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# On-line HPLC Analysis System for Metabolism and Inhibition Studies in Precision-Cut Liver Slices

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A novel approach for on-line monitoring of drug metabolism in continuously perfused, precision-cut liver slices (PCLS) in a microfluidic system has been developed using high-performance liquid chromatography with UV detection (HPLC-UV). In this approach, PCLS are incubated in a microfluidic device made of poly(dimethylsiloxane) (PDMS) by continuous, single-pass perfusion with fresh medium. Two syringe pumps are incorporated into the system to infuse substrates or inhibitors at varying concentrations into the perfusion medium just before the chip entrance. The medium containing the metabolites produced by the PCLS is directed toward an injection loop. Once filled, the content of this injection loop is automatically injected onto an HPLC for analysis. The on-line analysis of metabolites was tested by using the substrate, 7-hydroxycoumarin (7-HC). Rapid switching between substrate and solvent control was possible, and a direct metabolic response of the liver slice to perfusion with substrate was detected. Very stable phase II metabolism over a period of 24 h was observed. The inhibitory effect of phloxadine B on the formation of 7-hydroxycoumarin glucuronide (phase II product of 7-HC) was also investigated. Phloxadine B was injected into the incubation medium in increasing concentrations varying from 0 to 200  $\mu$ M. The results showed a concentration-dependent inhibition of 7-HC glucuronide formation and allowed the calculation of an IC<sub>50</sub> value (concentration in which 50% of the enzyme is inhibited) of  $\sim$ 85  $\mu$ M using one single liver slice. On-line detection was also shown to be advantageous for the detection of unstable metabolites. This was demonstrated by determination of the metabolites of the drug diclofenac. The reactive metabolite, acyl glucuronide, was detected at relatively high concentrations which remained very constant over a period of 4 h. In contrast, only low and decreasing amounts of diclofenac acyl glucuronide could be measured in the conventional well-plate incubation system. The advantages of this novel on-line analysis system for PCLS include the capability to obtain direct information about tissue function, assess the concentration dependence of drug–drug interactions in one single slice, and detect unstable metabolites. The

system also enables fast analysis without the need to store samples, thus eliminating the associated freeze–thaw problems, and allows the simultaneous analysis of multiple metabolites.

Studies on drug metabolism in the liver have most often employed microsomes prepared from liver cells, as they are easy to handle and one of the best characterized in vitro systems. However, for proper prediction of metabolism, it is a disadvantage that microsomes do not contain all the metabolizing enzymes present in the cells and that the cofactors are not present at their physiological levels. The use of primary hepatocytes or precision-cut liver slices (PCLS) is therefore preferred for quantitative analysis. One of the advantages of PCLS over hepatocytes is that all the different cell types are present, including their metabolizing enzymes, in their natural tissue-matrix configuration. This makes liver slices a good organomimetic model complete with intact cell–cell interactions, reflecting a more physiological situation. Commonly, liver slices are incubated in well plates or in dynamic organ culture systems.<sup>1</sup> However, recently we and others have reported examples of incubation systems with continuous liver slice perfusion.<sup>2–4</sup> The incubation environment is kept stable over time due to the flow, with a continuous influx of nutrients and removal of waste products. Another advantage of using perfusion is that it allows easy coupling to other flow-based analytical methods such as high-performance liquid chromatography (HPLC) to perform on-line analysis. Fractions collected at the outlet of the incubation system can in principle be injected directly onto an HPLC system to analyze the metabolites formed. Various on-line analysis systems have been developed for microsomal fractions using liquid chromatography<sup>5–7</sup> and capillary electrophoresis.<sup>8,9</sup>

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Sung et al. even showed in-line monitoring of cytochrome P450 activity of a hepatoma cell line (HepG2/C3A) via fluorescence detection on chip.<sup>10</sup> Until now, however, no examples exist for the on-line analysis of metabolites using precision-cut tissue slices (PCTS), though this capability clearly would facilitate metabolism studies. We recently developed a novel microfluidic biochip for the perfusion and incubation of precision-cut tissue slices.<sup>4</sup> The results showed that it is possible to keep liver slices viable for at least 24 h and intestinal slices for at least 7 h in the biochip and that 7-ethoxycoumarin metabolism is comparable with the conventional well-plate system and thus with the in vivo situation as well.<sup>4,11,12</sup> Analysis, however, was still done off-line.

The aim of this study is to develop an on-line analysis system for the microfluidic biochip to perform metabolism and inhibition studies on-line in PCLS. With this system, it is possible to (1) inject a substrate into the medium just before the inlet of the biochip and (2) measure the metabolism in liver slices directly by automatic injection of fractions collected at the chip outlet onto an HPLC system. Switching between substrates, or addition of an inhibitor, is accomplished by two syringe pumps incorporated into the system. These pumps also allow the injection of a substrate or inhibitor multiple times at increasing concentrations to measure concentration-dependent effects on metabolism. Other advantages of this system are the speed of analysis and elimination of the necessity to store samples, enabling the analysis of unstable metabolites. The quantification of these types of unstable compounds in conventional well-plate incubations is impeded, as detectable concentrations are not achieved due to the relatively large incubation volume and decomposition during the incubation. In addition, PCTS function and stability of metabolic enzymes can be monitored directly over time. To show the applicability of the system, the phase II metabolism of 7-hydroxycoumarin (7-HC) into 7-hydroxycoumarin sulfate (7-HC-S) and 7-hydroxycoumarin glucuronide (7-HC-G) was determined over time.

