An alternative approach based on microfluidics to study drug metabolism and toxicity using liver and intestinal tissue
Midwoud, Paul Marcel van
A Microfluidic Approach for *In Vitro* Assessment of Interorgan Interactions in Drug Metabolism Using Intestinal and Liver Slices

Paul M. van Midwoud
Marjolijn T. Merema
Elisabeth Verpoorte
Geny M.M. Groothuis

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Abstract
Over the past two decades, it has become increasingly clear that the intestine, in addition to the liver, plays an important role in the metabolism of xenobiotics. Previously, we developed a microfluidic-based *in vitro* system for the perifusion of precision-cut liver slices for metabolism studies. In the present study, the applicability of this system for the perifusion of precision-cut intestinal slices, and for the sequential perifusion of intestinal and liver slices, all from rat, was tested to mimic the *in vivo* first pass situation. Intestinal and liver slices, exposed to the substrates 7-ethoxycoumarin (7-EC), 7-hydroxycoumarin (7-HC) and lidocaine (Li), exhibited similar metabolic rates in the biochip and in the well plates for periods of at least 3 h. The metabolic rate remained the same when two slices were placed in adjacent microchambers and perifused sequentially. In addition, the system has been adapted to sequentially perifuse intestinal and liver tissue slices in a two-compartment co-culture perfusion system with a continuous flow of medium. It becomes possible to direct metabolites or other excreted compounds formed by an intestinal slice in the first compartment to the second compartment containing a liver slice. The intestine does not influence liver metabolism for these substrates. The interplay between these two organs was demonstrated by exposing the slices to the primary bile acid, chenodeoxycholic acid (CDCA). CDCA induced the expression of fibroblast growth factor 15 (FGF15) in the intestinal slice, which resulted in a stronger down-regulation of the enzyme, cytochrome P450 7A1 (CYP7A1), in the liver slice in the second compartment than when the liver slice was exposed to CDCA in a single-microchamber biochip. We thus demonstrate in this paper that intestinal slices, in addition to liver slices, remain functional in the biochip under flow conditions, and that the two-microchamber biochip has great potential for the study of interorgan effects. This is the first example of the incorporation of both liver and intestinal slices in a microfluidic device. Use of this microfluidic system will improve our insight into interorgan interactions and elucidate as yet unknown mechanisms involved in toxicity, gene regulation and drug-drug interactions. Moreover, human-specific metabolism can be studied when human slices are available.
Introduction

It is generally known that the liver plays a major role in drug metabolism. However, in the last twenty years it has become increasingly clear that the role of the intestine in drug metabolism cannot be neglected. Increased insight into intestinal drug metabolism and active transport processes has revealed the importance of the intestine not only for drug absorption, but also for the metabolism of xenobiotics. As studies on drug metabolism in the intestine are difficult to perform in vivo, especially in man, various in vitro systems have been developed to study intestinal metabolism, as was recently reviewed by Van de Kerkhof et al. These include subcellular fractions, cell lines, primary isolated enterocytes or intact tissue preparations. However, subcellular fractions and cell lines have their limitations for drug metabolism studies, since they do not contain all enzymes at the proper concentrations. Therefore, primary cells or intact tissue are to be preferred for these studies. However, the isolation of primary cells results in the loss of normal cell-cell and cell-matrix contacts, which may induce changes in cellular function. Precision-cut intestinal slices, on the other hand, have proven to be a versatile in vitro system for metabolism studies and show a good correlation with the in vivo situation. They closely resemble the complex architecture of the organ, with all cell types present in their natural tissue-matrix configuration. The common method to incubate intestinal slices is to place them in medium-filled well plates. However, in this in vitro system, the medium is usually refreshed every 24 h, resulting in decreasing concentrations of nutrients and accumulation of metabolites and waste products. To prevent such variations in culture conditions, we recently developed a new system for the incubation of rat liver slices, consisting of a perfused microfluidic biochip where the liver slice is continuously perfused. (Note: the term “perfusion” is used to emphasize that the medium flows around the tissue slice rather than through it, whereas the term “perfusion” applies to the medium flow through the microchamber.) The microenvironment of the slice could be very well controlled in this microfluidic system, and liver slices maintained their viability and metabolic function for at least 24 h. These results were as good as those obtained in optimized, conventional well plate assays, where drug metabolism rates are similar to those in the organ in vivo. Such a microflow system offers a flexible platform for experimentation, as medium composition and flow can easily be varied throughout the course of an experiment, allowing the study of dose dependence and drug-drug interactions in one slice.

