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A comparative study of precision cut liver slices, hepatocytes, and liver microsomes from the Wistar rat using metronidazole as a model substance

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1. Metronidazole is metabolized by rat liver *in vitro* models to form a hydroxy metabolite, an acetic acid metabolite, a glucuronic acid conjugate, and a sulphate conjugate.

2. Four different *in vitro* systems for investigation of drug metabolism based on liver preparations from the male Wistar rat have been investigated.

3. An incubation system where liver slices are incubated in 12-well culture plates was evaluated with respect to metabolism of metronidazole. Optimal viability was observed for a time period of up to 24 h. The Michaelis–Menten parameters for the metabolism of metronidazole in liver slices were calculated and the intrinsic clearance values compared with the values determined in hepatocytes incubated in suspension. It was found that the intrinsic clearance with respect to formation of oxidative metabolites, the hydroxy metabolite, and the acetic acid metabolite correlated, whereas the intrinsic clearance with respect to formation of the glucuronic acid conjugate was lower in slices compared with hepatocytes.

4. The metabolism of metronidazole in liver slices, in hepatocytes in primary monolayer culture, in hepatocytes incubated in suspension, and in liver microsomes was compared. All the incubations were performed under identical incubation conditions including the same incubation medium. The trend observed was that the initial metabolic rates of the production of the hydroxy metabolite, the glucuronic acid metabolite, and the acetic acid metabolite of metronidazole were higher in microsomes than in the other liver preparations. The metabolic rates in hepatocytes in primary culture and in suspension with respect to the oxidative metabolites were higher than in liver slices. The metabolic turnover observed in liver slices was predicted to correlate with *in vivo* data earlier obtained for rat.

Introduction

In vitro models have been widely used for the assessment of xenobiotic metabolism. Isolated hepatocytes either in primary culture or in suspension are often used and is a very successful tool in drug development for evaluation of the metabolic patterns for new drugs (Bissel *et al.* 1973, Guzelian *et al.* 1977, Sandker *et al.* 1994, Pahernik *et al.* 1995). It has recently been proposed that the use of liver slices can serve as a reliable alternative method (Barr *et al.* 1991, Dogterom 1993, Dogterom and Rothuizen 1993, Miller *et al.* 1993, Salyers *et al.* 1994, Smith *et al.* 1986, 1987, Vickers *et al.* 1993) and the method is becoming increasingly utilized. Liver slices have the advantage relative to hepatocyte cultures that they maintain tissue architecture. Heterogeneity of the liver composition is maintained so that all liver cell types are present and functional heterogeneity is maintained because of cell-to-cell interactions (Smith *et al.* 1986, 1987). Liver slices are therefore thought to provide patterns of drug metabolism that are more similar to *in vivo* metabolism than other *in vitro* liver models such as hepatocytes or liver microsomes. Hepatocytes

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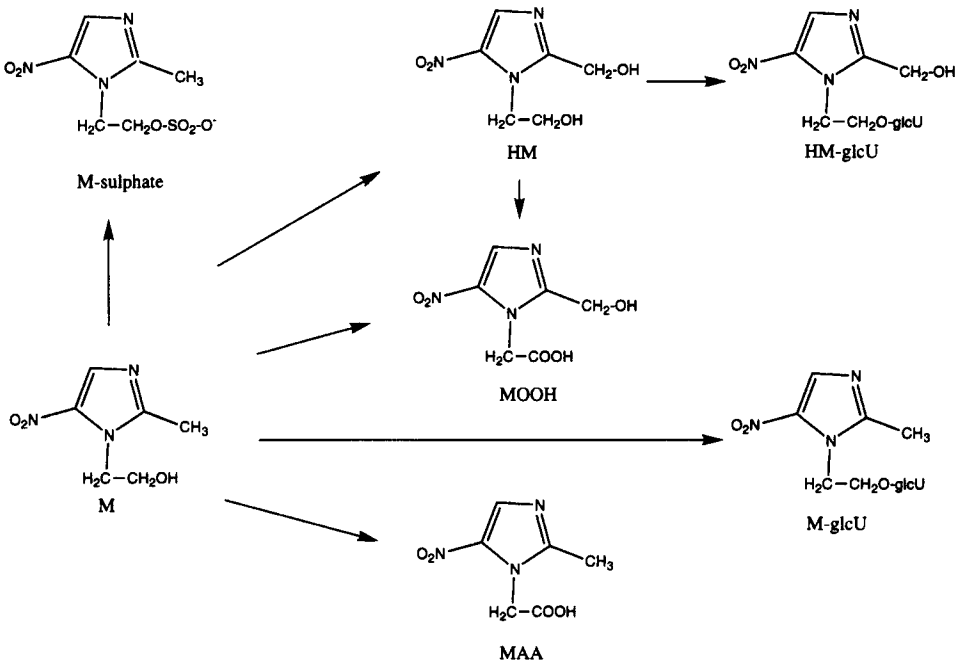
are usually suspended by collagenase digestion. This damages the cell membranes and disrupts cell-to-cell contact and thus the intercellular transport systems (Dich and Grunnet 1990). The cell membranes may however be regenerated during early stages of culture in monolayer cultured hepatocytes (Bissel *et al.* 1973, Guzelian *et al.* 1977, Fry and Bridges 1979, Dich and Grunnet 1990). Furthermore, changes in the characteristic functions of cultured differentiated hepatocytes such as a decline in cytochrome P450 content have been repeatedly demonstrated (Guzelian *et al.* 1977, Fry and Bridges 1979). This was explained to be the consequence of the loss of cell-to-cell contact and/or the absence of an extracellular matrix. Recently, it has however been postulated that the decline in cytochrome P450 enzymes additionally may result from the absence of endogenous inducers in the incubation medium and that also liver slices show a decline in P450-enzymes over a 24-h incubation (Wright and Paine 1991).

