Chapter 2

*In vitro* methods to study intestinal drug metabolism

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Chapter 2

Abstract
Although the liver has long been thought to play the major role in drug metabolism, the metabolic capacity of the intestine is increasingly recognized. In vivo studies eventually pointed out that significant first-pass metabolism by the intestinal wall has implications for the bioavailability of several compounds. Furthermore, the relevance of drug transporters in this process has been proven. Only a few methods are presently available to study drug metabolism in vivo or in situ in the gut. With most of these methods it is generally difficult to discriminate between the contribution of liver and extrahepatic tissues. Consequently, direct measurement in intestinal tissue is critical. To study intestinal drug metabolism in vitro, apart from subcellular fractions, several intact cell systems are nowadays available.

This review discusses the currently available in vitro methods to study intestinal drug metabolism. The advantages and limitations of the intact cell systems (isolated intestinal perfusion, everted sac, Ussing chamber preparations, biopsies, precision-cut slices, primary cells); subcellular fractions (S9 fractions, microsomes) and that of intestinal cell lines (Caco-2, LS180 cells amongst others) are discussed. Their applicability to study phase I-III metabolism/transport and drug-drug interactions in various species are summarized. Furthermore, potential causes of variation within and between methods are discussed, while metabolic rates obtained with the different methods are compared.

It is concluded that subcellular fractions and cell lines seem to be rather efficient methods to study drug metabolism at the individual enzyme level, but that the isolated intestinal perfusion, everted sac and Ussing chamber preparations appear to be particularly useful for studying overall drug metabolism and interactions with transporters. Tissue biopsies, precision-cut intestinal slices and primary cells seem most appropriate to study metabolism of slowly metabolized drugs as well as the induction of the various induction pathways involved.

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1. Introduction

Although the liver has long been thought to play the major role in drug metabolism in the body, the metabolic capacity of the intestine in this respect is increasingly recognized. This is supported by the demonstration of high expression of drug metabolizing enzymes (DMEs) \[1,2\]. \textit{In vivo} studies eventually proved the significance of first-pass metabolism by the intestinal wall for the bioavailability of cyclosporine, verapamil and midazolam among others \[1-3\]. However, the information on the relative importance of human intestinal drug metabolism from \textit{in vivo} studies is scarce, since these studies are technically and ethically complex. Therefore, \textit{in vitro} investigations are required to extend the current knowledge in this field. The aim of this review is to provide an overview of current \textit{in vitro} methods to study drug metabolism and transport in the intestine, discussing both the advantages and limitations of the test systems. In this review, we focus on the use of both rat and human tissue. Rat data are useful in the light of the widely used animal metabolic studies with this species, while large species differences in these aspects require also studies in human tissue for a proper prediction. Furthermore, this review provides extensive information to facilitate interpretation of data obtained with intestinal \textit{in vitro} methods.

For the interpretation of \textit{in vitro} drug metabolism studies, several aspects of the physiology of the intestine need to be taken into account. First of all, the intestine is not a homogeneous organ. The expressions of DMEs and drug transporters (DTs) follow different patterns both on the crypt-villus axis and along the length of the intestine. For DMEs, the activity has been reported to be the highest in the differentiated epithelial cells of the villus region and to decline progressively towards the crypt region \[4,5\]. This heterogeneity is introduced by cell migration from crypt to villus, which appears to be a concerted program of differentiation leading to apoptosis and cell extrusion into the lumen \[6\]. The migration from crypt to villus axis takes place in approximately 3 days in human and 2 days in rat intestine \[7\].

Along the tract, in general, the metabolic activity of phase I and II metabolism is higher in the duodenum and jejunum than in the ileum and colon in both rat \[8-10\] and human \[11-14\]. Yet, the gradient along the intestinal tract is not similar for all members of the CYP family. For example, CYP3A5 is predominantly present in human colon tissue \[15\]. Also for transporters, many different gradients along the tract have been described as has been summarized by Pang \[16\]. In human tissue for example, in distal direction the expression levels of some transporters increase (MDR1 (Pgp/ABCB1) \[17-19\], MRP1 (ABCC1 \[19\]), OCTN2 \[20\]), decrease (BCRP (ABCG2 \[21\]), MRP2 (ABCC2 \[19,22\]) or remain constant (OATP2B1 \[20,22\]). Furthermore, MRP3 is the highest in colon \[19,22\], whereas ASBT is the highest in ileum in comparison with both duodenum and colon \[20,23\]. The localization of the transporters in relation to the DMEs is important for bioavailability and metabolism of both drugs with high permeability and low solubility (class 2 of the BCS) and with high solubility and low permeability (class 3) \[15,24\]. Thus for interpretation of \textit{in vitro} data, it is very important to take into consideration from which part of the intestine the chosen \textit{in vitro} preparation is derived.

Apart from the DMEs present within the enterocytes one should realize that also enzymes present within the lumen might contribute to drug metabolism in the intestine. These comprise both mammalian enzymes (enzymes from gastric and pancreatic secretions and intestinal cells) as well as bacterial enzymes (concentrated in ileum and colon) \[25\] and ref herein. The latter have been shown, for instance, to be involved in reduction of azo and
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nitro groups and in deconjugation of glucuronides. The influence of these enzymes should be further investigated to appreciate their contribution to drug metabolism, but is outside the scope of this review.

The pH gradient along the intestinal tract is another important aspect in intestinal physiology. The luminal pH is greatly dependent on the intestinal segment involved [26] (in humans: duodenum: 5-7, jejunum: 6-7, ileum: 7, colon: 5.5-7 and rectum: 7), whereas the pH of the blood ranges in between 7.2 and 7.4, indicating that under physiological conditions the pH at the basolateral side is higher than the pH at the luminal side [27]. The pH determines the charge of many acidic and basic drugs and therefore determines the rate at which a drug enters the lipophilic cell membrane simply via passive diffusion as described by the pH-partition hypothesis or whether carriers for the charged drug molecules are needed. Furthermore, the function of the carriers can be pH-dependent [28], but the pH dependency is different for various compounds. The permeability of tacrolimus, for example, is not affected by pH [29], but the permeability of angiotensin II antagonist HR720, increases drastically when lowering the apical pH from 7.4 to 6.0 [30]. Therefore, the incubation conditions in in vitro studies may largely influence the outcome of the experiment.

In principle, the contribution of the intestine to drug clearance from the blood and the rate of absorption from the lumen may be influenced also by the intestinal blood flow. In contrast to liver, the blood flow in the intestine is distributed: the majority of the resting intestinal blood flow (60-70%) is distributed to the submucosa/mucosa, but considerably less (5-30%) of the intestinal blood flow perfuses the enterocytes. However, after a meal, the blood flow to the intestine is increased, which decreases the transit time and chance of metabolic conversion of a drug within the intestinal tissue, thereby, increasing the bioavailability of drugs, as was convincingly shown in clinical studies [31].

Apart from drug metabolizing enzymes, luminal directed drug efflux transporters, such as MRP2 and Pgp, form another obstacle for drug absorption [32] by excreting drugs back from the enterocyte to the gut lumen, thereby at least partly preventing their entrance in the circulation [33-35]. The hypothesis that intestinal secretion of drugs and/or its metabolites is an important elimination pathway [32,36] was confirmed in experiments using the in situ perfusion technique, where inhibition of Pgp increased the apparent permeability of tacrolimus in rat ileum and colon [29]. However, the exact contribution of Pgp to the barrier function of the intestine is in most cases difficult to assess and considered to be rather limited [37]. However, it should be realized that transporters are needed to excrete drug conjugates from the enterocyte into the blood stream or back into the lumen.

The significance of the intestine in determining the fate of drugs within the body is not only expressed by its high capacity to metabolize drugs, but also by its sensitivity to induction and inhibition of drug metabolizing enzymes [15,38-41]. It is suggested that intestinal enzymes respond to a greater extent than hepatic enzymes to orally administered inducers because of more direct exposure to higher concentrations of these substances [42]. Over 20 clinically significant drug interactions have been identified in which intestinal metabolism was implicated as a major contributor [31]. The major pathways for drug induction (via AhR or nuclear receptors PXR, CAR or GR [43]) are all present and functioning in the intestine as shown in different in vitro intestinal cell systems [44-48].
2. **In vivo and in situ methods to study intestinal drug metabolism**

2.1. **In vivo methods**

The possibilities to study drug metabolism in the intestine *in vivo* in the intact organism are indirect and scarce and above all, they often do not provide adequate intestinal drug metabolism data because of the difficulty to discriminate between liver and intestinal contributions. With a very sophisticated, but scarcely available method, drugs are administrated intraduodenally during the anhepatic phase of liver transplantation. Blood samples are collected from the portal vein and with this technique, significant human intestinal first-pass metabolism was proven for cyclosporine A [3] and midazolam [49]. The latter study also showed, that the intestine is able to metabolize midazolam after intravenous dosing [49]. In recent years, studies using either human intestinal tissue mounted in Ussing chamber set-ups [50] or Caco-2 cells [51], have revealed that most of the formed 1’OH-midazolam is excreted from the enterocyte into the lumen, indicating that the contribution of the intestine to metabolize these compounds is most probably even higher than estimated from such *in vivo* studies. Measurements of metabolic plasma clearance during an anhepatic phase also provided data on the metabolic capacity of all extrahepatic organs together [52-54]. However, this again does not offer a solution for determining the intrinsic metabolic capacity of the intestine.

