Innovative methods to study human intestinal drug metabolism in vitro:

Precision-cut slices compared with Ussing chamber preparations

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List of non-standard abbreviations:
Abstract

Predictive *in vitro* methods to investigate drug metabolism in the human intestine using intact tissue are of high importance. Therefore, we studied the metabolic activity of human small intestinal and colon slices and compared it with the metabolic activity of the same human intestinal segments using the Ussing chamber technique. The metabolic activity was evaluated using substrates to both phase I and phase II reactions: testosterone, 7-hydroxycoumarin (7HC) and a cocktail of CYP substrates (midazolam, diclofenac, coumarin and bufuralol). In slices of human proximal jejunum, the metabolic activity of several CYP mediated and conjugation reactions remained constant up to 4 h of incubation. In the colon slices, conjugation rates were virtually equal to those in small intestine; while CYP mediated conversions occurred much slower. In both organs, morphological evaluation and ATP content implied tissue integrity within this period. CYP conversions using the Ussing chamber technique showed that the metabolic rate (sum of metabolites measured in apical, basolateral and tissue compartments) was constant up to 3 h. For 7HC conjugations, the metabolic rate remained constant up to 4 h. The distribution of the metabolites in the compartments differed between the substrates. Overall, metabolic rates were surprisingly similar in both techniques and appear similar to or even higher than in liver. In conclusion, this study shows that both human intestinal precision-cut slices as well as Ussing chamber preparations provide useful tools for *in vitro* biotransformation studies.
Although the liver has long been thought to play the major role in drug metabolism, the metabolic capacity of the intestine has been increasingly recognized (von Richter et al., 2001) (Glaeser et al., 2004).

In the seventies of the 20th century, intestinal metabolism was already reported (Stohs et al., 1976). Nonetheless, until recently the clinical significance of intestinal drug metabolism remained under debate (Lin et al., 1999) (Doherty and Charman, 2002). Although basic knowledge concerning human intestinal drug enzyme and transporters expression has been collected over the last decade (Obach et al., 2001) (Thorn et al., 2005), the ultimate proof for their significance has been given by in vivo studies. For several compounds, such as cyclosporin, verapamil and midazolam, in vivo studies have shown significant first-pass metabolism by the intestinal wall (Kolars et al., 1991) (Glaeser et al., 2004) (von Richter et al., 2001). 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 (Pelkonen et al., 2001), in addition to the interplay between transporters and metabolic activity (Suzuki and Sugiyama, 2000) (Benet et al., 2004) (Jeong et al., 2005).

The challenge in studying human intestinal metabolism nowadays is to develop in vitro systems in which intact cells remain functioning, since quantitative in vivo studies are difficult to perform. Subcellular human intestinal fractions, such as microsomal preparations and S-9 fractions, have been used up to 30 minutes of incubation (Wynalda et al., 2003). However, in addition to their limited lifespan, they lack the operable cell-membrane transporters and are often incubated with unphysiological concentrations of co-factors. Furthermore, Caco-2 cells have been proven to be useful for absorption studies (Artursson and Karlsson, 1991; Ungell and Karlsson, 2003). However, interlaboratory reproducibility of Caco-2 cells is problematic and they are lacking normal levels of important metabolic enzymes such as CYP3A4 (Sun et al., 2002).
Until now, several intact cell systems exist to study intestinal metabolism. Recently, the precision-cut slice technique was applied to rat intestinal tissue and these studies showed that intestinal precision-cut slices are superior to so-called intestinal tissue biopsy punches (de Kanter et al., 2005). In addition, they exhibit drug metabolism at a constant rate for at least 3 h of incubation (van de Kerkhof et al., 2005). With the very efficient and rapid slice technique, many slices can be produced from a small tissue sample maintaining the cells in their physiological context with respect to metabolic enzymes, membrane transporters and co-factors. The applicability of this system for human tissue, however, has not been validated to date. The Ussing chamber technique, in which a small piece of mucosal tissue is mounted such that mucosal and serosal side are separately exposed to incubation medium, has been proven to be useful in absorption studies (Ungell et al., 1998) and successfully predicts human intestinal permeability \textit{in vivo} for several compounds (Lennernas et al., 1997). However, to our knowledge, studies using Ussing chamber preparations were mainly focused on the prediction of the fraction absorbed, whereas metabolic capacity and disposition of the formed metabolites has not been reported. Nonetheless, indications exist that when only intestinal transport is measured, the metabolic extraction of the tissue might lead to false predictions of fraction absorbed (Ungell, 2005).

In this study, we adapted the precision-cut slice method to human tissue previously developed for rat intestinal tissue to study drug metabolism. The viability of slices was evaluated by measuring intracellular ATP levels, morphology and metabolic stability during 4 h of incubation. Slices of proximal jejunum and colon tissue, were incubated with high concentrations of testosterone (TT) or 7-hydroxycoumarin (7HC) to assess phase I and respectively phase II capacity at $V_{\text{max}}$. Additionally, slices were incubated with a cocktail of CYP substrates (midazolam, coumarin, diclofenac...
and bufuralol) at concentrations below Km. These compounds are probes for CYP3A4/5, CYP2A6, CYP2C9 and CYP2D6. Secondly, from the same proximal jejunum tissue samples we prepared mucosal sheets for the Ussing chamber set-up. These sheets were incubated up to 4 h with the cocktail or with 7HC, while the viability was monitored by electrical parameters (Polentarutti et al., 1999). This enabled a direct comparison between the metabolic activity of human intestinal tissue either mounted in Ussing chambers or in the form of precision-cut slices.
Materials and Methods

