detailed review where a large number of different approaches to visualize limiting molecular properties for oral absorption and oral bioavailability were discussed (Ritchie et al., 2011a). They also applied a 'traffic light' system to identify API developability (Ritchie and Macdonald, 2009; Ritchie et al., 2011b). They analysed whether the developability of 280 GSK compounds (at different stages of development, from preclinical candidate selection to proof of concept) was linked to the number of aromatic rings. One of the properties examined was the solubility and based on log*P* and number of aromatic rings the authors could differentiate between compounds having a poor solubility due to high lipophilicity from those suffering from high aromaticity. Their findings, together with previously published efforts to

Table 3
Traffic light values used in the 'Oral Physchem Score'.

Value	$Solubility_{pH6.5}$	ClogP	Mw	PSA	Rotatable Bonds
	μg/mL			$Å^2$	
0	≥50	≤3	≤400	≤120	≤7
1	10-50	3-5	400-500	120-140	8-10
2	<10	>5	>500	>140	>11

Decision system established at Bayer (Lobell et al., 2006). Abbreviations used: Clog*P*, calculated log*P*; Mw, molecular weight; PSA, polar surface area.

identify molecular features producing a strong crystal lattice (Wassvik et al., 2008; Mahlin et al., 2011), facilitate the identification of APIs with solid-state limited solubility, i.e. compounds for which strong interactions in the crystal lattice is the main factor limiting the solubility (sometimes described as 'brick dust' molecules). Overall, the GSK studies conclude that the developability of molecules significantly decrease when the number of aromatic rings is >3. Factors that led to this conclusion were solubility, which significantly decreased with the number of aromatic rings, and lipophilicity, serum albumin binding and CYP3A4 inhibition, all of which significantly increased with increased number of aromatic rings. In addition, a trend (although not statistically significant for the dataset explored) for increased hERG binding and hERG toxicity was observed as a function of an increased number of aromatic rings (Ritchie and Macdonald, 2009; Ritchie et al., 2011b). The studies also revealed that the number of aromatic rings decreased for the GSK compounds when advancing from preclinical candidate selection (aromatic rings per molecule = 3.3) to the late stage proof-of-concept studies (aromatic rings = 2.3). These counts of aromatic rings should be compared with the number of aromatic rings found in marketed oral drugs which is 1.6.

To summarise, several rule-based systems and predictive models to forecast absorption exist. These are based on rapidly and often easily calculated molecular descriptors and can inform the medicinal chemists on likely ADMET properties and developability of compound series. In the present literature the following cut off values have been identified to produce APIs with acceptable biopharmaceutical profile:

- MW < 500 (Lipinski et al., 1997)
- log*P* < 5 (Lipinski et al., 1997)
- Hydrogen bond donors < 5 (Lipinski et al., 1997)
- H acceptors < 10 (Lipinski et al., 1997)
- Number of rotatable bonds < 10 (Veber et al., 2002; Blake, 2003; Wenlock et al., 2003)
- $\log S_{pH6.5}$ > 10 mg/L (Lobell et al., 2006)
- TPSA < 140 Å^2 (Palm et al., 1997; Clark, 1999)
- Aromatic rings < 4 (Ritchie and Macdonald, 2009)

It should be mentioned that all the above listed properties cannot be strictly applied to all targets and the impact of each of them may need to be weighted depending on the receptor family explored (Lipinski and Hopkins, 2004). These properties will also inform the formulators on what hurdles to expect during development. However, the current computational models provide only rough estimations of the essential physicochemical properties (e.g. solubility, log*P*, melting point (Tm) and pK_a). To further improve these models it is important to include the chemical space of drug discovery compounds in the training sets. This is the main reason for why the pharmaceutical industry develops their own in-house programs for prediction of e.g. log*P*, pK_a, solubility and permeability, i.e. that the chemical space of their libraries then will be covered by the model used. The improvement obtained in these predictions by this approach was recently clearly seen for pK_a predictions of Roche compounds. By including experimentally determined pK_as of a subset of Roche discovery compounds in the model development, structural features and ionizable groups not well represented in the literature dataset had the chance to influence the model development. This approach resulted in that the Root Mean Square Error improved from 1.09 (without Roche structures included in the training) to 0.49 (with Roche structures included in model development) when the obtained model was challenged with another Roche dataset (Milletti et al., 2010). Still these properties need to be determined experimentally during the preformulation stage to obtain accurate values of e.g. log*P*, pK_a and solubility. Furthermore, computational models predicting, e.g. dissolution profiles under physiologically relevant conditions or the impact of excipients on API performance *in vivo* are rare (Fagerberg et al., 2010; Fagerberg et al., 2012; ADMET Predictor from SimulationsPlus, CA), and therefore these important factors currently need to be measured experimentally.

4.2. Early physicochemical profiling methods

4.2.1. Traditional approaches

The physicochemical properties that are measured traditionally as a part of the preformulation package include solubility (*S*), dissociation constant (pK_a), melting point (Tm) and log*P*. Intrinsic solubility (S_0, see Table 4 for definition), pK_a and log*P* can be predicted within a reasonable range using computational models and molecular descriptors (Johnson and Zheng, 2006; Norinder and Bergstrom, 2006; Mannhold et al., 2009; Rupp et al., 2011) and therefore general information of these properties is usually available when entering into preformulation assessments. However, the current computational models for solid state properties such as Tm only provide categorical predictions (e.g. high/intermediate/low melting point) rather than absolute values (Bergstrom et al., 2004). Therefore, to obtain accurate prediction of biopharmaceutical performance, it is essential that all physicochemical and solid state properties of the API are determined experimentally.

4.2.1.1. pKa. The pK_a and pK_a type (acidic or basic) of a molecule is used to determine its charge state at a particular pH in solution. The charge state is known to have an impact on absorption, distribution metabolism, elimination (ADME) properties and the pharmacokinetic profile of a molecule (Avdeef, 2003). For example, the charged state of a compound will have a positive effect on the dissolution rate, as the solubility of ionizable compounds is known to increase proportionally with the amount of the charged species as a consequence of pH (Box et al., 2006). Conversely, the charged state of a compound will have a negative effect on passive permeability across a membrane, as lipophilicity is inversely proportional to the amount of charged species (Comer, 2006). Hence, the pK_a, solubility and log*P* will help evaluate the propensity of a dosed API to dissolve and achieve the required concentration in GI fluid and subsequently partition into the systemic circulation. In other areas, the charged state of a molecule will influence binding to transporters, enzymes, the site of action and off target receptors (Manallack, 2008).

Typically, three techniques are used; acid–base (potentiometric) titration, UV-spectrophotometry (photometric) or capillary electrophoresis. The potentiometric technique is based on measurements of the consumption of a titrant as a function of pH during an acid–base titration of an ionizable sample and is regarded as the gold standard for pK_a measurement. Typically sample concentrations of 0.5–1 mM are required to determine pK_as with values between 3–11 and hence less than a milligram is commonly needed for a standard titration in 1–2 mL of an aqueous medium. More sample will be required for pK_as at extreme values (3 > pK_a > 11). For poorly soluble compounds, cosolvents may be required to present the sample in sufficient concentration for pK_a determination. Cosolvents often result in a shift to the pK_a and

Table 4
Solubility definitions.

Thermodynamic solubility	Saturated solution in equilibrium with the thermodynamically stable polymorph
Intrinsic solubility	Equilibrium solubility at pH where the API is in its neutral form
Apparent solubility	Solubility measured under given assay conditions

therefore the aqueous pK_a-value is extrapolated from several results determined over a wide range of cosolvent concentrations (Takács-Novák et al., 1997). An attractive alternative to the potentiometric technique is the photometric technique, which also requires an acid–base titration of the sample, but here, the UV absorbance is measured as a function of pH. In order to determine the pK_a by this method, a change in the UV absorbance signal from the sample must be observed during the titration. Hence, the compound must have a chromophore and it should be within close proximity (up to 3–4 bond lengths) to the ionization center. The photometric technique is more sensitive than the potentiometric technique, requiring a sample concentration of ~30 μM. Also in this method cosolvents can be used for very poorly soluble compounds and extreme pK_a values are more easily determined with the photometric technique as compared to the potentiometric. A third technique that can be used for pK_a determinations is capillary electrophoresis (CE). CE, which can be coupled to mass spectrometry to increase the sensitivity, measures pK_a through the mobility of the compound under an applied potential. With this methodology, migration times of the sample are measured in a number of different pH buffers. The migration time will depend upon the charge state of the molecule and when plotted against pH, should depict the ionization profile of the sample and hence determine the pK_a (Wan et al., 2003). In fact, any other sample property that changes with pH can, in principle, be used to determine a sample's pK_a. For example, NMR and conductivity have been used to measure pK_a. Conductivity has been found to have limited applicability at high and low pH, due to the high conductivity of the medium at these pH's (Albert and Sergeant, 1984). NMR, however, can be useful for site-specific identification of ionizable groups when multiple ionization centers are present, although relatively high sample concentrations are needed (Box et al., 2008).