With a pharmacokinetic method the significance of intestinal metabolism was proven after concomitant administration of grapefruit juice (which inhibits intestinal CYP3A) and midazolam to healthy volunteers, showing an increase of midazolam plasma concentrations after oral administration [55]. This study, however, could not elucidate the relative quantitative contribution of the intestine to midazolam metabolism, because the extent of inhibition of metabolism in the various organs involved is unknown. It should be noted that, a comparison of AUCs after oral and i.v. administration does not provide exact information about the contribution of the intestine to drug clearance and bioavailability, since the assumption that only liver contributes to drug metabolism after i.v. dosing has been proven to be not valid [56].

2.2. **In situ methods**

Intestinal drug metabolism can also be investigated by *in situ* perfusion techniques. The *in situ* human regional jejunal perfusion technique is mainly used for permeability studies [57], and showed that the intestine is capable of significant metabolism of several compounds, such as verapamil [1,2], talinolol [58], sulforaphane and quercetin-3,4’-glucoside [59] as well as amoxicillin and amiloride [60]. Although this technique has major advantages, such as intact physiology and blood flow, it has been scarcely used due to the ethical issues and limited availability of healthy volunteers and in patients.

The *in situ* perfusion technique in rats, as reviewed by Yu [61], can be used for up to 2-3 h [62,63]. Using this method, the involvement of Pgp in determining bioavailability of drugs has been studied. Among others, it has been shown that inhibition of Pgp induces the apparent permeability of tacrolimus [29]. Kavin et al. showed that viability of this preparation is maintained up to 1-2 h of perfusion, as appeared from oxygen and glucose utilization, active transport of glucose from the bowel lumen and from its morphology [63]. Rupture of
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intestinal micro vessels during perfusion may interfere with the results [62], but can largely be prevented by addition of dexamethasone and norepinephrine to the perfusion media. With this adaptation, the viability of the isolated gut can be maintained up to 5 h. This was shown by evaluating morphology showing intact epithelium with brush borders, and by maintenance of oxygen consumption, motility, water transport, lymph flow and vascular responsiveness to nor epinephrine [64]. However, addition of dexamethasone to the incubation medium, most probably influences drug metabolism [46,65].

A modification of this method is the in situ perfused rat intestine-liver preparation [66] in which single-pass perfusions [62] better mimic the in vivo first-pass effect [67] in comparison with circulating perfusions. Using this method, it has been shown that the intestinal wall extracts 4-methylumbelliferone [67] and salicylamide [68] and that its contribution to the total metabolic clearance is dependent on the intestinal blood flow. This method has the advantage that metabolism and absorption can be measured at the same time and that the blood flow remains intact. Disadvantages of this method are the limited viability and the used of anesthetics that can influence the outcome of the experiments [69]. In addition, it is a very labor intensive and technically difficult procedure with a high number of animals needed.

Thus, to quantitatively investigate drug metabolism within intestinal tissue, in vitro methods are evidently required. The limited access to human small intestinal tissue [70] stresses the urgency for availability of highly efficient in vitro methods. Also for animal studies, in vitro methods are needed to discriminate between the contribution of intestine and liver, to study regional differences and to reduce the number of animals needed for experiments as much as possible. For induction studies, such in vitro examinations form a good alternative, provided that the test system maintains its viability for sufficient incubation times.

3. In vitro methods to study drug metabolism

Although at this moment in vitro models do not reflect in vivo situations to the extent where they can replace in vivo investigations, they are very useful to address more specific questions in studies in which conditions can be more easily controlled than in vivo [71,72]. The general concern for in vitro studies is the sensitivity of the intestine to oxygen deprivation and the accompanying preservation problems. Fisher et al. [73] advised to perfuse the lumen with oxygen-saturated buffer prior to interrupting the circulation of the intestinal wall, to maintain tissue integrity. This idea has been supported by Plumb et al. [74], showing that intestines, deprived from oxygen for only 4 min, already show severe villus disruption and edema after only 20 min of reperfusion, while intestines that are constantly perfused and are never deprived of oxygen supply, maintain their structural integrity even after perfusion for 1 h. However, the extraction ratio of 1-naphthol and the appearance of 1-naphthol glucuronide were not affected when carbogen was replaced by air in the isolated perfused intestine [75]. Furthermore, for everted sac preparations the time required from dissection and preparation should not exceed 8 min [76] and the tissue should be kept in the oxygenated buffer solution continuously [76]. Also the intactness of Ussing chamber preparations, which can be demonstrated by the stability of electrical parameters in this system, is dependent on the overall ‘pre-chamber’ treatment. It depends on the anesthesia
of the rats prior to the mounting of the tissue, while the rat tissue should be kept in constantly oxygenated KBR solution directly after excision [77]. Surprisingly, preparations of human, pig, rabbit and chicken intestine survive much longer than those of rodents (rat, mouse). The latter degenerate rapidly within a few hours after dissection [6].

There are several in vitro methods available, each having their own advantages and limitations. Information about these methods, but also their applicability to different species and to study phase I, II and III metabolism/transport and drug-drug interactions are evaluated in the following sections. Causes of variation within and between methods are discussed and metabolic rates obtained with the different methods compared. Since the availability of human tissue is limited, the amount of tissue needed for a particular technique should be as small as possible. To stress this point, the methods are discussed in the order of required amount of tissue, starting with the method requiring the largest amount.

3.1. Intact tissue methods
The general advantage of working with intact tissue systems is that cell-cell contacts remain intact, all cell types remain present and enzyme systems, co-factors and transporters are present in their physiological context.

3.1.1. Isolated intestinal perfusions
‘A preparation of surviving rat small intestine for the study of absorption’ was already available in 1949 [73]. For this method, a part of the intestine is removed from an anaesthetized rat. Then, the segment is placed in a bath filled with buffer and luminally perfused with the compound of interest [73]. This technique has now evolved so that the vasculature can be remained intact during the excision procedure and allows not only perfusion of the luminal, but also of the vascular side [62,78].

Working with an isolated perfused intestine requires several experimental precautions. As already discussed in the introduction to this section, the uninterrupted perfusion of the lumen with oxygenated buffers is necessary to maintain viability of the preparation [73,74]. Andlauer et al. [79] have used a perfused intestine for 60 minutes after 30 min of equilibration and have shown intact perfusion pressure, lactate-pyruvate ratio, oxygen uptake, acid-base homeostasis and the genistein flux, but it is not clear whether their system could be used for longer time-periods. Other investigators state that viability is maintained using this method for up to 2-3 h, revealed by the same parameters and furthermore that the appearance rate of 1-naphthol glucuronide remained constant between 0-45 min [75]. No information, however, was provided about longer incubation times.

The buffer system seems critical in studies with perfused intestines. Using the ‘standard’ Krebs-Henseleit bicarbonate solution equilibrated with carbogen (95% O₂ and 5% CO₂), no difference in morphological appearance between a non-perfused section of jejunum and a section perfused for one hour could be detected. In simple Krebs-Henseleit phosphate medium or a Tris-phosphate medium, perfusion for only 20 min already causes loss of structural integrity of the intestine [74].

Using the isolated perfusion technique, biotransformation of several compounds has been studied in combination with the direction of metabolite excretion. For example, genistein glucuronide is recovered on both the luminal and serosal side after perfusion with genistein [80]. Furthermore, vascularly administered 1-naphthol is metabolized to 1-naphthol...
glucuronide, which is mostly excreted to the serosal side [75]. Many investigators have used this technique to study metabolism (phase I and II [75,79,81]), transport [82], but also inhibitory drug-drug interactions [83,84]. The isolated perfusion technique thus offers several benefits. Variables such as temperature, pH, osmolality, blood pressure and flow can be controlled [63]; both sides (luminal and vascular) of the intestine can be studied and exposed to different pH [69,78] and the anoxic period is minimal [78]. The major drawback of this method, however, is that it is applicable only to animal tissue [69] and that the method can only be used for short-term incubation studies, impeding its application in, for example, induction studies and studying slowly metabolized drugs. Furthermore, the technique uses high luminal perfusate flow rates which dilutes the samples which might complicate the analysis [78].

