The Suitability of Hepatocyte Culture Models to Study Various Aspects of Drug Metabolism

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Summary
Since the liver is the main organ involved in the metabolism and the toxicity of xenobiotics, isolated rat hepatocytes have been increasingly used in recent years as a model to identify pharmacological and toxicological responses of drugs. However, it is generally recognised that isolated hepatocytes retain most of their functions only for a short period. For this reason, numerous models and techniques have been developed to study and improve the metabolic capacity of hepatocytes in vitro over an extended time period and in application for drug metabolism studies. In the present study, we compared four different cell culture models to fulfill these requirements and have therefore harvested hepatocytes and cultured them in different culture systems over two weeks. In order to prove certain advantages or disadvantages of each model, we compared the metabolic capacity, albumin secretion, the release of cytosolic and mitochondrial enzymes, as well as the capacity to metabolise diclofenac (DF).

We found that rat hepatocytes in all studied culture models (except the Unisyn Bioreactor) were able to metabolise DF to the same extent as found in vivo. However, the concentration of metabolites was found to decrease with culture time using the monolayer although the DF metabolite level in the collagen Sandwich culture was higher than that of the monolayer culture. The 3D-membrane bioreactor preserved the metabolic capacity for a prolonged period of time. The concentrations of DF metabolites in the Unisyn hollow fiber bioreactor were below the detection limit, which corresponded to other parameters such as albumin secretion and cytochrome P450 activity, disqualifying this culture system clearly for the use of in vitro primary hepatocyte cultures. The other three systems all have their place in drug metabolism with different advantages. However, our studies clearly showed that hepatocytes cultured within a collagen sandwich or in the 3D-membrane bioreactor qualify to study various aspects of drug metabolisms over a long time period. Further studies are needed to prove if the later two culture models may really help to reduce animal testing.

Zusammenfassung: Die Verwendbarkeit verschiedener Hepatozyten – Kulturmodelle für in vitro Metabolismusstudien


Keywords: 3R, in vitro, hepatocytes, drug screening, cell culture
1 Introduction

The liver is one of the main organs involved in the biotransformation of xenobiotics into pharmacologically inactive, active or sometimes toxic metabolites. Although the whole liver consists of different cell types, several authors have clearly demonstrated that freshly isolated parenchymal cells, mainly hepatocytes, can express most of the functions of the intact liver and are thus suitable for biotransformation studies of xenobiotics (for review see Castell et al., 1997). In general, these studies are performed either directly on freshly isolated cells or in short-term cultures due to the rapid degeneration of hepatocytes in vitro (Dich et al., 1988). Due to the rapid loss of hepatocyte function in vitro investigators have searched for alternative approaches to address relevant questions related to drug development. The use of microsomes, recombinant enzymes or the over-expression of drug-metabolising enzymes in cell lines are helpful to acquire a maximum of information. However, these "systems" will not provide sufficient information about possible reactions (e.g., formation of phase II metabolites) or interactions of drug candidates with an "in tact" liver cell. Therefore, the use of hepatocytes is indispensable to obtain all possible information about metabolising pathways of a new possible drug candidate (for review see Castell et al., 1997).

In order to maintain the phenotype of normal hepatocytes in culture, several approaches have been employed to improve their function including the co-culture of hepatocytes with liver-derived endothelial, endothelial cells or epithelial-like cell lines (Guguen-Guillouzo et al., 1983), or the use of matrigel (Sinclair et al., 1979). An appropriate in vitro model would have many advantages such as the reduction of animal assays, saving time when evaluating new drug candidates or the development of safer drugs. However, major limitations are set on the use of hepatocyte cultures to evaluate drug candidates. In particular the loss of cytochrome P450 (CYP) and it's associated monoxygenase activities as well as albumin secretion (Acosta et al., 1979; Clayton and Darnell, 1983; Skett, 1994). It is speculated that the loss of CYP 450 in hepatocytes is due to an adaptive response to the culture environment (Holme, 1985). To overcome this deficit, several defined culture media were developed (Nelson et al., 1982; Narita et al., 1985; Lindsay et al., 1991; Dich et al., 1988; Isoms et al., 1985).