Adequate nutrient and oxygen exchange is crucial for the viability of *in vitro* liver preparations. Therefore different incubation systems have been developed for the incubation of liver slices. The dynamic organ culture system (Smith *et al.* 1986, Barr *et al.* 1991) in which rotating vials are used and the more simple incubation system proposed by Dogterom (1993) in which 12-well tissue culture plates shaken on a gyratory shaker are used. By comparison, hepatocytes in primary culture are usually incubated in a static manner on collagen coated Petri dishes. Hepatocytes in suspension are incubated in culture plates, in vessels or in flasks on a gyratory shaker similar to the liver slice incubations.

The aim of the present study was to investigate the metabolism of a probe drug in precision-cut liver slices. The metabolism in rat liver slices was then compared with rat liver hepatocytes in primary monolayer cultures, in hepatocytes in suspension and with rat liver microsomes under exactly the same incubation and incubation medium conditions. Metronidazole (M) was used as a model drug because it has previously been used as a probe drug when investigating cytochrome P450 isoenzymes. Thus, the metabolic fate of the drug has been extensively studied in both rat and man (Ings *et al.* 1975, Tempelton 1977, LaRusso *et al.* 1978, Allars *et al.* 1985, Loft and Poulsen 1989, Loft and Poulsen 1990), but never in long-term incubation systems (incubations > 2–3 h) such as hepatocytes in monolayer culture or in precision-cut liver slices.

It has been demonstrated by autoradiography of rat that the liver plays a major role in the metabolism of the drug (Ings and McFadzean 1975, Tempelton 1977). Oxidative metabolism is the major route for biotransformation of metronidazole in man and three major oxidative metabolites are found, namely the hydroxy metabolite, 1-(hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole (HM), the acetic acid metabolite, 2-methyl-5-nitroimidazole-1-acetic acid (MAA), and the 2-carboxy metabolite, 1-(hydroxyethyl)-2-carboxy-5-nitroimidazole (MOOH) (scheme 1). Both metronidazole and its hydroxy metabolite are conjugated with glucuronic acid (M-glcU and HM-glcU) (Loft and Poulsen 1989, Thomsen *et al.* 1995). Furthermore, formation of the sulphate conjugate of M has been proposed (Loft and Poulsen 1989, 1990). In rat the predominant route of biotransformation is conjugation with glucuronic acid forming M-glcU, but the oxidative metabolites are also observed. In the male rat urine following relative amounts of metabolites has been observed (% dose), 25% M-glcU, 4% HM, 5% MAA, 0·2% MOOH, and 2% sulphate conjugate (Ings and McFadzean 1975, Tempelton 1977, LaRusso *et al.* 1978, Allars *et al.* 1985).



Scheme 1. Metabolic pattern of metronidazole.

Materials and methods

Chemicals

Metronidazole and the metabolites HM, MAA, MOOH were kindly donated by Steffen Loft, Department of Pharmacology, University of Copenhagen, and Dumex Ltd (Copenhagen, Denmark). M-glcU was previously bio-synthesized in rat liver microsomes and purified by preparative hplc as described previously (Thomsen *et al.* 1995). Disodium UDP-glucuronate (UDP-GA), glucose 6-phosphate, glucose 6-phosphate dehydrogenase and $NADP^+$ were purchased from Boehringer (Mannheim, Germany). Gentamycin sulphate and Waymouths medium was purchased from Sigma Chemicals Co. (St Louis, MO, USA). *N*-cetyl-*N*,*N*,*N*-trimethylammonium (CTMA) bromide analytical grade was purchased from Merck (Darmstadt, Germany). All other chemicals were of high purity grade and purchased from commercial sources.

Animals

Laboratory bred male Mol:Wist (Hannover) rats, weighing 200–250 g, were housed under constant temperature and humidity conditions with a 12-h light/dark cycle. The animals were allowed free access to food (Altromin®, Chr. Petersen A/S, Ringsted, Denmark) and water.

Slice preparation

Immediately after the livers had been removed from the rats, liver slices were prepared in ice-cold Krebs–Henseleit buffer (4°C, pH 7·4) on a Krumdieck tissue slicer (Alabama Research and Development Corp., Munford, AL, USA) as described earlier (Krumdieck *et al.* 1990, Smith *et al.* 1986, Barr *et al.* 1991). The setting for slice thickness on the tissue slicer was varied from 100 to 500 μm and liver cores of 8 mm in diameter were made with a sharpened stainless steel tube. The protein content in the slices was determined after homogenization by the Lowry method (Lowry *et al.* 1951). The slice characteristics from the metabolic experiments, where a slice thickness of 200 μm was used, were the following: wet weight, 11·9 (±1·76) mg (n = 10); protein content, 0·12 (±0·02) mg protein/mg slice (n = 10).