The first aim of this paper was to investigate whether intestinal tissue slices can also be cultured in the biochip, and to compare their metabolic activity with slices incubated in the conventional well plate system. To the best of our knowledge, this is the first report of the incorporation of intestinal tissue slices into a microfluidic device.

In vivo metabolites formed by the intestine and excreted to the basolateral side
are transported via the portal blood to the liver. It can be hypothesized that these metabolites are further converted in the liver and/or influence liver function. However, these so-called interorgan effects have proven difficult to verify in in vitro experiments. In well plates, two different tissue slices can be placed in one well; however, mutual interactions can take place in this case, and it would be impossible to conclude which organ was responsible if interorgan effects were to be observed. Thus, the second aim of this study was to construct a new in vitro system to sequentially perifuse precision-cut tissue slices (PCTS) from the intestine and the liver, to gain new insight into these potential interorgan effects.

There have been to date only a few examples of co-culture flow systems incorporating cells derived from different tissues that allow the investigation of interorgan effects. None of these has used freshly isolated cells, relying instead on cell lines. Flow systems containing Caco-2 cells (a colon-derived cell line, differentiated into small intestinal-like cells) and HepG2 cells (a liver carcinoma cell line) have been reported for absorption and biotransformation studies. Recently, Mahler et al. developed a microscale cell culture analog (µCCA) with Caco-2 and HepG2 cells. Due to the low volume, this µCCA allowed for near in vivo fluid-to-tissue ratios and cellular shear-stress values. These co-culture systems yielded interesting results for absorption and toxicity studies. However, the lack of normal expression levels of important metabolic enzymes in Caco-2 and HepG2 cells makes this system less predictive for metabolism studies. Moreover, interorgan effects due to mediators excreted by cell types other than the enterocytes will not be detected. To ensure availability of these endogenous enzymes, the use of primary cells, or even better, tissue, is preferred in in vitro systems. Chen and Pang showed the sequential ex vivo perfusion of intact rat intestine and liver preparations for metabolism studies, and studied the effect of flow on first-pass metabolism. However, two rats are needed for one experiment, one as intestine donor and one as liver donor, making the system labour-intensive and not particularly attractive from an animal usage point of view. Moreover, this technique is not applicable to the human case, since it is clearly ethically impossible to perform this experiment in the human context. To date, no microfluidic in vitro system has been developed which incorporates both liver and intestinal primary cells or tissue explants, such as biopsies or slices. Such a system would greatly increase our understanding of interorgan interactions as well as substantially reduce animal use.

We have therefore developed an in vitro microflow system for the purpose of investigating interorgan effects, made by coupling two microchambers, one containing an intestinal tissue slice, the other a liver slice, which can be sequentially perfused. In this way, metabolites formed by the intestine in the first chamber can be directed to the liver in the second chamber for further metabolism, thereby mimicking in vivo, first-pass metabolism. In addition, the possible influence of metabolites or
other mediators formed by the intestine on the liver can be studied, providing insight into interorgan effects.

To demonstrate the feasibility of the microfluidic system for metabolism studies, we tested whether rat liver and intestinal slices remain functional in such a sequential perfusion set-up. The metabolism of several model compounds was measured in a single liver slice, a single intestinal slice, two sequentially coupled liver slices, two sequentially coupled intestinal slices and a sequentially coupled intestinal and liver slice. The compounds 7-ethoxycoumarin, 7-hydroxycoumarin and lidocaine were used as model substrates to probe the activity of several phase I cytochrome P450 isoenzymes, and phase II UDP-glucuronyltransferase and sulfotransferase. Since the well plate provides an excellent benchmark with high viability for short-term metabolism studies, these experiments were also performed in well plates as control. It was not expected that these compounds would show interorgan effects.

To demonstrate the application of the developed system to the investigation of interorgan effects, the interplay of liver and intestine in the regulation of bile acid homeostasis was simulated. In addition to the liver, the intestine also plays an important role in the regulation of enzymes and transporters involved in bile acid transport and metabolism. One of the key events in the regulation of bile acid homeostasis is the regulation of the enzyme, cytochrome P450 7A1 (CYP7A1), expressed in the liver and the rate-limiting enzyme for the synthesis of bile acids from cholesterol. Several mechanisms have been shown to explain the bile acid-induced down-regulation of CYP7A1. In the first place, bile acids bind to the nuclear receptor, farnesoid X receptor (FXR), in the liver, thereby inducing the expression of small heterodimer partner (SHP), which in turn down-regulates the expression of CYP7A1 and hence the synthesis of bile acids. In addition, primary bile acids, like chenodeoxycholic acid (CDCA), induce the expression of fibroblast growth factor 15 (FGF15) in the rodent intestine (FGF19 in the human intestine). This results in the formation of the FGF15 protein, which is transported in vivo via the portal vein to the liver. In the liver, this protein causes an additional down-regulation of CYP7A1. In this study, the interorgan interaction was confirmed in the biochip incorporating sequential perfusion of precision-cut liver and intestinal slices by measuring the gene expression of FGF15 and CYP7A1 using real-time PCR.