4.2.1.2. Lipophilicity. The lipophilicity of a drug molecule represents its affinity for a water-immiscible organic phase in comparison to the aqueous environment (van de Waterbeemd et al., 1997). Using this property, it is possible to assess the affinity of the molecule for low dielectric media such as biological membranes, enzymes, carriers and target sites and log*P* has been shown to be related to biological activity for many compounds (Meyer, 1899; Leo et al., 1971). The log*P* and the distribution coefficient (log*D*) is typically determined using *n*-octanol and water. The concentration of the analyte is found in both phases of a *n*-octanol:water system, and log*P* is defined as the concentration ratio of the neutral species, whereas log*D* is the concentration ratio of all charge states. The distribution of ionizable species varies with pH as a consequence of the pK_a and hence, log*D* may vary with pH while log*P* is constant (Comer and Tam, 2001). Other partition solvents such as cyclohexane, dodecane, *n*-hexane and hexadecane have also been used to determine partition and distribution coefficients (Zissimos et al., 2002), but the original solvent *n*-octanol remains the favored system.

The standard reference method for log*P*/*D* determination involves shaking or agitating a flask containing the solvents and analyte for 24 h or until equilibrium is achieved. The API, in both solvents, is then quantified using an appropriate technique (e.g. UV, LC-UV, LC-MS/MS). The dynamic range of this method is dependent on the detection limits of the analytical method and so the volume ratio of *n*-octanol to water should be adjusted to account for this. Another widely used technique involves a pH titration of ionizable samples in the *n*-octanol:water system (Slater et al., 1994). The partitioning of the sample into *n*-octanol will cause a shift in the pK_a which is proportional to the lipophilicity of the neutral state. This shift is used to determine the log*P* of the compound in accordance with Le Chatelier's "equilibrium principle". Volume ratios of *n*-octanol:water can be adjusted to determine APIs of different levels of lipophilicity e.g., hydrophilic compounds may need larger volume of *n*-octanol than lipophilic compounds, for an equilibrium shift to be observed. Other techniques for determining lipophilicity have used chromatography where retention or migration times of the sample are compared to standards of known lipophilicity. These methods include using octanol coated columns (Lombardo et al., 2001; Gocan et al., 2006) or the Chromatographic Hydrophobicity Index (Valko et al., 1997). Capillary electrophoresis has also been used to assess distribution using a micellar based system for studying membrane partitioning (Wong et al., 2004).

4.2.1.3. Solubility. Solubility is one of the most important properties of an API regardless of the route of administration. For oral medications, dissolution is required before a molecule can permeate across a biological membrane to be absorbed into the systemic circulation and elicit its pharmacological response. Without sufficient solubility, molecules can suffer from solubility-limited absorption and/or non-linear pharmacokinetics and dose responses. This is particularly problematic when trying to develop formulations to assess safety issues. In the last decade the number of poorly soluble APIs reaching clinical development has increased as increased potency has often been pursued by incorporating lipophilic regions into molecules (Keseru and Makara, 2009). Hence, solubility enhancement and enabling formulation technology as a means to increase API concentration levels in the lumen and thereby enhance absorption have gained much attention (see Section 4.6).

Thermodynamic solubility of an API is defined as the concentration reached at equilibrium between the solid drug substance (*solute*) in a liquid solvent to form a homogeneous solution of the solute in the solvent (for solubility terminology see Table 4). Typically, pharmaceutical scientists want to know the solubility in an aqueous-based system and dissolution media have been designed to mimic gastric and intestinal fluids under fasted and fed conditions as discussed in detail in Section 3. For formulation purpose, the impact of excipients and additives on solubility are important. In aqueous-based systems the pH of the medium is also crucial in assessing the solubility of ionizable drugs as it influences the degree of ionization and the proportion of neutral and charged forms present. The neutral drug species is much less soluble than the ionized species or salt forms of the drug as described by the Henderson–Hasselbalch equation.

Traditionally, thermodynamic solubility has been determined using the shake-flask technique. The golden standard protocol for solubility measurements includes equilibrating the pure, crystalline API for several days in a saturated suspension containing the medium of interest. Samples are withdrawn at multiple time points to determine if equilibrium has been reached, which involves separating solid from liquid phase by centrifugation or filtration and determining API concentration in the liquid phase. In general, API chemical stability is assessed throughout the equilibration time, the pH of the liquid is measured after equilibrium is reached and finally, the solid state form of the remaining solid in the vessel can be analysed to reveal whether or not a solid-to-solid phase transition occurred during the experiment (Bergstrom et al., 2002; Glomme et al., 2005). This procedure is usually performed during late discovery/early development stage, but is time consuming and therefore not useful for medium to high throughput screening. For this reason, a large number of scaled down methods exist for the determination of intrinsic (S_0) and apparent solubility (S_{app}) making the protocols applicable to the discovery phase when only small amounts of compound are available. These methods range from miniaturised shake-flask methods (0.05–2 mL (Bergstrom et al., 2002; Glomme et al., 2005; Wyttenbach et al., 2007)) over potentiometric titration (1–15 mL, (Avdeef, 1998; Stuart and Box, 2005)) to small-scale dissolution baths (<20 mL) with real time concentration determination *in situ* (Avdeef, 2007;

Avdeef and Tsinman, 2008). In addition to solubility the intrinsic dissolution rate (IDR) is of importance. This property is typically measured under physiologically relevant conditions, e.g. at the pH of the small intestine, but may also be determined in the presence of excipients. IDR is typically measured in standardised USP dissolution baths using the rotating disc method. These vessels require large solvent volumes and are therefore not applicable in discovery and the early stages of development. As a response to this several new small scale dissolution baths have recently been developed, and e.g. the μDISS Profiler can be used for small scale IDR measurements from discs (Avdeef, 2007; Avdeef and Tsinman, 2008). Together, the IDR and solubility measurements will inform on the likelihood of solubility or dissolution limited absorption. In addition, the IDR measurements can be used to guide formulation scientists on expected improvement in concentration obtained from e.g. particle size reduction or improved dispersion.

4.3. Current physicochemical profiling to increase throughput and applicability

Multiple methods have been established to determine solubility and dissolution rate at various stages during the research and development process (Table 5) within pharmaceutical companies (Murdande et al., 2011).

During the early discovery stage only a few milligrams of API is available for solubility determination. The material is of limited purity and with unknown solid state characteristics (amorphous/crystalline, salt/neutral) and may contain residual solvents. Consequently, solubility values obtained during the early stages are not very reliable and therefore find limited applicability in biopharmaceutical predictions. Rather, they are performed to obtain initial information of the solubility range of a compound or a compound class. With this aim in mind, the methods typically bin compounds into classes of 'less than' or 'greater than' an acceptable value. At this stage the methods are automated, performed in microtiter plates and commonly stock solutions of DMSO are used although protocols for measurements starting from the powder exist (Alsenz and Kansy, 2007; Zhou et al., 2007; Wan and Holmen, 2009). The time interval for shaking/mixing the API and solvent will be different in different laboratories, and currently, no standardised protocols exist. Alternatively turbidimetric solubility determination can be performed in which the solubility is determined based on API precipitation from a DMSO/water supersaturated solution (Lipinski et al., 2001). However, these methods have sometimes provided values greater than 50-fold higher the saturation solubility of the thermodynamically stable polymorph (Stuart and Box, 2005).

4.4. Physiologically relevant dissolution profiling

To better mimic the *in vivo* dissolution profiles for orally administered API and/or formulations thereof, dissolution profiling has evolved towards using physiologically relevant media as reviewed in Section 3. The impact of the pH on the solubility and the dissolution rate has been emphasized by the requirement of complete dissolution of the maximum oral dose in the pH range of 1–6.8/7.5 to flag high solubility as proposed in the Biopharmaceutics Classification System (BCS) (FDA, 2000; EMA, 2010). Further, the simulated intestinal fluids have made it possible to analyse the dissolution effects of the colloidal lipid structures naturally present in the intestine to solubilise poorly soluble, lipophilic compounds. These media have been extensively applied in measurements of S_{app}, determination of IDR and/or dissolution rate determinations. A majority of the studies published on dissolution profiling have reported dissolution and/or apparent solubility for a single API or formulations thereof, but during the last couple of years larger datasets have also been profiled in detail for dissolution behaviour and apparent solubility of APIs in fasted and fed state simulated media (Fagerberg et al., 2010, 2012; Ottaviani et al., 2010; Söderlind et al., 2010; Zaki et al., 2010; Clarysse et al., 2011). In some of these papers, the obtained data have been compared with dissolution profiles obtained in aspirated HIF (Söderlind et al., 2010; Clarysse et al., 2011). One finding in these comparisons was that the FaSSIF-V2 better predicted the solubility in HIF for neutral compounds than did the original FaSSIF, while they did not differ in their ability to forecast HIF solubility of acids and bases. Several papers have indicated that for API with log$P$ > 3–4, or log$D_{6.5}$ > 2, the mixed micelles composed of lipids and bile acids present in jejunal fluid may significantly increase the S_{app} of the drug in the intestine. (Dressman and Reppas, 2000; Bergstrom et al., 2007; Fagerberg et al., 2010; Gamsiz et al., 2010; Ottaviani et al., 2010) This simple rule of thumb can be used to determine whether a solubility measurement should be performed in a more complex biorelevant medium containing mixed micelles rather than in pH-adjusted buffers.