### 3.1.2. Everted sac

Everted sacs are commonly used for absorption studies. Wilson and Wiseman first introduced this method in 1952 to study absorption of compounds along the intestine of rat and golden hamster [85]. Two different everted sac methods are described to date. With the first method, the intestine is everted and closed on both sides actually creating a closed sac [86]. With the second method, the intestine is everted, canulated on both sides and perfused [72,76,87]. In brief, the intestine is excised after cervical dislocation, washed, everted over a glass rod, filled with fresh oxygenated culture medium and divided into sacs [88], which can differ in length between 1 cm [8] and 10 cm [89].

As shown by evaluating everted sac preparations, the period of time of dissection including preparation should not exceed 8 min and the tissue should be kept in the oxygenated buffer solution all the time [76]. Furthermore, freezing and thawing should be prevented, since this greatly damages the metabolizing capacity of everted sac preparations as shown by a 44% decrease of 6β-hydroxytestosterone formation [90].

Under optimal conditions, the everted sacs remain viable up to approximately 120 min [69,76,86,88-93]. Farthing et al. conclude that their sacs remain viable up to 2 h after measuring a clear glucose concentration gradient over the intestinal wall after 30 min which keeps increasing up to 2 h [92]. Rehner et al. use the everted sac technique up to 60 minutes because of maintained morphology up to at least 2 h, and constant oxygen uptake during this incubation period [76]. Ballent et al. conclude maintenance of tissue viability up to 70 min based on increasing glucose levels in the serosal media [91] and Emoto et al. show a constant rate of 6β-hydroxylation of testosterone up to 3 h [90], measured on the serosal side.

Several buffers are used for the perfused intestine technique such as Krebs bicarbonate buffers [76,89,92], medium 199 solution [91,94] or potassium phosphate buffer [90], sometimes in combination with a NADPH-generating system [8,90]. The pH is usually 7.2-7.4 [87,90,93] and buffers are normally oxygenated with carbogen [e.g. [76,87,91,93]]. Incubation under air, however, has been shown to maintain 6β-hydroxylation of testosterone constant in time up to 3 h [90].

This method has been described to be applicable to rat [85,87,93] and mouse [90]. It has been used to study phase I [89,90,92], phase II [8,95] and phase III of drug metabolism [91,94] as well as inhibition of for example CYP3A [89] and Pgp [91]. With this method it has been discovered that secretion of androstenedione (a metabolite of testosterone) is directed to both the luminal and serosal side, which is in line with *in situ* findings in the same study.
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[92]. The excretion of the metabolite of buproprion, hydroxybuproprion (CYP2B1) [96] and the metabolite of dextromethorphan, 3-methoxymorphinan (formed by CYP2D1/6) are directed to both the luminal as well as the serosal side [93] as revealed by this system. This system is fast [88] and inexpensive [69,88]. It can be used to measure the influence of regional differences [88] such as the decreasing gradient along the intestinal tract for many P450 reactions in mice [90]. The small volume inside the sac offers analytical advantages [76,88]. In addition, all cell types and the mucus layer are present [69]. A limitation of this method is that practically it is only applicable to animal tissue [69]. Furthermore, compounds of interest have to cross all layers of the small intestinal wall including the muscle layer in order to be detected at the serosal side [69,88]. Further, it should be noted that the preparation has a relatively short life-time [88] (up to 3 h), which limits the investigation of enzyme induction and of slowly metabolized compounds.

In some cases, the in vitro - in vivo extrapolations with this system have shown to be inadequate. Farthing et al. [92], for example, showed quite large differences in testosterone metabolite formation between in vivo studies and everted sacs. The lower oxidation-reduction potential caused by anoxia in in vitro tissue has been suggested be responsible for the formation of 5α-testosterone found in vitro and not in vivo [92]. On the other hand the contribution of Pgp to the intestinal secretion of ivermectin as observed in vitro by Ballent et al. was confirmed in vivo [91].

No reports could be found investigating the influence of the thickness of the intestinal wall to its viability. Stripping of the muscle layers, as preferably done in Ussing chamber preparations, is no common practice for everted sacs. Since this has been shown to be beneficial for Ussing chamber preparations [97], it could be a way to improve the viability of the everted sac during longer incubation and diminish the limitation of crossing all cell layers before reaching the serosal side.

3.1.3. Ussing chamber

Ussing chamber preparations are widely used to study transcellular permeation and less frequently to study metabolism of drugs in the intestinal wall. In 1951, Ussing and Zehren invented the Ussing chamber to study the transport of sodium in frog skin [98]. In the Ussing chamber preparations, mucosal sheets are mounted in between two buffer compartments (chambers). The luminal (apical) and serosal (basolateral) chambers are usually supplied with continuously oxygenized Krebs-Ringer bicarbonate (KRB) buffer [88]. Compounds of interest can be applied to either the luminal and/or serosal side [69] and transport to either side can be measured.

Two versions are currently in use, which differ in the size of the tissue sample. The amount of tissue necessary for one set-up ranges from 3.8 mm² [99] – 5 mm² [100] in the smaller version, to 0.65-1.78 cm² in the larger version [77,97,101-103]. The smallest chambers (mm²-range) have, up to now, only been used for absorption studies [99,100] and are very promising in the light of the scarce availability of human tissue.

Human and pig intestinal walls are too thick to be used directly in the Ussing chamber technique and therefore, the serosa and muscle layers need to be removed [97]. The effect of this stripping procedure has been tested on rat tissue. Improvement of tissue quality has been shown as the potential differences (PD) remain significantly higher between 80 to 120 min of incubation, yet passage of mannitol is significantly enhanced during this time period.
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Apparentlly, the facilitated oxygen and nutrient supply weigh out the possible negative effects of disrupting tissue integrity [97]. The viability of Ussing chamber preparations has been thoroughly validated. The chambers have been successfully used for up to 90 min [104], 120 min [97,103], 180 min [50,57,77] and even for 8 h as reported by Lampen et al. [105] who showed that the conductivity of the tissue remains constant up to 8 h [105]. Polentarutti et al. have validated their Ussing chamber preparations thoroughly and based on the potential difference (PD) (> 4mV small intestine; > 6mV colon), short circuit current (SCC), resistance, permeability for propranolol and mannitol as well as morphological evaluation, they have concluded that tissue integrity is maintained up to 180 min [77]. Soderholm et al. have included the permeability of $^{51}$Cr-EDTA as viability parameter and have reported that this is unchanged up to 120 min. In addition, mucosal ATP and lactate levels are stable up to 180 min and transmucosal glucose flux up to 240 min. Lactate dehydrogenase leakage is limited during 120 min and transmission electron microscopy showed preserved ultrastructure up to 120 min. However, light microscopy shows epithelium lifting from the basal lamina at 90 min [104]. From the latter study, it can be concluded that the choice of viability parameter drastically influences the period in which the tissue is considered ‘viable’. In most other studies, the PD and SCC have been used and these remain stable up to 120-240 min [30,50,77,106].

Ussing chamber preparations are generally incubated under carbogen [97,105,106] with KRB buffer solutions at pH 7.4 [30,77,105] as incubation medium [30,50,77,97,103,105,106], Ussing chamber preparations have also been used at pH 5.5 (luminal side) and pH 7.4 (serosal side) [107] or pH 6.0 both sides [103]. The bidirectional supply of glutamine to the incubation medium has been shown beneficial for using chamber viability, as it helps to maintain the rat enterocyte ATP content in this system [102].

The Ussing technique has been applied to human tissue [50,97,106,108], and to various animal species including rat [57,77,97,109], pig [97,105,110] and rabbit [101,111] to study permeability [30,100], phase I and II metabolism [50,101,105,108,110] as well as drug transport (phase III) [103].