This study demonstrates the first example of metabolism studies in intestinal slices under flow conditions. Moreover, this is also the first example of sequential perfusion of precision-cut tissue slices from intestine and liver to investigate interorgan interactions.

Material and Methods

Chemicals
Sodium chloride, low-temperature-gelling agarose, 7-ethoxycoumarin (7-EC), 7-
hydroxycoumarin (7-HC), 7-hydroxycoumarin sulfate (7-HC-S), 7-hydroxycoumarin glucuronide (7-HC-G), and D-glucose monohydrate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Chenodeoxycholic acid (CDCA) was obtained from Calbiochem (San Diego, CA, USA). Gentamicin, Williams Medium E with Glutamax-I, and amphotericin B (Fungizone) solution were purchased from Gibco (Paisley, UK). Lidocaine (Li) was obtained from Centrachemie (Etten-leur, The Netherlands). Monoethylglycinexylidide (MEGX) was a kind gift from AstraZeneca (Södertälje, Sweden).

Animals
Male Wistar rats (HsdCpb:WU) weighing ca. 300 g were obtained from Harlan (Horst, The Netherlands). The rats were maintained under a 12-h light/dark cycle in a temperature- and humidity-controlled room with free access to food (Harlan chow no. 2018, Horst, The Netherlands) and tap water. The use of animals for these experiments was approved by the Animal Ethics Committee of the University of Groningen.

Preparation of precision-cut tissue slices
After anaesthetizing the rat with isoflurane/O₂, the intestine and liver were excised and placed in ice-cold oxygenated Krebs-Henseleit buffer (pH = 7.4) and ice-cold Viaspan organ preservation solution (University of Wisconsin (UW) solution, Du Pont Critical Care, Waukegab, IL, USA), respectively. Liver slices were made as described previously.⁹ Slices were approximately 4 mm in diameter, 100 µm thick (~6 cell layers), and had a wet weight of about 3 to 4 mg. Intestinal slices (jejunum and ileum) were made as described by Van de Kerkhof et al.⁸ The jejunum slices were used for metabolism studies, and ileum for the regulation of bile acids. Briefly, the intestine was cut into 3 cm segments and flushed with Krebs-Henseleit buffer. Segments were ligated at one end, and filled with 3% (w/v) agarose solution in 0.9% sodium chloride at 37°C. Subsequently, the segments were placed in ice-cold Krebs-Henseleit buffer to solidify the agarose. The gel-filled intestinal segment was then embedded in 37°C agarose solution, and sliced after gelling at 4°C. The total time needed for the preparation of the gel-filled intestinal segment is around 30 minutes, and slicing of 10-20 slices takes about 10-15 min. Intestinal slices were approximately 400 µm thick and had a wet weight of approximately 2 mg.

Microdevice
Figure 1 shows a schematic view of the microdevice made of 10 layers of polydimethylsiloxane (PDMS). Details of the biochip fabrication and medium distribution through the chamber have been extensively described elsewhere.⁹ The biochip used in this study is the same, with one minor modification. Previously, the chips contained
4 microchambers. For this study, we designed biochips where each chip contained 6 microchambers, all of which could be perfused separately. The medium inlet at the bottom of the chip was connected via a microfluidic channel (500 µm x 100 µm (W x H)) with the tissue chamber. A polycarbonate filter (10 µm thick, 8 µm pores) was integrated at the entrance to the tissue chamber (Ø4 mm x 2 mm, 25 µL). This filter acts as an inverse showerhead, and ensures an equal distribution of medium flow towards the tissue slices, as described previously. At the top of the microchamber a second polycarbonate filter was integrated, to ensure that the tissue slices are suspended in medium and that the medium flows all around the tissue slice (perfusion). The microchamber was connected to the outlet via a microfluidic channel. Connecting this outlet to the inlet of a neighbouring chamber allows sequential perfusion of these two chambers. An important feature of this device is the integration of two PDMS membranes to act as aeration windows to maintain a highly controlled microenvironment for tissue-slice incubations. PDMS is known to be gas permeable, and by integrating 250-µm-thick PDMS membranes, oxygen and carbon dioxide from the environment can penetrate the PDMS and enter the medium. This enables the regulation of oxygen and carbon dioxide (important to maintain the pH at 7.4) concentrations in the medium.