Physiological dissolution profiling has traditionally been performed at a reasonably large scale (500–900 mL) typically using dissolution baths of USP II and IV type. Such assays become expensive because of the large amount of pure BS and PL required for the biorelevant medium and further the general applicability is limited because of the large amount of API required to saturate the medium. Hence, large scale dissolution profiling methods are not suitable when a series of compounds is to be assessed or when the amount of API is limited. Currently, several miniaturised methods such as the μDISS, the T3 platform and miniaturized USP dissolution apparatus II are present and these can be used to measure both intrinsic dissolution rate from small discs in small volumes of biorelevant dissolution medium or measure dissolution and apparent solubility from powder (Avdeef, 2007; Avdeef and Tsinman, 2008; Gravestock et al., 2011; Zecevic and Wagner, 2013). These innovations have resulted in the ability to test dissolution in volumes of only 10–20 mL, which in turn have shifted the use of simulated intestinal fluids to the stage of early preformulation. It needs to be mentioned that during powder-based dissolution assays the surface area of the particles is constantly changing. In addition, the amount of the material weighed into the dissolution vial, the wettability and the dispersion of the particles will influence the determined dissolution rate. *In vivo,* the size of the particle when introduced to the intestinal fluid may influence the dissolution and thus absorption after oral administration of solid API. A theoretically calculated critical particle size below which the drug absorption is no longer dissolution rate limited has been suggested (Oh et al., 1993; Butler and Dressman, 2010), however, thus far, experimental knowledge on biological relevance of such a calculated particle size value is limited. Only a few *in vivo* animal pharmacokinetic (PK) studies investigating the influence of particle size on *in vivo* dissolution and absorption have been published, each study using different experimental conditions and doses, sometimes with only two particle size fractions compared to each

Table 5
Solubility measurements and method applicability.

	Solubility in discovery	Solubility in development
Assay	Turbidimetric solubility	Equilibrium solubility
API form	Non crystalline/polymorphic	Crystalline
Solid state	Not characterized	Polymorphs characterized
API form	Dissolved in DMSO	Solid used
Method	Added to stirred medium	Equilibrating with medium
Time scale	10's of minutes	24–48 h
Applicability	Suitable in early discovery	Essential in development
Used for	*in vivo* animal SAR	MAD, dissolution, salt screen
Amount	Micrograms	Milligrams

Abbreviations used: Dimethylsulfoxide (DMSO), structure activity relationships (SAR), maximum absorbable dose (MAD)

other (Liversidge and Conzentino, 1995; Jia et al., 2002; Scholz et al., 2002; Jinno et al., 2006; Hanafy et al., 2007; Xia et al., 2010). A qualitative correlation has been observed in which dissolution rate increased with decreased particle size as would be expected from the Noyes–Whitney relationship. However, a particle size at which further reduction does not yield further increase in dissolution *in vivo* has not been possible to extract from these experimental investigations.

While (intrinsic) dissolution rate and apparent solubility is commonly studied in biorelevant dissolution media it may be more difficult to investigate the tendency of the API to precipitate in the intestinal fluid. Precipitation may occur for APIs administered in solid-state manipulated materials such as amorphous systems, cocrystals or salt, all of which result in supersaturation and eventually precipitation. However, from a non-modified API perspective, precipitation *in vivo* is mainly a concern for poorly soluble weak bases which, during the transit from the stomach to the jejunum will become significantly less soluble due to the increased pH. Although precipitation risk may be identified from the pH-dependent solubility profile or *in vitro* precipitation studies, these have been found to over-predict the precipitation tendency *in vivo* (Carlert et al., 2010). This may partly be explained by the experimental set up *in vitro* (Augustijns and Brewster, 2012; Bevernage et al., 2013), which amongst others does not take into account the disappearance of the drug from the intestine. Another explanation is the fact that supersaturation may be obtained long enough in the jejunum to allow absorption to occur. This could, for instance, be a result of food components remaining in the intestine and potentially acting as precipitation inhibitors. However, *in vitro* precipitation data have been used to establish theoretical models for calculation of crystallization rates which successfully evaluated the risk for *in vivo* precipitation (Carlert et al., 2010).

4.5. Impact of solid state transformations occurring during storage and dissolution

New API is usually presented after synthesis as a solid, classically produced by a crystallization process to improve purity. This solid state controls dissolution and solubility since it must be thermodynamically favorable for the molecule to leave the solid and enter the solvent for dissolution to occur (Yalkowsky, 1999). The free energy values associated with the solid form are determined by the crystal structure and as small molecules have the ability to exist in multiple solid state forms or polymorphs as well as solvates, salts, co-crystals or the disordered amorphous state, control is the key to ensure reproducible performance. The API solid state and its associated properties is a critical factor controlling pharmaceutical developability (Huang and Tong, 2004). The ability to predict an API's solid state based on its molecular structure is a developing field (Price, 2004) based around searching for the most stable lattice structure that can be formed by a molecule. The early attempts tended to overestimate the possibility of polymorphism and did not incorporate any consideration of kinetic factors during the crystallization process controlling nucleation and growth. In addition simple rigid model molecules were studied to aid predictive capabilities rather than the more complex flexible multi-component structural features present in currently discovered APIs. Refinements have been introduced which are improving the predictive properties of these techniques but the ability to absolutely *de novo* predict crystal structure and polymorphism remains elusive (Price, 2008; Abramov, 2012). The current computational approaches cannot accurately predict crystal structure or its effect on solubility or the mechanical properties, the latter being crucial for formulation processes (Shariare et al., 2012). Prediction of structure is therefore advancing as one of the methods for solid form discovery (Llinas and Goodman, 2008), however at present it only complements current solid form screening techniques (Aaltonen et al., 2009) by confirming already known crystal forms and potentially pointing to missed forms.

In general, about 80% of small molecule APIs are polymorphic, i.e. the same chemical structure can display different crystal lattice arrangements, with the majority appearing in two or three different polymorphs (Grunenberg, 1997). Even though the chemical structure is the same, visual appearance (colour, crystal habit) and physicochemical properties (e.g. Tm, hygroscopicity, S_{app}, IDR, density, hardness, stability) may differ significantly between different polymorphs. To avoid polymorphic transition throughout manufacturing and storage, and the change in solubility and bioavailability associated with such solid state transformation (Singhal and Curatolo, 2004), immediate release tablets are generally developed using the API in its thermodynamically stable form. However, this crystal form is the least soluble form and not always identified at early development stages. Indeed, occasionally new polymorphs are not identified until after the medicine is marketed with the most famous example being ritonavir (Bauer et al., 2001). For this drug, only one polymorph was known when it was launched (1996), and it was marketed as a liquid-capsule filled with the drug dissolved in a mixture of ethanol, surfactant and water. Two years after ritonavir was marketed a new and thermodynamically more stable polymorph, which was four times less soluble than the marketed polymorph, was identified. This resulted in a temporary withdrawal of ritonavir followed by subsequent relaunch in 1999. Two approaches were taken to manage this relaunch. The synthesis scheme was refined to identify two different processes resulting in pure polymorph I (the metastable polymorph) or pure polymorph II (the thermodynamically stable polymorph). Furthermore, a formulation was designed to accommodate the less soluble polymorph II (Chemburkar et al., 2000; Bauer et al., 2001).

The ritonavir case shows that polymorphic transitions may have great impact on the performance of the drug product. However, the general consensus is that expected changes in S_{app} associated with polymorphic changes generally are low. Typically the S_{app} of the metastable polymorph as compared to that of the thermodynamically stable polymorph differ no more than a factor of two, although higher ratios have also been observed (Pudipeddi and Serajuddin, 2005). A similar trend has been observed for the S_{app} ratio of the thermodynamically stable anhydrate as compared to the solubility of the hydrate. However, the pseudopolymorphs tend to result in slightly higher ratios than those observed for non-solvated polymorphs. It needs to be stressed that when comparing the thermodynamically stable polymorph to other obtained polymorphs the stable polymorph has the lowest solubility whereas when comparing with hydrates, the latter has the lowest solubility in water. The same thermodynamic principles apply for dissolution rate meaning the stable polymorph dissolves the slowest.