The versatility of this system was further demonstrated by performing an inhibition study on-line in which phloxine B was infused together with 7-HC. Phloxine B is a phenyl-xanthene dye which is known to inhibit the enzyme UDP-glucuronosyltransferase (UGT), especially the isoenzyme UGT1A6.<sup>13,14</sup> This UGT enzyme is the main catalyst for the glucuronidation of 7-HC. According to Mizutani, phloxine B is a noncompetitive inhibitor.<sup>14</sup> In this study, the tissue slices were perfused with different concentrations of phloxine B to assess the IC<sub>50</sub> value (concentration in which 50% of the enzyme is inhibited) and confirm the mechanism of inhibition.

Finally, the ability to analyze unstable metabolites was demonstrated by incubating PCLS with diclofenac, a nonsteroidal anti-inflammatory drug for the treatment of inflammation and pain. In the rat, diclofenac is mainly converted into 4'- and 5-hydroxydi-

clofenac and diclofenac acyl glucuronide.<sup>15,16</sup> The last metabolite is considered responsible for serious idiosyncratic hepatotoxicity<sup>17</sup> but is, as many other acyl-glucuronides, chemically unstable. This impedes the quantification of this type of compound in conventional well-plate incubations, as discussed above. As a consequence, these metabolites can easily go undetected in the early stage of drug development, causing serious problems during clinical trials. Since the on-line system analyzes metabolites present in medium fractions directly after their formation, it is suitable to detect this unstable metabolite simultaneously with other metabolites.

## EXPERIMENTAL SECTION

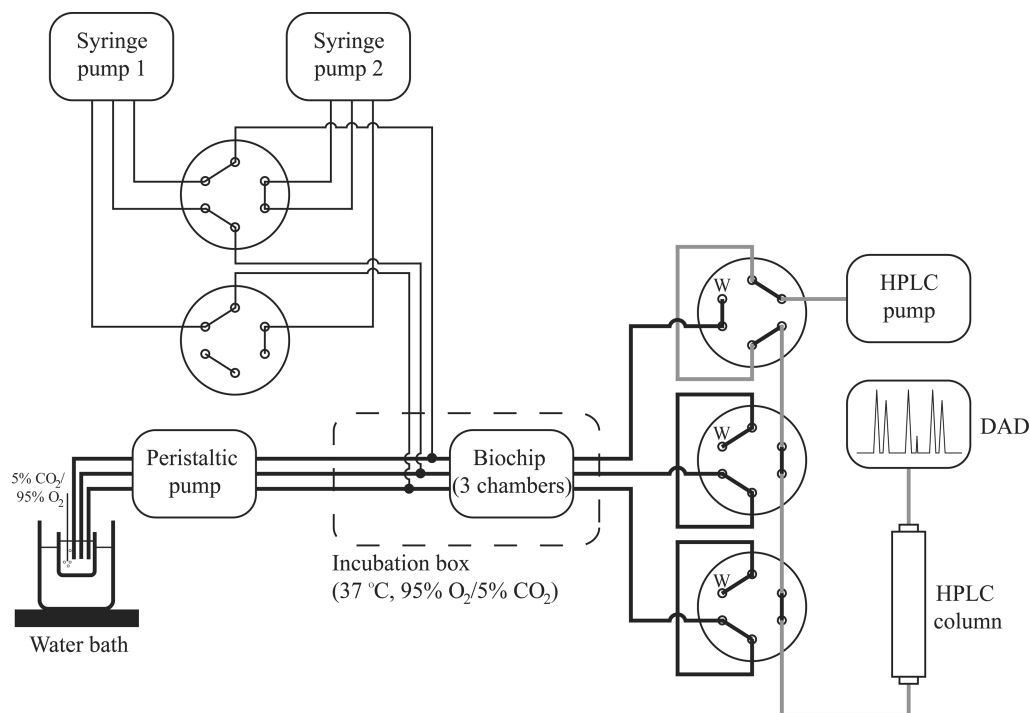
**Chemical Reagents.** Acetonitrile (ACN, HPLC grade) and methanol (MeOH, HPLC grade) were purchased from BioSolve B.V. (Valkenswaard, The Netherlands). Glacial acetic acid was obtained from VWR International B.V. (Amsterdam, The Netherlands). HPLC-grade water was prepared by passing demineralized water through an Arium 611 ultrapure water system (Sartorius, Göttingen, Germany). Gentamicin and Williams medium E (WME) with Glutamax-I were obtained from Gibco (Paisley, U.K.). D-Glucose monohydrate, ammonium formate, diclofenac (DCF) sodium salt, phloxine B, 7-HC, 7-HC-S, and 7-HC-G were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Diclofenac acyl glucuronide (DCF-AG), 4'-hydroxydiclofenac (4'-OH-DCF), and 5-hydroxydiclofenac (5-OH-DCF) were obtained from Toronto Research Chemicals (North York, Ontario, Canada). Stock solutions of 7-HC in MeOH (50 mM), DCF in DMSO (20 mM), and phloxine B in WME (20 mM) were prepared in-house. 7-HC and DCF solutions were stored at -20 °C, and phloxine B at 4 °C, until further use.

**Precision-Cut Rat Liver Slices.** Liver tissue from male Wistar rats (HsdCpb:WU) weighing ca. 300 g were used for the experiments in this study. Animal use was approved by the Animal Ethics Committee of the University of Groningen. PCLS were prepared as described before.<sup>4</sup> Slices had a wet weight of 5 mg and were approximately 4 mm in diameter and 250 µm thick (~14 cell layers). They were stored for a maximum of 2 h in ice-cold University of Wisconsin (UW) solution (DuPont Critical Care, Waukegan, IL, U.S.A.) until placement in the biochip.