![Figure 1](image_url)

**Figure 1. Schematic illustration and photograph of the PDMS biochip.** (a) Cross-sectional view of one chamber with the integrated polycarbonate (10 µm thick) and PDMS membranes (250 µm thick). The dimensions of the microchamber is Ø4 mm x 2 mm high. (b) Animated illustration of how liquid flows through the biochip. A substrate is added at the inlet and converted into metabolites by the liver slice, which are then transported to the outlet by the flow. (c) Photograph of the PDMS biochip containing six microchambers in the polycarbonate holder. The dimensions of one chip are 30 mm x 20 mm x 12 mm (L x W x H).
Incubation system
Well plates
Tissue slices were incubated in 12-well plates (Greiner bio-one GmbH, Frickenhausen, Austria) in 1.3 mL Williams Medium E with Glutamax-I. For both liver and intestinal slices, the medium was supplemented with 25 mM D-glucose monohydrate and 50 µg/mL gentamicin. In addition, media for intestinal slices and co-incubation of liver and intestinal slices were supplemented with 2.5 µg/mL amphotericin B. Well plates were placed in humidified plastic boxes which were continuously supplied with humidified carbogen to obtain an atmosphere of 95% oxygen and 5% CO₂. The boxes were shaken at 90 cycles per minute. All experiments were performed in an incubator at 37°C. Co-incubation of liver and intestinal slices was performed with both slices placed in the same well in 1.3 mL medium.

Biochip
The same medium was used for the biochip experiments as for the well plates. Glass syringes (ILS, Stützerbach, Germany) were filled with medium which was saturated with carbogen at 37°C (pH of 7.4). Filled syringes were placed in a syringe pump. PEEK tubing was used to connect the syringes with the biochip (150 mm x 0.75 mm ID) (Da Vinci Europe, Rotterdam, The Netherlands). To sequentially perfuse two chambers, the outlet of the first chamber was connected to the inlet of the second chamber with PTFE tubing (150 mm x 0.5 mm ID) (Polyfluor Plastics, Oosterhout, The Netherlands). PTFE tubing was also connected to the outlet of the chip (75 mm x 0.50 mm ID) to collect medium fractions. The chip was placed in a humidified plastic container with a continuous exchange of humidified carbogen gas, and the box was placed in an incubator set at 37°C. After flushing the system with medium for at least half an hour, tissue slices were placed in the microchambers. A schematic overview of the system set-up for the sequential perfusion of two microchambers containing slices is given in Figure 2. The flow rate was set at 10 µL/min, as pilot experiments showed that high flow rates (>50 µL/min) resulted in damage to the tissue, whereas low flow rates (<4 µL/min) may result in insufficient diffusion and refreshment rate (results not shown). A flow rate of 10 µL/min results in complete refreshment of the medium in the microchamber every 2.5 minutes.

Morphology
Non-incubated (0 h) precision-cut tissue slices and slices incubated in both well plates and biochip for 3 hours (3 h) were subjected to morphological evaluation. Slices were fixed in 4% formaldehyde in phosphate-buffered saline solution for 24 h at 4°C. Thereafter, slices were placed and stored in 70% ethanol until paraffin embedding, sectioning and haematoxylin and eosin staining (HE staining). HE staining was performed as described previously by De Graaf et al.21
Metabolite analysis
Slices were pre-incubated for one hour in well plates. They were then either incubated in well plates with medium containing 500 µM 7-EC or 500 µM 7-HC for 3 hours, or 5 mM Li for 1 hour, or transferred to a biochip and perfused with medium containing these substrates. After incubation, the medium samples were stored at -20°C until analysis. Analysis was performed on an HPLC system with UV detection. 7-EC, 7-HC, 7-HC-G and 7-HC-S were quantified as described previously, as was Li and its metabolite, MEGX. To compare the results obtained in well plates and biochip, and to compare two-slice with one-slice incubation, all results were reported in terms of amount of metabolite formed per minute per milligram protein in the tissue. Protein amounts in tissue slices were determined using the Bio-Rad protein assay dye reagent (Bio-Rad, Munich, Germany) using bovine serum albumin as standard.

Bile acid-induced regulation of gene expression
Ileum and liver slices were incubated singly in a biochip or sequentially perfused with or without 50 µM CDCA. After 7 h of incubation, the slices were taken out of the chips and snap-frozen in liquid nitrogen. Subsequently, RNA was isolated from the slices using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized using the Promega Reverse Transcription System (Promega, Madison, USA), in which 1.25 µL sample were used in real-time PCR reactions using Sensi-Mix SYBR green (Quantace, London, UK). The primers used for real-time PCR are listed in Table 1. The comparative threshold cycle (Cₜ) was used to quantitate the expression of the genes of interest. For intestinal slices, the expression was calculated...
relative to villin as reference gene (ΔCₜ), and for liver slices relative to GAPDH as reference gene. The effect of treatment with CDCA was calculated relative to the control slices (ΔΔCₜ) and expressed as fold expression according to 2^(ΔΔCₜ).