In contrast to the relatively modest changes in solubility observed for different polymorphs, solid state transformation producing the amorphous state results in significantly higher S_{app} as compared to that obtained for the stable crystalline polymorph (Hancock and Parks, 2000; Weuts et al., 2011). For example, the amorphous S_{app} can be calculated from Tm, enthalpy and entropy of fusion, and isobaric heat capacities, as described by Hancock and Parks (Hancock and Parks, 2000). For glibenclamide, this resulted in a ratio (calculated amorphous solubility over the solubility of the crystalline material) of 1600 whereas the experimentally measured ratio was much lower most likely due to strong tendency for re-crystallization during the dissolution. In any case, the amorphous state shows great promise increasing the dissolution rate and apparent solubility and hence, early information on the inherent propensity of the API to remain in its amorphous form is warranted. It has recently been shown that this property can

accurately be predicted from molecular structure (Mahlin et al., 2011). In addition, molecules with molecular weight >300 have been suggested as possible to transform to the corresponding amorphous state by standard amorphization technologies such as spray drying or melt quenching (Mahlin and Bergstrom, 2013).

From the material presented above it becomes clear that it is of utmost importance to characterize the solid state of the API batch and relate the solubility and/or dissolution rate that is determined to the solid form that was present. Further, the solid state needs to be characterized throughout the development process to keep track on possible solid state transformations that may occur during storage or during scale-up. Similarly, there is increasing evidence in the literature of solid state transformations occurring also during dissolution. To further improve the mechanistic understanding of the dissolution process, the experimental setup has recently been extended with visualization techniques (ActiPix Technology, Sirius) and Raman spectroscopy to identify solid state transformations during dissolution *in situ* (Rantanen, 2007; Strachan et al., 2007; Savolainen et al., 2009). The dissolution and solubility measurements will become more complete through these approaches.

4.6. Early assessment of supersaturation and excipient effects during dissolution

An important aspect of API interrogation associated with solubility is the tendency of the API to supersaturate and if it does supersaturate, to evaluate whether this effect is long-lived. Several assay methodologies have been developed in this context including the solvent shift/quench paradigms at 10 mL scale using medium to low throughput (Vandecruys et al., 2007; Warren et al., 2010; Bevernage et al., 2013), 96-well high throughput approaches tied to this concept (Brewster et al., 2011; Yamashita et al., 2011) and titration based approach for ionisable compounds, implemented on the Sirius T3 platform (Box et al., 2006; Box and Comer, 2008; Hsieh et al., 2012). The same techniques have also been applied to assess the effect of excipients on tendency of API's to supersaturate with reference to supersaturation extent and duration (Warren et al., 2013). The solvent shift method relies on supersaturation generated by adding a concentrated solution of the poorly water-soluble drug of interest in a water-miscible organic solvent to an aqueous media (Bevernage et al., 2013). The rate and extent of precipitation is assessed either in a simple buffer or media or in the presence of an excipient that may act to inhibit nucleation and/or crystal growth. The titration based protocol is a potentiometric procedure which rapidly measures the equilibrium aqueous solubility of organic acids, bases, and ampholytes that form supersaturated solutions. In this procedure, the equilibrium solubility is actively determined by changing the concentration of the neutral form by adding acidic or basic titrants and monitoring the rate of change of pH due to precipitation or dissolution (Box et al., 2006). Supersaturation evaluations have been conducted using buffers, simulated fluids including biorelevant fluids as well as aspirated HGF and HIF (Bevernage et al., 2010, 2011, 2012; Psachoulias et al., 2011). The influence of an absorption component (e.g. Caco-2 cells) on the maintenance of supersaturation has been investigated and this experimental set up reduce the precipitation *in vitro* (Bevernage et al., 2013).

4.7. Wettability

Wettability is important as it is a prerequisite for processes such as disintegration, dispersion, solubilisation and dissolution (Lippold and Ohm, 1986; Buckton and Darcy, 1995; Brown et al., 1998; Cheema et al., 2007; Tian et al., 2007; Buch et al., 2011) and can have direct implication on drug stability (Morris et al., 2001; Ohta and Buckton, 2005). The wettability of a certain API will also influence the manufacture processes of drug products indirectly, e.g. granulation and coating (Asthana and Sobczak, 2000), and ultimately impacting the drug product quality specification and its clinical behaviour. Wettability is described by the wetting angle, θ, of the liquid to the solid in the presence of the gas as defined by the Young equation (Young, 1805). As the tendency of a drop to spread out over a flat, solid surface increases, the contact angle decreases. If $\theta < 90^\circ$ wetting of the surface is favourable, whereas if $\theta > 90^\circ$ (i.e. the fluid minimizes the surface contact through formation of a compact liquid droplet) wettability is poor (Shafrin and Zisman, 1960).

Four principal wetting models to determine θ with solids are described in the literature: the spread or sessile, the capillary rise, the immersional and the condensational or adsorptive (Lazghab et al., 2005) of which the spread/sessile and capillary rise methods are the most commonly used for pharmaceutical materials. The spread wetting procedure involves deposition of a given amount of liquid on the solid API. The liquid will spread over the surface of the API and θ is determined at the three phases (solid/liquid/gas) contact line. In the capillary rise method the API powder is packed under controlled conditions in a cylindrical cell with a porous bottom. The wetting is based on the capacity of the liquid to rise in the powder bed and θ is then calculated from the rate of liquid penetration using the Washburn model (Washburn, 1921; Galet et al., 2010). Sessile methods have been used for measurement of θ of large single crystals and have been widely applied to compressed discs of pharmaceutical powders (Harder et al., 1970; Buckton and Newton, 1986; Puri et al., 2010). The advantage of this method is that the θ can be determined directly by measurement of the shape of the drop using microscopic visualisation methods, e.g. Environmental Scanning Electron Microscopy ESEM (Jenkins and Donald, 1999), Atomic Force microscope (AFM), or goniometry (Extrand, 2004). The drawbacks with the sessile method is that it is sensitive to the conditions used and the results are dependent on e.g. polymorphic changes on the surface during compression (Buckton and Newton, 1986), drop volume, rate of dropping and distance between dropper and surface (Baki et al., 2010). Immersing approaches based on the Wilhelmy plate method have been published where the plate has been made of the compound under investigation and the θ is then determined upon drawing the plate up from the liquid investigated (Chawla et al., 1994; Sheridan et al., 1994). The Wilhelmy plate method has also been modified to use a glass slide onto which drug compound powder is glued. In this modified method θ is measured after displacement on the liquid (Dove et al., 1996; Pepin et al., 1997). Dove and coworkers reported that the two Wilhelmy plate techniques produced comparable results (Dove et al., 1996).

4.8. Permeability

4.8.1. Physicochemical tools

4.8.1.1. Surface activity profiling (SAP). Surface activity profiling (SAP) describes the relationship between drug concentration and the surface pressure of the drug solution and it has been postulated that SAP can be used to assess API permeation across biological membranes. Physiological membranes consist of a hydrophobic core region and hydrophilic surfaces and are therefore described as amphiphilic systems. This barrier is appropriate to view as an anisotropic interfacial system (Bassolino-Klimas et al., 1993; Eytan et al., 1996). Amphiphilic substances are able to penetrate into membranes, with the penetration efficiency partly determined by their ability to position themselves at the lipophilic–hydrophilic interface (Tejwani et al., 2011). This latter aspect can be characterized by the cross-sectional area of the drug at the interface (Petereit et al., 2010), while the incorporation of the drug into

the interface can be measured by the change in the layer pressure. Analogous to the importance of substance parameters such as hydrogen bonding potential, molecular size, charge and lipophilicity for permeation processes through membranes (van De Waterbeemd et al., 1996; Pauletti et al., 1997; Camenisch et al., 1998), these parameters are also important for surface activity in solutions and for interfacial orientation (Seelig et al., 1994). Amphiphilic substances organize themselves at the air/water interface in an anisotropic manner. For the hydrophobic effect, polarities of the different phases are important. The dielectric constant of the lipid bilayer interior ($\varepsilon = 2$) and of air ($\varepsilon = 1$) are comparable, which is in contrast to that of water ($\varepsilon = 80$). Thus it is postulated that the orientation of amphiphilic substances at the air/water interface is similar to their orientation at the luminal/lipid bilayer interface (Gerebtzoff et al., 2004). Seelig et al. proposed that by correlating surface activity values, such as CMC with membrane permeation data, it would be possible to predict the ability of APIs to cross membranes (Seelig et al., 1994). Other relevant parameters describing surface activity are the interfacial air/water partitioning coefficient (K_{AW}^{-1}) and the cross sectional area (A_S). These parameters can be derived from Gibbs' adsorption isotherm (Suomalainen et al., 2004; Petereit et al., 2010), which in dilute systems utilize the concentration (C) of the API instead of the chemical potential (Seelig et al., 1994). In addition the surface excess can be calculated from Avogadro constant and the area requirement of the surface active molecule at the interface (Gerebtzoff et al., 2004). In other experiments, the surface pressure (Π) rather than surface tension (γ) is measured, and plotted versus ln C to derive $d\Pi/d\ln C$ and calculate CMC and K_{AW}^{-1} of an amphiphilic substance (Fig. 11) (Suomalainen et al., 2004).