**Instrumentation.** A schematic view of the developed on-line analysis system is given in Figure 1. The system consists of five different parts: (1) a peristaltic pump (medium delivery), (2) an incubation box including the microfluidic biochip which has three chambers to perfuse three slices simultaneously, (3) three injection valves, (4) two syringe pumps (substrate/inhibitor delivery) connected to two two-position micro electric actuator injection valves, and (5) an HPLC system. An Ismatec IPN peristaltic pump (Ismatec SA, Zurich, Switzerland) equipped with Pharmed tubing (i.d. 0.25 mm, VWR International B.V., Amsterdam, The Netherlands) was used for medium flow (10 µL/min). The medium itself was placed in a water bath kept at 37 °C. Carbogen (95% O<sub>2</sub>/5% CO<sub>2</sub>) was continuously bubbled through the reservoir containing medium to maintain a constant pH of

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**Figure 1.** Schematic overview of the on-line analysis system for metabolism and inhibition studies. The system consists of a peristaltic pump for medium flow (10  $\mu\text{L}/\text{min}$ ), two syringe pumps for substrate or inhibitor introduction (100  $\text{nL}/\text{min}$ ), two substrate valves to switch between syringe pumps, temperature-controlled humidified incubation box containing the biochip, and three injection valves to inject the samples onto an analytical HPLC column. A more detailed description of the system may be found in the text. Note that the topmost injection valve has been switched to bring the injection loop in-line with the HPLC pump for introduction of this sample onto the HPLC column: W = waste; DAD = diode array detector.

7.4 and a high level of oxygen. Williams medium E with glutamax-I (WME), supplemented with 50  $\mu\text{g}/\text{mL}$  gentamicin and 25 mM D-glucose, was used as medium for all the experiments described. The medium was delivered to a plastic container (incubation box, 200 mm  $\times$  100 mm  $\times$  100 mm (L  $\times$  W  $\times$  H)) by means of Pharmed tubing. Inside this incubation box, a PEEK T-piece (Upchurch Scientific, Murrieta, CA, U.S.A.) was placed right before the inlet of the biochip to rapidly mix the medium with substrate added from the syringe pump(s). PTFE tubing (0.50 mm i.d., Polyfluor Plastics, Oosterhout, The Netherlands) connected the T-piece with the biochip. The incubation box was equipped with a Peltier element (Okaphone, Groningen, The Netherlands), a temperature controller (Horst temperature regulator HT 30, Sigma-Aldrich, Zwijndrecht, The Netherlands), and two computer ventilators to maintain a temperature of 37  $^{\circ}\text{C}$ . The box was continuously supplied with humidified carbogen to ensure a well-controlled environment for tissue slice incubations. Individual slices were introduced into each of the three chambers of the poly(dimethylsiloxane) (PDMS) chip, which was then placed in the incubation box. The fabrication of the PDMS chip has been described extensively elsewhere.<sup>4</sup> The chip incorporates microfluidic channels to deliver medium to each of the three microchambers containing slices. Two polycarbonate membranes (10  $\mu\text{m}$  thick) form the ceiling and floor of each microchamber to realize an even distribution of medium flow around the tissue slice and to ensure that the slice is horizontally suspended in the flow (see the Supporting Information, section S.1). The chip also contains two 250- $\mu\text{m}$ -thick PDMS membranes above and below each chamber to act as

“breathing” membranes to keep the pH and oxygen levels stable in the incubation environment. The outlet of each chamber was connected with PTFE tubing to a separate injection valve (automated cartridge exchange unit, Spark Holland, Emmen, The Netherlands). The injection valves were equipped with 75  $\mu\text{L}$  PEEK injection loops (0.50 mm i.d.). At a flow rate of 10  $\mu\text{L}/\text{min}$ , it takes 7.5 min to fill each loop with medium containing substrate and metabolites.

To add substrate to the medium via the T-piece as mentioned above, two New Era syringe pumps (New Era Pump Systems Inc., Farmingdale, NY, U.S.A.) equipped with 500  $\mu\text{L}$  Hamilton syringes (Hamilton, Bonaduz, Switzerland) were used. The syringe pumps were computer-controlled to deliver 7-HC, phloxine B, or solvent as control (MeOH or WME) at a flow rate of 100  $\text{nL}/\text{min}$ . Silica capillaries (i.d. 75  $\mu\text{m}$ , Aurora Borealis, Assen, The Netherlands) were used to connect the syringes to the two-position micro electric actuator injection valves (VICI Valco, Schenkon, Switzerland). These valves were used to switch between the two syringe pumps to realize a rapid change from one reagent solution to the other. After stopping a syringe pump, there is always pressure on the syringes, resulting in continued delivery of a solution for a short time. With the substrate valves, substrate delivery could be instantaneously discontinued when the pump was switched off. Silica capillaries were used to connect the substrate valves with the T-pieces located just before the inlet of the chip.

Samples from each chamber were injected sequentially onto an HPLC system by switching the corresponding injection valve to bring the 75  $\mu\text{L}$  PEEK injection loop in-line with the HPLC system.