Slice incubation

The slices produced were incubated in Waymouths medium, without phenol red, containing 10% (v/v) foetal calf serum and 84 μg gentamycin sulphate/ml. Experiments were performed using six rats and two incubations were performed from each rat (two slices were incubated separately in separate

wells). After slicing the slices were transferred to 12-well culture plates containing 3 ml medium in each well. The plates were placed on a gyratory shaker (100 rpm) in an incubator (37°C) saturated with a gas mixture of 5% CO_2 and 95% air. After 60 min (lag time found via viability tests) of pre-incubation the incubation medium was changed to Waymouths medium containing metronidazole.

Slice viability

As a reflection of membrane integrity and thus viability of the cells the intracellular amount of K^+ was measured, as described by Dogterom and Rohuizen (1993) on a IL model 143 flame photometer (Milan, Italy). The ATP level in the cells reflects cell integrity as well as the metabolic state of the cells and was measured by hplc as described earlier (Jones 1981, Dogterom 1993). Lactate dehydrogenase (LDH) release was measured spectrophotometrically by the method of Bergmeyer *et al.* (1965) as the rate of NADPH consumption in the presence of pyruvate. All the incubations were performed over 24 h. The K^+, ATP and LDH level was determined after 1, 2, 4, 6, 8 and 22 h. The number of experiments performed corresponded to six slices from one rat and the slices were incubated separately in separate wells.

Investigation of metabolism of metronidazole in liver slices

Linearity studies with respect to metabolic turnover as a function of slice thickness were performed with metronidazole (0·4 mM) in the incubation medium. The choice of a substrate concentration of 0·4 mM was based on results obtained from the investigations of the Michaelis–Menten parameters estimated here and previously (Loft and Poulsen 1990). This substrate concentration was below the K_m for the formation of all the metabolites of M. The setting of slice thickness on the Krumdieck tissue slicer was varied in order to cut slices with varying thickness (microtone setting of 100–500 μm) and the protein content was estimated by the method of Lowry *et al.* (1951). The linearity of HM formation with respect to number of slices in each incubation well was similarly investigated. The number of slices was varied from 1 to 6 in each incubation well and the setting of slice thickness was 200 μm.

Michaelis–Menten parameters were calculated as previously described (Loft and Poulsen 1989) and the concentration of metronidazole in the incubation medium was varied from 0·1 to 10 mM during these studies. Slices and media were harvested separately after 1 h and analysed.

The metabolic time profile of metronidazole was investigated over a period of 22 h. The substrate concentration in the incubation medium was in this case 0·4 mM, slice thickness was 200 μm and one slice was incubated in each incubation well. Slices and medium were harvested separately after 1, 2, 4, 8 and 22 h.

Experiments with hepatocytes in primary monolayer culture

The hepatocytes were cultured on collagen (from rat tail) coated Petri dishes (60 mm i.d.) in 3 ml Waymouths medium containing 10% (v/v) foetal calf serum and 84 μg gentamycin sulphate/ml. The liver cells were prepared by liver perfusion *in situ* as described earlier (Braakman *et al.* 1989, Dich and Grunnet 1990), the hepatocytes were then isolated by centrifugation (Dich and Grunnet 1990) and approximately 6×10^6 hepatocytes were plated on Petri dishes. A viability > 85% was assessed by tryphan blue exclusion. The Petri dishes were placed in an incubator saturated with a gas mixture of 5% CO_2 and 95% air (37°C) and the culture medium was changed every second day. The metabolic experiments were performed 48 h after plating according to the appropriate lag time during which metabolic balance as well as cell attachment is restored. When hepatocytes in monolayer culture are used for investigations of drug metabolism lag times of 48–72 h are often used (Dich and Grunnet 1990, Bader *et al.* 1993, Pahernik *et al.* 1995) and this preincubation time was therefore applied in the present studies where comparisons of *in vitro* models used in investigations of drug metabolism were performed. It was reasonable to compare these *in vitro* models even though different preincubation times were used as the studies should reflect the *in vitro* models used in drug research today. These *in vitro* models will be affected by the decline in P450 enzymes during incubation. Three animals were used (two incubations were performed for each rat in separate Petri dishes) and the hepatocytes were incubated with metronidazole (0·4 mM). Aliquots of 200 μl were taken from the Petri dishes after 1, 2, 4, 8 and 24 h and were analysed.

Experiments with hepatocytes incubated in suspension

The hepatocyte were isolated as described earlier (Braakman *et al.* 1989, Dich and Grunnet 1990) and were suspended in Waymouths medium containing 10% (v/v) foetal calf serum and 84 μg gentamycin sulphate/ml. The cell concentration was approximately 6×10^6 hepatocytes/ml with a viability of > 85%, determined by tryphan blue exclusion. After 15 min pre-incubation at 37°C, the cells were transferred to 12-well culture plates and Waymouths medium containing metronidazole was added. Each well contained approximately 6×10^6 hepatocytes and the total volume in each well was 3 ml. The

concentration of metronidazole was 0·4 mM and the hepatocytes were incubated for 60 min in an incubator saturated with 95 % air and 5 % CO_2, at 37°C. Three animals were used (two incubations were performed for each rat in separate wells).