4.8.1.1.1. Surface tension measurement. Two different applications of surface tension measurement techniques are currently used; the dynamic surface tension (DST) which measures the change in surface tension during the formation of a new liquid/air surface over time and static surface tension (SST) which measures surface tension of an already-formed interface. DST is typically measured with the bubble pressure tensiometer (Eastoe and Dalton, 2000; Fainerman and Miller, 2004), whereas SST typically is measured with the Wilhelmy plate method (see Section 4.7) and the Du Nouy Ring method. The DST and SST methods are time-consuming and require relatively large liquid volumes ($\geqslant$10 mL) and are therefore not suitable in early physicochemical profiling. A 96-well multichannel microtensiometer has therefore been developed by Kibron (Finland). This method is based on the Du Nouy ring method, but instead of rings the probes are designed as eight needles, each of which is attached to a balance measuring the individual force exerted. The measurements are automated and are performed in a medium throughput mode requiring sample volumes of only 50 μL (Kansy et al., 1998; Suomalainen et al., 2004).

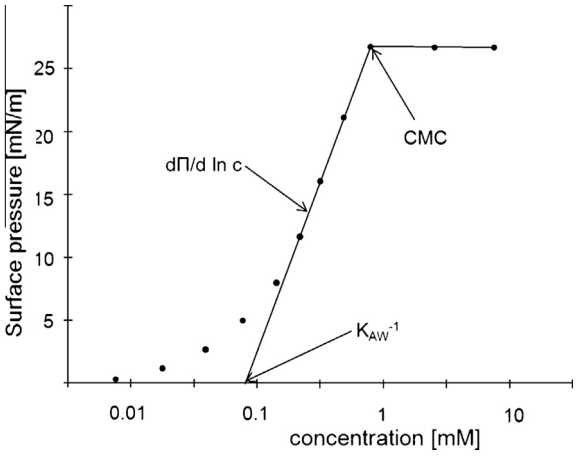

Fig. 11. Substance concentration versus the corresponding surface pressure value. Adopted from Suomalainen et al. (2004). Abbreviations used: CMC, critical micellar concentration; K_{AW}^{-1}, interfacial air/water partitioning coefficient; (Π), surface pressure; C, concentration.

4.8.1.2. Artificial membranes. Physicochemical methods such as the SAP mentioned above and artificial membranes depend on the assumption that transcellular passive diffusion across the intestinal epithelium is the main route of absorption (Sugano et al., 2010). Examination of the molecular properties of registered oral drugs supports this assumption since the majority of these drugs have physicochemical properties that are optimal for rapid diffusion into and out of the cell membranes (Wenlock et al., 2003). The most common artificial membranes are composed of phospholipids often in combination with physiological proportions of other membrane lipids (Kansy et al., 1998; Sugano et al., 2004; Avdeef and Tsinman, 2006). These lipids are deposited onto membranes such as the Transwells, where the lipid fills the pores of the filter membranes used. Typically, the assay is considered a simplified permeability assay, in which the API is dispensed onto the lipid membrane and the concentration obtained in the acceptor chamber is determined after a certain time interval. The obtained results are then used to bin compounds into classes such as good/intermediate/low permeability and/or to rank compound libraries for their permeability properties. One draw-back with the artificial membranes is that the length of the pores of the filter support, which will be filled with the lipid solution used, is much longer than the length of a cell membrane lipid bilayer and hence, will accommodate many multiples of such layers in a series. This creates an *in vitro* artifact that slows down the permeation across these membranes as compared to the permeation found in e.g. the enterocyte. Both theoretical calculations and experimental approaches, such as pH shifts have been used to rectify this problem (Avdeef et al., 2005; Avdeef et al., 2007). Application of these approaches is required for quantitative extrapolation to *in vivo* permeability but requires expert knowledge. Often, such quantification is not necessary to perform during the early stages of drug discovery where it is considered sufficient to bin compounds with regard to high or low permeability.

4.8.2. Cell-based models

More than two decades after their introduction (Hidalgo et al., 1989; Artursson, 1990; Hilgers et al., 1990), cultures of cell monolayers, such as Caco-2, are still routinely used in drug development to assess intestinal drug permeability. Although the cell-based models cannot compete with artificial membranes with respect to speed and price, they are applied when more quantitative information is required. Since Caco-2 monolayers can withstand quite vigorous stirring conditions, good estimates of passive permeability coefficients in the vicinity of those observed in human *in vivo* can be obtained (Artursson and Karlsson, 1991; Lennernäs et al., 1996). For compounds displaying an intermediate passive permeability, it is more difficult to predict the intestinal permeability *in vivo* since in all systems, including the perfused human intestine, there is a steep relationship between permeability and absorption (Lennernäs, 1998; Matsson et al., 2005). In this permeability range, compounds tend to be more polar and have a slower distribution into the cell membranes and/or they may be subjected to active transport processes. In this case, cell monolayers are useful since, in contrast to artificial membranes, they host these alternative drug permeability pathways. One such alternative is the paracellular route in which the drug molecule crosses the monolayer by passing through the tight junctions between the cells. This route is considered to have limited contribution to the overall permeability,

although it may be of importance for small hydrophilic molecules (Adson et al., 1994, 1995). For studies of the impact of the paracellular route, it is important to recognize that different cell lines express different pore populations, which is in agreement with the *in vivo* situation. Thus, Caco-2 cells mainly express a paracellular pore population that mimics the smallest of the two pore populations present in the human small intestine (Linnankoski et al., 2010). This pore population is too small to allow identification of molecules with significant paracellular permeability *in vivo*. In contrast, the conditionally immortalized rat fetal intestinal cell line 2/4/A1 (Paul et al., 1993) mainly express the larger of the two pore populations found in human and may therefore be a more suitable tool for studies of the paracellular pathway (Linnankoski et al., 2010). Interestingly, the leakier paracellular route in the 2/4/A1 cell line has resulted in drug permeabilities of low permeable drugs that are in good agreement with the corresponding human intestinal permeabilities (Tavelin et al., 2003).

The contribution of the many transport proteins present in the intestinal epithelium is considered to be of minor importance for the average oral drug, and also for drug molecules that are substrates to particular transporters. This is because the average drug concentration in the intestinal lumen is considered to be sufficiently high during the major part of the absorption phase to saturate possible active drug transport pathways (Sugano et al., 2010). Further, the expression of many drug transporting proteins seems to be too low to be of significance (Hilgendorf et al., 2007), although this needs to be verified when emerging protein expression data obtained from proteomics analyses of tissue from human intestine can be accounted for. Recently, a new approach for calculating the maximal transport activity for a particular transport protein in a particular organ was proposed (Karlgren et al., 2012). Notably, there are important exceptions in that polar drugs with limited membrane distribution and structural resemblance of nutrients or small peptides (e.g. molecules resembling small amino acids and di-or tripeptides) can also permeate the enterocyte via transport proteins. These transport proteins can actively transfer the compounds across the membrane or facilitate other influx mechanisms (Steffansen et al., 2010). Also several drug efflux proteins of the ABC-transporter family are highly expressed in the enterocytes and these are known to be transporters that accept a variety of structurally different substrates (Raub, 2006; Giacomini et al., 2010). In general, the efflux transporters do not constitute a threat to the absorption of the average dosed (highly permeable) oral drug (Fenner et al., 2009). For drugs given at low doses and that have small therapeutic indices, the efflux transporters may limit systemic exposure and may be sensitive to drug–drug interactions (DDIs) potentially resulting in reduced drug safety (Englund et al., 2004). An example of such a drug is digoxin, for which regulatory agencies require DDI studies, starting with *in vitro* assessment of permeability in the presence of an ABC-transporter such as P-glycoprotein (Giacomini et al., 2010; Food and Drug Administration, 2012; European Medicines Agency, 2013). To perform DDI investigations, cell models that express sufficiently high amounts of the protein need to be used, and for this purpose Madin Darby Canine Kidney (MDCK) cells over-expressing transport proteins are often applied.

A drawback shared by the cell monolayer cultures discussed above is that they lack a mucus layer produced by goblet cells; an epithelial cell population different from those represented in the commonly used cell monolayer models. Mucus layer producing goblet cell populations have been cloned from the human intestinal epithelial cell line HT29 (Wikman et al., 1993; Behrens et al., 2001). Using such cell lines and isolated pure mucin and reconstructed mucus (Larhed et al., 1997), it was shown that the mucus layer does not provide a significant barrier to the average orally administered drug. Although a small influence on lipophilic drugs could be observed, this was not considered to be significant. In contrast, a significant effect could be observed for larger drugs such as vasopressin and cyclosporine A (Larhed et al., 1997). For targets that have ligands with physicochemical properties beyond the Lipinski rule-of-five space it is likely that the mucus layer may influence the final absorption. In analogy, large aggregates of drugs, e.g. provided by formulations may get trapped in the mucus layer. Co-cultures of Caco-2 and mucus producing HT29 cells are not successful due to insufficient mixing between the cell lines in monolayers (Wikman-Larhed and Artursson, 1995). Although this was reported almost 20 years ago, cell-based models that better mimic the *in vivo* situation have not yet been established.