One promising approach to maintain hepatocyte functions over an extended period of time was the establishment of a so called "Sandwich" culture where hepatocytes are embedded between two extracellular collagen matrices. This three-dimensional collagen sandwich technique aims to reconstitute the in vivo microenvironment of hepatocytes in the intact liver to maintain their phenotype, morphological, metabolic capacity, and stable albumin secretion (Dunn et al., 1989; Bader et al., 1992; Michalopoulos et al., 1993; Kern et al., 1997). These experiments demonstrated that environmental conditions close to those existing in the intact liver must be mimicked to maintain hepatic functions. Additional factors were identified to further improve in vitro culture conditions which included a continuous supply of carbon dioxide and oxygen supply, and metabolite removal. Moreover, a "perfect" in vitro system would facilitate cell sampling and provide sufficient metabolic capacity to study various aspects of liver functions. To meet these criteria, various hollow fiber bioreactor systems were developed using various hepatocyte species (see for review Busse et al., 1999). In these systems, cells attach to the surface of fibers or membranes and reorganise themselves into three-dimensional structures that may result in a hepatocyte microenvironment closely related to the physiological situation. However, several problems still occur with the use of various cell culture systems: i) most systems are pilot models; ii) they are rarely evaluated in other laboratories; iii) they are only suitable for certain cell types and iv) culture conditions are often poorly defined.

To study various aspects of liver diseases or the biotransformation of xenobiotics investigators use different hepatocyte species. We selected the rat as an experimental model since this species is widely used, easy to maintain in most laboratories, and the hepatocyte isolation is a standard procedure guaranteeing a high degree of reproducibility. We here evaluate four different cell culture models (monolayer cultures, sandwich cultures, and two different bioreactors) with regard to various aspects of drug metabolism. We measured the membrane leakage of transaminases (LDH, AST, ALT), as well as for how long the culture systems would retain their metabolic capacity (EROD, ECOD), and albumin secretion. To compare the culture systems with regard to the possible use as a drug screening system we chose diclofenac-sodium (DF-Na) since a considerable amount of pharmacokinetic and pharmacological data exist about this drug allowing a fair comparison with existing in vivo data.

2 Animals, materials and methods

2.1 Materials

William's medium E, penicillin-streptomycin, HEPES and calf serum were purchased from GIBCO BRL (Paisley, Scotland). Percoll was obtained from Pharmacia (Uppsala, Sweden). Collagenase H was obtained from Boehringer Mannheim (Mannheim, Germany). LDH, AST, and ALT test kits were purchased from Sigma (Deisenhofen, Germany). All other reagents were also obtained from Sigma unless indicated otherwise. Omeprazol-Na was kindly provided by Astra GmbH (Wedel, Germany).

2.2 Animals

Adult male Sprague-Dawley rats weighing 250±50 g were obtained from Charles River, Sulzfeld, Germany. Animals were kept at constant temperature, humidity, and fed commercial rat chows. Animals fasted 12 hours prior to perfusion, and had continuous free access to tap water ad libitum. The animals were maintained in accordance with the institutional animal welfare guidelines of the University.

2.3 Isolation of rat hepatocytes

A perfusion solution (Buffer 1), consisting of 0.142 M NaCl, 6.7 mM KCl, 10 mM HEPES was prepared and adjusted to pH 7.5. Prior to use EGTA (2.5 mM final concentration) was added to withdraw calcium and the solution warmed to 37°C. The second perfusion solution (Buffer 2) contains the following: 6.7 mM NaCl, 0.67 mM KCl, 0.48 mM CaCl2, 10 mM HEPES and 0.5% Albumin and adjusted to pH 7.5. Prior to use, collagenase type H was dissolved at a final concentration of approximately 20 mg/100 ml and the solution warmed to 37°C. Culture medium con-
sisted of modified William's medium E, and was supplemented with 1 μM insulin, 15 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 1.4 μM hydrocortisone, and 10% fetal calf serum. Rat hepatocytes were isolated by a modification of the two-step in situ collagenase perfusion technique as described (Seglen, 1976). The liver was perfused in situ with buffer 1 for app. 15 minutes, then explanted from the cavity, and perfused with buffer 2 for an additional 20 minutes. After dissociation of hepatocytes from the tissue, the crude cell suspension was filtered through a sterile gauze, and centrifuged at 50 g for 5 min at 4°C. In order to achieve high purity, the cell pellet was re-suspended and passed over a 30% Percoll gradient (50 g, 25 min at 4°C) to remove non-parenchymal and dead cells. The final cell pellet was re-suspended in culture medium and hepatocyte purity was assessed under light microscopy and viability was measured by trypan blue exclusion. Using this isolation procedure the viability of cells was always higher than 95% and we isolated app. 1.6 x 10⁸ hepatocytes from one rat liver.