Table 1. Primer information of the rat genes used for real-time PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’ )</th>
<th>Reverse primer (5’-3’ )</th>
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<tr>
<td>GAPDH</td>
<td>CGCTGGTGCTGAGATGTGCG</td>
<td>CTGTGTCATGAGCCTTCC</td>
</tr>
<tr>
<td>Villin</td>
<td>GCTCTTTGAGTGCTCCACCC</td>
<td>GGGGTGGGTCTTGGATTT</td>
</tr>
<tr>
<td>CYP7A1</td>
<td>CTGCATACACAAAAGTCTATGTCA</td>
<td>ATGCTTCTGTGTCAAATGCC</td>
</tr>
<tr>
<td>FGF15</td>
<td>ACCGCTGATTCGCTACTC</td>
<td>TGTGCCCCAAACAGTCCATTCCT</td>
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</table>

Statistical evaluation

Significant differences between control and treated slices were determined using the Student’s t-test, with p < 0.05 considered as significantly different. For metabolism studies, it was of interest to determine what, if any, significant differences existed between experimentally determined metabolic rates; for gene expression, ΔΔCₜ values were statistically evaluated.

Results and Discussion

Liver slices

Previously, we showed that liver slices remain viable for 24 h in the microchamber of our biochip. Here, it was important to ensure that a liver slice in the second microchamber, perfused with outflow medium from the first microchamber, retained its viability and received sufficient nutrients and substrates. Hence, the viability of a liver slice in the second chamber was compared with that in the first chamber.

Figures 3a), c) and e) show the morphology of liver slices directly after slicing and after 3 h incubation in well plates and in the biochip, respectively. No differences in liver morphology were observed in the biochip and well plates when compared to fresh tissue. The morphology was also not changed when two liver slices were coupled and sequentially perfused, indicating intact cells.

The metabolic characteristics of the slices in the first microchamber were subsequently compared with those in the second. The metabolic rates of liver slices after incubation for 1 h with 5 mM Li, and for 3 hours with 500 µM 7-EC or 500 µM 7-HC, are given in Figure 4 for single liver slices in the well plate and biochip, and for two liver slices sequentially perfused in the biochip. The substrate concentrations used were well above the concentrations needed to achieve the maximum metabolic rate, resulting in a constant rate of metabolism despite a small decrease in substrate concentration during incubation in the well plates. This allows us to compare the metabolic rate in well plates with that in the biochip, where substrate concentrations remain constant.
The formation of MEGX was $2.6 \pm 0.2$ nmol/min/mg protein, and was not significantly different in the well plates and biochip (Figure 4a)), indicating a similar viability in both systems. In addition, the formation rate of MEGX expressed per mg of protein was identical in one liver slice and two sequentially perifused liver slices. Therefore, we can conclude that the consumption of nutrients and substrates by the first slice did not negatively affect the metabolic rate of the second slice. Moreover, the substrate concentration in the second chamber was still high enough for the second slice to operate at the maximum metabolic turnover rate, despite the consumption of substrate in the first chamber. The same results were observed for 7-EC and 7-HC (Figure 4b) and 4c)). No significant differences were observed in specific metabolic activity between slices in well plates and in the biochip, and between one slice and two slices perifused sequentially. The slight, but not significant, difference in the level of 7-HC between the biochip and well plates, observed previously,\textsuperscript{9} might indicate a somewhat more efficient metabolism of 7-HC into 7-HC-G in the biochip. The total metabolic conversion of 7-EC in rat liver slices was $218 \pm 10$ pmol/min/mg protein,
and of 7-HC was $594 \pm 30 \text{ pmol/min/mg protein}$. These metabolic rates were comparable with metabolic rates found previously,\textsuperscript{16} and thus correlate well to the \textit{in vivo} situation.\textsuperscript{16, 24}

Figure 4. Metabolism of (a) 5 mM Li, (b) 500 µM 7-EC, and (c) 500 µM 7-HC in single liver slices and in two liver slices perifused sequentially in the biochip as indicated in Figure 2. Results are given as the mean ± standard error of the mean (SEM) of 3-4 rats, with 3 slices per experiment. No significant differences were observed in metabolic activity between liver slices perifused singly or sequentially, or between experiments in well plates and biochips.
**Intestinal slices**