The main advantage of the Ussing chamber technique is that bidirectional transport and metabolism can be studied at specific intestinal locations. The compounds can be added on luminal and/or serosal side and this method is applicable to both animal and human tissue [69]. Furthermore, the direction of excretion of the metabolites can be studied. Lampen et al. have shown that > 90% of the sirolimus and tacrolimus hydroxylated metabolites are directed to the luminal side in pig tissue [105,110]. Using human intestinal tissue, 1'OH-midazolam (CYP3A4/5), 4'OH-diclofenac (CYP2C9), OH-bufuralol (CYP2D6), but also 7-hydroxycoumarin sulphate are mostly excreted to the luminal side [50]. On the contrary, 7-hydroxycoumarin glucuronide is equally directed to both the serosal and luminal side [50]. Rogers et al. have reported that excretion of sulphated and glucuronidated ethynylestradiol and paracetamol are directed to both the luminal and serosal side [108]. This indicates that also in vivo a significant amount of the metabolites formed in human intestine ends up at the luminal side. Whether these luminally excreted metabolites are re-absorbed (either as such or after deconjugation) later on in the intestinal tract, is unknown and remains an important aspect to investigate.

A limitation of the Ussing chamber technique is that tissue viability is generally limited to 2-4 h [69]. However, this is long enough to study metabolism, transport and absorption of many
compounds. It is worthwhile to study whether drug interactions such as induction can be studied in this system, since such interactions have been detected even after only 5 h of incubation in rat intestinal precision-cut slices (chapter 4) and in Caco-2 cells already after 2 h [59]. A distinct limitation of the Ussing chamber technique is that the outcome of absorption rate studies is per definition an underestimation, since there is no intact blood flow and the compound has to cross all intestinal cell layers to reach the serosal chamber [69], but this aspect may vary depending on the drug studied.

3.1.4. Biopsies
Biopsies collected for diagnostic procedures or that are ‘punched’ out of excised tissue sheets have been used to study drug metabolism [112-116]. Intestinal biopsies (2 [114] – 21 mm² [115]) are either prepared from ‘whole thickness’ intestinal tissue [116] or from tissue remaining after stripping off the muscle layer [113,114] and then cultured. The full thickness of the human intestinal wall has been estimated 2 to 7.5 mm [117], indicating that diffusion of substrates, metabolites, nutrients and oxygen might be a limiting factor during incubation. Separation of the mucosa from the outer serosal layers has been found beneficial [6]. In contrast with the latter finding, De Kanter et al. showed that full thickness rat biopsies (so-called punches) possess higher ATP levels after 3 h of incubation in comparison with punches without muscle layer [118].

Biopsies of intestinal tissue have been cultured in dynamic organ cultures for 24 h [112] or in 6-wells plates for 3 h [118]. Others have cultured intestinal biopsies for 12 h [119], 48 h [114,115] or even 14 days [120]. In the latter study, samples from human colonic mucosa were incubated in a specialized medium exposed to a carbogen (5% CO₂ and 95% O₂) atmosphere consisting of Waymouth MB752/1, ascorbic acid, hydrocortisone, ferrous sulphate, penicillín-streptomycin, mycostatin and fetal calf serum. The medium was refreshed after 24 h and thereafter every 48 h [120]. Although tissue morphology was properly maintained, the functional integrity of the preparations has not been demonstrated. In another study, CMRL Medium 1066 was shown to be superior to Trowells T8, L15, Medium 199 and DMEM in supporting the viability and maintaining the differentiation of adult rat jejunal enterocytes in full thickness biopsies that were cultured during 21 h [116]. However, even when the optimal CMRL medium 1066 was used, dramatic morphologic changes appeared in time with a disproportional greater loss of epithelial cells then of other cell types [116]. The addition of dexamethasone, insulin or EGF to the incubation medium seems to improve the cell viability markedly [6].

Other media used for biopsy incubations are Waymouth's media [112], Williams Medium E with glutamax [118], DME with additives [119] and Leibovitz L15 medium [115]. In all studies, the medium is saturated with carbogen. It should be noted that the media used for biopsy incubations are surprisingly different from those applied to everted sac or Ussing chamber, in which relatively simple media are used.

In one study, the viability of precision-cut intestinal slices (precision-cut rings of intestinal tissue, described in the section ‘precision-cut slices’) and ‘biopsies (with muscle layer) were directly compared [118] and the metabolic rates were not significantly different between the two preparations during 3 h of incubation. The precision-cut slices, however, showed higher tissue levels of ATP during these 3 h of incubation and had a higher quality and content of isolated RNA after 6 h of incubation in comparison with biopsies, indicating that viability of
slices is better maintained, which is particularly important for longer periods of incubation [118].

Biopsies have been used for phase I [113,115,116] and phase II metabolism studies [118,121], as well as for inhibition [122] and induction studies [115,116,119]. Biopsies have been prepared from intestinal tissue of different origin. Besides normal intestinal tissue [119,121], also fetal tissue has been used [115] as well as tumor tissue [121] and inflamed tissue [114]. This technique is in principle applicable to all species, but mostly human [112,114,115] and rat [113,118,119] tissue have been used.

Using tissue biopsies, Vickers et al. showed that cyclosporine A metabolism in human colon biopsies is comparable to liver [113]. In another study, intestinal biopsies were shown to be responsive to 1α,25-dihydroxyvitamin D3 as mRNA levels of CYP24 were induced by this compound [119]. Furthermore, 1α,25-dihydroxyvitamin D3 increased the expression levels of CYP3A4 in human proximal and distal intestine [115]. Addition of dexamethasone to the medium increased CYP3A mRNA and protein in biopsies cultured for 21 h [116].

Major advantages of the biopsy method are that it is quick, efficient and in principle applicable to all species. A limitation of this method is its impossibility to study the direction of excretion of metabolites or drugs. Activity of transporters has not been studied with this system yet, but might be possible by using transporter specific inhibitors.

3.1.5. Precision-cut slices

The use of liver slices was first published in 1923 by Otto Warburg [71], however, its application was abandoned due to the large variability and short-term viability of these hand-cut slices. Hand-cut intestinal slices have been used in the eighties by Farthing et al. to study testosterone metabolism [92] using KRB buffer with glucose without added cofactors for incubations of only 60 min. Plumb et al. characterized the structural integrity of intestinal rings incubated in either tris-phosphate solution at pH 7.2 or 5.0, or Krebs-Henseleit solution showing severe epithelial damage after only 2 min of incubation [74], which may be explained by the difficulty to prepare reproducibly thin slices.

With the introduction of the Krumdieck tissue slicer [123] and later the Vitron slicer, it has become possible to prepare precision-cut slices with a thickness of between 100 μm up to 500 μm. These precision-cut slices are widely used in the field of drug toxicity and metabolism as has been recently reviewed [124]. This technique has recently been adopted to enable slicing of intestinal tissue [118] in which the tissue is flushed after excision. In order to facilitate proper cutting the segment was filled with a low gelling temperature agarose solution at 37°C and directly cooled to allow gelling of the solution. Then, the agarose filled segment is embedded in agarose using a tissue-embedding unit and subsequently sliced using a Krumdieck tissue slicer (Alabama Research and Development Corp. Munford, AL, USA). The slices (thickness: 350-450 μm and wet-weight 2-4 mg) are individually incubated in 6- or 12-wells plates [9,125] using Williams medium E with glutamax supplemented with gentamicin (antibiotic) and amphotericin B (anti fungal) ([9,118,126]) at pH 7.4 under carbogen. Martignoni et al. supplement their medium with insulin, hydrocortisone, methionine and fetal calf serum [47]. The precision-cut slices have been used up to 4 [50], 6 [47] and 24 h of incubation [9,118](chapter 4).

The viability of rat and human small intestinal and colon slices has been extensively examined. Slices show intact morphology [9,50,118], constant intracellular levels of ATP [9,118], a constant metabolic rate for both phase I and phase II substrates up to 3 h [9] and
Chapter 2

good quality of RNA isolated after 24 h of incubation [118]. The morphology of both human proximal jejunum and colon slices has been shown to remain intact during 4 h of incubation, whereas intracellular ATP levels in proximal jejunum remain stable [50]. Furthermore, the mucus layer has been shown to stay present during at least 3 h of incubation [9], indicating that the physiological microclimate is retained. Metabolic rates decrease moderately after 8 h of incubation depending on the enzyme studied to about 40-50% of the initial value after 24 h of incubation (chapter 4).

This technique can in principle be applied to all species and until now it has been proven useful for human [50], rat [9] and mouse [126] intestinal tissue. It has been validated for several purposes: phase I and II metabolism studies [50,118] up to 3/4 h; drug clearance studies up to 24 h [127,128] and induction studies up to 24 h [9,47]. Using this technique, gradients in metabolic activity for both phase I and II substrates along the rat intestinal tract have been assessed [9]. Furthermore, it has been shown that rat [9,47], but also mouse [126] intestinal tissue is responsive to inducing stimuli as has been measured at mRNA level [9,47], but also at the enzyme activity level [9].