Experiments with liver microsomes

Microsomes were prepared separately from six rats by differential centrifugation (9000 *g* for 15 min followed by 100000 *g* for 60 min) in 0·25 M sucrose solution and was stored at −80°C until used. The microsomes were incubated at 37°C for 60 min in an incubator saturated with 95 % air and 5 % CO_2. The incubation mixture (total volume of 500 μl) was as follows: 100 μl rat liver microsomes (11 mg protein/ml), in 400 μl Waymouths medium (pH 7·4) containing 10 % (v/v) foetal calf serum, 0·4 mM metronidazole and a NADPH generating system (0·2 μmole $NADP^+$, 2 μmol glucose 6-phosphate and 2 μmol $MgCl_2$). The incubations were initiated by the addition of 3 U glucose 6-phosphate dehydrogenase and 2 mg UDP-GA and was later terminated by the addition of 500 μl methanol.

Analysis of metabolites

Analysis of the metabolites formed during the incubations were performed as earlier described (Thomsen *et al.* 1995) using a dynamically modified silica system (system 1), additionally the samples were analysed using a reversed phase chromatographic system on a C_{18} chromatographic column (system 2).

All samples were stored at −20°C until analysed. Aliquots of medium from the liver slice incubations and the incubations with hepatocytes in monolayer culture could be analysed directly after centrifugation for 10 min at 1500 *g*. The amounts of metabolites remaining within the slices were analysed as follows: the slices were removed from the incubation medium, washed in a saturated saline solution and transferred to a vial containing 950 μl distilled water and 50 μl perchloric acid (70 %). The slices were homogenized and the homogenate was centrifuged for 10 min at 1500 *g*. To 250 μl of the supernatant, 50 μl 3 M KOH was added to precipitate perchloric acid as potassium perchlorate before the sample was centrifuged and the supernatant analysed by hplc.

Aliquots from the microsomes and hepatocytes incubated in suspension were diluted 1:1 with methanol, centrifuged at 9000 *g* for 10 min and then analysed.

The hplc equipment was a Waters (Milford, MA, USA) liquid chromatographic system consisting of a model 6000 A pump, a 715 ultra WISP auto injector, and a 490E programmable multi-wavelength detector. Data were collected using Maxima 820 software. A Shimadzu (Kyoto, Japan) CTO-6A oven was used for setting the temperature of the columns. A saturation column (150 × 4·6 mm i.d.) dry packed with LiChroprep Si 60 (15–25 μm) (Merck, Darmstadt, Germany) was installed between the pump and the auto injector in system 1.

The chromatographic conditions for system 1 were as follows: The analytical column was a Knauer column (120 × 4·6 mm i.d.) (Berlin, Germany), slurry packed with LiChrosorb Si 60 (5 μm) (Merck, Darmstadt, Germany), the temperature of the column oven was set at 35°C. The eluent consisted of methanol–0·2 M potassium phosphate buffer (pH 7·0)–water (25:30:45; by vol.) with 2·5 mM *N*-cetyl *N,N,N*-trimethylammonium bromide (CTMA) added (Thomsen *et al.* 1995). The flow rate was initially 0·5 ml/min, after 9 min it was increased to 1·0 ml/min. The UV detector was set at 312 nm. The chromatographic conditions for system 2 were as follows: the analytical column was a Knauer column (120 × 4·6 mm i.d.) slurry packed with Spherisorb ODS-2 (Phase Separation Ltd, Queensferry, UK), 5 μm, with an eluent consisting of acetonitrile–methanol–0·01 M potassium phosphate (pH 3·5) (3:1:96; by vol.). The flow rate was 1·0 ml/min and UV-detection was at 312 nm.

Results

Viability of liver slices

The results of the intracellular K^+ measurements in homogenized rat liver slices as a function of time (24-h incubation) indicated that the slices were viable for at least 24 h (table 1). During the first 60 min the K^+ content increased to about 32 μmol K^+/g slice reflecting the cells recovering from the slicing procedure. The intracellular K^+ level remained constant for the rest of the incubation period. Immediately after slicing the ATP content in the slices was low (5 nmol ATP/mg protein) but a recovery phase (2 h) was observed and a maximum of 10 nmol ATP/mg protein was reached (table 1). Towards the end of incubation the ATP concentration decreased (6 nmole/mg protein at $t = 24$ h). Lactate dehydrogenase

Table 1. Viability parameters for precision cut liver slices during incubation.

	During the first hour	After 2 h	22–24 h
K^+ (μmol K+/g slice)	8–20	30	32
ATP (nmol/mg protein)	5·5	10	6
LDH (Racker units/mg protein)	0·08–0·12	< 0·02	0·05

K^+- and ATP-contents as well as LDH release during the 24-h incubation of liver slices in culture plates.

release from the cells was used as another sensitive indicator of slice viability. The release of LDH expressed in Racker units/mg protein as a function of time was followed (table 1). LDH was released into the medium (0·08–0·12 Racker Units LDH/mg protein) during the first 2 h of incubation and 0·12 was the maximum amount of LDH released to the medium during the incubation period. From 2 to 8 h only small amounts of LDH were released (< 0·02 Racker Units LDH/mg protein). Towards the end of incubation the LDH release to the medium increased reaching 0·05 Racker Units LDH/mg protein.