Chemicals

Dimethyl sulfoxide (DMSO), tri fluoro acetic acid (TFA), low gelling temperature agarose (type VII-A), amphotericin B solution (250 µg/ml), D-glucose, 7-hydroxycoumarin (7HC), 7-hydroxycoumarin-glucuronide (7HC-GLUC), HEPES (N-(2-hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid)), sodium bicarbonate, β-glucuronidase, sulfatase, testosterone, 11β-hydroxytestosterone (11β-TOH), 6β-TOH and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO). Williams Medium E with Glutamax-I (WME) and gentamicin (50 mg/ml) solution were obtained from Invitrogen (Paisley, UK). Formaldehyde solution (3.8%) was purchased from Mallinckrodt Baker B.V. (Deventer, The Netherlands). Hydroxy-bufuralol maleate salt, bufuralol hydrochloride salt, 1-hydroxy midazolam and 7-hydroxycoumarin-sulphate (7HC-SULF) were purchased from Ultrafine (Manchester, UK). 4-Hydroxy-diclofenac was obtained from Gentest (Woburn, USA). Midazolam was purchased from Lipomed AG (Arlesheim, Switzerland). Sodium azide was obtained from Kebo lab (Spånga, Sweden). Sodium chloride, EDTA (ethylene diamine tetra-acetic acid), formic acid, tris-HCl, dichloromethane and sodium acetate were obtained from Merck (Darmstadt, Germany). Acetonitril (ACN) and methanol (MeOH) were purchased from Rathburn (Walkerburn, Scotland). Ethanol was obtained from Kemetyl (Haninge, Sweden). 2α-, 16α-, 11α-TOH were obtained from Steraloids (Newport, RI).

Human tissue

Human tissue originated from surgical resections with approval from the regional ethical committee in Gothenburg (Sweden) and from each of the individual patients; proximal jejunum was obtained from obesitas patients from Sahlgren’s University.
hospital (Sweden) and the colon resections from patients suffering colon carcinomas from East Hospital (Gothenburg, Sweden). Donor characteristics are listed in Table I.

After surgical removal, tissue was directly stored in ice-cold constantly oxygenated (with carbogen, 95% O₂ and 5% CO₂) Krebs-Bicarbonate Ringer (KBR) solution (pH 7.4) with the composition reported earlier (Polentarutti et al., 1999).

**Preparation and incubation of human tissue**

Directly after tissue arrival in the laboratory, the tissue was put in fresh oxygenated ice-cold KBR solution and the muscle layers and serosa were carefully removed. In all cases, preparation of mucosal tissue was finished within 1-2 h calculated from excision. The end of mucosal preparation is referred to as t=0 in experiments.

*Preparation of precision-cut slices*

Mucosal tissue was transferred to ice-cold oxygenated Krebs-Henseleit buffer (containing 10 mM HEPES and 25 mM D-glucose, pH 7.4). Tissue was cut in pieces of 7-9 mm width and variable length and were subsequently vertically embedded in 3% low-gelling temperature agarose in 0.9% NaCl solution (37°C) to ensure that every slice consisted of all cell layers present in the mucosa. Then, the agarose solution was allowed to gel, using a pre-cooled (0°C) tissue embedding unit (Alabama Research and Development, Munford, AL). After the agarose solution had gelled, precision-cut slices (thickness about 400 µM) were cut using a Krumdieck tissue slicer as described previously (de Kanter et al., 2005).

*Incubation of precision-cut slices*

Slices were incubated individually in 12-wells culture plates (Costar no 3512, Corning Incorporated, NY, USA) in 1.3 ml of Williams Medium E with Glutamax-I (WME), supplemented with D-glucose (final concentration 25 mM), gentamicin (final
concentration 50 µg/ml), amphotericin B (final concentration 2.5 µg/ml). The culture plates were placed in a pre-warmed cabinet (37°C) in plastic boxes. Slices were incubated under humidified carbogen (95% O₂ / 5% CO₂) and shaken using an orbital shaker at approximately 45 rpm.

**Preparation and incubation for Ussing chambers**

Mucosal tissue was mounted onto the Ussing chamber segment holder as flat sheets with a surface area of 1 cm². Each of the two chamber compartments on both sides of the segment was subsequently filled with 10 ml oxygenated KBR (room temperature) continuously gassed with carbogen (95% O₂ / 5% CO₂), stirred with approximately 300 rpm and slowly warmed until 37°C by water jackets. The tissue was mounted and placed in the Ussing chamber set-up after which the tissue was allowed to stabilize for 30 minutes to recover from the preparation and to equilibrate in the chambers.

**Viability testing of precision-cut slices**

**Morphology**

The condition of precision-cut slices was evaluated after 4 h of incubation by morphology. After incubation in triplicate per experiment, slices were stored in 3.8% formaldehyde solution at 4°C. In addition, directly after tissue arrival in the laboratory, a piece of tissue was put in ice-cold 3.8% formaldehyde solution as a control. Samples were sent to HistoCenter AB (Box 138, 421 22 V. Frölunda, Sweden) for further processing and haematoxylin and eosin staining. Colon slice incubations for morphology were performed in two experiments with tissue from two subjects in triplicate; small intestinal slice incubations were performed in 5 experiments with tissue from five subjects in triplicate.
**Intracellular ATP levels**

Intracellular ATP levels were evaluated to judge the overall metabolic condition of the tissue after 4 h of incubation. Directly after tissue preparation, 3 pieces of tissue were snap-frozen as controls. Intracellular ATP levels were determined according to the method described by de Kanter et al. (De Kanter et al., 2002). Directly after incubation, slices were put in 1 ml ice-cold 70% ethanol containing 2 mM EDTA (pH 10.9) to precipitate the proteins, and directly snap-frozen in N2(l) and stored at –80 °C until further use. Subsequently, samples were thawed on ice and homogenized by sonication. Samples were centrifuged (3 min, 4°C, 10,000 g) to spin down the proteins, after which the supernatant was transferred to a new cup and the pellet was used for protein determination. The supernatant was diluted with 100 mM Tris-HCl, 2 mM EDTA solution (pH 7.8) and analysed using the ATP Bioluminescence Assay Kit CLS II (Roche Applied Sciences, Mannheim, Germany) measuring luminescence using a plate reader (Spectramax microplate reader, Molecular devices, Sunnyvale, California). ATP was determined in four experiments using tissue from four different donors in triplicate.