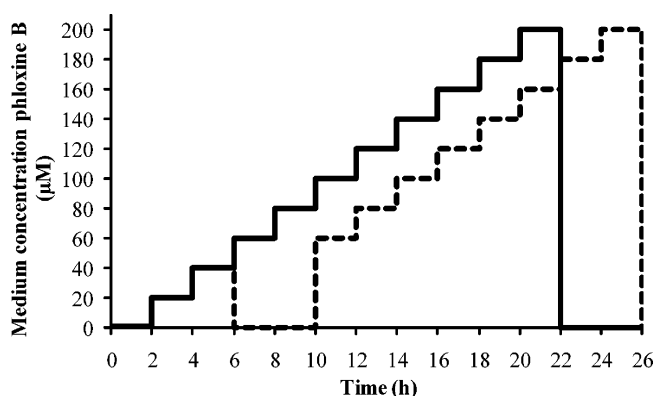


**HPLC Analysis of 7-Hydroxycoumarin Metabolites.** The analysis was performed on an automated HPLC-UV system consisting of an Agilent 1100 series LC system with low-pressure mixing (500  $\mu\text{L}/\text{min}$ ), column thermostat (30  $^{\circ}\text{C}$ ), and diode array detection (DAD) at 320 nm (Palo Alto, CA, U.S.A.). Separations were performed on a Hypersil 3.2 mm  $\times$  100 mm (5  $\mu\text{m}$ ) BDS C18 column (Alltech, Breda, The Netherlands), preceded by a 2.1 mm  $\times$  12.5 mm XDB-C8 guard column (Agilent, Palo Alto, CA, U.S.A.). After switching the injection valve, the sample was loaded onto the trap column with 85% solvent A (3% (v/v) ACN, 1% (v/v) glacial acetic acid, and 5 mM tetrabutylammonium hydrogen sulfate in water (pH 2.5)), and 15% solvent B (50% (v/v) ACN, 1% (v/v) glacial acetic acid, and 5 mM tetrabutylammonium hydrogen sulfate in water (pH 2.5)). With a flow rate of 500  $\mu\text{L}/\text{min}$  it takes 9 s to flush the injection loop. After injection, gradient elution of the analytical column was performed starting at 15% solvent B (85% solvent A) and increasing to 100% solvent B (0% solvent A) over 5 min. Before the next sample was injected, the column was flushed with 15% solvent B and 85% solvent A for 4 min. The injection valve was then switched back to the load position to refill the sample loop, and the next injection valve was switched to inject the next sample. The analysis of one sample takes 10 min. The procedure was performed sequentially for the three sample loops, which meant that the contents of each loop was injected once every 30 min.

**Metabolism Studies with 7-Hydroxycoumarin.** Slices were perfused with medium containing  $\sim 500$   $\mu\text{M}$  7-HC. This was achieved by filling the three substrate syringes with stock solution containing 50 mM 7-HC dissolved in MeOH. Because the infusion flow rate of this solution was 100-fold lower than the medium flow, the final concentration of 7-HC in the perfusion medium before entering the chip was  $\sim 500$   $\mu\text{M}$ . This concentration was used to be able to compare the results obtained with this new system with data obtained with liver slices in the conventional well-plate system, reported previously.<sup>12,18</sup> The second syringe contained MeOH as solvent control.

During a metabolism experiment, the slices were pre-incubated for 2 h in medium without substrate. The substrate valves were then switched to deliver 7-HC, and slices were further incubated with this compound for 2 h. Thereafter, the slices were incubated with medium containing a small amount of solvent control (1%) for 4 h and then again incubated with 500  $\mu\text{M}$  7-HC for 2 h. This procedure for 7-HC delivery was performed four times over a period of 24 h. The analysis of one sample takes 10 min. The procedure was performed sequentially for the three sample loops, which meant that the contents of each loop was injected once every 30 min. At the end of the experiment, the slices were taken out of the biochip and subjected to total protein content determination. Each experiment involved measurements in triplicate using three slices from one rat and was performed with slices from three rats. Before and after each experiment, a calibration curve was made by injecting 7-HC, 7-HC-G, and 7-HC-S standards onto the HPLC column.

For the inhibition studies, the system was slightly modified, since the intention was to increase the concentration of the inhibitor phloxine B over time. The substrate valves to switch



**Figure 2.** Calculated concentration of phloxine B in medium over time when changing the flow rates of syringe pumps 1 and 2. Though the total flow rate remained constant at 100 nL/min, individual pump flow rates were changed every 2 h to create a stepwise phloxine concentration gradient with a range from 0 to 200  $\mu\text{M}$ . The dashed line represents the concentration profile of phloxine B in the experiment incorporating the 4 h pause in phloxine delivery (from the 6 h to 10 h time point), performed to determine if this inhibitor is competitive or noncompetitive.

between solvents were replaced with PEEK T-pieces, making it possible to infuse substrates from both syringe pumps at the same time. One syringe pump was equipped with three syringes containing 20 mM phloxine B, the other syringe pump with three syringes containing solvent control (WME). Since phloxine B is light-sensitive,<sup>14</sup> all samples and syringes were wrapped in aluminum foil and no light was added to the incubation boxes. The syringe pumps were programmed to change the flow rate every 2 h. The flow rate of the pump delivering phloxine B was increased stepwise, while the flow rate of the pump delivering the solvent control was decreased accordingly. In this way, the concentration of phloxine B was changed every 2 h in steps of 20  $\mu\text{M}$ , while the overall flow rate (100 nL/min) was kept constant. The concentration of phloxine B was varied between 1 and 200  $\mu\text{M}$  (final concentration) (Figure 2). The slices were continuously perfused with WME containing 100  $\mu\text{M}$  7-HC. A concentration of 100  $\mu\text{M}$  was chosen instead of 500  $\mu\text{M}$  as in the previous experiments, to prevent cofactor depletion during the experiment. Slices were first incubated with 7-HC only, that is, without inhibitor. When stable metabolite formation was obtained, as determined by the on-line analysis system, the syringe pump delivering the inhibitor, phloxine B, was started.

Experiments were performed without inhibitor to measure the stability of 7-HC-G production over time, and with increasing phloxine B concentrations over time, as shown in Figure 2. To determine whether the inhibitor was competitive, experiments incorporating a pause in phloxine B delivery were also performed. This was accomplished after a 2 h incubation with 40  $\mu\text{M}$  phloxine B by switching the syringe pumps back to  $\sim 100\%$  solvent control for 4 h. Slices were perfused with medium containing 100  $\mu\text{M}$  7-HC during this time. After this 4 h period, the syringe pump program was resumed to further increase the concentration of phloxine B. Again, each experiment involved measurements in triplicate using three slices from one rat and was performed with slices from three rats.