2.4 Cultivation of rat hepatocytes (see table 1)

(i) Monolayer cultures of rat hepatocytes: Freshly harvested hepatocytes were seeded out onto 1% collagen pre-coated sterile 6-well culture plates (1.5 x 10⁶ cells/well; Becton Dickenson, F-Le Pont de Clai) and incubated at 37°C in a humidified incubator at 95% air 5% CO₂. Approximately six hours later, medium and unattached hepatocytes were removed and replaced with 2.0 ml fresh medium. Then, the medium was changed daily. Aliquots of supernatants were taken at different time intervals, and stored at -80°C for further analysis (see Figure 1A).

(ii) Sandwich cultures of rat hepatocytes: Primary rat hepatocytes were cultured in sterile 6-well culture plates (Becton Dickenson, F-Le Pont de Clai) between two layers of collagen as described (Michalopoulos et al., 1993). The two collagen layers (each layer 0.7 ml thick) consist of ten parts collagen solution (Vitrogen 100, Collagen Corporation, Fremont, CA, USA) and one part of 10x Waymouth medium (Life Technology GmbH, D-Karlsruhe). The collagen concentration was app. 2.6 mg/ml. The gel mixture was evenly spread on a culture plate, and polymerisation was allowed for 20-30 min at room temperature before hepatocytes were seeded onto the collagen layer. Following an additional cell attachment period, excess medium was removed and cells were covered with a second collagen layer. Then, after a second period of polymerisation, cultures were covered with 2.0 ml culture medium and placed in a humidified incubator at 95% air 5% CO₂, at 37°C. The medium was changed daily, and supernatants were collected and stored at -80°C for further determination (see Figure 1B).

(iii) The Unisyn-Hollow fiber bioreactor: The Unisyn bioreactor (kindly provided by In Vitro Systems & Services Inc., D-Osterode, see Figure 1C): The reactor and tubing circuits were placed in an apparatus with temperature control (37°C), supply of oxygen, carbon dioxide and air, in order to reach a final equilibrium of 95% air 5% CO₂ and a roller pump for medium perfusion. In order to set up the system, about 250 ml culture medium was used to flush the system (cell compartment and tubing) for 24 hours before equilibrating the system with fresh medium. In each experiment 1.6 x 10⁸ rat hepatocytes were introduced into the reactor. During the first six hours, the reactor was constantly gently rotated to allow an even cell distribution throughout the intercapillary network. Constant circulating medium supply occurred in a closed system at a perfusion rate of 10 ml/minute, and was changed daily. The culture conditions were exactly as suggested by the manufacturer. At different times aliquots were taken and stored at -80°C for the further analysis.

(iv) 3D-membrane bioreactor (Figure 1D): This reactor was filled with 1.0 x 10⁸ rat hepatocytes. During the first six hours, the reactor was manually turned upside down every half hour in order to achieve an even cell distribution within the capillary bed (Gerlach et al., 1994; Zeilinger et al., 2000). Within the bioreactor, three independent capillary systems provide gas exchange, medium supply and removal of cell debris and metabolites. In detail, the reactor circuit was controlled by precisely regulated pump units for bioreactor perfusion and medium supply. A temperature-controlled heating unit and flow-meter for oxygen/carbon dioxide and air exchange, medium...

![Figure 1: Scheme of the four culture systems used in the present study. ICS: Intra Capillary Space; ECS: Extra Capillary Space.](image-url)

**Table 1: Summary of the investigated culture models**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Monolayer Culture</th>
<th>Sandwich Culture</th>
<th>Unisyn Bioreactor</th>
<th>3D Membrane Bioreactor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell number per well or unit</td>
<td>1.5 x 10⁸</td>
<td>1.5 x 10⁸</td>
<td>1.6 x 10⁸</td>
<td>1.0 x 10⁸</td>
</tr>
<tr>
<td>Medium [ml]</td>
<td>2.0</td>
<td>2.0</td>
<td>250</td>
<td>100</td>
</tr>
<tr>
<td>Cells per ml</td>
<td>0.75 x 10⁶</td>
<td>0.75 x 10⁶</td>
<td>0.84 x 10⁶</td>
<td>1 x 10⁶</td>
</tr>
<tr>
<td>Flow rate</td>
<td>No</td>
<td>No</td>
<td>Circulating rate</td>
<td>5 ml/h</td>
</tr>
<tr>
<td>Medium change</td>
<td>Daily</td>
<td>Daily</td>
<td>Daily</td>
<td>Continuous</td>
</tr>
</tbody>
</table>

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able constant temperature and gas exchange. The total amount medium in the system (bioreactor and tubing) was 100 ml. The perfusion rate was 8 ml/minutes and the fresh medium was continuously added at a flow rate of 5 ml/hour finishing in a complete medium exchange within 24 h. At different times aliquots were taken and stored at -80°C for the further analysis.