Subsequently, we tested the incorporation of intestinal tissue slices in the biochip. As far as we know, this is the first attempt to perifuse intestinal slices and measure metabolism in a perifusion system. The viability and function of intestinal slices incubated in well plates up to 24 h have been shown by us and others.\(^{25,26}\)

In general, the morphology of intestinal slices (jejunum) as evaluated by an independent pathologist was retained intact compared to fresh intestinal slices, as can be seen in Figures 3b), d) and f). However, after incubation for 3 h in well plates, the villi, present at the apical side of the intestine, appeared somewhat flattened and broadened, as was also observed in earlier studies.\(^6\) The villi of most of the intestinal slices perifused in the biochip were less flattened compared to those in the well plates (see Figure 3f)), although this was not the case for all slices evaluated. No changes in slice morphology were observed when incubating two intestinal slices instead of one, independent of whether incubation was performed under flow or no-flow conditions.

Figure 5 shows the metabolism of 5 mM Li, 500 µM 7-EC and 500 µM 7-HC by small intestinal slices (jejunum) incubated in well plates or perifused in the biochip, in a single microchamber and in two microchambers perfused sequentially. The formation of MEGX in the small intestine under all incubation conditions was 140 ± 38 pmol/min/mg protein. When intestinal slices were exposed to 7-EC, the metabolites 7-HC and 7-HC-G were formed, but 7-HC-S was undetectable (see Figure 5b)). When slices were exposed to 7-HC, both 7-HC-G and 7-HC-S were formed. The total metabolic rate for 7-EC in the intestine was 21 ± 2 pmol/min/mg protein, and for 7-HC 304 ± 40 pmol/min/mg protein, comparable to the rate found previously by Van de Kerkhof \textit{et al}. in well plates.\(^{26}\) As with liver slices, the specific metabolic rate of two intestinal slices perifused sequentially did not significantly differ from one individual slice, and no significant difference was observed between the well plates and the biochip. This indicates that intestinal slices in the biochip retain a similar viability as in well plates for at least 3 h.

The total phase I metabolism of 7-EC and Li in intestinal slices was 10- and 18-times lower, respectively, than in liver slices. This is in line with the ratio of the 10-20 fold difference in cytochrome-P450 protein expression in the liver and intestine as found by Matsubara \textit{et al}.\(^{27}\) and Lin \textit{et al}.\(^{28}\) The rate of formation of 7-HC-S from 7-HC in the intestinal slices was comparable to the liver slices, while the rate of glucuronidation was only two times lower. This indicates a high activity of sulfotransferase and glucuronyltransferase in the intestine as observed previously.\(^6,8\) The lack of detection of 7-HC-S after incubation with 7-EC can be explained by the low phase I biotransformation rate by cytochrome P450 isoenzymes, and thus low intracellular concentration of the phase I metabolite 7-HC.
Figure 5. Metabolism of (a) 5 mM Li, (b) 500 µM 7-EC, and (c) 500 µM 7-HC in single intestinal slices and in two intestinal slices perifused sequentially. Results are the mean ± SEM of 3-4 rats, 3 slices per experiment. No significant differences were observed between intestinal slices perifused singly or sequentially, or between experiments in well plates and biochip. ND: not detectable.

Intestinal and liver slices
Sequential perifusion of intestinal slices and liver slices in the biochip can serve as a model to study first-pass metabolism. The weight of the tissue slices used for the experiments was 2 mg for intestine and 3-4 mg for liver. This ratio reflects the organ weight ratio in the rat in vivo.\textsuperscript{16} Again, the morphology did not change in the two
incubation systems during the co-incubation of a liver and intestinal slice. Slice morphology in both the liver and intestinal samples was comparable with single-slice incubation or perfusion, as shown in Figure 3. This indicates that both liver and intestinal slices remain intact in the well plate system as well as in the biochip. Figure 6 shows the metabolism of the three substrates after co-incubation of liver and intestinal slices in well plates or sequential perfusion in biochips (indicated as “measured”). In addition, the data are given for single-incubation experiments with separate liver and intestinal slices, after which the total metabolic rate of a liver and an intestinal slice was calculated and corrected for inter-experimental differences in protein content of the slices (indicated in Figure 6 as “calculated”).