To verify the IC<sub>50</sub> value obtained in the on-line system, the inhibition experiment was also performed in a conventional 12-well-plate incubation system. For this purpose, PCLS were pre-

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incubated for 2 h in wells filled with 1.3 mL of WME supplemented with 25 mM D-glucose and 50  $\mu\text{g/mL}$  gentamicin. Subsequently, slices were transferred to prewarmed plates into wells containing fresh WME, 100  $\mu\text{M}$  7-HC, and various concentrations of phloxadine B (0, 20, 40, 60, 80, 100, 150, 200  $\mu\text{M}$ ) and incubated for 4 h. Samples were stored at  $-20^\circ\text{C}$  until analysis by HPLC. In this case, three PCLS were required for each concentration of phloxadine B so that each experiment required 24 slices from one rat. Three experiments, one for each of three rats, were performed.

**Metabolism Studies with Diclofenac.** The equipment used to separate and detect DCF and its metabolites was similar to the separation and detection of 7-HC, with the exception of the column. Isocratic separation was performed on a 150 mm  $\times$  4.6 mm Hypersil gold column with 5  $\mu\text{m}$  C18 particles using a mobile phase consisting of 25% (v/v) ACN and 0.05 M ammonium formate (pH 6.2) dissolved in water. The flow rate was set at 1 mL/min, and the UV detector was set at 282 nm. The run time was 30 min.

Pilot experiments showed that DCF was not adsorbed onto the peristaltic tubing used for the medium delivery. Therefore, DCF was added directly to the medium at a final concentration of 200  $\mu\text{M}$ , and slices were continuously exposed to this substrate for 4 h. The three injection valves were switched sequentially, once every 30 min, to inject samples of medium containing DCF and its metabolites onto the HPLC. Again, one experiment was performed per rat, with each experiment involving the simultaneous testing of three liver slices in the trichamber microfluidic biochip device. Three rats were used.

As control, 12 liver slices per experiment were also incubated for 4 h in well plates containing 200  $\mu\text{M}$  DCF. Every hour, three slices were taken out of their respective wells, and the medium was directly collected and stored in the freezer at  $-20^\circ\text{C}$ . Samples were taken out of the freezer the day after incubation and analyzed with the same HPLC as was used for on-line analysis. Three experiments, one for each of the rats used in the microfluidic system and each employing 12 slices, were carried out.

## RESULTS AND DISCUSSION

**On-line System Diagnostics.** The HPLC methods used for the analysis of the metabolites produced by 7-HC and DCF were optimized to obtain fast and efficient separations. The reader is referred to section S.2 (Supporting Information) for more information on the methods.

Phenol red was present in the medium in all experiments to monitor the pH. The presence of the phenol red peak in the chromatogram could be used to advantage to assess the stability of both the HPLC run and the flow rate of the peristaltic pump. When blockage or leakage in the system occurred, it was immediately detected as a decrease in the peak area of phenol red, since the injection loop was not completely filled with medium in these situations. In all the experiments described in this report, the relative standard deviation (RSD) of the area of the phenol red peak recorded over a 24 h period did not exceed 1%, with a RSD of  $<0.05\%$  in retention time. This indicates a robust HPLC analysis over the 24 h period of the measurement.

To check the stability of the syringe pumps, the addition of 7-HC substrate was also monitored, since a change in the ratio of the peak areas for substrate and phenol red enabled the detection of potential problems. An increase in the peak area (substrate)/peak area (phenol red) ratio indicated a problem with medium

delivery, whereas a decrease indicated a problem with the substrate addition. A decrease in the areas of both peaks generally meant that leakage had occurred after the incubation chamber. If both peak areas remained constant but the metabolite peak areas were low, the tissue slices were no longer viable.