2.5 Measurement of hepatocellular damage and analysis of albumin secretion
To evaluate the hepatocellular damage following various treatments and culture times, culture supernatants drawn from hepatocytes incubated for different time periods were tested for lactate dehydrogenase (LDH), aspartate transaminase (AST), and alanine transaminase (ALT) release using a commercially available ELISA kit (Sigma). The continuous albumin synthesis in the culture systems was measured by the release of albumin into the culture medium using a NEPHRAT albumin test kit (Exocell, INC. Philadelphia, USA).

2.6 Induction and enzyme activity of cytochrome P-450
   (i) CYP 1A: Rat hepatocytes were incubated in the presence or absence of 3-methylcholanthrene (3-MC) or Omeprazol dissolved in culture medium at a final concentration of 2.5 μM and 250 μM, respectively. The culture medium was changed daily for 14 days in the presence or absence of inducing agents. CYP 1A activity was assessed by determination of the associated deethylation of ethoxyresorufin (EROD) in all cell cultures (Bruke and Mayer, 1974). Briefly, cells were washed and incubated with fresh culture medium and subsequently incubated with 25 μM ethoxyresorufin and 1.5 mM salicylamide for two hours. Salicylamide was added to prevent conjugation of 7-hydroxy metabolites of 7-ethoxyresorufin (Burke and Orrenius, 1978). Then, the supernatant was transferred to a microtiter plate to measure the formation of 7-hydroxyresorufin using a Fluorescent Spectrophotometer with an excitation of 530 nm and an emission wavelength of 590 nm. Standard curves were generated in culture medium spiked with 7-hydroxyresorufin. EROD activity was calculated from this curve using the BMG software and was expressed in picomoles resorufin/min/10⁶ cells.

(ii) CYP 3A: Rat hepatocytes were incubated in the presence or absence of 2.5 μM 3-MC or 2 mM phenobarbital. The medium was changed daily for 14 days in the presence or absence of the inducing agents. Following a defined induction period, CYP 3A activity was measured employing the ethoxycoumarin-O-deethylation (ECOD) assay (Fry et al., 1980). Briefly, cells were washed and incubated with fresh culture medium and subsequently incubated with 25 μM ethoxycoumarin and 1.5 mM salicylamide for two hours. Salicylamide was added to prevent conjugation of 7-hydroxy metabolites of 7-ethoxycoumarin (Burke and Orrenius, 1978). Then, the supernatant was transferred to a microtiter plate to measure the formation of 7-hydroxycoumarin using a Fluorescent Spectrophotometer with an excitation of 390 nm and an emission wavelength of 460 nm. Standard curves were generated in culture medium spiked with 7-hydroxycoumarin. ECOD activity was calculated from the standard curve using the BMG software and was expressed as formed 7-hydroxycoumarin in picomoles/min/10⁶ cells.

2.7 Metabolism of diclofenac-sodium (DF-Na; MW 318.1) in rat hepatocyte cultures
In the first set of experiments we tried to decipher the optimal DF-Na concentration which would give sufficiently high concentrations of DF-metabolites to analyse in culture supernatants. Therefore, hepatocytes cultured in the monolayer- and sandwich system were incubated with 0.01, 0.05, and 0.1 mM DF-Na for 24 h. Then, supernatants were collected and stored at -80°C until final analysis. Based on these results we decided to use 0.1 mM DF-Na for all further experiments since at this concentration we observed a sufficiently high metabolism rate and quantity of the drug in the studied cell culture systems. In order to investigate the rate and extent of DF metabolism following prolonged cultivation of hepatocytes, cells were exposed to 0.1 mM DF on days 2, 5, 8, and 14. Samples were drawn from the systems immediately (= 0 h) as well as at 4 h, 8 h, 12 h and 24 h after addition of

Figure 2: Time-dependent LDH, ALT, and AST membrane leakage from rat hepatocytes cultured in different models. The medium was changed as described before and samples collected for enzyme leakage at the indicated time points. Data are always given as the mean ± SE of 4 to 6 experiments done in triplicates. For the bioreactors, results represent the mean ± SE of 3 experiments. All data are expressed as mU/24 h/10⁶ cells.
2.8 Determination of diclofenac and the metabolites 4'-hydroxy and 5-hydroxy diclofenac