**Stability of drug metabolism rate**

The stability of metabolic rate was determined between 0 h and 4 h of incubation. To evaluate phase I metabolism, slices were incubated with TT (final concentration 250 µM, 1% MeOH) or a cocktail of 4 compounds: midazolam (3 µM), diclofenac (5 µM), bufuralol (10 µM) and coumarin (3 µM) with 0.35% MeOH as a final solvent concentration. As control, medium was incubated with substrate but without slice. After 1, 2, 3 and 4 h of incubation with testosterone, slice and medium were harvested together and stored at –20°C until further use. In earlier rat studies, we found that significant amounts of testosterone metabolites were retained within the slice (de Kanter et al., 2005). Therefore slice and medium were analysed together.
For cocktail incubations, no metabolic interactions were detected between substrates and these concentrations (T. Andersson, AstraZeneca, Mölndal (personal communication)). Further, pilot experiments showed that only minor amounts of the cocktail metabolites were retained in the slice that were barely detectable. Therefore, only the medium samples were analysed. During 0-4 h of incubation, medium samples (100 µl) were taken every hour. The last sample contained slice tissue and remaining medium. Samples were stored at -20°C until further use.

To evaluate phase II metabolism, slices were incubated with 7HC (500 µM, 1% MeOH) and as a control, medium was incubated with substrate. Medium samples (200 µl) were taken every hour between 0 h and 4 h and stored at -20°C. After incubation, slices were stored at -20°C for protein determination.

Metabolism experiments with proximal jejunum were performed on tissues from 5-8 donors in triplicate. Metabolism experiments in colon were performed on tissue from 5 donors in triplicate.

**Viability testing Ussing chambers**

*Electrical parameters*

Viability was monitored by measuring electrical parameters as described before (Polentarutti et al., 1999): the Potential Difference (PD) is reflecting the voltage gradient generated by the tissue, the short-circuit current (SCC) is reflecting the ionic fluxes across the epithelium and the resistance (R) is reflecting tissue integrity.

In brief, a four electrodes system recorded electrical parameters during the experiment. The Potential Difference (PD) was recorded using calomel electrodes connected by agar/NaCl-bridges. Every 5 minutes, 5 current (I) pulses (0, ±15, ±30 µA) were sent via AgCl electrodes through the chamber after which the voltage response (U) was measured. The current (I) - voltage (U) pairs form a linear plot from which the PD can be deduced. PD was obtained from the intersection with the
voltage axes, when I=0, the resistance (R) of the tissue segment was the slope of this plot. R was corrected for external, non-tissue resistance by the recorder system. The short-circuit current (SCC) was calculated from Ohm’s law: I = U/R.

**Stability of drug metabolism rate**

Tissue from four individual donors was used to prepare both precision-cut slices and Ussing chamber preparations to enable a direct comparison between the metabolic activity in the Ussing chamber preparations and precision-cut slices. In table I it is indicated which tissue was used for both experiments.

For a set of 8 chambers per subject and per day, two experimental set-ups were chosen: I) Four chambers were incubated with the cocktail of the four CYP substrates: midazolam (3 µM), diclofenac (5 µM), bufuralol (10 µM) and coumarin (3 µM). II) Four chambers were incubated with 7HC (500 µM, 1% MeOH) to assess phase II metabolism. The metabolic reactions were monitored during 4 hours of incubation. At 1, 2, 3 and 4 h of incubation with the model substrates from one of the chambers, tissue and medium (both apical (A) and basolateral (B) individually) were harvested. This terminated the incubation of one chamber at that specific time point. Samples were stored at 4°C until the end of the experiment and subsequently stored at –20°C until further use.

**Metabolite analyses**

**Testosterone sample analysis**

After thawing (at 4°C), samples were homogenized using a sonicator. Then, 10 ml 11β-TOH (internal standard, 0.2 mg/ml) and 6 ml dichloromethane were added. The total mixture was vortexed and centrifuged using a Beckman-Coulter CS-6KR centrifuge (Beckman Coulter, Fullerton, CA) (10 min, 4°C, at 800 g). The water layer was removed and the dichloromethane evaporated overnight. The residue was
dissolved in 130 µl of 50% MeOH and then analysed by HPLC as described previously (van ’t Klooster et al., 1993). The concentration range of the standard curve was 2-10 µM. The LLOQ (Lower Limit of Quantitation) was 0.1 µM. The intraday and interday variability (2 µM) were resp. 6.1 and 8.8 %CV (coefficient of variation).

Cocktail sample analysis
Slice samples containing medium and tissue were homogenized by sonication. Ussing chamber tissue samples were homogenised in 2 ml fresh WME using a potter Elvehjen. Subsequently, two volumes of ACN were added to precipitate the proteins. Then, samples were centrifuged (10 min, 20 °C, 4000 g). The supernatant, used for metabolite analysis, was diluted with 2 volumes of water (final concentration of ACN: 30%). The precipitate was stored at −20°C for protein determination. Medium samples were directly pipetted into a 96-wells plate together with standards (1’ OH-midazolam, 4’ OH-diclofenac, OH-bufuralol and 7’ OH-coumarin) dissolved in both water and 30% ACN. The plate was centrifuged for 20 min (4000 g, 4°C) before injection into a LC-MS occurred. For OH-bufuralol, the concentration range of the standard curve used was 0.05-0.25 µM and the interday variability (0.25 µM) was 5.6%CV. For 4’OH-diclofenac and 1’OH-midazolam the concentration range of the standard curves was 0.25-1 µM and the interday variability (1 µM) was resp 3.4 and 1.3%CV. The LLOQ was lower than 5 nM for OH-bufuralol, 1’OH-midazolam and 4’OH-diclofenac.