Metabolism of metronidazole in vitro

Metronidazole was metabolised in the *in vitro* rat liver preparations to form both phase I (HM and MAA) and phase II (M-glcU) metabolites. The chromatograms obtained from incubations of metronidazole with rat liver slices for 8 h are shown in figure 1. In figure 1A the sample was analysed with the dynamically modified silica system (system 1). This system is optimal for analysing glucuronic acid conjugates (M-glcU) and the acidic metabolites (MAA), but the peak corresponding to the hydroxy-metabolite (HM) tends to become a shoulder on the peak corresponding to metronidazole, as the concentration of metronidazole is high in the incubation media relative to the concentrations of the metabolites formed. In order to analyse HM accurately the reversed phase chromatographic system, system 2 was used (figure 1B). This system is however not appropriate for analysis of the acidic metabolites as they elute with the solvent front and the peak corresponding to the glucuronic acid conjugate suffers from tailing.

The sulphate conjugate was found to correspond to the peak observed at t_R = 10·6 min in chromatographic system 1 (figure 1A). This peak disappears after the sample has been treated with a mixture of β-glucuronidase and sulphatase (the β-glucuronidase activity was inhibited by D-saccharic acid 1,4-lactone) for > 24 h at 37°C (data not shown). But as the sulphate conjugate of metronidazole was not available as a reference substance, no absolute proof of this fact was obtained and no attempts were made to quantify this metabolite in the present work. No spontaneous degradation of metronidazole itself was observed throughout an incubation period of 24 h when no slices were added to the incubation medium (data not shown). The amount of metabolites remaining in the slices was negligible (data not shown), so the results were based on the amount of metabolite released to the incubation medium. The amount of M remaining in the slices was constant during the incubation period and corresponded to 3·6 nmol/mg protein.