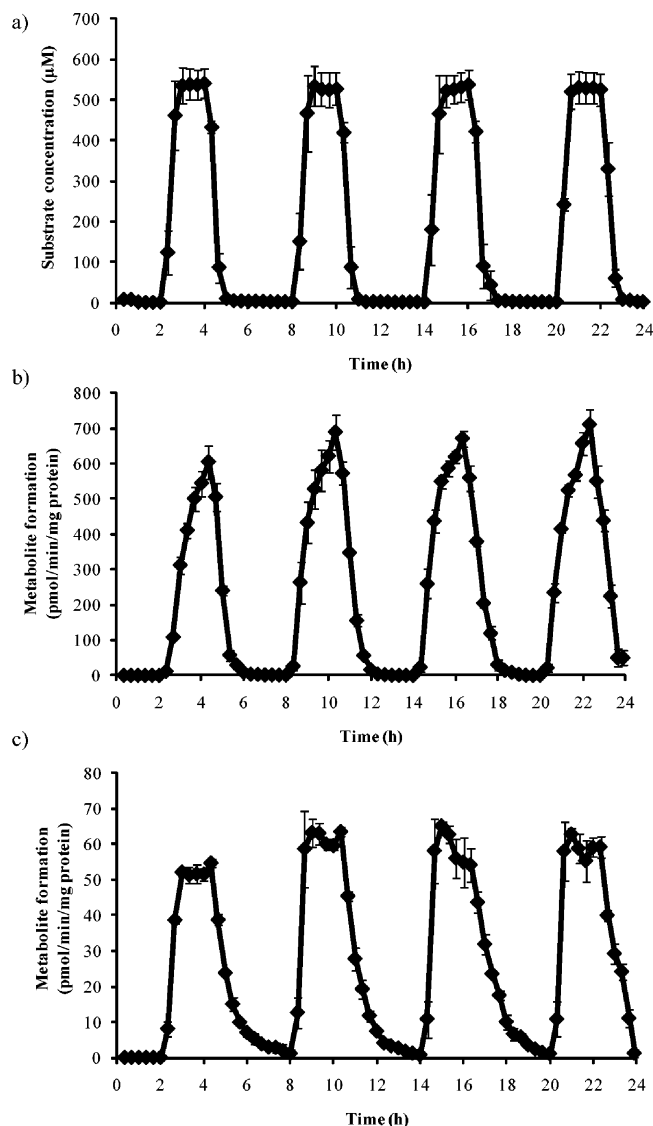
**7-Hydroxycoumarin Metabolism.** In well plates, slices are normally pre-incubated in medium without added xenobiotics to replace the tissue-slice storing solution (UW) with medium and remove the damaged cells on slice surfaces which inevitably arise from the slicing procedure.<sup>19</sup> Enzymes released by damaged cells, like proteases, might negatively affect liver function.<sup>20</sup> Therefore, this pre-incubation was also performed in our on-line analysis system. UW solution, cell debris, and leaked enzymes were flushed out of the microchamber with the continuous flow over the course of 2 h before metabolism experiments were started. The use of flow also has an advantage over well plates at this stage of these experiments, since slices remain in the same chambers after pre-incubation; a simple switch of a valve or pump upstream is enough to change the solution to which the slices are exposed. In contrast, slices must be removed from wells after pre-incubation and placed in new wells containing fresh medium when well plates are used, an extra handling step which is eliminated in the microflow case. The pre-incubation period also serves as a control for peaks in the chromatogram due to endogenous metabolites or medium components. No extra peaks were apparent during these 2 h compared to the blank runs recorded at 320 nm in the absence of tissue in the microchamber.

After 2 h, the syringe pump with 50 mM 7-HC was turned on to start the addition of 7-HC to the medium at 500  $\mu\text{M}$  final concentration. The profile of 7-HC concentration in the outflow during the experiment is given in Figure 3a. The calculated time needed to transport the substrate through the tubing and chambers and to fill the injection loops was 21 min (dead time). In practice, after the initial 2 h equilibration period, the 7-HC concentration achieved its maximum value within 30 min and was stable for the 2 h incubation period. This is in line with the calculated dead time. After 2 h, the syringe pump with 7-HC solution was turned off and the syringe pump with solvent controls was turned on, resulting in a direct decrease in the measured 7-HC concentration. This on-line substrate injection procedure was repeated three times with very repeatable results, as can be seen in Figure 3a.

When incubating the liver slices with 7-HC, the two phase II products were formed, as expected.<sup>12</sup> During the addition of substrate, the formation of 7-HC-G (Figure 3b) and 7-HC-S (Figure 3c) could be clearly detected. At this high concentration of 7-HC, the glucuronide is the major metabolite formed, as described previously.<sup>18</sup> Moreover, the metabolic rate for glucuronidation was around 600 pmol/min/mg of protein, which was similar with rates found previously.<sup>12,18</sup> Importantly, the formation of metabolite was found to be repeatable over the 24 h period measured. 7-HC was administered for four 2 h periods, and the same pattern for the formation of 7-HC-G was obtained, indicating that the slices remain functionally intact. Surprisingly, the peak area of 7-HC-G continuously increased over the 2 h period though the 7-HC concentration

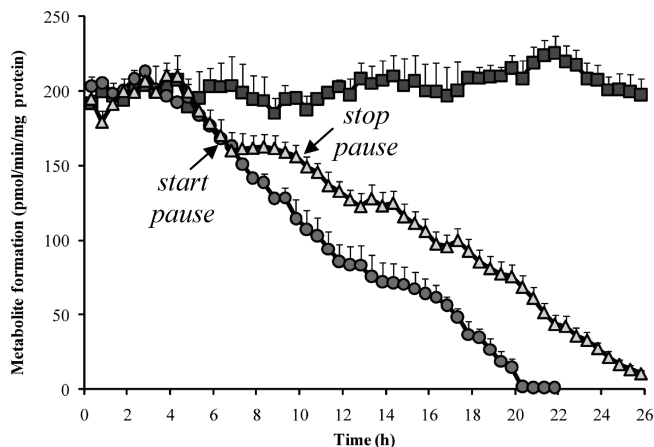
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**Figure 3.** On-line metabolism study of 500  $\mu\text{M}$  7-hydroxycoumarin in rat liver slices. (a) 7-Hydroxycoumarin (average  $\pm$  standard deviation) is converted into (b) 7-hydroxycoumarin glucuronide and (c) 7-hydroxycoumarin sulfate. The results plotted are the mean  $\pm$  standard error of the mean (SEM) of three rats, three slices per rat.

was constant. The same trend was obtained in all three experiments and for all four 7-HC administrations performed per experiment, as can be seen in Figure 3b. An increase in 7-HC-G production over time has also been observed by others in rat<sup>21</sup> and human liver slices,<sup>22</sup> but the mechanism of this increase is not clear. The formation of 7-HC-S, on the other hand, was very stable over time. This metabolite was also formed immediately after the addition of 7-HC was started, and a plateau in concentration was observed. The amount of 7-HC-S formed was approximately 10 times lower compared to that of 7-HC-G, which can be explained by the well-known higher maximum turnover rate of UGT enzymes compared to sulfotransferase.<sup>23</sup>



**Figure 4.** On-line inhibition study with 100  $\mu\text{M}$  7-hydroxycoumarin (7-HC) and varying concentrations of phloxine B in rat liver slices. Squares represent the formation of 7-HC-G without inhibitor, dots represent the 7-HC-G formation with increasing phloxine B concentration ranging from 0 to 200  $\mu\text{M}$ , and triangles represent the 7-HC-G formation with increasing phloxine B concentration, including an interruption in phloxine delivery for 4 h at 40  $\mu\text{M}$  phloxine B (mean  $\pm$  standard error of the mean, for three rats, three slices per experiment). The start and end of the interruption in phloxine B delivery are indicated with arrows.