The frozen supernatants of hepatocyte cultures were thawed at 20°C and in the case of triplicate assays (monolayer and sandwich cultures) the supernatants were combined and mixed. Solid phase extraction columns (Bakerbond SPE Octadecyl-C18, J.T. Baker Inc., Phillipsburg, NJ, USA, V =3 ml), containing 200 mg RP-C18 resin were conditioned with 3 ml methanol and 3 ml 0.1 N HCl. One ml of the samples was transferred into a separate vial, mixed with 50 μl ISTD (1.586 ng flufenamic acid) and placed onto a SPE column. The resin was washed with 3 ml 1 mM K-phosphate buffer, pH 5.0. Elution of the analytes was effected with 2x2 ml butylmethylether/ cyclohexane (2:1 v/v). The solvent was removed under a stream of nitrogen and the residue was reconstituted by addition of 350 μl H2O/methanol (80:20) and 5 min ultrasonic treatment. Sixty μl thereof were subjected to LC-MS/MS analysis employing a triple stage quadruple mass spectrometer (Quattro II, Micromass, UK-Altrincham) equipped with a Jasco HPLC system (HPLC-pump PU 980, Degasser DG 980-50, Gradient Unit LG 980-02 and Autosampler AS 950, Jasco, D-Groß-Umstadt). Separation of analytes was achieved on a Purospher RP18e (75x4 mm i.d., dp=5 μm) with 20 mM ammonium acetate, pH 6.9/acetonitrile (35/65, isocratic mode) at a flow rate of 1 ml/min. Ionisation was performed applying the electrospray mode (ESP+, source temperature: 150°C, probe temperature: 400°C, drying gas: 400 l N2/h, sheath gas: 100 l N2/h, collision gas: argon) Detection of diclofenac, its metabolites and the internal standard was achieved by multireaction monitoring. Collision induced dissociation (CID) was employed with argon as collision gas for generation of product ions from precursor ions (CID-pressure 2.5*10^-3 mbar). Monitored pairs of m/z values were 296.0 > 215.0 (diclofenac, DF), 312.0 > 293.0 (4'-hydroxy-diclofenac) and 282.0 > 264.0 (flufenamic acid). Prior to the analysis of samples from hepatocyte cultures, calibrations curves for diclofenac (373.3-14,930 ng/ml) and 4'-hydroxy-diclofenac (53.2-2,128 ng/ml) were established.

Figure 3: Albumin secretion of rat hepatocytes cultured in different models. Medium was changed as indicated before and samples collected at the indicated time points. Results are expressed as μg albumin/h/10⁶ cells. Data are always given as the mean ± SE of 4 to 6 experiments done in triplicates. For the bioreactors, results represent the mean ± SE of 3 experiments.

3 Results

3.1 Effects of culture time on transaminase membrane leakage in the investigated cell culture systems

Hepatocyte enzyme leakage decreased with culture time in monolayer and sandwich cultures. However on day five, hepatocytes cultured as monolayer showed an increase of enzymes leakage (Figure 2A). Hepatocytes embedded in collagen showed a continuous decline of LDH activity over the first eight days, thereafter the levels were below the detection limit. The levels of AST and ALT did not change significantly over the investigated study period (Figure 2B). Hepatocytes cultured in the Unisyn bioreactor showed little change in transaminase activity (Figure 2C). Cells cultured in the 3D-membrane bioreactor showed one initial peak of LDH in the supernatant 24 hours after hepatocytes were seeded out, then, the levels dropped continuously over the next...
3.3 Effects of culture time on cytochrome P450 inducibility in rat hepatocytes

3.3.1 Induction of CYP 1A1/2 activity measured as EROD activity in rat hepatocytes following Omeprazol, and 3-MC incubation

Rat hepatocyte's basal and induced EROD activity was measured in the presence and absence of Omeprazol or 3-methylcholanthrene (3-MC). Our data clearly show that basic CYP1A activity distinctly decreased with culture time in rat hepatocytes cultured as monolayer or embedded in collagen. The loss of basic hepatic EROD activity in monolayer cultures was similar to that observed in sandwich cultures (Figure 4A and B). As shown in Figure 4A and B, maximum induction of this CYP isoform was reached when cells were continuously exposed to either drug during three days. Once hepatic peak CYP 450 1A1/2 was reached, a rapid decline of the activity was observed, although the cells were further exposed to CYP inducer. In monolayer cultures, peak EROD activity reached 4.0- and 5.7-fold over untreated controls when exposed to Omeprazol and 3-MC, respectively. Hepatocytes embedded in a collagen matrix showed peak EROD activity of 2.8- and 9.2-fold when exposed to Omeprazol or 3-MC, respectively. Basic EROD activity in the 3D-membrane bioreactor remained the same over two weeks and increased to a maximum of approximately threefold when cells were exposed to 3-MC (Figure 4C).