LC separations were performed on a reversed-phase HyPURITY C_{18} analytical column (50 mm x 2.1 mm i.d., 5 µm, ThermoQuest, Runcorn, UK) with a HyPURITY C_{18} guard column (2.1 x 10 mm, 5 µm) at 40°C. The mobile phase consisted of a mixture of (A) 0.1% formic acid in water and (B) 0.1% formic acid in ACN. A gradient elution containing solvents A and B was performed: solvent A was linearly decreased
from 95% to 20% during 3 min, where it remained isocratic for 0.2 min. Then A was increased to 95% during 0.1 min and re-equilibrated at 95% A for 2.5 min. A flow-rate of 0.75 ml/min was used and an aliquot of 15 µl was injected for analysis. The mass spectrometric analyses were performed with a triple quadrupole mass spectrometer, API4000, equipped with electrospray interface (Applied Biosystems/MDS Sciex, Concord, Canada). The instrument settings were optimized using the individual analytes. The MRM transitions of the precursor ions to the corresponding product ions were monitored for the analytes. Instrument control, data acquisition and data evaluation were performed using Applied Biosystems/MDS Sciex Analyst 1.4 software.

**Deconjugation of cocktail samples**

To test whether further conjugation of the hydroxy-metabolites of the cocktail compounds had occurred, Ussing chamber medium samples after 2 h of incubation were deconjugated. For this, 40 µl medium was added to 65 µl 1 N sodium acetate buffer (pH 4.5) containing 5480 U/ml β-glucuronidase and 59 U/ml sulfatase and incubated for 2 h at 37°C. The reaction was terminated by precipitation of the proteins adding 2 volumes of ACN. After vortexing, the samples were centrifuged and diluted twice with water, before analysis. Analysis occurred as described in the section cocktail sample analysis.

**7HC sample analysis**

After thawing, sodium azide (final concentration 0.1 mg/ml) was added to the samples to prevent bacterial growth. Subsequently, the samples were centrifuged (3 min, 4 °C, 10,000 g) and metabolites were determined by HPLC. 7HC, 7HC-GLUC and 7HC-SULF were used as references. Chromatography was performed on a Zorbax Column SB-C18 (4.6 mm x 15 cm, 5µm, Hewlett Packard (ChromTech AB, Hägersten, Sweden). The mobile phase consisted of A) 0.1% TFA and B) 0.1% TFA
in 100% ACN. The separation run lasted 16.0 minutes, including column equilibration. The gradient was as follows: 0% B for 1 min, followed by linear increase to 20% B over 4 min, followed by a step gradient to 85% B for 6 min, remained at 85% B for 3.5 min and returned to 0% B for 1.5 min. Per sample, 50 µl was injected and the flow rate was 0.8 ml/min. The concentration range of the standard curves of 7HC-GLUC and 7HC-SULF was 0.25-1 µM and the interday variability (1 µM) was resp 0.5 and 3.4%CV. The LLOQ was lower than 0.25 µM.

**Protein determination**

Since slices had somewhat different sizes, the protein content was determined on all slice samples after ATP analysis, 7HC incubations or cocktail incubations to enable expression on protein basis. Protein values were determined on all Ussing chamber tissue samples as well. After incubation samples were stored at –20°C until further use. Subsequently, samples were thawed and 5 N NaOH was added: 20 µl 5 N NaOH for slice samples used for ATP determination and 7HC metabolism in eppendorf cups and 200 µl 5 N NaOH for cocktail slice samples and Ussing chamber tissue samples in centrifuge tubes. Fourty min of incubation at 37°C followed to dissolve the tissue. Then, water was added to obtain a final concentration of 0.1 N NaOH needed for the analysis. Tissue was homogenized using sonication and further diluted to determine the protein content using Bio-Rad protein assay dye reagent (Bio-Rad, Munich, Germany) with bovine serum albumin as standard. For testosterone experiments, the protein content could not be determined, since the proteins were removed together with the water layer during extraction. Therefore, the average of the protein values of 7HC incubations was used. Per experiment, 79-97% of the slices had a protein content ranging from 50% to 150% of the average protein content.
Statistics

Statistical significance of the difference between Ussing chamber preparations and precision-cut slices was determined using a paired Student’s t test. The linearity of metabolic rates in time was determined using linear regression analyses.
Results

Viability assessment of small intestinal and colon slices

Three different parameters were assessed to evaluate the viability of small intestinal and colon slices after 4 h of incubation: morphology, ATP levels and metabolic stability. Small pieces of non-incubated tissue served as controls for morphology and ATP levels.

Morphology

Although the villi showed somewhat flattening, the overall tissue morphology and the morphological appearance of the colonocytes/enterocytes of colon and proximal jejunal slices remained intact during 4 h of incubation in comparison with non-incubated control tissue. No cell swelling or necrosis was observed. The morphological evaluation indicated that the number of intact epithelial cells was not reduced during this incubation period (fig 1).

Intracellular ATP

General viability of small intestinal slices was assessed in four experiments by measurement of intracellular ATP levels. The level of ATP in tissue pieces harvested directly after preparation of the mucosal sheets was 1.2 nmol/mg protein and increased significantly during the first 4 h of incubation to 3.3 nmol/mg protein (p < 0.02).