The calculated and measured metabolite formation rates did not significantly differ for any of the measured substrates. The metabolizing enzymes in the liver slices were thus functioning at their optimal turnover rate. This means that the consumption of nutrients and the excretion of waste products by the intestinal slice did not affect liver-slice metabolism during the 3 h of incubation. We conclude from these data that coupling two chambers sequentially did not affect the metabolic competence of slices in the second chamber. From these results, we can also conclude that the intestine does not influence the liver’s metabolism of the three substrates used in this study, at least not under the applied concentrations. Again a small, but now significant, difference was found for the amount of 7-HC measured in the well plates compared to the biochip. However, the total phase I metabolic rate, which is the sum of 7-HC-G, 7-HC-S and 7-HC, was not significantly different. As the 7-HC-G formed was slightly higher in the biochip compared to the well plates, the lower 7-HC concentration may be caused by a more efficient formation of 7-HC-G by the liver slice in the biochip.

From the data presented in Figures 4, 5 and 6, it can be concluded that the contribution of the small intestine to the metabolism of 7-EC and Li is small compared to the liver at the applied substrate concentrations. However, this may be different at lower substrate concentrations, where the ratio of the metabolic rate in the liver to that in the intestine is smaller, as shown by Martignoni et al.25 The contribution of the small intestine to the phase II metabolism of 7-HC, on the other hand, was high at the concentration tested.

Interorgan interaction
The ability to monitor interorgan interactions was assessed by studying the bile acid-induced regulation of the enzyme, CYP7A1, responsible for bile acid synthesis. Single liver and intestinal slices were incubated with CDCA, to measure the direct effect of CDCA on the expression of CYP7A1 in the liver and FGF15 in the intestine. In addition, to determine the effect of products formed by the intestine on bile acid treatment of the liver, the outlet of the chamber containing an intestinal slice was
Figure 6. Metabolism of (a) 5 mM Li, (b) 500 µM 7-EC, and (c) 500 µM 7-HC in liver slices and intestinal slices perifused sequentially or incubated together in the same well (measured). For the calculated values, the results for individual slice incubations were used. Results are the mean ± SEM of 3-4 rats, 3 slices per experiment. No significant differences were observed between calculated and measured metabolic rates. Significant difference between well plate and biochip is indicated with * (p < 0.05).

connected to the inlet of a chamber containing a liver slice. When intestinal slices were treated with CDCA, a significant up-regulation of FGF15 was observed (see Table 2). As expected, results obtained with single intestinal slices were not
significantly different from those in co-incubated slices, since the intestinal slice was always perfused in the first compartment during sequential perfusion. As shown in Table 2, the fold expression of FGF15 of the three rats was highly variable. This variability is probably due to the gradient of FGF15 expression along the intestinal tract\(^{19}\) as it is difficult to prepare the slices from exactly the same location of the intestine. However, for all three rats there is a clear and significant up-regulation of FGF15 in ileum slices when perfusing them with CDCA, as shown by others.\(^{29}\)

**Table 2. Fold change of the expression of FGF15 in rat ileum slices after 7 h of exposure to CDCA compared to untreated control slices.**

<table>
<thead>
<tr>
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<th>Rat 1</th>
<th>Rat 2</th>
<th>Rat 3</th>
<th>Mean</th>
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<tbody>
<tr>
<td>Single</td>
<td>32</td>
<td>332</td>
<td>134</td>
<td>166</td>
</tr>
<tr>
<td>Co-incubated</td>
<td>30</td>
<td>290</td>
<td>134</td>
<td>151</td>
</tr>
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</table>

When perfusing intestinal and liver slices sequentially without the addition of bile acids, the CYP7A1 expression in the liver was comparable to that in single-liver-slice incubation (see Figure 7). This indicates that intestinal slices do not release compounds which affect the expression of CYP7A1 in the liver under control conditions. The incubation of single liver slices with CDCA resulted in a small (25%) but not significant down-regulation of CYP7A1.\(^{17,18}\) However, after exposure of sequentially perfused intestinal and liver slices to CDCA, the CYP7A1 expression in the liver slices was decreased to 40% of its control value. Since exposure of liver slices alone to CDCA and sequential perfusion of intestinal and liver slices without CDCA did not result in such low CYP7A1 expression, this effect is most likely due

![Figure 7. Expression of CYP7A1 in liver slices treated with CDCA after single perfusion and after sequential perfusion with an intestinal slice. Results are the mean ± SEM of 3 rats, 3 slices per experiment. Significant difference with respect to control is indicated with * (p < 0.05).](image-url)
to a product formed by the intestine upon exposure to CDCA. As shown by Inagaki et al., bile acids induce the expression of FGF15 in the intestine and this FGF15 can be transported in our microfluidic system to the liver by continuous perfusion. The exposure of the liver slices to both CDCA and FGF15 formed by the intestine resulted in the strongly reduced expression of CYP7A1. This will in turn result in a decrease of the synthesis of bile acids from cholesterol in the liver. This clearly demonstrates, for the first time, the utility of the dual-chamber microfluidic biochip for the investigation of the interplay between the intestine and liver, in this case for the control of bile acid synthesis.