Intestinal permeability of API is often determined by calculating the concentration and surface area normalized permeability coefficient. Due to this normalization, comparison between different models for assessment of permeability is possible. For instance, human intestinal permeability can be compared with that obtained in a cell monolayer and has in many cases been found to be in good agreement (Tavelin et al., 1999, 2002; Stenberg et al., 2001). This is in contrast to the traditionally applied flux equation commonly applied by physiologists and pharmacologists, which is system dependent due to the different area available in different systems. Another factor that impacts the permeation across various *in vitro* membranes is the aqueous boundary (unstirred water) layer adjacent to the cells (Loftsson et al., 2007). In the unstirred situation, a highly permeable compound may obtain a measured value that is 20-fold lower than the unbiased permeability obtained during stirring. Standardized approaches for permeability coefficient determinations have been proposed (Hubatsch et al., 2007) and would be of great assistance for comparison of data between laboratories and development of *in silico* models of predefined quality. Unfortunately, to date there is no standardized way to determine these permeability coefficients in the scientific community. Thus, variable units, *in vitro* artifacts and precision of the measurements will impact on the determined 'permeability'. The level of precision required is often dependent on the stage of compound development. In discovery stages, a permeability measurement producing a categorical number (high/low) above or below a certain value may be sufficient to assure that absorption is not permeability-limited, while in drug development a more exact value may be required to model pharmacokinetic parameters such as T_{max} and C_{max}.

4.9. Analysis of drug absorption using physiology-based pharmacokinetics (PBPK)

Since the early 1960s it has been possible to extract rudimentary absorption data from an oral dose pharmacokinetic profile (Wagner and Nelson, 1963). The predictions of *in vivo* performance of drug products advanced with the introduction of non-compartmentally based pharmacokinetic models in the 1980s (Veng-Pedersen, 2001) and recent advances include the ability to deconvolute drug input functions providing *in vivo* dissolution information and improved *in vitro in vivo* correlations (IVIVC) (Huehn and Langguth, 2013). The availability of enhanced computational tools has permitted the 'whole body systems approach' to be refined for *in vivo* prediction using physiology-based pharmacokinetic (PBPK) modelling. In PBPK properties of the drug and the system (i.e. the body) are considered in a particular structural model (Rowland et al., 2011). As an example, a more advanced dissolution absorption and metabolism model that was recently introduced, incorporates API related factors (e.g. solubility and dissolution) along with biopharmaceutical features (e.g. intestinal permeability, influence of transporter and enzymes) and effects of GI tract motility (Jamei et al., 2009). Computational models combining these pieces of information permit predictions of plasma profiles to be generated and the influence of solubility and dissolution to be assessed along

with the effect of modifications to these values (Wagner et al., 2012; Willmann et al., 2012). In early stage development however, human pharmacokinetic parameters are unlikely to be available and scaling from animal data will be required. For a more detailed background on drug absorption, PBPK and the impact of API properties on these outcomes, the reader is referred to a recent review (Kostewicz et al., 2013).

5. Preformulation assays – an industrial perspective

The preformulation work in the pharmaceutical industry is defined by the way the process has been organized and is normally divided into discovery, development and commercial phases. The profiling program varies from company to company, however, whilst there is no formal definition of the process and the description of the milestones can vary between companies, there is a common understanding of key deliverables in terms of material characterization required to advance to the next stage. In the drug discovery process the purpose of investigating physicochemical and pharmaceutical parameters are to predict the behaviour of the compounds and identify risks. In the development program physicochemical parameters are determined to provide sufficient understanding for the chemical processing so that consistent quality can be obtained and to optimise the formulation. A number of different preformulation assays have been developed by the industry to comply with the needs defined by the organization, as described in the previous sections. This section will rather describe the assumptions for the preformulation scientist in the different phases.

Data generated during profiling of compounds in the preformulation phase have many applications and general goals are to (i) identify developability and potential downstream issues, (ii) provide data to understand *in vitro*/*in vivo* results and to perform structure property analysis, (iii) predict *in vivo* performance and facilitate selection of compounds for preclinical and clinical investigations, and (iv) guide molecular structure modifications. The ready availability of preformulation data during the drug discovery phase allows project teams to make informed decisions on compounds and formulations to advance to PK, biology and exploratory toxicology studies. A robust preformulation program will also provide data to underpin candidate selection for progression to clinical development and address pharmaceutical factors which could potentially lead to attrition at a later stage of the development process.

5.1. Target selection to lead finding

Target selection and lead finding deals with pharmacological and molecular biological identification of the intended target and disease state for the project (Tamimi and Ellis, 2009). Most of the work is done *in vitro* and based upon available compound libraries and custom synthesized libraries – this is where the project is defined. At this early stage typical preformulation data are based either on *in silico* predictions or simplified methods to measure the aqueous solubility, log*P* and pK_a. Further, medicinal chemists focus on identifying good chemical starting points for lead optimization to avoid "molecular obesity" induced by high molecular weight and log*P* (Keseru and Makara, 2009) resulting in poor ADMET properties (Gleeson, 2008; Waring, 2009, 2010; Gleeson et al., 2011; Hann and Keseru, 2012). As mentioned previously, log*P* is used as an indicator for several other physiological properties, such as intestinal and blood–brain barrier permeability, P-gp efflux, metabolic liability, plasma protein binding and toxicological risk (Gleeson, 2008; Gleeson et al., 2011).

In silico models to predict solubility are often based upon internally developed programs, particularly within the larger pharmaceutical companies, but commercially available software packages capable of calculating different molecular descriptors and properties are also used (Elder and Holm, 2013). Experimental miniaturized high through-put screening (HTS) methodology are used and several screening methods have been developed, the most common being kinetic solubility determinations from a DMSO stock of the API (Kerns, 2001; Lipinski et al., 2001; Bevan and Lloyd, 2000; Avdeef, 2001; Pan et al., 2001; Chen et al., 2002; Bard et al., 2008). At this stage of the discovery process, the obtained kinetic solubility profile of lead compounds can provide some qualification for the developability of APIs. However, there is a tendency to overestimate solubility values when using kinetic solubility as compared to equilibrium solubility (Chen and Venkatesh, 2004; Kramer et al., 2010; Saal and Petereit, 2012). This is governed by the propensity of a compound to crystallize rather than its thermodynamic solubility and hence may mislead the optimization process (Saal and Petereit, 2012). In spite of these challenges, kinetic solubility is a good indicator of potential solubility issues that may occur during *in vitro* pharmacological experiments performed in downstream development. Since such assays use DMSO stock solutions and are conducted over a comparably short time interval, high numbers of compounds may be screened and eliminated from further development.

Lipophilicity, expressed as log*P*, can be predicted to a reasonable level of accuracy by *in silico* methods, however, at the early stages of discovery two main types of experimental methods are also used (Hageman, 2010). These include determination by chromatographic methods, where several procedures have been published (Boyce and Milborro, 1965; Braumann et al., 1983; Kaliszan, 1990, 1992; Dorsey and Khaledi, 1993; Lambert, 1993; Pagliara et al., 1995; Lombardo et al., 2000; Valko et al., 2001; Yamagami et al., 2002; Gocan et al., 2006) or miniaturization of an octanol/water shake flask (Gulyaeva et al., 2003). The chromatographic process cannot directly model the bulk organic-water partitioning process since the non-polar stationary phase is an interphase (immobilized at one end) and not a bulk medium (Nasal and Kaliszan, 2006). The API interacts with the bonded hydrocarbon layer in the column (Tchapla et al., 1984). Therefore, the retention is affected by the surface density of the bonded alkyl chains (Sentell and Dorsey, 1989). Despite these methodological differences, the data from the classical shake-flask log*P* determination and the retention factor obtained from reversed-phase HPLC chromatographic systems comprising of a hydrocarbon silica stationary phase and an aqueous mobile phase (log k) generally correlate reasonably well (Novotny et al., 2000; Dai et al., 2001).

Software packages are used to predict pK_a (Wan and Ulander, 2006), however, pK_a determinations by CE, which only requires small amounts of compound, is also feasible at this early stage (Ishihama et al., 1994; Pang et al., 2004; Poole et al., 2004; Jia, 2005; Zhou et al., 2005). Determination of pK_a by RP-HPLC has also been explored, wherein the determination of the capacity factor (k') from retention time (t) of the API as a function of the pH is used to calculate the pK_a value (Kaliszan et al., 2004; Wiczling et al., 2004). Both the CE assay and the HPLC-based methods are separation techniques, which can reduce the effect of impurities on the pK_a measurement. This may be a beneficial feature when determining pK_a in the early stages of drug discovery. However, numerous measurements are required, the mathematical treatment is typically a manual process and the results will have poor accuracy due to variable column/capillary interactions at different pH.