Viability in Ussing chamber preparations

To evaluate the tissue integrity of Ussing chamber preparations, electrical parameters were monitored.
Electrical parameters

In figure 2, the electrical parameters of Ussing chamber preparations are shown during 4 h of incubation with substrates. The potential difference (PD), reflecting the voltage gradient generated by the tissue, was 7.9 mV and decreased significantly (p < 0.003) to 4.5 mV after 4 h of incubation. The short-circuit current values (reflecting the ionic fluxes across the epithelium) were 2 µA/mm² at the beginning of the experiment and after a small increase to 2.3 µA/mm² decreased non-significantly to 1.2 µA/mm² after 4 h of incubation. The resistance (R), reflecting tissue integrity, increased non-significantly from 42 to 48 Ohm x cm² after 4 h of incubation.

Stability of metabolic rate in precision-cut slices

Testosterone metabolism

The rates of testosterone metabolite formation in human proximal jejunum are shown in figure 3. Four different metabolites could be quantified: androstenedione, 16α-, 6β- and 2β-hydroxytestosterone. Androstenedione and 16α-TOH were formed at a constant rate up to 4 h of incubation (R² > 0.93). For 6β- and 2β-TOH the highest formation rate was detected between 0 and 1 h of incubation. In order from highest to lowest formation rate: androstenedione (316 pmol/min/mg protein), followed by 6β-TOH (107 pmol/min/mg protein in first h), 2β-TOH (25 pmol/min/mg protein in first h) and 16α-TOH (9 pmol/min/mg protein).

Hydroxylation of midazolam, diclofenac, bufuralol and coumarin

Slices were incubated with a cocktail of drugs (midazolam, diclofenac, bufuralol and coumarin) to further evaluate the stability of CYP mediated reactions. The metabolite formation in time is depicted in figure 4A-C. No 7’OH-coumarin metabolite formation could be detected in the slices. 1’OH-midazolam (R² > 0.92), 4’OH-diclofenac (R² > 0.97) and OH-bufuralol (R² > 0.93) formation were time-dependent up to 4 h of
incubation. The fastest metabolic hydroxylation rate was detected using midazolam as a substrate (11.6 pmol/min/mg protein), followed by diclofenac (7.7 pmol/min/mg protein) and bufuralol (1.3 pmol/min/mg protein). In figure 4, the metabolite values in the slices are depicted as mean values of 4 experiments using tissue from 4 different donors. These were performed simultaneously with Ussing chamber experiments. Two additional slice experiments were performed with the cocktail. The obtained results were in line with the results depicted in figure 4 (data not shown). The range of metabolite formation rates in slices in the 6 experiments were 8.3 - 17 pmol/min/mg protein (1′OH-midazolam), 4.4 – 9.4 pmol/min/mg protein (4′OH-diclofenac) and 0.2 – 2.2 pmol/min/mg protein (OH-bufuralol).

In colon slices, the hydroxy-metabolite formation for all substrates was below the detection limit.

7HC conjugation

Figure 5 shows that for both glucuronidation and sulphation the metabolic rates were constant during the first 4 h of incubation in both small intestine (open symbols, R² > 0.98) and colon (closed symbols, R² > 0.98).

In small intestinal as well as in colon slices, the glucuronidation rate is higher than the sulphation rate. Comparing colon and small intestinal slices, comparable rates were found for both glucuronidation (small intestine: 315 pmol/min/mg protein, colon: 377 pmol/min/mg protein) and sulphation (small intestine: 52 pmol/min/mg protein and colon: 62 pmol/min/mg protein).

Stability of metabolic rate in Ussing chamber preparations

Hydroxylation of midazolam, diclofenac, bufuralol and coumarin

In the Ussing chamber incubations, metabolites which were detected in the apical chamber (A), basolateral chamber (B) and in the tissue (T), are indicated in figure 6. The sums of the metabolites in these three compartments corresponds to the total
metabolite formation in the Ussing chamber incubations and are depicted in figure 4A-E.

Like in the slices, 7′OH coumarin could not be detected in Ussing chamber experiments after 4 h of incubation neither in compartment A nor B. However, low amounts of the metabolite could be detected in tissue, indicating a low activity of the CYP2A6 enzyme.

1′OH-midazolam ($R^2 > 0.95$) formation and 4′OH-diclofenac ($R^2 > 0.99$) formation were linear up to 3 h and OH-bufuralol formation up to 4 h of incubation ($R^2 > 0.98$) (fig 4A-C). As for slices, the highest metabolite formation was 1′OH-midazolam (9.3 pmol/min/mg protein), followed by 4′OH-diclofenac (6.9 pmol/min/mg protein) and OH-bufuralol (1.3 pmol/min/mg protein). All detectable hydroxy-metabolites were excreted in a higher extent into the apical compartment compared to the basolateral compartment (significant for 1′OH-midazolam ($p > 0.005$) and 4′OH-diclofenac ($p > 0.007$), but not significant for OH-bufuralol ($p > 0.11$) (fig 6A-C).

The total amount of metabolites that accumulated in the tissue remained constant during incubation: after 1 h of incubation 22-34% of the formed metabolites were retained within the tissue and after 4 h of incubation 10-13%.

De-conjugation of cocktail metabolites

To evaluate possible conjugation of the hydroxy-metabolites formed, media of Ussing chamber preparations (after 2 h of incubation) from both A and B compartments were de-conjugated and re-analysed (table 2). In the A as well as the B compartment no relevant conjugation could be detected for 1′OH-midazolam, OH-bufuralol or 7′OH-coumarin (remained below the detection limit). However, the amount of 4′OH-diclofenac increased to 133% in A and to 249% in B after deconjugation, resulting in an increase in total metabolism of 145%. 
7HC conjugation

For 7HC, tissue glucuronidation and sulphation rates were constant during 4 h of incubation in the Ussing chambers ($R^2 > 0.95$) (fig 4D and E). As for slices, glucuronidation (415 pmol/min/mg protein) occurred faster than sulphation (66 pmol/min/mg protein). The excretion of both conjugates to the A and B compartments differed for the two conjugates. For 7HC-GLUC, a slightly higher excretion to B than to A was observed (non-significant: $p > 0.08$) (fig 6D). For 7HC-SULF, more metabolite (although non-significant: $p > 0.08$) was excreted towards A compared to B (fig 6E). After 1 h of incubation, 18% of 7HC-GLUC and 0% 7HC-SULF is retained within the tissue. However, after 4 h of incubation the total amount retained in the tissue was low in relation to the amount excreted (0% for 7HC-SULF, 2.5% for 7HC-GLUC).