The finding that metabolite formation was constant indicates that this on-line system is suitable for induction and/or inhibition studies.

**7-Hydroxycoumarin Glucuronide Inhibition.** The formation of 7-HC-G was relatively constant during 24 h of continuous perfusion of PCLS with 100  $\mu\text{M}$  7-HC, although again a slight increase over time was observed (Figure 4, squares). We can conclude that slices are viable and exhibit a constant rate of metabolism over 24 h under these conditions, which is a prerequisite for the inhibition studies performed in the present on-line set-up.

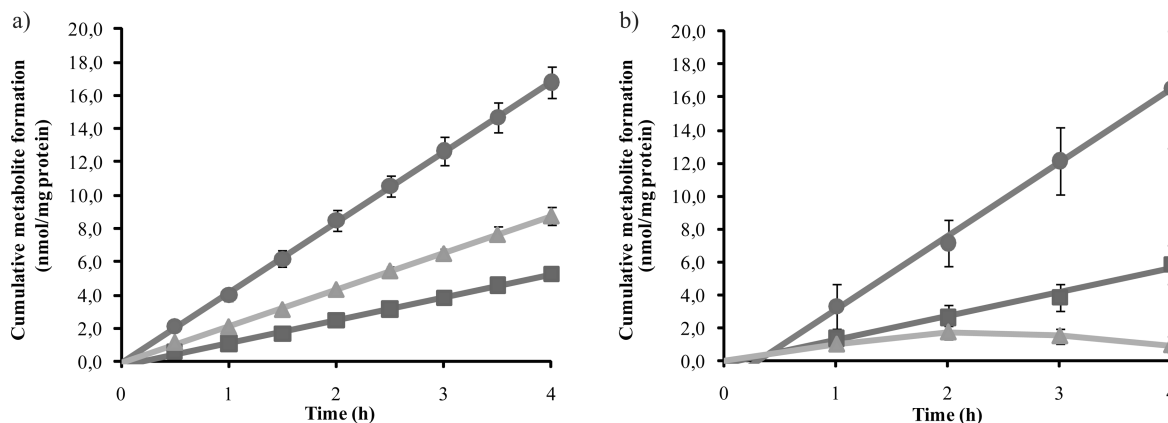
When starting the syringe pump program for phloxine B substrate addition, the metabolite formation was constant for 4 h (Figure 4, dots). This corresponds to a phloxine B concentration of 20  $\mu\text{M}$ , indicating that at this concentration 7-HC-G formation was not inhibited. When the concentration was increased to 40  $\mu\text{M}$  phloxine B, the metabolite formation began to decrease gradually and continued to do so as the phloxine B concentration was increased stepwise every 2 h. After 20 h, when the phloxine B concentration had reached 200  $\mu\text{M}$ , hardly any 7-HC-G metabolite was detected. An  $\text{IC}_{50}$  value (phloxine B concentration at which 50% of the enzyme is inhibited) of  $81 \pm 5 \mu\text{M}$  was calculated from this data. In a second experiment, the influx of phloxine B was interrupted after the 40  $\mu\text{M}$  infusion period (indicated with arrows in Figure 4) and slices were perfused with only medium with 7-HC for 4 h. The 7-HC-G formation remained constant during this period, and the observed metabolite formation did not increase. If inhibition were competitive, we would expect 7-HC-G formation to increase again up to the original metabolic rate as substrate gradually displaced inhibitor. However, the phloxine B inhibitor apparently remained bound to the enzyme even when no phloxine was being added, leading to the conclusion that phloxine B is not a competitive inhibitor. This data is in agreement

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**Figure 5.** Results of a metabolism study of 200  $\mu\text{M}$  diclofenac (DCF) for 4 h in (a) the biochip and (b) well plates. DCF is mainly converted into 4'-hydroxydiclofenac (4'-OH-DCF) (dots), 5-hydroxydiclofenac (5-OH-DCF) (squares), and diclofenac acyl glucuronide (DCF-AG) (triangles). The results plotted are the mean  $\pm$  standard error of the mean (SEM) of three rats, three slices per experiment.

with the observations made by Mizutani that phloxine B is a noncompetitive inhibitor for the human UGT1A6 isoenzyme.<sup>14</sup> However, from the data obtained with the on-line analysis system, no conclusion can be drawn as to whether phloxine B inhibition is reversible or irreversible. When the infusion of phloxine B was resumed in the experiment, the rate of formation of 7-HC-G continued to decrease, to the same extent as for experiments performed without interruption of inhibitor delivery (see Figure 4, triangles vs dots). The IC<sub>50</sub> value measured with the interruption was  $89 \pm 5 \mu\text{M}$ , which was not significantly different from the value obtained with continuous infusion. The IC<sub>50</sub> value measured for human UGT1A6 was  $40 \mu\text{M}$ ,<sup>13,14,24</sup> which is lower than the 80–90  $\mu\text{M}$  found in this study for rat liver UGT1A6 enzymes. Possibly, this reflects a species difference in the affinity of the phloxine B for the respective isoforms of UGT. To verify the IC<sub>50</sub> value obtained in the on-line system, comparable experiments were performed in well plates, with a co-incubation of 7-HC (100  $\mu\text{M}$ ) and phloxine B concentrations ranging from 0 to 200  $\mu\text{M}$ . An IC<sub>50</sub> of  $83 \pm 6 \mu\text{M}$  was found (data not shown), confirming our biochip data. Therefore, it can be concluded that the IC<sub>50</sub> of phloxine B for rat UGT1A6 is around 85  $\mu\text{M}$ .