The use of intestinal, but also liver, lung, kidney and colon slices to predict whole body metabolic clearance has been reported by De Kanter et al. [127,128]. In these studies, by measuring drug depletion using concentrations << Km and by subsequent PBPK scaling of the data, whole body clearance of several compounds, such as testosterone and 7-hydroxycoumarin was adequately predicted. In addition, these studies provide information about the contribution of the different organs to total body clearance. For example, it has been shown that the small intestine may markedly contribute for over 50% to total body clearance of testosterone. For some compounds the clearance is under-predicted using the slice system [128], unless albumin is added to the incubation medium [127].

Metabolic rates obtained with human proximal jejunum precision-cut slices and Ussing chamber preparations have been compared and appear to be very similar in the two systems [50]. The precision-cut slice technique is highly efficient considering the large amount of slices that can be prepared even from small tissue samples (>=1 cm²) [9] and the number of slices (>100) that can be easily handled per person per day. This indicates that slices from different organs can be used from the same animal, which is an advantage for the above-mentioned whole body clearance experiments, but it also contributes to the reduction of the number of experimental animals and costs needed for safety studies, and to the efficient use of human tissue [71].

Similar to biopsies, the slice method cannot be used to study direction of excretion of metabolites or drugs to either luminal or serosal side. Transporter-mediated uptake or excretion can, in principle, be measured using specific inhibitors, but this application still requires investigation.

3.2. Primary cells

Primary cells have been used to assess drug metabolism in a limited number of studies. Nevertheless, they could serve as a tool in drug metabolism as is shown in several studies [129-131]. Specialized isolation procedures are required to obtain functioning enterocytes and two distinct methods have been described here.
**Enterocyte elution:** With this method, enterocytes are gently eluted from the intestinal wall [132] using repeated incubation of tissue sheets with buffers containing phenylmethylsulfonylfluoride (PMSF), dithiothreitol (DTT) and/or EDTA and heparin [132-135]. A pure epithelial cell fraction is isolated by this method and serosal and interstitial cells are excluded, as confirmed by morphological evaluation [134,136-138]. Differences between enterocyte elution in rat and man have been described. In rat intestine, 1.5 mM EDTA effectively removes both villus and crypt cells when incubated for 1 h [136]. In human intestine, 5 mM EDTA only removed all villus cells, but only 10% of the crypt cells after a similar incubation period. Therefore, separation of human villus and crypt cells can only be achieved by first eluting the villus cells with EDTA and then releasing the residual crypt cells mechanically by scraping [134].

Another, less frequently used method, is based on the standard hepatocyte isolation procedure, using pronase and collagenase in the incubation medium. In this manner 100-150 x 10^6 viable primary porcine enterocytes were isolated in one isolation procedure [130].

**Shed enterocytes from human perfusion techniques:** Perfusates obtained from human perfusion experiments contain shed cells. During 2 h of perfusion, circa 5 mg protein (= ca 50-60 x 10^6 cells) can be harvested with 84 % villin positive cells (a marker for enterocytes) and 64% of the cells being non-apoptotic [70]. Shed cells have been used to study mRNA and protein expression of DMEs and DME activity [2,70]. Isolated cells from pig [129,130], guinea pig [139] and rat [131] have been used for drug metabolism studies. The alkaline phosphatase activity in cultured porcine enterocytes remains constant up to 8 days of culturing, but the percentage of viable enterocytes is only 50% after 72 h as was concluded based on trypan blue exclusion [129,130]. Isolated pig enterocytes have been proven useful for phase I metabolism studies using, amongst others, 7-ethoxycoumarin [129] and tacrolimus [130] as model compounds, and for phase II drug metabolism i.e. glucuronidation and sulphation of 1-naphthol [139]. In addition, induction of metabolism has successfully been studied in these cells in culture. For example, tacrolimus and 7-ethoxycoumarin metabolism was induced by respectively dexamethasone and 3-methylcholantrene or β-naphthoflavone up to 36-fold after incubation for 24-48 h [129,130]. Also the inhibition of 7-ethoxycoumarin and ethoxyresorufin metabolism by α-naphthoflavone has been reported [131].

### 3.3. Subcellular fractions

Subcellular fractions can be obtained by further processing of the eluted enterocytes or collected shed cells, as described above. In addition, two other methods are commonly used to obtain subcellular fractions.

**Tissue homogenization:** With this method, intestinal tissue is homogenized directly in a buffer containing sucrose, EDTA and PMSF using a potter-elvehjem [140,141]. Intestinal S9 fractions and microsomes prepared via this method consist of cell fractions of all cell layers present in the intestinal wall.

**Mucosal scraping:** With this method, immediately after excision of the intestine, a segment is placed on an ice cold stainless dish and cut longitudinally and is then washed in the
presence of PMSF for 1 min. Subsequently, mucosal cells are gently scraped off with a
glass slide [142-145]. The scrapings are dipped in buffer containing PMSF, trypsin inhibitor,
leupeptin, aprotinin, bestatin, glycerol and microsomes are prepared [144]. Microsomes
yielded via this method have been described to contain low concentrations of P450s and
appreciable contamination with hemoprotein [135].

3.3.1. S9 homogenates
From preparations obtained by direct tissue homogenization, mucosal scraping, elution of
enterocytes or from shed enterocytes, S9 fractions can be prepared by centrifugation (at
9,000 g for 20 min, the supernatant fraction contains cytosol and microsomes [140]). S9
fractions are generally incubated between 5 min [143] and 30 min [140] and co-factors (such
as NADPH generating system, UDPGA, PAPS etc) are added to the incubation medium
[140]. No information is provided stating whether S9 fractions can be incubated for longer
incubation periods.
The method is in principle applicable to all species and can be used to study phase I
[70,140,142] and phase II drug metabolism (glutathione transferase, phenol sulfotransferase
[140]) and for inhibition studies. Using rat S9 fractions, gradients of testosterone metabolism
and of CDNB conjugation along the intestinal tract have been identified [140]. In S9
homogenates of human shed enterocytes, verapamil metabolism was detected [70].
The advantages of this method are that it is very efficient to study drug metabolism and
inhibition and applicable to all species and for studying regional differences along the rat
intestinal tract [140]. The limitations of this method are that co-factors are not present in
physiological concentrations and the system lacks DTs.

3.3.2. Microsomes
After direct tissue homogenization, mucosal scraping, elution of enterocytes or collecting
shed enterocytes, microsomes can be prepared by differential centrifugation. Prior to this
centrifugation step, eluted cells are first homogenized using a potter-elvehjem homogenizer
and sonicated [136].
The commonly used incubation buffers contain a NADPH-generating system and when
glucuronidation is studied, UDPGA is added [146]. Incubations vary between 10-45 min
[126,131,144,146].
The preparation of intestinal microsomes is considered to be more subject to artefacts than
preparation of hepatic microsomes. The exposure of intestinal microsomal membrane
material to abundantly present intestinal proteases during isolation is considered to cause
this difference. Therefore, the addition of protease inhibitors during preparation such as
trypsin inhibitor, leupeptin, aprotinin and bestatin, has been found beneficial for CYP3A
activity recovery [90]. As noted by Kaminsky and Zhang, the many contradictions in the
published data on intestinal P450 composition are probably partly due to the differences in
the procedures used to prepare enterocyte microsomes [7]. Furthermore, evidence exists
that freezing and thawing may cause loss of activity of microsomes, since one freeze/thaw
cycle already destroys over 50% of the testosterone 6β-hydroxylase activity (CYP3A) [8,90].

A direct comparison between microsomes (with added co-factors) and eluted cells showed
that the Km values for metabolism of drugs were similar for the two preparations. In contrast,
the Vmax was higher in microsomes which may possibly indicate rate limitation by co-factors
In vitro methods to study intestinal drug metabolism

in the intact cells. The authors have corrected for the recovery of 45% of microsomes during the isolation procedure [131]. In a direct comparison of mouse everted sacs and intestinal microsomes, both obtained from the upper part of the intestine, the everted sac exhibited higher testosterone 6β-hydroxylation (CYP3A), tolbutamide methyl hydroxylation (CYP2C) and bufuralol 1’ and 6’ hydroxylation (CYP2D) capacity; the microsomes showed higher chlorzoxazone 6-hydroxylation (CYP2E1) [90]. Furthermore, Lampen et al. have reported qualitatively similar metabolic patterns of metabolites from tacrolimus and sirolimus in Ussing chamber preparations and microsomes [105,110].