Comparison of metabolic rates in precision-cut slices and Ussing chamber preparations

Proximal jejunum tissue used in the Ussing chamber incubations as well as precision-cut slices were prepared at the same time from tissues of four donors. For 1’OH-midazolam formation, slices had a slightly, but significantly higher metabolic rate ($p < 0.03$ tested with a paired 2-tailed student’s t-test) compared with Ussing chamber incubations (fig 4A). For 4’ OH-diclofenac and OH-bufuralol formation (fig 4B and C), the metabolic rates were similar for precision-cut slices and Ussing chamber preparations.

For 7HC conjugation (fig 4D and 4E), the metabolic rates in Ussing chamber preparations were slightly, but significantly higher ($p < 0.02$ tested with a paired 2-tailed student’s t-test) than in precision-cut slices.
Discussion

Recently, rat intestinal precision-cut slices were presented as a tool to study intestinal metabolism (Martignoni et al., 2004; de Kanter et al., 2005; van de Kerkhof et al., 2005). In addition, the rat and human intestinal Ussing chamber technique has been optimized as a tool to predict the absorption of the original compound (Ungell, 2005) and not for metabolism. Since predictive in vitro methods are of high importance to investigate human intestinal drug metabolism, we characterized the viability and metabolic activity of human intestinal slices and compared it with the metabolic activity in Ussing chamber preparations.

In slices, the intracellular ATP levels and morphology indicated that they were viable up to 4 h of incubation. The increase of intracellular ATP levels during 4 h of incubation can be explained by the temperature change from 4°C (storage) to 37°C during incubation, allowing ATP levels to be re-established. This result is in line with earlier observations obtained with human liver, lung and kidney slices (De Kanter et al., 2002). In Ussing chamber preparations, the electrical parameters indicate that the viability and integrity were maintained for at least 4 h, since the tight-junctions (reflected by the resistance) remained intact, which is a high-energy process. However, a small decrease of ion transport activity (PD and SCC) occurred, which is probably due to some loss of cells and surface area, as has been described by Polentarutti et al (Polentarutti et al., 1999).

The metabolic stability was our major focus during these studies. Therefore, proximal jejunal slices were incubated with TT to test phase I activity. Formation rates of 6β-TOH and 2β-TOH, both mediated by CYP3A4 (Yamazaki and Shimada, 1997), tended to decline after 1 h. In contrast, androstenedione formation (mediated by 17β-HSD (Farthing et al., 1981) and CYP2C19 (Yamazaki and Shimada, 1997)) and 16α-
TOH formation were constant up to 4 h. This may indicate different half-lives of the individual (iso)enzymes (Renwick et al., 2000) or product inhibition.

The decision to use a cocktail of model substrates was made to increase the amount of information that could be obtained per donor tissue: Midazolam 1’hydroxylation (CYP3A4/3A5), diclofenac 4’hydroxylation (CYP2C9), coumarin 7’hydroxylation (CYP2A6) and bufuralol hydroxylation (CYP2D6) were chosen based on substrate specificity (Bjornsson et al., 2003). Slice formation rates of 4’OH-diclofenac, 1’OH-midazolam, OH-bufuralol, 7HC-GLUC and 7HC-SULF were constant up to 4 h of incubation. The difference in the stability of 1’OH-midazolam (non-saturated conditions) and 2β-TOH and 6β-TOH formation (saturated conditions) could be explained by the exhaustion of co-factors and/or product inhibition of 2β-TOH and 6β-TOH.

Only for 7HC-GLUC formation a lag-phase was observed. Pre-incubation of slices for 1 h did not influence this lag-phase (pilot experiment, data not shown). No lag-phase was observed in Ussing chamber experiments with 7HC, since both medium and tissue were harvested. In these tissues a constant amount of metabolite was detected up to 4 h of incubation, indicating that accumulation of the metabolites in slices may explain the lag-phase. Therefore, metabolic rates used for comparison are expressed as metabolite formation measured between 1 and 4 h of incubation.

Hydroxylation of cocktail compounds in colon was undetectable and thus much slower compared with proximal jejunum. For 7HC-sulphation and 7HC-glucuronidation, the metabolic rates expressed per mg protein were similar in colon and proximal jejunum as was also found in rat (van de Kerkhof et al., 2005). In previous rat studies, intestinal slice incubations with 7HC showed higher glucuronidation rates (750 (colon) and 977 pmol/min/mg protein (proximal jejunum)) compared to human slices (377 and respectively 315 pmol/min/mg protein). The glucuronidation/sulphation ratio was higher in rat (colon: 16 and proximal jejunum:
23) than in human (colon and proximal jejunum: 6) (van de Kerkhof et al., 2005), suggesting a clear species difference. This is in contrast with liver in which the glucuronidation/sulphation ratio is higher in human (19) than in rat (15) (De Kanter et al., 2002). However, in future studies the test group should be expanded and also intestinal tissue from healthy subjects should be taken into account, to proof the existence of this species difference. To our knowledge, it is unknown whether obesity influences drug metabolism in the small intestine.

When Ussing chamber preparations were incubated with the cocktail of CYP substrates or 7HC, the metabolite formation was linear up to 3-4 h of incubation. The distribution of metabolites in the A and B compartments, however, differed clearly per substrate. This may indicate a clear role of transporters. Possibly, metabolites appear at the apical site due to metabolism in slough-off cells. To exclude this, we sampled medium after incubation and incubated these for another 3 h. No further metabolism was detected in these incubations confirming that the formation of the metabolites occurred in cells within the tissue and was then excreted into the apical compartment.