3.3.2 Cytochrome 3A activity measured as ECOD activity in rat hepatocytes following the exposure to phenobarbital and 3-MC

Basic and induced hepatic ECOD activity was evaluated using control medium or medium containing 2 mM phenobarbital or 2.5 μM 3-MC over several days. As depicted in Figure 5A and B, the basic ECOD activity declined with culture time in all culture models. In monolayer cultures hepatic ECOD activity peaked on day two and reached a 2.9- and 7.5-fold
increase when exposed to phenobarbital or 3-MC, respectively. Hepatocytes embedded in collagen showed also a peak activity on day two with a maximum of 2.2- and 3.4- fold increase by phenobarbital or 3-MC, respectively. Hepatocytes embedded in collagen showed also a peak activity on day two with a maximum of 2.2- and 3.4-fold increase by phenobarbital or 3-MC, respectively. Taken together, the metabolic capacity to metabolise certain drugs through CYP 450 3A seems to be lower in hepatocytes embedded in collagen rather than cultured as monolayer. It is interesting to note, that ECOD activity in the 3D-membrane bioreactor showed almost no changes in the presence of the CYP 450 inducer 3-MC as compared to untreated control cultures (Fig. 5C). In general, it is noteworthy, that ECOD levels were very low in this system which maybe related to possible interactions (e.g., absorption) between collagen, tubing, membranes and the CYP inducers as well as substrates (Zeilinger et al., personal communication). No basic or inducible ECOD and EROD activity was observed when rat hepatocytes were cultured in the Unisyn bioreactor using either 3-MC, Omeprazol-Na or phenobarbital as CYP inducer (data not shown).

3.4 Metabolisation of Diclofenac in the different cell culture systems
As demonstrated in Fig. 6 and Fig. 7, hepatocytes cultured as monolayer, between rat collagen layers as well as in the 3D-membrane bioreactor were able to metabolise DF-Na. Fig. 6 shows that only a small portion of DF-Na was metabolised to the main metabolites 4'-OH-DF (Fig. 6B), and 5-OH-DF (Fig. 6C) as compared to the added amount (Fig. 6A). The later result, however, might be bias since we also observed that the two main metabolites were only stable over a short time period when incubated in medium without cells. The main metabolites 4'-OH-DF, and 5-OH-DF disappeared in the culture medium in the absence of cells. The metabolite 5-OH-DF completely disappeared within 96 h while 4'-OH-DF disappeared to 30% probably by binding to components of the culture medium (Table 1). Furthermore, it can not be excluded that additional interactions might exist between the collagen, plastic, cells and the metabolites. In Fig. 7 we show the time course of metabolite production (4'-OH-DF) and the reduction of originally added DF-Na over 24 hours in the 3D membrane bioreactor. On day 8 and 14 we found a continuous decline of DF-Na which was paralleled by a continuous increase of the main metabolite 4'-OH-DF. The metabolite 5-OH-DF was not detected which might be related to the stability of the metabolite in the medium or to its interactions with the tubing or membranes.

4 Discussion
The objective of the present study was to compare four commonly used cell culture models in order to determine which of these systems might be most suitable to investigate certain aspects of drug metabolism in the liver, and may lead to the reduction of in vivo animal testing without interfering with the quality of the required results.

The choice of EROD and ECOD as a parameter of proper metabolic capacity of liver cells was made since the methods assess different families of CYP activity. While EROD assesses mainly CYP 1A...
activity, ECOD activity is catalysed by a broader number of CYP proteins. Together these two CYP isoforms comprise 60-90% of the total CYP proteins in the liver (Parkinson, 1996). Thus, the measurement of these two activities in basal and induced states provide a fair estimate of the drug metabolising capacity of isolated liver cells. Several studies have indicated that isolated hepatocytes retain a number of structural and functional characteristics as observed in vivo. However, one variable that is drastically altered very early in culture is the microsomal mixed-function oxidase (MFO) system (Acosta et al., 1979). It was observed that the initial EROD and ECOD activity continuously decreases with culture time and that the loss was slower in hepatocytes cultured in a collagen sandwich rather than in monolayer cultures. Using CYP inducer such as 3-MC this tendency was partially reversed.