In a direct comparison of rat everted sac and intestinal microsomes, testosterone 6β-hydroxylation (CYP3A), testosterone 16α-hydroxylation (CYP2B), 7-ethoxycoumarin O-deethylation (CYP1A,2B), 4-nitrophenol glucuronidation (UGT1A1, 10x higher) and morphine 3-glucuronidation (UGT2B1/12) activities have been reported to be higher in everted sacs. In contrast, ethoxyresorufin O-deethylation and pentoxyresorufin O-depentylation (CYP2B) activities have been reported to be higher in microsomes and 1’OH-bufuralol formation (CYP2D) and 1-naphthol glucuronidation (UGT1A6) have been shown to be processed in similar rates [8].

The method is in principle applicable to all species with data available from rat [135,146,147], mouse [144], rabbit [146] and human intestinal microsomes [145,148] and can be used to study phase I metabolism [46,147-150], glucuronidation [146,149] and inhibition [144,149].

The overall advantage of this method is that it is very efficient to study drug metabolism and inhibition and applicable to all species. Moreover, the preparations are commercially available. The limitations of this method are that cytosolic DMEs and DTs are lacking and that co-factors are not present at physiological conditions. This makes it difficult to compare metabolic rates obtained with microsomes and intact cell systems. It should be noted that the activities are often lower than in the intact preparations (see section 3.6).

3.4. Cell lines

3.4.1. Caco-2 cells

Caco-2 cells are derived from human adenocarcinoma cells from colon [69] and it has been demonstrated that these cells polarize and differentiate in long-term culture, both morphologically and functionally [151]. However, it is still a matter of debate whether Caco-2 cells represent the human small intestine or colon. They do form tight-junctions and express many brush border enzymes, such as the typical membranous peptidases and disaccharidases [69]. Furthermore, many DMEs and DTs are expressed in Caco-2 cells, but the level of expression differs quite extensively from both human duodenum and colon tissue [152]. Caco-2 cells lack normal expression levels of important metabolic enzymes such as CYP3A4, CYP2B6, CYP2C9 and CYP2C19 [28,88,152-154]. In table I, the expression levels of the most important DMEs and DTs in human intestinal tissue and Caco-2 cells are summarized.
### Table I: Comparison of drug metabolizing enzymes and drug transporters detected in human intestinal tissue and cell lines

The table depicts information concerning the presence of DMEs and DTs in human small intestinal tissue (SI: duodenum, jejunum and ileum), colon tissue, Caco-2 and LS180 cells detected at mRNA level, protein level (P), activity (A) or not detectable (ND). Where available, expression levels in Caco-2 cells and LS180 cells were expressed as % of the expression in small intestine (small intestine is 100%). (+ is present; - is tested but not present; ± is tested, but the presence is not consistent between reports)

<table>
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<th>Colon</th>
<th>Caco-2 cells</th>
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The Caco-2 cell line has been proven to be useful for absorption studies [155,156]. Transport studies are usually performed with physiological salt solutions buffered with 10 mM HEPES (pH 7.4) or 10 mM MES (pH 6.0) [28]. To mimic the acidic microclimate of the small intestine, the pH on the apical side is kept at 5.5 [107] - 6.5 [27,29,152], whereas the pH at the basolateral side is kept on 7.4 [27,29,152]. Caco-2 cells are in fact enterocyte-like cells and therefore lack mucus-secreting capacity. To better simulate the actual microclimate present in vivo, co-cultures have been developed in which Caco-2 cells and mucus secreting cells (such as HT29-MTX) are co-incubated [69,157], but whether the results are better predictive for the in vivo situation was not elucidated. Because of the large differences between the expression of DMEs and DTs in vivo and in Caco-2 cells, the use of these cells for qualitative/quantitative prediction of drug metabolism is not justified. However, Caco-2 cells can be used to study induction of DMEs [151,158], although the low level [159] or lack of expression of the nuclear receptor PXR [160] impedes studying PXR-mediated induction. Recently, a new technique has been developed in which Caco-2 cells are transfected with orphan nuclear receptor PXR [161]. Other nuclear pathways seem to be functioning in Caco-2 cells since their sensitivity to 1α,25-dihydroxyvitamin D₃, β-naphthoflavone, 3-methylcholanthrene [151], 2,3,7,8-tetrachlorodibenzo-p-dioxin [162], rifampicin [159] and dexamethasone has been shown [158]. In another study, however, no inducing effect of phenobarbital (CAR-mediated) has been observed [163]. To date, the presence of CAR in Caco-2 cells was only confirmed in Caco-2 TC7 cells after 15 days of post-confluent culturing [164]. One of the difficulties of working with Caco-2 cells is the problematic interlaboratory reproducibility of Caco-2 cells [28], which is expressed by variable amounts of DMEs (phase I, and phase II) and DTs between passage numbers [165] and during cultivation [22,28,166,167]. For example, CYP3A4 expression in low passages (20-30) is four times lower in comparison with high passage numbers (92-105) [165] and MDR1 expression increases 3-4 fold between 4 and 21 days of culturing [22]. Furthermore, Caco-2 cells require a long culture time (20-days) to differentiate in standard culture medium after confluence is reached [69] and this may form a drawback for use for screening purposes [88]. Another bottleneck may be the absence of the mucus layer and differences between regions of the intestinal tract cannot be studied with this cell system [28]. Nevertheless, the Caco-2 cells also offer several advantages. The method is simple and in principle can be used for some aspects of metabolic drug screening. In addition, it is applicable to study mechanisms of transport and compounds of interest can be added on either sides [69]. Caco-2 cells are commonly applied for studying the influence of Pgp on the secretion of drugs [88].

### 3.4.2. LS180 cells

The LS180 cell line is also a human-derived colon adenocarcinoma cell line and is commercially available [168]. The characterization of the expression of DMEs and DTs has not been done as extensively as for Caco-2 cells, but the information available is added to table I. The level of expression of MDR1 and CYP3A4 is comparable in Caco-2 and LS180 cells [160]. In addition, in both cell lines CYP3A4 can be up-regulated by 1α,25-dihydroxyvitamin D₃ [169], but induction of MDR1 has only been shown in LS180 cells [159]. BCRP expression is much lower in LS180 cells in comparison to Caco-2 cells. A comparison
between metabolic capacities of LS180 and Caco-2 cells has not been reported. Contrary to Caco-2 cells, LS180 cells do express PXR [48]. LS180 cells can be used to study induction of DMEs and has been shown to be responsive to amongst others 1α,25-dihydroxyvitamin D₃ [159,169], 3-methylcholanthrene [168,170], 2,3,7,8-tetrachlorodibenzo-p-dioxin [168,170], dexamethasone [45,48], rifampicin [45,48,159] and phenobarbital [45,48]. Thus, LS180 cells are preferred over Caco-2 cells for induction studies.

3.4.3. Other intestinal cell lines
Apart from the Caco-2 cell line, Caco-2 TC7 cells (Caco-2 cells that have been exposed to methotrexate) are used for absorption studies. In comparison with Caco-2 cells, TC7 cells have been reported to express higher levels of CYP3A4 and lower levels of MDR1 [69]. On the other hand, others have reported that TC7 cells express MDR1 at a higher level and CYP3A4 at a lower level than both LS180 and Caco-2 [159]. The Vmax of terfenadine hydroxylation is much lower in Caco-2 TC7 cells than in human jejunum tissue. This indicates that CYP3A4 activity levels are low in Caco-2 TC7 cells and it has been suggested that CYP3A5 is most probably present in TC7 cells instead of CYP3A4 [171]. In TC7 cells, CYP3A4 can be induced in response to 1α,25-dihydroxyvitamin D₃, but not MDR1, which is in line with the findings in Caco-2 cells [159].

Several established cell lines exist that are derived from the entire small intestine of newborn, germ-free rats e.g. IEC-6 (rat duodenum) and IEC-18 (rat ileum) [6,172]. Cell lines of human origin as HUTU 80 (duodenum) [172], HCT 8 (ileum/cecum) [172] and FHS 74 (fetal small intestine) [172] are used as well. Only a few reports have been published concerning drug metabolism in these cell lines. It has been reported that in IEC-18 cells, induction of CYP1A1 is found after β-naphthoflavone exposure [173]. FHS 74 cells have been shown to express CYP3A4 protein [172] but in IEC-6, IEC-18, HUTU 80 and HCT 8, CYP1A1, CYP1A2, CYP2C9/10 and CYP3A proteins could not be detected [172].