The role of transporters in uptake and excretion of compounds in the intestine has been extensively discussed (Benet et al., 2004) (Ito et al., 2005). The co-localization of CYP3A4 and Pgp in the villi tips of human intestine has also been reported (Watkins, 1997) indicating a collaborative role between enzymes and efflux transporters in the gut. In Ussing chamber studies using pig intestine, extensive luminal efflux of the oxidative product hydroxy-sirolimus was reported (Lampen et al., 1998). For glucuronide and sulfate conjugates formed in rat enterocytes, a significant extrusion into the intestinal lumen has been reported as well (Adachi et al., 2005). Apical excretion was shown to be facilitated by MRP2 in Caco-2 cells (Suzuki and Sugiyama, 2000), which is in line with localisation of MRP2 at the apical membranes of enterocytes (Borst and Elferink, 2002). The present study showed for the first time a clear luminal efflux of hydroxy-metabolites in human intestine. Elucidation of the
identity of the transporters involved in the apical and basolateral excretion of the metabolites will be the focus of further research.

A direct comparison of metabolic activity between the methods could be made, since tissues from the same donors were used to prepare both slices and Ussing chamber preparations. The two techniques offer different advantages and limitations. The slice technique requires only 4 mg of tissue per slice and is technically easier to use. So, much more experimental variations may be tested within one experiment. More intestinal tissue is needed to mount tissue in the Ussing chamber set-up (around 160 mg mucosal tissue per set-up). However, the latter has the major advantage that vectorial transport of drugs and metabolites can be studied. The incubation conditions for both systems were also slightly different. In fact, these were chosen on the basis of optimized conditions developed for the two systems separately. Surprisingly, in spite of these differences similar metabolic activities were found for all reactions (phase I and II) tested.

After intensive literature search, we feel that we are unable to compare the obtained results with earlier published microsomal data, since no exact scaling factor is known. Although certain reports give the microsomal protein yield after isolation, the loss during isolation has not yet been reported. Furthermore, for intestinal preparations, the origin of the tissue (location along the tract, patients/donor background) and/or the storage and preparation techniques used, will anyhow introduce differences in the metabolic rates. Only when studies are performed with the same donor material, metabolic rates of different methods can be compared. This might be a subject for future studies.

To underline the intrinsic importance of the enterocytes in drug metabolism, we compared the intestinal metabolic rates with activities obtained using human liver.
First of all, a remarkably low interindividual variation was seen in this study using gut tissues. For testosterone hydroxylation only a 2-fold difference was detected between six donors. This is not in line with the observation of others that intestinal metabolism is highly variable (Lin and Lu, 2001), but may be related to the relatively small number of obese donors used in our study. Liver slice values of human TT hydroxylation have been reported to be: 65 (6β-TOH), 24 (2β-TOH), 223 (Androstenedione) and 5 (16α-TOH) pmol/min/mg protein (De Kanter et al., 2002). The proximal jejunum rates detected in the present study were resp. 1.6, 1, 1.4 and 1.8 times higher compared with the liver activity. However, these activities cannot be directly ascribed to the activity of liver hepatocytes and intestinal enterocytes. In liver, hepatocytes represent more than 80% of wet weight of which about 50% is contributing to the metabolic activity in slices of 250 µm thickness (de Graaf et al., 2006), indicating that hepatocyte activity is 2.5 times the tissue value. In intestinal mucosa, enterocytes account for approximately 25% of total wet weight (morphological observation), indicating that the enterocyte activity is 4 times the tissue value. From these observation, we come to the remarkably conclusion that the phase I metabolic rate in enterocytes is estimated to be 1.7 to 2.9 times higher than that in hepatocytes.

7HC-glucuronidation and 7HC-sulphation rates in human liver slices have been reported to amount 880 and 33 pmol/min/mg protein for 7HC-GLUC and 7HC-SULF respectively (de Kanter et al., 2002). If the above mentioned assumptions are taken into account, glucuronidation rates in enterocytes in the present study reach 60-70% of the hepatocyte values. Enterocyte sulphation rates are approximately 250-300% of the sulphation rate in hepatocytes. We conclude that sulphation rates in enterocytes of proximal jejunum as well as colon are higher than in hepatocytes, but glucuronidation rates are lower.
From the present study it can be concluded that both precision-cut intestinal slices as well as Ussing chamber preparations are valuable tools to study human intestinal drug metabolism. Both systems remain viable up to 4 h and exhibit drug metabolism at similar rates. Interestingly, we estimate that human enterocytes exhibit at least comparable to even higher metabolic rates in comparison with hepatocytes.

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References


Footnotes:

a) unnumbered footnote providing the source of financial support

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b) name + full address of person to receive reprint requests

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Legends for figures

Figure 1
Micrographs of small intestinal tissue as control (A) and slices after 4 h of incubation (B). In addition, micrographs of colon tissue as control (C) and colon slices after 4 h of incubation (D). Staining: Haematoxylin and eosin (100x for small intestine; 200x for colon). Representative micrographs from 5 experiments (small intestine) and 2 experiments (colon) using 3 slices per incubation.

Figure 2
Electrical parameters of human intestinal segments in the Ussing chamber technique during 4 h of incubation: potential difference (PD) (A), shortcircuit current (SCC) (B) and resistance (R) (C). Results show mean ± SEM of 4 donors; each time point represents 6-32 chambers.

Figure 3
Metabolic activities as detected in precision-cut slices toward testosterone prepared from small intestine: (A) Androstenedione and (B) 6β-TOH, 16α-TOH and 2β-TOH. Results show mean ± SEM of 5-7 donors; in each experiment 3 slices were incubated per time point.