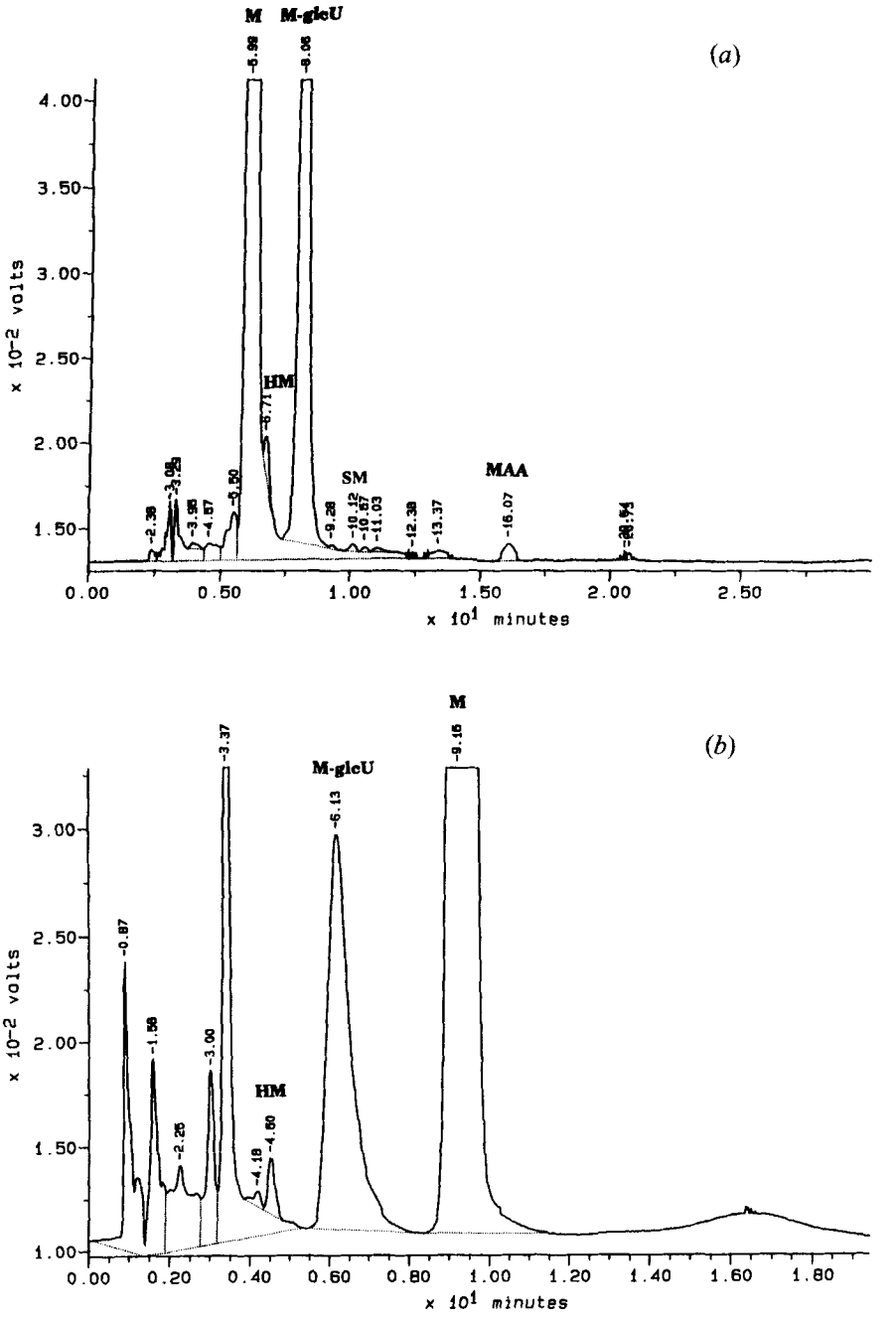


Figure 1. Chromatograms of samples from incubations of metronidazole with rat liver slices after 8-h incubation. Incubations were performed in 12-well culture plates at 37°C. Two different chromatographic systems were used. (A) The dynamically modified silica system (system 1) was as described in the Materials and methods. The retention times for metronidazole (M), its hydroxy metabolite (HM), the glucuronic acid conjugate (M-glcU) and the acetic acid metabolite (MAA) were $t_{R(M)} = 6{\cdot}0$ min, $t_{R(HM)} = 6{\cdot}7$ min, $t_{R(M\text{-}glcU)} = 8{\cdot}1$ min, and $t_{R(MAA)} = 16{\cdot}1$ min. (B) The chromatographic system corresponding to system 2 was also described in the Materials and methods. The retention times observed were $t_{R(M)} = 9{\cdot}2$ min, $t_{R(HM)} = 4{\cdot}5$ min, and $t_{R(M\text{-}glcU)} = 6{\cdot}1$ min.

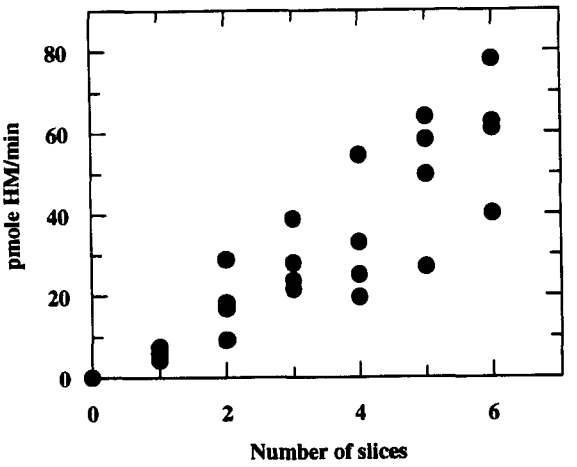


Figure 2. Relationship between the number of slices in each well and metabolic rate towards formation of the hydroxy metabolite (HM). Concentration of metronidazole in the incubation medium was 0·4 mM and incubations were performed at 37°C in 12-well culture plates and slice thickness corresponded to a setting on the Krumdieck Tissue Slicer of 200 μm. The incubation time was 60 min.

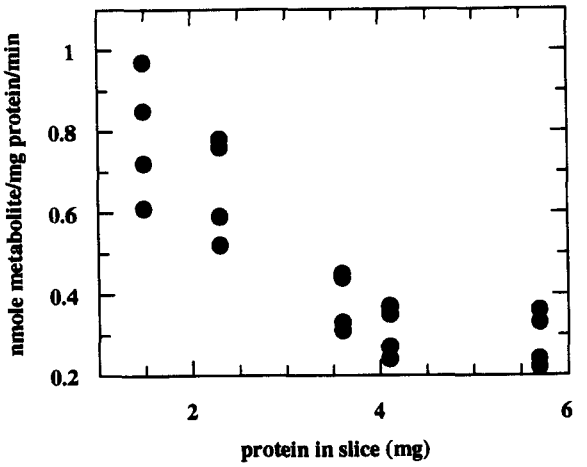


Figure 3. Relationship between hydroxy metabolite formation and slice thickness (expressed in mg protein/slice). Concentration of metronidazole in the incubation medium was 0·4 mM. Incubations were performed at 37°C in 12-well culture plates. Incubation time was 60 min.

Linearity studies

The effect of increasing the number of slices in each incubation well was investigated with respect to release of HM to the medium. The relationship between metabolic rate (formation of metabolites of metronidazole per min) and the number of slices in each incubation well is given in figure 2. There seems to be a linear relation between amount of metabolite formed and the number of slices incubated (the linear correlation coefficient was 0·95). Different settings on the Krumdieck tissue slicer were used to produce slices of varying thickness and their protein content was determined. Their metabolic activities towards formation of HM as a function of protein content is presented in figure 3. A decrease in metabolic activity

Table 2. Michaelis–Menten parameters obtained for metronidazole when incubated with liver slices.

	HM	M-glcU	MAA
K_m (mM)	0·8 ± 0·1	7·6 ± 1·3	0·6 ± 0·2
V_{max} (pmol/min/mg protein)	15·5 ± 2·0	405 ± 35	7·6 ± 0·7
$Cl_i = V_{max}/K_m$ (nl/min/mg protein)	19·3 ± 7·6	53·3 ± 16·5	12·7 ± 6·9

Substrate range was 0·05–10 mM and the slices were incubated in 12-well culture plates, one slice was incubated in each well. Samples were analysed after 60 min of incubation. Six rats were used with two separate incubations for each rat; the standard deviations are given above.