3.5. Sources of variability of drug metabolism and enzyme expression in vitro
Variability of metabolic rates of drugs and of expression levels of DMEs and DTs in intestinal preparations (>30-fold for CYP3A content and Vmax [145]) has been repeatedly reported [145,166,174-176]. On the other hand, some reports show a relatively low variation in CYP3A4 (only a factor 2 at protein level, but a factor 5 at mRNA level), CYP2C8 (1.5-fold at protein level) and CYP2D6 (approximately 10-fold at protein level) [2] and in the hydroxylation activities of midazolam (2-fold), diclofenac (2-fold) and bufuralol (10-fold), as measured in precision-cut slices from patients suffering from obesity [50].

When measuring drug metabolic rates in vitro, the outcome is determined by individual parameters (polymorphism, age, gender, ethnic origin, disease, diet) as well as by differences in functionality introduced by the method (duration of warm/cold ischemia, preparation procedure, incubation procedure). To predict drug metabolism, it is very important to take these potential sources of variability into account.

3.5.1. Individual parameters influencing drug metabolism
Polymorphism has been described in all phases of drug metabolism and transport (phase I-III) [177] and varies between ethnic groups [177-179]. Differences in metabolic rates due to polymorphisms have, amongst others, been described for CYP2D6, CYP2C9 and
CYP2C19, influencing the plasma levels of respectively traxoprodil, tolbutamide and omeprazole [180,181].

Furthermore, although only tested in liver tissue, differences in expression levels of CYP3A4, CYP3A5 and CYP3A7 between Japanese and Caucasians have been reported [182]. This may partly be introduced by diet factors. In rodents, differences between rat strains and mice strains have also been described and represent a source of interlaboratory variation [183,184]. For example, hepatic microsomes from Sprague-Dawley rats possess about 200-fold higher diazepam p-hydroxylation activity than Wistar rats [184]. Polymorphisms, ethnic differences and strain differences should be considered as potential sources of variability in intestinal metabolism.

Also, food and drug intake are known to influence metabolic rates in intestinal tissue. For example, healthy volunteers treated with rifampicin (orally) have induced mRNA levels of CYP3A4, CYP2C8 and CYP2C9 [44]. GSTA1 and UGT1A1 mRNA levels are increased in shed enterocytes obtained after oral administration of sulforaphane (an isothiocyanate found in broccoli) and quercetin (a flavonoid glycoside occurring in onion) [59].

Age and gender have been described to influence the rate of several metabolic reactions. As tested in human duodenum biopsies, CYP3A and Pgp mRNA expression differ as a function of age during the first 6 years of life [185]. In rat, both age and gender have been proven to influence drug metabolism [186]. The rat intestinal CYP3A activity, however, does not change significantly with age [187].

The state of health can further change the CYP expression in patients [121], thereby causing individual differences between patients. Patients with Crohn’s disease, exhibit an increased expression of CYP3A4, CYP3A5 and MDR1 compared with the control group [188] and in patients with Barrett’s epithelium GSTP1 levels are decreased [174].

3.5.2. Differences introduced by methodology

Apart from the interindividual differences, the in vitro methodology used can be a source of variability of functionality. For example, the clinical standard for harvesting of human small intestine consists of only a vascular flush with UW solution as part of multi visceral organ procurement [189]. After excision, a phase of cold ischemia is unavoidable and results in the gradual deterioration of absorptive properties, eventually progressing into irreversible damage [189]. Oxygen deprivation of a rat intestine for only 4 minutes followed by reperfusion introduces severe damage [74] and although human small intestine seems intrinsically more resistant to ischemic injury in comparison with smaller animals like rat [6,190], the excision procedure may greatly influence functionality of human small intestine preparations. Luminal flushing improves small bowel morphology after storage [190], but is no standard to date. In addition, it has been shown to be beneficial to constantly oxygenize the excised tissue as has been common practice for Ungell et al. [77], since flushing once does not provide enough oxygen during storage [189]. Furthermore, Celcior (an organ preservation solution with an extra-cellular salt composition and low viscosity used for kidney preservations) exceeds the preservation capabilities of UW (University of Wisconsin preservation solution used for liver transplantation) for rat small intestine [191]. Taken together, more investigation is required to optimize the harvesting conditions of human small intestines in order to decrease the loss of functionality of the tissue.

Another possible cause of variability in functionality is the preservation time. In current reports, few authors mention the duration of preservation pre-treatment and if so, reported
preservation times appear to be highly variable. For example, Zhang et al. reported that the cold ischemic time before cell harvest was less than 24 h [134], whereas Paine et al. reported that the time elapsed between vascular cross clamp (start cold ischemia) and freezing of mucosal scrapings was < 5 h [145]. In the studies of Van de Kerkhof et al., the time between tissue excision and preparation was 1-2 h, while care was taken that the tissue, obtained from surgery, was directly and constantly oxygenized after excision [50]. Nevertheless, no studies have been performed showing the impact of cold storage on the functionality of DMEs and DTs in human intestine. Therefore, it remains unclear whether these 1-24 hours of cold storage influence the final outcome of drug metabolism studies.

The preparation protocol of the various techniques can introduce variation in the measured metabolic rate as well. Several investigators show that only one freeze-thaw cycle diminishes the metabolic activity of everted sacs, but also of microsomes where freezing and thawing is common practice [8,90]. Furthermore, medium additives, such as sodium taurocholate, inhibit the function of MRP3 efflux carriers [28].

Furthermore, it is practically impossible to obtain uniform biopsy tissues from different patients with respect to the location of the samples within the 6 m of the small intestine [175]. Gradients of CYP [11-13], conjugating enzymes [11,174,192] and transporters [16] therefore form another source of variability between different donors.

Finally, the methodology of incubation of cellular and subcellular fractions most probably is a source of data variation, since different co-factor concentrations are present. Furthermore, drug concentration at the site of DMEs may vary between these cellular and subcellular systems, since it is dependent of passive diffusion, influx and efflux transporters in intact cell systems.

### 3.6. Comparison of metabolic rates obtained with different methods

In this section, we compare the metabolic rates obtained with different methods. Since individual differences between donors highly influence the results obtained with human tissue, we have limited these comparisons to rat data.

To adequately compare metabolic rates obtained with different methods, all data were recalculated and expressed in the same units: pmol/min/mg intestinal protein (total intestinal protein of tissue including muscles). Data from studies with precision-cut slices are usually expressed per mg intestinal protein; data from microsomes prepared after scraping (rat intestinal microsomes after scraping procedure (RIM S)) are usually expressed per mg of mucosal microsomal protein, whereas data obtained with microsomes prepared after elution (rat intestinal microsomes after elution procedure (RIM E)) are expressed per mg of enterocyte microsomal protein.

Koster et al. published that 18.1 mg protein per g intestinal wet weight originates from the epithelial cells [10]. Taking into account a total protein content of 121 mg/g wet weight in intestinal tissue [128] of which 18.1 mg consists of epithelial protein, this indicates that 15% of the intestinal tissue consists of epithelial cells (scaling factor 0.15). Of these epithelial cells, 13.3% of the cellular protein is microsomal protein ($10^6$ enterocytes contain 0.45 mg of cellular protein, of which 0.06 mg is microsomal protein: scaling factor 0.13) [10]. For scraped microsome and S9 preparations, the whole intestinal wall is used with the exception of the muscle layers. The muscle layer in rat intestine is approximately 20% of the tissue.
weight (own morphological observation; scaling factor 0.8 x 0.13 = 0.10). For S9 fractions obtained after the scraping procedure, cytosolic (approximately 50% of all cellular proteins) plus microsomal proteins (13.3%) are present of the mucosal layer (80% of total intestinal tissue) (scaling factor 0.5 x 0.13 x 0.8 = 0.5). As an exception, Borm et al. expressed the metabolic rates per pmol/min/mg intestinal tissue weight [131]. Then, the published metabolic rates were corrected for the protein content in the intestine (12.1% [118], scaling factor (100/12.1 = 8.3).

All scaling factors used are listed in table II.