**Diclofenac Metabolism.** A study investigating the metabolism of DCF, a nonsteroidal anti-inflammatory drug taken to reduce inflammation and pain, was undertaken, as this drug is known to produce a chemically unstable but presumably hepatotoxic metabolite, DCF-AG. Other important metabolites formed when liver slices are incubated with DCF are 4'-OH-DCF and 5-OH-DCF. The metabolite formation over a period of 4 h for the biochip on-line analysis system is shown in Figure 5a, and that for well-plate incubations is shown in Figure 5b. Both phase I products, 4'-OH-DCF and 5-OH-DCF, were formed at a constant rate for at least 4 h in the on-line system and the well plates, with comparable formation rates in both systems. DCF-AG was also formed at a very constant rate in the biochip, as can be seen in Figure 5a (triangles). In well plates, however, the amount of DCF-AG decreased over time. To determine the cause of the disappearance of DCF-AG in well plates, this compound was incubated at 37 °C in WME for 5 h in the absence of liver slices. Samples were taken and analyzed by HPLC. Results showed that the DCF-AG

concentration decreased over time with a half-life of 30 min, with the simultaneous appearance of new peaks in the chromatogram (data not shown). A shoulder was also observed on the DCF peak. This clearly indicated that the disappearance of DCF-AG is due to chemical decomposition rather than inhibition of the acyl glucuronidation in the liver slices. Similar peaks also appeared in the chromatograms recorded for medium samples collected after incubation of slices with DCF in well plates. It is likely, then, that these peaks are due to breakdown products of DCF-AG. These extra peaks did not appear when medium samples for incubation of slices with DCF were analyzed using the on-line system. It can therefore be concluded that the DCF-AG formed and excreted by the liver slices decomposes in the medium in the well-plate system and is not formed by the liver slices. Decomposition products may include DCF and glucuronic acid, as mentioned by Grillo et al.<sup>25</sup>

## CONCLUSION

A novel on-line HPLC system for the analysis of the metabolism of precision-cut liver slices was developed and tested with two substrates and a UDP-glucuronyltransferase inhibitor. Slices were incubated in small microchambers in a flow-through microfluidic device. The use of microfluidic technologies meant that metabolite concentrations were constant in the medium outflow of the device. Moreover, metabolite concentrations were not diluted in the early stages of an experiment as they are in well plates, ensuring concentrations which were high enough for detection with a conventional HPLC-UV system over all time points of the experiment. Although the system used in this study employed a UV detector, detection sensitivity could also be improved in the future through use of fluorescence detection or mass spectrometry (MS). MS would additionally allow the identification of metabolites of new chemical entities on-line. Since medium samples do contain salts, the possibility of ion suppression would need to be taken into account when using MS, but there are simple approaches for solving this problem (e.g., nanoLC<sup>26</sup>).

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With the on-line system, it was possible to monitor the conversion of 7-HC to its metabolites by liver slices over time, with phase II metabolism of 7-HC proving to be very stable over a period of at least 24 h. Given this level of performance, the system should allow the investigation of circadian rhythms,<sup>27</sup> and in fact, a small circadian variation could be observed for 7-HC-G. The ability to measure an inhibition-type drug–drug interaction on-line was demonstrated using the inhibition of rat UGT1A6 by phloxicine B as example. The decrease in metabolite formation was not competitive in nature, and an IC<sub>50</sub> value of ~85  $\mu$ M was determined for this particular inhibitor. The IC<sub>50</sub> value was verified with the conventional well-plate system, which also yielded an IC<sub>50</sub> value of 85  $\mu$ M. Interestingly, 24 slices per experiment were needed to obtain the same result in the well-plate system study as in the on-line system, where only three slices were used per experiment. This system is thus also very interesting for experimentation with tissue which is not readily available, such as human material.

Due to the incorporation of two syringe pumps, it was possible to switch easily between two substrates with a relatively fast response (<30 min of dead time). The ability to mix two solutions at different flow rate ratios to create a concentration gradient over time was also demonstrated with the rat UGT1A6–phloxicine B enzyme inhibition example. This demonstrates the flexibility of this system and its potential suitability for many other applications, such as the measurement of enzyme conversion rates at different substrate concentrations with one tissue slice.

The ability to measure unstable metabolites was demonstrated with the incubation of PCLS with DCF. The three major metabo-

lites, 4'-OH-DCF, 5-OH-DCF, and DCF-AG, were detected. The on-line system clearly revealed the very stable formation of the DCF-AG metabolite. This is in contrast to well plates, in which DCF-AG was observed at much lower initial concentrations which then also decreased over time. As the peak area corresponding to DCF-AG decreased, other unidentified peaks appeared in the chromatograms. With the on-line system, it was possible to show that these unknown peaks were not metabolites produced by the liver but, most likely, breakdown products of the DCF-AG formed in the medium.

Advantages of this on-line analysis system include speed of analysis, direct information about tissue slice activity, elimination of sample storage problems, and the capability to detect unstable metabolites. Use of an automated system for switching syringe pumps and injection valves means that much of the original manual labor associated with this type of analysis is circumvented. The use of HPLC enables the analysis of complex mixtures for a number of components simultaneously over a wide concentration range. Several measurements can be performed with one slice, which is beneficial for applications requiring scarce tissue. This on-line system therefore also could potentially contribute to the reduction of the number of experimental animals needed for drug development.

#### **SUPPORTING INFORMATION AVAILABLE**

Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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