In the present study, we found that hepatocyte cultures treated with Omeprazol and 3-MC or phenobarbital and 3-MC showed a sustained increase of ECOD and EROD activity. The inducible metabolic capacity was observed over 14 days in sandwich cultures and the 3D-membrane bioreactor, while in monolayer cultures the peak was on day five, and non-existent in the Unisyn bioreactor. Furthermore, our results clearly showed that hepatocytes cultured between two collagen layers maintained their inducible CYP capacity over 14 days. 3-MC consistently stimulated the EROD and ECOD activity stronger than the apparent more specific inducers Omeprazol or phenobarbital, which is in agreement with previous findings (Haugen et al., 1975; Thor et al., 1976; Diaz et al., 1990; Donato et al., 1993; Lubinski et al., 1994). This suggests that different mechanisms of induction might be involved. The CYP 1A family consists of two genes CYP 1A1 and CYP 1A2 (Nelson, 1993). While the CYP 1A1 gene is highly inducible in many organs including the liver, CYP 1A2 is constitutively expressed in the liver and almost not detectable in other organs (Tai et al., 1993). The CYP 1A2 gene can be increased by compounds such as Omeprazol (Diaz et al., 1990). EROD activity was not detectable in the Unisyn bioreactor while an activity increase was seen in the 3D-membrane bioreactor when cells were exposed to 3-MC.

Table 2: Metabolite stability of 4'-OH-DF, and 5-OH-DF in culture medium

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>4'-OH-DF</th>
<th>5-OH-DF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>peak area</td>
<td>rel. Content [%]</td>
</tr>
<tr>
<td>0 h</td>
<td>198,096</td>
<td>100.0 ± 0.3</td>
</tr>
<tr>
<td>24 h</td>
<td>158,851</td>
<td>80.2 ± 3.4</td>
</tr>
<tr>
<td>96 h</td>
<td>126,740</td>
<td>64.0 ± 2.1</td>
</tr>
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</table>

Results of investigations on the stability of 4'-hydroxy-diclofenac and 5-hydroxy-diclofenac in the absence of cells in Williams' medium E during incubation at 37°C.
The CYP 3A family is the major steroid inducible cytochrome P450 monooxygenase in rat, rabbit and humans. Induction of CYP 3A, measured as ECOD activity has been shown to be inducible by 3-MC and phenobarbital in isolated liver cells (Thor et al., 1976). The stimulation of enzyme activity by 3-MC in rat liver is reported twice than that seen with phenobarbital (Jacobson et al., 1974). We found that both 3-MC and phenobarbital were able to induce hepatic O-deethylation activity, although 3-MC stimulated this enzyme consistently stronger. These results indicate that CYP 3A induction by 3-MC and phenobarbital may proceed through different pathways reflecting the diversity seen in CYP 3A subfamilies (Haugen et al., 1975). No CYP 3A activity was seen in the Unisyn bioreactor while the 3D-membrane bioreactor showed very 'small' changes when cells were exposed to 3-MC. This low level of metabolisation or the formation of certain DF metabolites might be explained by the stability of the metabolites in the culture medium as well as the absorption and/or interactions with the tubing or membranes of the bioreactor.

Protein degradation is a dynamic process, whose rate can be influenced by a drug and/or the metabolic status of the cell (Guguen-Guillouzo et al., 1983). Albumin secretion, which is a specific function of hepatocytes in vivo, rapidly declines in monolayer cultures while in the 3D-membrane bioreactor the highest degree of synthesis was observed. According to the literature, albumin storage and synthesis in vitro is only possible for a short time since cells have no continuous supply of nutritional agents (Peters, 1977). In contrast, the 3D-membrane bioreactor functions with an open circuit to continuously perfuse hepatocytes and to aliment the system with necessary agents to maintain a continuous albumin synthesis. In addition, the system provides positive aspects to mimic the in vivo situation of hepatocytes in the liver which might be an advantage for drug metabolism studies (Zeilinger et al., 2000). Since albumin secretion in normal hepatocytes co-cultured with epithelial cells lasts for several weeks, it is conceivable that albumin secretion is related to the production of an extracellular matrix and thus important for pharmacological studies (Guguen-Guillouzo et al., 1983). In the present study, hepatocytes embedded in rat tail collagen preserved a prolonged survival rate and stability in albumin secretion as compared to cells cultured as monolayer. In sandwich cultures, both bottom and top surfaces of cells attach to a collagen matrix which may provide environmental conditions that resembles the in vivo geometry. When hepatocytes were cultured as monolayer, the basic surfaces of hepatocytes attach to the plastic while the top is directly in contact with culture medium, due to the characteristics of plastic, cells exhibit phenotypic changes which may lead to partial or complete loss of highly differentiated functions (Bissell, 1981).