Figure 4
Metabolic activities detected using precision-cut slices and Ussing chamber preparations prepared from proximal jejunum. Phase I reaction rates were assessed with a cocktail of 4 compounds: midazolam towards 1’OH-midazolam (A), diclofenac towards 4’OH-diclofenac (B), bufuralol towards OH-bufuralol (C) and coumarin towards 7’OH-coumarin (not detectable). Phase II reactions were monitored using
7HC towards 7HC-glucuronidation (D) and 7HC-sulphation (E). Results are mean ± SEM of 4 donors; in each experiment 3 slices and 1 Ussing chamber preparation were incubated per time point.

**Figure 5**

Conjugation activity toward 7HC was measured using precision-cut slices prepared from small intestine (open marks) and colon (closed marks). 7HC-glucuronide (♦) and 7HC-sulphate formation (▲) were measured during 0-4 h of incubation. Results are mean ± SEM of 5-7 donors; in each experiment 3 slices were incubated.

**Figure 6**

Distribution of formed metabolites over the apical (A), basolateral (B) and tissue (T) compartment was measured using human proximal jejunum in the Ussing chamber set-up. Phase I reaction rates were assessed with a cocktail of 4 compounds, formation of 1’OH-midazolam (fig 6A), 4’OH-diclofenac (fig 6B), OH-bufuralol (fig 6C) and 7’OH-coumarin (not detectable). Phase II reactions were monitored using 7HC towards 7HC-glucuronide (fig 6D) and 7HC-sulphate (fig 6E). Results are mean ± SEM of 4 donors; in each experiment 1 Ussing chamber preparation was incubated per time point.
Table 1 – Donor Characteristics

**Proximal jejunum obtained from surgical resection from patients suffering obesity**

<table>
<thead>
<tr>
<th>Number</th>
<th>Age</th>
<th>Gender</th>
<th>Medication</th>
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<tbody>
<tr>
<td>1</td>
<td>46</td>
<td>W</td>
<td>Unknown</td>
</tr>
<tr>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42</td>
<td>W</td>
<td>Unknown</td>
</tr>
<tr>
<td>3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>49</td>
<td>M</td>
<td>Lansoprazol</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
<td>W</td>
<td>Unknown</td>
</tr>
<tr>
<td>5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39</td>
<td>W</td>
<td>Venlafaxine, Metoprolol</td>
</tr>
<tr>
<td>6</td>
<td>54</td>
<td>M</td>
<td>Chlorpromazin, Metformine, Rosiglitazon</td>
</tr>
<tr>
<td>7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31</td>
<td>M</td>
<td>Unknown</td>
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</tbody>
</table>

**Colon obtained from surgical resection from patients suffering colon carcinoma**

<table>
<thead>
<tr>
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<th>Age</th>
<th>Gender</th>
<th>Region</th>
<th>Medication</th>
</tr>
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<tbody>
<tr>
<td>8</td>
<td>68</td>
<td>W</td>
<td>Caecum</td>
<td>Lactulose, Ferrous sulphate, Paracetamol</td>
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<tr>
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<td>Caecum</td>
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<tr>
<td>10</td>
<td>56</td>
<td>M</td>
<td>Sigmoideum</td>
<td>Metoprolol, Lisinopril</td>
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<tr>
<td>11</td>
<td>59</td>
<td>M</td>
<td>Sigmoideum</td>
<td>non</td>
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<tr>
<td>12</td>
<td>79</td>
<td>W</td>
<td>Unknown</td>
<td>Unknown</td>
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</tbody>
</table>

<sup>a</sup> Approval from regional ethical committee and individual patients was obtained for all investigations

<sup>b</sup> Donor tissue used for Ussing Chamber and precision-cut slice incubation
Table 2 – % increase in metabolite concentration after incubation with deconjugating enzymes. Deconjugated samples were Ussing chamber medium samples incubated with cocktail of compounds for 2 h (n=4, mean ± SEM)

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Apical Chamber</th>
<th>Basolateral chamber</th>
</tr>
</thead>
<tbody>
<tr>
<td>1’OH-midazolam</td>
<td>108 ± 6.8 %</td>
<td>110 ± 4.6 %</td>
</tr>
<tr>
<td>4’ OH-diclofenac</td>
<td>133 ± 11.6 %</td>
<td>249 ± 30.8 %</td>
</tr>
<tr>
<td>OH-bufuralol</td>
<td>108 ± 4.8 %</td>
<td>92 ± 8 %</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2

A

PD (mV)

0 60 120 180 240

B

SCC (µA/mm²)

0 60 120 180 240

C

R' area (Ohm x cm²)

0 60 120 180 240

Incubation time (min)
Figure 3

A

- Androstenedione

R² = 0.9594

B

- 6β-TOH
- 2β-TOH
- 16α-TOH

R² = 0.9324

Incubation time (h)

Metabolite formation (nmol/mg protein)
Figure 4

A: 1’OH-midazolam

B: 4’OH-diclofenac

C: OH-bufuralol

D: 7HC-glucuronidation

E: 7HC-sulphation

Metabolite formation (nmol/mg protein) vs. Incubation time (h)

R² values for each graph:
- A: R² = 0.9256
- B: R² = 0.9709
- C: R² = 0.9368
- D: R² = 0.9446
- E: R² = 0.9635
Figure 5

- glucuronidation colon
- sulphation colon
- glucuronidation proximal jejunum
- sulphation proximal jejunum

Metabolite formation (nmol/mg protein)

Incubation time (h)

R² = 0.9829
R² = 0.9877
R² = 0.9866
R² = 0.9998
Figure 6

A: 1’OH-midazolam

B: 4’OH-diclofenac

C: OH-bufuralol

D: 7HC-glucuronidation

E: 7HC-sulphation