5.2. Lead optimisation

In the lead optimisation phase, preformulation input is important in order to optimise the physicochemical properties of a chemical series with the objective of improving the developability

characteristics of the lead series. Lead optimisation may partly be achieved with *in vitro* measurements of physicochemical properties such as those described in the previous section but *in vivo* experiments to differentiate the properties of a lead series are an important component of this phase of the discovery process. During the lead optimisation phase, more API is available, which allows a company to choose to continue with screening methodologies or switch to lower throughput but more rigorous preformulation screening assays. From a preformulation perspective, solubility, lipophilicity and pK_a are the most important physicochemical parameters for the same reasons as previously stated. Also permeability is usually included at this stage and is determined by artificial membranes or cell-based methods, all dependent upon the need in the project. Lipophilicity is usually determined both as log*P* and log*D*, either using high-throughput (Section 5.1), shake-flask or potentiometric titration methods (Section 4.2.1.2). Studies at Lundbeck have indicated that for compounds with log*P* > 5 the shake-flask method is the most precise. High quality data is also warranted for pK_a during this stage, because of the large influence the charged species will have on the pharmacokinetic profile. Most commonly, potentiometric and/or spectrophotometric methods are used to measure pK_a. The availability of the API as solid material means that thermodynamic solubility, typically in a phosphate buffer at pH 6.5 or 7.4, is determined and used as a developability index. While the equilibrium solubility measurements most accurately represent the thermodynamic solubility, the resulting value will be dependent on e.g. solute purity, solvent, chemical/physical stability in solution, time, temperature, mixing conditions, solvent purity, adsorption to surfaces, pH and methodology, all of which are factors that may vary in the discovery phase ((Alsenz and Kansy, 2007; Di et al., 2012). Typically only one time point is used to measure the 'thermodynamic' solubility in the discovery phase whereas several time points may be measured at later stages of development to ensure that the equilibrium solubility is reached. Interestingly, Saal and Petereit (2012) recently investigated the thermodynamic solubility of 465 research compounds. They reported that ~60% of drug residues examined after thermodynamic solubility were crystalline, 22% amorphous and the remainder of indeterminate crystallinity, emphasizing the need for solid state characterization during solubility assessment.

The lead optimisation phase may also include *in vivo* screening studies where ensuring adequate systemic drug exposure is critical to assess efficacy and toxicity. Early *in vivo* studies employ different animal species (mouse, rat, rabbit, dog, guinea-pigs, monkeys, etc.), as well as different routes of administration (oral, intraperitoneal, intravenous, subcutaneous, etc.). Primarily subcutaneous and oral routes are preferred, but the selected route for administration is highly dependent upon the preferred experimental procedures in a company, and the targeted disease. Consequently, the test formulation has to accommodate a variety of species, routes of administration, dose volumes, stability, pH, viscosity, osmolality, buffer capacity and biocompatibility, whilst minimising local toxic effects and avoiding interaction with pharmacological behaviour models (Bittner and Mountfield, 2002; Lee et al., 2003; Neervannan, 2006). Guidelines for both dosing volumes and excipient acceptability in different preclinical species are available in the literature (Gough et al., 1982; Masini et al., 1985; Diehl et al., 2001; Gould and Scott, 2005; Gad et al., 2006; Neervannan, 2006; Rowe et al., 2006; Li and Zhao, 2007; Stella and He, 2008), but much of this information is also kept within organisations as there are disease model dependencies and different traditions with regard to vehicles employed. For instance, the vehicle requirements for a single dose pharmacokinetic investigation are much less stringent than those defined for long term dosing in regulated toxicity studies. The rising awareness of the biopharmaceutical challenges posed by current discovery pipelines (Lipinski, 2000; Lipinski and Hopkins, 2004), places increased emphasis on ensuring that the development and composition of these early formulations adequately consider the biopharmaceutical properties of the selected API. The formulation systems are normally based upon investigation of the thermodynamic solubility in the vehicle and all the classical solubilisation techniques that are currently employed. These include pH adjustment, cosolvents, cyclodextrin complexation, surfactants, but also more sophisticated pharmaceutical systems such as amorphous solid dispersions and lipid-based formulations (Neervannan, 2006; Li and Zhao, 2007; Maas et al., 2007; Porter et al., 2007; Al-Ghananeem et al., 2010; Gopinathan et al., 2010; Kwong et al., 2011; Higgins et al., 2012; Zheng et al., 2012). Recently the use of suspensions, in particular nano-suspensions, in the discovery phase has gained interest (Merisko-Liversidge and Liversidge, 2008; Kesisoglou and Mitra, 2012; Talekar et al., 2012).

5.3. The drug discovery/development interface and preclinical development

To cross the interface between drug discovery and drug development the number and sophistication of the measured parameters increases significantly. The pharmaceutical developability of a lead candidate is evaluated to provide a project risk assessment of formulation development. The initial work performed during lead optimization will hopefully be of sufficient quality to provide guidance for the selection of enabling technologies with the highest chance of success.

Larger amounts of pure compound are now available allowing re-investigation of log*P*/*D* and pK_a. A complete pH-solubility profile can be determined and combined with high quality permeability measurements performed in Caco-2 or MDCK cell lines to provide a preliminary BSC/ Developability Classification System (DCS) classification (Amidon et al., 1995; Larhed et al., 1997). Typically dissolution and solubility are assessed in simulated GI fluids to provide more realistic estimations of the intestinal solubility (and stability) than that obtained using pure buffers. The thermodynamic solubility protocol at this development stage normally includes investigation of the solid residue by XRPD to identify which solid form is in equilibrium with the solvent. A mini-salt screen may be conducted to evaluate the salt forming potential of a lead compound and provide options for purification during crystallization. The timing of salt (and also solid form) selection will typically depend on the development challenges facing the compound. In some cases, extensive screens will be deferred until after proof-of-concept in man, e.g. when the biopharmaceutical profile of the lead candidate is considered to pose low risk in several areas. In cases where a salt is important to allow proof-of-concept in man, e.g. to increase dissolution rate or supersaturation propensity, salt screening will get higher priority. The interested reader is referred to recently published reviews in the field (cf. Elder et al., 2013; Kumar et al., 2007). Stress stability studies will also be initiated to identify chemical instability and light sensitivity. For these studies the compound in solution is normally exposed to oxygen, light and a wider pH range (1–10) at elevated temperature in solution. If significant degradation is observed, a more thorough investigation of the impact on *in vivo* bioavailability is conducted. Typically, chemical stability in biorelevant media at more relevant *in vivo* concentrations are performed and includes examination of the degradation kinetics, elucidation of degradation mechanism and determination of pH where stability is at a maximum.

The pharmaceutical developability assessment conducted in several different companies has been published (Balbach and Korn, 2004; Balani et al., 2005; Li and Zhao, 2007; Maas et al., 2007; Ku, 2008; Kawakami, 2009; Saxena et al., 2009; Palucki et al., 2010). This consists of *in vivo* determinations of the bioavailability and

plasma half-life as an investigation of biopharmaceutical properties. For this purpose a decision tree in which both solutions and suspensions are dosed in animals has been used to investigate the feasibility of using traditional dosage forms. This has been exemplified by Branchu et al. (2007), who found that enabling techniques were possible to forecast for APIs based on log dose number, hydrogen bond donors (HBD), log*D* and molecular surface area. Furthermore, standardization of screening approaches to identify the need for enabling formulations has been suggested (Mackie et al., 2008). Here, the API is dosed to rats as either a suspension in methocel or as a solution in 20% aqueous 2-hydroxypropyl-β-cyclodextrin (dose of 10 mg/kg). This is compared to a 2.5 mg/kg i.v. typically dosed in a cyclodextrin vehicle. A similar protocol can be used in dogs at a dose of 5 mg/kg. Dosing of solution and suspension may lead to equivalent exposure with high absolute oral bioavailability (defined as a solution/suspension AUC ratio of between 0.8–1.2 and an oral bioavailability >0.6), which aligns with a DCS/BCS category of class 1 or class 1-like. Similarly, exposures between the solution and suspension can be similar but with an overall low BA suggesting either a BCS 3 or 3-like material, or significant first pass metabolism. In some cases, the AUC generated by the solution may be much higher than that associated with the suspension. Mackie and colleagues (2008) defined this as a solution versus suspension exposure ratio >3. This ratio is used to identify APIs for which solubility or dissolution rate of the solid API is limiting oral BA. For other APIs, the suspension may produce a higher exposure than the solution. This may encounter for APIs that are chemically degraded under the acidic conditions in the stomach or for those that precipitate from solution in a less soluble form than that present in the suspension. Those compounds exhibiting similar exposure from solution and suspension can use conventional formulation strategies. However, those APIs with a high solution/suspension exposure ratio are likely to require enabling formulations strategies, whereas compounds with a low solution/suspension exposure ratio need enteric coating or other technology approaches. A similar approach was taken in a recent IVIVC study in which certain dose / solubility ratios of the API at various pH were to identify whether solubilisation technology would be needed during future development of solid oral dosage forms (Muenster et al., 2011). In this study, a correlation of dose/solubility ratio at various pH with *in vivo* dissolution data in rat was found. A good IVIVC was observed at pH 4.5 and 7, but it was much less predictive at pH 1. This was expected, since rat stomach pH is known to be between 3.8 and 5 (Kararli, 1995). Hence, it was reported that 50% drug release in the rat GI tract corresponded to almost complete *in vivo* dissolution in humans.