It is well established that hepatocytes do not survive for more than a few hours in suspension (Guilouzo et al., 1993), and that the adherent culture is superior in terms of metabolism and survival time. The adhesion of hepatocytes influences the survival time and metabolic functions of the cells (Gerlach et al., 1989). The comparison of the two different bioreactors confirmed these observations. When hepatocytes were seeded into the Unisyn bioreactor, many hepatocytes did not attach to the fiber and subsequently the viability and metabolic functions were far behind the results obtained with the other three culture systems.

The liver is quantitatively the most important site of drug metabolism. However, several hundred drugs are known to cause hepatic injury. The release of cytosolic enzymes into the incubation medium is a sign of cellular injury (Bauer et al., 1985). Hence, the degree of cellular injury or a time course of enzyme leakage is a useful way of monitoring the progress of damage caused by drugs (Berry et al., 1991). Comparing the results of enzyme leakage obtained with the different cell culture systems, it was found that DF-Na does not show direct significant hepatotoxicity when used at 0.1 μM. DF-Na is a non-steroidal anti-inflammatory drug. The pharmacokinetic and metabolism of DF-Na has been investigated in a number of species. DF-Na is rapidly and completely absorbed after administration in all species and metabolised by direct conjugation, or hydroxylation of the aromatic rings. DF is metabolised in mammals into two main products: 4'OH-DF and 5OH-DF which can be found in blood, urine, and bile. The reduction of metabolites as seen over time in all systems is due to the constitutive reduced metabolic capacity, however, it can be also explained by the interaction of metabolites with the medium, the collagen or the plastic since in cell free systems metabolites of DF-Na disappear (see for review Stierlin and Faigle, 1979, Nussler and Albrecht, unpublished results).

The present study demonstrates a possible link between drug metabolism and cytochrome P450 activity in vitro cultures. Several facts support this assess-
ment. First of all, we found that basic CYP 1A and CYP 3A activities decreased distinctly with culture time in all cell culture models. Furthermore, we observed that the basic CYP 1A activity could be maintained on the same level over two weeks in the 3D-membrane bioreactor. In monolayer and collagen sandwich cultures, the concentration of DF-Na metabolites decreased with culture time. In contrast, hepatocytes retained practically the same metabolic capacity in the 3D-membrane bioreactor over two weeks metabolising DF-Na. On the other hand, the results also show that basic CYP 1A and CYP 3A activity in monolayer cultures was lower than that observed with hepatocytes embedded in collagen although, we noted that the concentration of DF metabolites was higher in cells cultured in collagen than in monolayer culture. Considering these results, there is a strong indication that the metabolism of DF is associated with cytochrome P450 enzyme capacity confirming recent results (Bort and co-workers, 1999).

Early knowledge of drug metabolic pathways and metabolites is important for the interpretation of pharmacological and toxicological data obtained in drug development studies. With the use of isolated hepatocytes, metabolic pathways and the potential formation of metabolites could be studied before clinical tests may take place. Under these circumstances, the use of human hepatocytes in sandwich cultures or in the 3D-membrane bioreactor will finally give further insights on the positive effects of these systems in preclinical drug development.

However, the present study raises some essential questions about the benefit of the described systems for the study of drug metabolism. Analysing all presented data it seems that for short term cultures the use of monolayer culture are sufficient to analyse questions related to drug metabolism. In order to address questions raised by long term cultures it seems that the here applied sandwich culture technique is the most convenient system to obtain a maximum of information on long term cultures. This is also underlined by the cost/benefit ratio of the two studied bioreactor systems. The Unisyn bioreactor clearly does not provide any information for drug metabolism studies. Although, the 3D membrane bioreactor provides some valuable information with regard to drug metabolism, the amount of cells to be used in this system is far higher than the benefit drawn from monolayer or sandwich cultures. This fact is particularly interesting with regard to the use of human hepatocytes in bioreactors since this species is difficult to obtain for these research purposes.

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