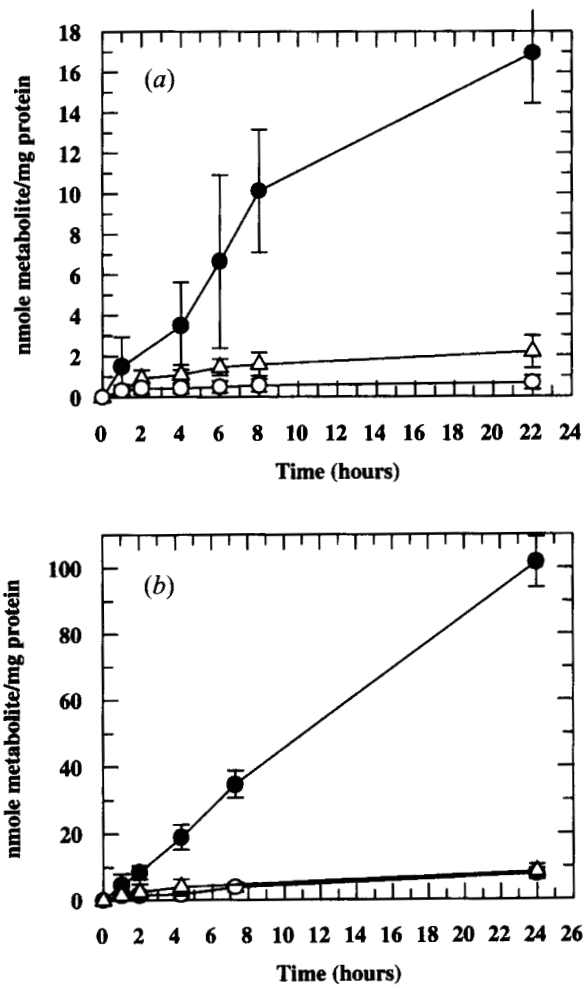


Figure 4. Relationship between incubation time and metabolism of metronidazole in the incubation media. Concentration of metronidazole in the incubation medium was 0·4 mM and the incubations were performed at 37°C. (A) Metronidazole was incubated with slices in 12-well tissue culture plates, one slice in each well; slice thickness was 200 μm. (B) Metronidazole incubated with hepatocytes (primary monolayer culture) in 60-mm Petri dishes. Six male rats were used with two incubations from each rat and the errors presented are the standard deviations. ●, M-glcU; ○, HM; △, MAA.

Table 3. Formation rates for metabolism of metronidazole estimated after 1-h incubation.

	Rate (pmol/min/mg protein)			
	Incubation in liver slices	Hepatocytes in monolayer culture	Hepatocytes in suspension	Microsomes
M-glcU	25·1 ± 11·3	31·0 ± 6·4	35·0 ± 12·2	54·8 ± 18
HM	5·4 ± 4·5	11·5 ± 2·7	14·3 ± 4·4	36·1 ± 5·0
MAA	4·4 ± 3·0	13·6 ± 1·9	14·7 ± 3·1	45·6 ± 4·2

Metronidazole (0·4 mM) was incubated with four different *in vitro* rat liver preparations. The standard deviations are presented. Six rats were used with two separate incubations from each rat.

with increasing slice thickness was observed from 1·5 to 4·0 mg protein per slice and > 4·0 mg protein a plateau was observed.

Michaelis–Menten parameters

The metabolic activity towards formation of M-glcU, HM, and MAA was investigated in rat liver slices over a substrate concentration range of 0·1–10 mM metronidazole after 60-min incubation (metabolism of M is linear within the first 60 min of incubation). The apparent V_{max} and K_m were calculated and the intrinsic clearance from liver slices was estimated (table 2). From these results it is seen that metronidazole is a low clearance substance from the rat liver with respect to formation of the metabolites analysed.

Metabolic time profiles in different in vitro *preparations*

The formation of the major metabolites of metronidazole (HM, M-glcU, and MAA) was investigated in two different long-term *in vitro* rat liver preparations (liver slices and hepatocytes in monolayer culture) over a period of 22 and 24 h respectively and the results are shown in figure 4. Figure 4A depicts the amounts of metabolites formed as a function of time, when metronidazole was incubated with rat liver slices. In figure 4B the metabolic time profile when metronidazole was incubated with rat hepatocytes in primary monolayer culture is shown. The percentage of metabolites formed by the hepatocytes in monolayer culture relative to the total amount of metronidazole and metabolites after 8 h was MAA–HM–M-glcU (1·3, 1·2, 11 %). In comparison the amounts formed after 8-h incubation with liver slices were MAA–HM–M-glcU (1·1, 0·4, 7·0 %).

The initial metabolic rates (obtained from 0 to 60-min incubation) towards formation of the three metabolites in rat liver slices and in rat hepatocytes in monolayer culture were compared with the initial metabolic rates of the short-term incubation *in vitro* liver preparations namely hepatocytes incubated in suspension and rat liver microsomes. The results are presented in table 3. The trends are that the metabolic rates observed with hepatocytes in primary culture and in suspension are comparable with the rates observed in liver slices with respect to formation of M-glcU. However, the formation rates of the oxidative metabolites HM and MAA are larger by a factor of two in hepatocytes compared to the rates in liver slices.

The metabolic rates observed in rat liver microsomes were higher than the rates observed in the other liver *in vitro* preparations.

Discussion

In the present studies different rat liver preparations were compared with respect to investigation of the metabolism of metronidazole *in vitro*. The results presented demonstrates that the liver slice model can be used for the study of metabolic profiles even for a low clearance substance and includes the possibility of incubating up to six slices in each well when using the 12-well culture plate incubation model. The results found for ATP levels, the K^+ content in the cells and the LDH-release to the incubation medium are in agreement with results found earlier for liver slices incubated both in incubation wells and in rotating vials (Dogterom 1993, Miller *et al.* 1993). The slices incubated in culture plates seemed to exhibit a lag time of 1–2 h with respect to restoring intracellular K^+ level and ATP concentration and the decrease in LDH release to the medium. The medium in the slice incubations was therefore changed to a medium containing metronidazole after 1 h. The slices show optimal viability for at least 8 h. After 8 h the viability and metabolic state of the slices appeared to slowly decrease corresponding to the decrease in ATP concentration in the slices and LDH release to the medium. Also a decrease in the rate of formation of metabolites was observed after 8 h of incubation (figure 4A).