Table II: Summary of the scaling factors used to recalculate the current expression units to pmol/min/mg intestinal protein

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Current unit</th>
<th>Ref</th>
<th>Scaling factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slices/biopsies</td>
<td>Per mg whole tissue protein</td>
<td>[50]</td>
<td>1</td>
</tr>
<tr>
<td>Mucosal cells</td>
<td>Per mg enterocyte protein</td>
<td>[131]</td>
<td>0.15</td>
</tr>
<tr>
<td>Micromes Scraped (RIM-S)</td>
<td>Per mg mucosal microsomal protein</td>
<td>[8]</td>
<td>0.1 (0.13 x 0.8)</td>
</tr>
<tr>
<td>Micromes Eluted (RIM-E)</td>
<td>Per mg enterocyte microsomal protein</td>
<td>[145,148,201]</td>
<td>0.02 (0.13 x 0.15)</td>
</tr>
<tr>
<td>S9 fractions after scraping</td>
<td>Per mg mucosal cytosol plus microsomal protein</td>
<td>[140]</td>
<td>0.5 ((0.5 + 0.13) x 0.8)</td>
</tr>
</tbody>
</table>

In table III, the formation rates of 6β-hydroxytestosterone and metabolism of 7-ethoxycoumarin at V_max (assuming that 100 µM of testosterone and 7-ethoxycoumarin already saturates the enzymes involved) obtained with various methods using rat tissue have been summarized.

Table III: Metabolic rates obtained with various in vitro methods applied to rat tissue (PCS = precision-cut slices; RIM = rat intestinal microsomes; S = after scraping procedure)

<table>
<thead>
<tr>
<th>6β-hydroxytestosterone (CYP3A)</th>
<th>PCS</th>
<th>Biopsies</th>
<th>S9 fractions S</th>
<th>RIM S</th>
<th>Everted sac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration testosterone (µM)</td>
<td>100</td>
<td>250</td>
<td>250</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Ref</td>
<td>[126]</td>
<td>[118]</td>
<td>[140]</td>
<td>[8]</td>
<td>[8]</td>
</tr>
<tr>
<td>Recalculated rate (pmol/min/mg protein)</td>
<td>25</td>
<td>33</td>
<td>0.6</td>
<td>4.2</td>
<td>8.3</td>
</tr>
<tr>
<td>Fold change compared with PCS</td>
<td>1</td>
<td>1.3</td>
<td>0.02</td>
<td>0.2</td>
<td>0.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>7-ethoxycoumarin (CYP1A, 2B)</th>
<th>PCS</th>
<th>Biopsies</th>
<th>Mucosal cells</th>
<th>RIM E</th>
<th>RIM S</th>
<th>Everted sac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration 7EC (µM)</td>
<td>500</td>
<td>500</td>
<td>200</td>
<td>200</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Ref</td>
<td>[9]</td>
<td>[118]</td>
<td>[131]</td>
<td>[131]</td>
<td>[8]</td>
<td>[8]</td>
</tr>
<tr>
<td>Recalculated rate (pmol/min/mg protein)</td>
<td>40</td>
<td>36</td>
<td>1.6</td>
<td>4</td>
<td>1.0</td>
<td>1.6</td>
</tr>
<tr>
<td>Fold change compared with PCS</td>
<td>1</td>
<td>0.9</td>
<td>0.04</td>
<td>0.1</td>
<td>0.03</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Over 100-fold differences between the different preparations in 6β-hydroxytestosterone formation and 7-ethoxycoumarin metabolism have been reported. Within one study directly comparing microsomes and everted sacs, both metabolic reactions (6β-hydroxytestosterone formation and 7-ethoxycoumarin metabolism) have a somewhat higher rate in everted sacs.
This is remarkable, since everted sacs are incubated without the addition of co-factors (that are supposed to be present at physiological concentrations within the cells) and intracellular drug concentrations may be rate limiting due to activity of influx or efflux transporters. The S9 fractions have surprisingly lower activity levels than microsomes for CYP3A conversion. 7-ethoxycoumarin metabolism (CYP1A, 2B) occurs in mucosal cells and eluted microsomes at the same rates, indicating that the transporters and the concentration of co-factors are not the rate limiting factor. Eluted microsomes have 3-times higher activities in comparison with the scraped microsomes, which supports the hypothesis that CYPs are better preserved using the eluting procedure. Precision-cut slices and biopsies show the highest formation rate for both reactions, suggesting that this method preserves the CYPs the best.

3.7. Final remarks and future perspectives

Only a few methods are available to study intestinal drug metabolism in vivo or in situ and are technically and ethically difficult in man. When performed, it remains difficult to discriminate between the contribution of liver and extrahepatic tissues. In contrast, in vitro, several intact cell systems are nowadays available to study drug metabolism in the intestine such as the isolated intestinal perfusion, everted sac, Ussing chamber preparations, biopsies, precision-cut slices, but also primary cells. Furthermore, subcellular fractions, such as S9 fractions and microsomes are used to study drug metabolism as well as cell lines, such as Caco-2, Caco-2 TC7 and LS180 cells. These methods have been described in this overview and their advantages and limitations are discussed. The choice of the method should therefore depend on the question of interest.

The S9 fractions as well as the microsomes do not possess the DMEs and co-factors in physiological conditions and lack the important interaction with influx and efflux transporters. Microsomes lack many conjugating enzymes as well. For phase I and certain phase II metabolism studies Vmax and Km values can be determined and this technique can be efficiently used for inhibition studies. But, these preparations cannot be used for studying drug-induced induction in vitro. In addition, the possible interaction with transporters that determine the exposure of the DMEs to the drug (class 2 and 3 of the BCS) [15,24] is lacking in this system, possibly hampering the extrapolation to in vivo metabolic rate. Finally, the comparison with other techniques point to a lower metabolic rate per mg tissue, resulting in under prediction of in vivo metabolism.

Since the available intestinal cell lines express DMEs and DTs to a different extent than human intestinal tissue, cell lines do not provide good models to study drug metabolism. On the contrary, the cell lines have been proven very useful for absorption studies and the mechanism of interactions between transporters such as Pgp and DMEs. For studying drug-induced induction of DMEs, the LS180 cell line has been proven to be useful as well. Caco-2 cells, on the other hand, lack PXR and depending on culture conditions also CAR and are therefore only applicable for the investigation of certain induction pathways.

All intact cell systems are, in principle, applicable for drug metabolism studies (phase I and II), some up to 2-4 h, and others up to 24 h of incubation. The isolated intestinal perfusion as well as the everted sac and Ussing chamber technique have been used over years to study mainly drug absorption, but it seems worthwhile to reconsider these techniques for drug metabolism studies. The major advantage of these techniques is the possibility to study the direction of excretion of the formed metabolites. Whether luminally excreted metabolites are
re-absorbed later on in the tract, for example, is unknown and remains an important aspect to investigate. The isolated perfusion and everted sac technique, however, remain only applicable to animal tissue, whereas the Ussing chamber technique is applicable to human tissue as well. The Ussing chamber preparations require more tissue in comparison with biopsies and precision-cut slices. These latter two techniques are used for longer incubation times up to at least 24 h of incubation and therefore offer the major advantage that drug-induced regulation of DMEs and DTs can be studied. This applicability, however, has not been fully explored yet and needs further examination, since knowledge on DME and DT regulation in the intestine is far from complete. Up to now, induction studies have only been performed with precision-cut slices and biopsies, where induction was shown on both mRNA and activity level. In comparison with biopsies, slices can be prepared from smaller amounts of tissue with reproducible size and thickness and after slice incubation qualitatively better mRNA is isolated. For the other intact tissue models, their applicability for induction studies has not been tested yet. DME induction in intestinal tissue has been shown already after 2 h of incubation at mRNA level using shed enterocytes. This suggests that all intact cell models are applicable for drug-induced induction studies at least on the mRNA level. Whether they can be used to study induction of enzyme activity remains to be shown.

The variation between intestinal *in vitro* methods is problematic for *in vitro* - *in vivo* prediction and they can at least partly be ascribed to variations in the preservation of human tissue. Since the preservation conditions of human small intestines have not been optimized yet, more investigation is required to evaluate and reduce the loss of functionality of stored tissue. The challenge of studying drug metabolizing rates to date is to get a grip on the variation present in the currently used methods prior to actually trying to predict the contribution of the intestine to the first-pass effect.

A completely different, more mechanism based method for the prediction of drug metabolism is computer-based modeling of the enzymatic rate based on Vmax, Km and abundance of the DMEs and DTs involved. However, this is outside the scope of this review and readers are referred to Rostami-Hodjegan and Tucker [193].

To conclude: whereas subcellular fractions and cell lines are efficient methods to study drug metabolism at the individual enzyme level, the isolated intestinal perfusion, everted sac and Ussing chamber appear particularly useful for studying overall drug metabolism and interactions with transporters. Biopsies, precision-cut slices and primary cells seem most appropriate to study induction and metabolism of slowly metabolized drugs.

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In vitro methods to study intestinal drug metabolism


Chapter 2


In vitro methods to study intestinal drug metabolism

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