**Conclusion**

We have developed a new *in vitro* system to study intestinal and liver metabolism in a microfluidic biochip under flow conditions. It was previously demonstrated that liver slices incorporated in this novel system retain their viability and functionality for at least up to 24 h. Now we show that it is also feasible to maintain intestinal slices in this biochip with high viability and functionality for at least 3 h. Morphological evaluation of intestinal slices indicates a slightly better preserved intactness of tissue in the biochip compared to the well plates. This is the first example of the incorporation of intestinal slices into a microflow system. The results obtained in this new *in vitro* system were verified with the optimized conventional well plate system, in which slices retain their *in vivo* metabolic rate at least for 8 h for intestine and 24 h for liver. The results were similar in both systems for the model substrates tested, which shows that the newly developed system is well-suited for metabolism studies. It was not the goal of the present study to test the maximum lifespan of the intestinal slices in the biochip. Rather, the aim was to show its applicability to metabolism studies for single intestinal and liver slice incubations, and for intestinal slices and liver slices in a multi-organ incubation set-up. Experiments to assess the lifespan of intestinal slices in the biochip are currently ongoing.

The main advantage of applying microfluidics to these studies is the excellent control of medium flow achievable and the possibility to easily control and alter the medium composition during an experiment. Due to this excellent control of liquid flow, it is possible to direct metabolites formed by tissue slices to a second compartment for further incubation, or for on-line metabolite detection. The present data show that the microfluidic approach used also enables microchambers to be linked to one another for sequential perifusion of tissue slices from the same or different organs while maintaining a high metabolic activity. The applicability of the system to the study of interorgan interactions was demonstrated by revealing the interplay between the intestine and liver in the regulation of bile acid synthesis. Upon exposure to the bile acid, CDCA, the intestine expressed FGF15, which caused a larger down-regulation of the expression of CYP7A1 in the liver than observed when
liver alone was exposed to CDCA. It is thus possible to study the effect of metabolites and/or (waste) products from one tissue slice on another. The coupling of two tissue slices has been shown now for small intestinal and liver slices. However, lung, kidney and colon slices could also be integrated into the system in the future, as precise slicing techniques have been developed for these organs as well. Therefore, this system makes it possible to investigate and gain insight into interorgan interactions in vitro. Another advantage of the continuous flow compared to the static well plate system is that the incubation environment is very stable over time. It is also possible to perform in- and on-line analysis in continuous flow systems, to study time-dependent drug-drug interactions and the concentration dependence of metabolism in one single slice, and detect chemically unstable metabolites (Chapter 4).

The advantage of the presented system compared to systems incorporating cell lines is the use of freshly isolated tissue, which retains the physiological expression of metabolizing enzymes. However, it should be noted that the apical and basolateral sides of the intestinal tissue were not perfused separately in our system, which means that the apically excreted metabolites and other excreted compounds are also supplied to the liver.

By using small precision-cut tissue slices, this system will contribute to the minimization of animal testing. Multiple experiments can be performed with only one rat, which is more efficient compared to the system used by Chen and Pang, for example, where two rats were used per experiment. Moreover, the ability to work with limited amounts of tissue enables the study of human-specific metabolism, when scarce human tissue is available.

As shown previously, incubation of tissue slices in well plates is a suitable in vitro system to measure the metabolism and toxicity of drugs with a good correlation to the in vivo situation. In this study, the results for the tested substrates in the metabolism studies performed in the biochip agreed consistently with those obtained in the validated well plate system. This is crucial for the validation of the biochip, assuring the reliability of future results obtained with this system for new substrates whose metabolism remains as yet uncharacterized in vitro or in vivo. Thus, we believe that this new biochip will serve as an excellent complementary system to the well plate system for three reasons: 1) questions related to the effect of flow on the viability and functionality of the tissue slices can be addressed, 2) the effect of intestinal response to drugs on liver metabolism and function can be investigated and 3) on-line analysis is facilitated, which also allows a better detection of chemically unstable, reactive metabolites. With this new tool, it should be possible to gain a better insight into interorgan interactions and elucidate unknown mechanisms involved in toxicity, gene regulation and drug-drug interactions. Accordingly, this microflow system will help minimize the gap between the in vitro and in vivo situation, and will moreover contribute to the reduction of the use of experimental animals.
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References
14. Wilkening, S.; Stahl, F.; Bader, A., Comparison of primary human hepatocytes and hepatoma cell line HepG2 with regard to their biotransformation properties. Drug Metab. Dispos. 2003, 31 (8), 1035-1042.

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