Linearity studies regarding the formation of the major phase I metabolite HM were used to investigate the appropriate incubation conditions, with respect to slice thickness and number of slices in each incubation well, in order to obtain comparable kinetic data. The conclusion of the linearity studies was that up to six slices could be incubated in each well. The decreased metabolic turnover observed when the slice thickness is increased indicates that as the slice thickness increases the diffusion of M into the inner liver cells of the slices is slow, which may explain the observed decrease in metabolic turnover with increasing amount of protein/slice. Dogterom (1993) postulated that the resulting decrease in metabolic turnover results from the fact that the metabolism only takes place in the surface liver cells of the slices. This may be confirmed here by the observation that metabolic turnover in terms of pmol/min does not change when the protein content in each slice is increased. The slice thickness (setting on Krumdieck slicer) should ideally be 100 μm (1·5 mg protein/slice) in order to obtain the largest possible metabolic turnover per mg protein, however an instrument setting at 200 μm (2·3 mg protein/slice) was chosen for the long-term incubations because the thinner slices tend to be more fragile when incubating in culture plates for longer periods of time (> 8 h). This setting for incubation of slices was used in the experiments for determination of the Michaelis Menten parameters and the comparative studies of the metabolic rates. One slice was here incubated in each incubation well, however in some situations it can be favourable to incubate more than one slice in each well in order to increase metabolic turnover and thereby overcome problems due to quantitation limits of the analytical system used.

The results obtained for the intrinsic clearance of metronidazole towards formation of its major metabolites in rats, are in agreement with the intrinsic clearance found in hepatocytes incubated in suspension (Loft and Poulsen 1990) with respect to formation of oxidative metabolites (8·5–28 nl/min/mg protein for

HM and 6–14 nl/min/mg protein for MAA). The intrinsic clearance of M-glcU is lower in liver slices than in hepatocytes in suspension where an intrinsic clearance of 194–808 nl/min/mg protein has earlier been observed (Loft and Poulsen 1991).

The metabolism of metronidazole observed using hepatocytes in primary culture were higher than the rates observed in the rat liver slice experiments. The effect is pronounced for the phase I metabolites HM and MAA. Hepatocytes in primary monolayer cultures are known to retain viability for several weeks (Bissel *et al.* 1973, Guzelian *et al.* 1977, Fry and Bridges 1979) and optimal viability is accomplished during the 24-h incubation period. The fact that the cells (corresponding to one liver slice in terms of mg protein) are spread out over a larger area (60-mm Petri dishes) in a monolayer means that the accessibility to the cells is better and the cells are in contact with a larger amount of incubation medium at the same time. A drawback of hepatocytes incubated in monolayer culture is that the Petri dishes are incubated in a static manner and the medium is only shaken as aliquots are taken out of the Petri dishes. Also hepatocytes in suspension would be expected to have a larger contact area with the medium than liver slices. In rat liver microsomes the rates observed for production of the three metabolites of metronidazole were significantly higher than in the other *in vitro* liver preparations. This is not surprising as the enzymes being responsible (cytochrome P450 enzymes and UDP-glucuronyl transferases) are concentrated in the microsomal fraction.

The initial metabolic rates towards formation of the three metabolites were compared for each *in vitro* model. The relative amounts were normalized so that the rate towards formation of M-glcU is set to 25%, in correlation with the amount observed for rats *in vivo* (Ings and McFadzean 1975, Tempelton 1977, LaRusso *et al.* 1978, Allars *et al.* 1985). The metabolic pattern of metronidazole can then be evaluated in each *in vitro* model. It follows from table 2 that the relative amounts of M-glcU–HM–MAA for liver slices was 25:5:4, in hepatocytes in monolayer culture 25:10:8, in hepatocytes in suspension 25:10:10 and finally in microsomes 25:17:21. *In vivo* studies on the metabolism of metronidazole in rat showed that the relative amounts of the three metabolites were 25:4:5 of M-glcU, HM and MAA respectively. These calculations indicate that the metabolic pattern found when metronidazole was incubated with liver slices resembles the *in vivo* data more than the other *in vitro* models tested.

The four different *in vitro* liver preparations described here each serve their own purpose. It is very time consuming to produce hepatocyte in monolayer cultures and precautions must be taken to prevent cell contamination. The hepatocyte cultures represent a more artificial *in vitro* system with respect to cell types and intercellular transport systems than liver slices, which may explain why the metabolic pattern observed in rat liver slices seems to reflect the metabolic pattern observed *in vivo* better than the other *in vitro* models tested here. Liver slices are easy to produce and the liver slice model is valuable for identification of metabolic patterns in up to 24-h incubations. For rapid screening tests with the purpose of metabolite identification, rat liver microsomes might be preferred as it is possible to obtain larger amounts of metabolites within a shorter time. The metabolic information obtained from microsomes however only concerns the metabolism in the sub-cellular liver fractions. Another aspect is the fact that for low clearance compounds like metronidazole screening of metabolism in long-term incubation systems are important as it sometimes can be difficult to get substantial turnover for analysis in incubation periods of 60–120 min.

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