Even though the process and stages at which *in silico*, *in vitro* and *in vivo* assays are performed may differ between various R&D organizations, all studies and results finally lead to an overall developability risk assessment. This risk assessment is the basis for management decisions on whether (or not) to proceed with a project into development. Furthermore, the risk assessment facilitates the project transfer from the research into the development program.

5.4. Clinical development program

At this stage, pharmaceutical formulations are prepared for administration to patients. Assessment of *in vivo* absorption enables the identification of compound/formulations liabilities and provides a basis for the early initiation of development strategies to overcome these problems. *In vitro* and *in vivo* characteristics of drug molecules gathered during drug discovery (e.g. dissolution, solubility, stability in fluids at the site of administration, PK in preclinical species), are useful inputs for subsequent clinical formulation development. Here, suitable formulation types and technologies are developed to meet biopharmaceutical targets for clinical development. Building the relationship between API properties and drug product performance is crucial at this stage to guarantee adequate product performance and clinical behaviour of a formulation *in vivo*.

The basic compound characteristics are normally re-evaluated during development using methods producing data with high quality. This includes redetermination of all the parameters defined in the previous section, in particular when the final solid form (e.g. salt and polymorph) has been selected and the clinical therapeutic dose is better defined. Also, investigations on compound/excipient compatibility are performed. In addition to the BCS, calculation of the Solubility Limiting Absorbable Dose (SLAD) (Butler and Dressman, 2010), Maximum Absorbable Dose (MAD) (Curatolo, 1998) and parameter sensitivity analysis are simple models used during formulation development to estimate the impact of certain API parameters like particle size distribution, solubility and permeability on absorption in relationship to the clinical dose. Models that estimate risk for solubility-related food effects are applied to investigate the need to develop a 'food resistant' formulation. These models should be applied as early as possible during the process to avoid reformulation and further clinical testing later in the development. Physiological models like Advanced Compartmental Absorption and Transit (ACAT) and PBPK have become an integral part of the development strategy in particular to predict impact of formulation on poorly soluble drugs. Such models rely on physicochemical parameters used as inputs and accurate measurements of these properties are required for successful simulations. Furthermore, the impact of excipients on e.g. dissolution rate and apparent solubility will improve the predictions of oral absorption and guide efficient clinical formulation development.

6. Physicochemical understanding of the API: future perspectives

Even though a large physicochemical screening platform is fundamental to the selection of preclinical drug candidates, API still often fail during preclinical testing and during first in man studies. Given the points discussed in this review increased efforts should be directed to obtain improved:

- *In silico* models for prediction of ADME properties of contemporary libraries. These need to be quantitative rather than qualitative and include physiologically relevant properties such as pH-dependent solubility, dissolution rate and apparent solubility in the context of the intestinal environment, intestinal permeability (passive and active) and toxicological risk assessment based on the absorbed amount to the systemic circulation.
- Miniaturized methods. As initial screening is conducted when the amount of compound is limited, methods to forecast *in vivo* performance need to be scaled down to become applicable at this stage. The ability to automate the established miniaturised methods is also important to consider. Desirable miniaturised methods include (i) simultaneous assessment of solubility-permeability interplay using simulated intestinal fluids and cell-based models, with the possibility to assess formulation performance and solid state characteristics of the resulting API *in situ*, (ii) *in vivo* relevant precipitation risk assessments and (iii) rapid and accurate physicochemical property screening of highly lipophilic compounds (log$P$ > 5) e.g. log$D_{6.5}$, log$D_{7.4}$, pK_a and pH-dependent solubility.
- Assessment of permeability. Highly accurate models for human small intestine permeability using cell models containing the correct contribution of passive and active transport

mechanisms need to be developed. These models should also include the impact of the unstirred water layer. In addition, models to better assess the potential for colonic absorption of APIs are of importance for development of controlled/extended release formulations.

- Physicochemical profiling used to predict *in vivo* performance. Many of the tools currently in use are based on knowledge of their accuracy to predict 'traditional' marketed oral drugs correctly, but other properties may be of greater importance for contemporary targets, such as deep intracellular nuclear targets, lipid metabolic pathways or neurotransmitter pathways, with highly lipophilic endogenous ligands.
- GI models to forecast interindividual variability and effects of disease states on absorption. This relates to further refinement of simulated fluids used to mimic the GI dissolution profiles, but also to the interplay between these fluids and cell monolayers to better mimic the absorption.
- *In vitro* models assessing *in vivo* relevant supersaturation and precipitation risk. The latter can be a result of the pH-change in the GI tract or the changed solubilisation capacity of a particular formulation. Improved models for these properties would increase the understanding of the pharmacokinetic profiles and facilitate the early formulation work during the pharmaceutical developability assessment.

In the IMI funded OrBiTo project, several of these issues will be addressed. Through the collaboration of a large number of pharmaceutical companies and academic groups a new database will be constructed in which the chemical space of the model compounds used will be expanded into the chemical space of ligands to contemporary targets. This database will be characterized for its physicochemical properties (e.g. log*P*, pK_a, solubility, permeability, etc.) and the resulting data will be used to develop new *in silico* models available to the common research community. Further, new models are targeted to better forecast important processes taking place during absorption, i.e. dissolution, solubilisation, wettability, precipitation and permeation. The measurement of these properties should also be conducted in the presence of excipients to help evaluate drug product performance. The aim of making the amendments discussed above is to improve the prediction of PBPK models, with emphasis on the role of absorption.

As briefly discussed above, permeability models and methods used to date have been developed based on drug compounds with reasonable lipophilicity. The much more lipophilic ligands explored by the industry in the post genome era may produce erratic results due to adhesion tendencies to the plastic components and/or entrapment in cell membranes. These processes can result in poor mass balance and a false low permeability. Further, the gut wall metabolism may be more significant for such molecules than for traditional drug-like molecules. Access to a large series of more diverse and lipophilic drug molecules is crucial to improve and update the methods to the chemical space currently being explored. A simplified system as compared to the cell models, to forecast permeability has been suggested to be the SAP method. In agreement with the problems experienced in the cell-based models, substances with non-amphiphilic, lipophilic structures with poor solubility in aqueous buffer solutions are the most challenging APIs for the SAP method. For such substances, Petereit recently established a modified SAP method, in which the influence of API incorporation during formation of micelles of 1,2-Dioctanoyl-sn-glcero-3-phospho-I-serine (a phospholipid) on the CMC was established (Petereit, 2011). The ratio of $CMC_{lipid}/CMC_{lipid+API}$ provide a good correlation to fraction absorbed ($R^2 = 0.83$) at the same time as the poor solubility was substantially overcome by the solubilizing effect of phospholipids. This method may be used to estimate not only the influence of self-association on API absorption, but also the influence of distribution between mixed micelles present in the intestinal fluid and membrane phospholipids, in order to provide a simple method for prediction of absorption.

Oral delivery of drug compounds is dependent upon dissolution in the intestinal fluids. A firm understanding of the variations in the intestinal content is therefore important for the robust development of a formulation. As the intestinal composition may vary in healthy and disease states it is important to understand the effect of such variations to produce simulated intestinal fluid relevant for the particular research program. If this is performed successfully the designed medium will provide a more realistic and physiologically relevant dissolution profiling of importance to specific patient populations. Further, refined tools to assess supersaturation of the drug candidate are important to forecast which enabling formulation type that would add most value to a specific API. Thus, weak bases that supersaturate may offer an advantage over those that do not and systems that can be influenced in their ability to supersaturate through the aid of excipients would have a higher chance of success during pharmaceutical drug development. Currently, most supersaturation assays are reasonably unsophisticated and the relative contributions of primary and secondary nucleation, crystal growth, hydrodynamic and mechanical stress cannot be revealed and the impact of the absorption of the drug (i.e. disappearance of API from the solution) is not correctly mimicked. Based on the limited data available, it appears that these simple systems are over discriminating compared to the *in vivo* situation, which is why there is a need for the development of more accurate methods. The use of biorelevant media, appropriate hydrodynamics and the incorporation of an absorption component may rectify these issues. However, the establishment of new tools allowing *in vitro* prediction of *in vivo* supersaturation and precipitation will be a complex task.

The current challenge is to generate new and improved understanding of the physicochemical parameters that are important for drug performance. This will lead to better selection of compounds in drug discovery, a better quantitative prediction of drug absorption and hence a better and more efficient pharmaceutical development process. The points discussed above are part of the work that will be performed in the OrBiTo project with the expectation that the joint efforts of the pharmaceutical industry and academic partners will successfully rectify some of the current problems identified in API pharmaceutical profiling.

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