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CHARACTERIZATION OF MIDAZOLAM METABOLISM USING HUMAN HEPATIC MICROSOMAL FRACTIONS AND HEPATOCYTES IN SUSPENSION OBTAINED BY PERFUSING WHOLE HUMAN LIVERS*

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Abstract—Isolated human hepatocytes provide a useful model for studying xenobiotic metabolism. However, *in vitro* studies using human hepatocytes are scarce due to the limited availability of this material.

A new methodology is described for obtaining hepatocytes from a whole adult human liver. This procedure is based on (i) the rapid and intense *in situ* washing step of the organ with Eurocollins then glucose supplemented HEPES buffer (10 mM, pH 7.4) at 4° in order to both minimize the warm ischemic period and remove erythrocytes, and (ii) a perfusion of collagenase solution (0.05% in 10 mM HEPES buffer at 37°) throughout the portal vein according to a recirculated model. All perfused buffers are oxygenized. Hepatocyte viability averaged 85% as determined by Trypan Blue dye exclusion. The ability of these hepatocytes to catalyze certain metabolic transformations such as Phase I and Phase II reactions has been particularly investigated using the benzodiazepine drug, midazolam, as a substance probe. Freshly isolated human hepatocytes in suspension retained the ability to metabolize midazolam to its different hydroxylated derivatives—mainly the 1-hydroxy-midazolam—which was further conjugated with glucuronic acid.

For a better understanding of the cytochrome P-450 mediated reactions, we studied the metabolism of midazolam in microsomal fractions prepared from twelve human livers. It was concluded that human microsomes (i) exhibited a Type I binding spectrum upon midazolam addition (K_s = 3.3 μM) and (ii) intensively metabolized the drug to its different derivatives. Furthermore, and since we demonstrated that midazolam was predominantly transformed by a single cytochrome P-450 enzyme, we could attribute the large inter-individual variations in midazolam metabolism to differences in human liver cytochrome P-450 content.

Due to the fact that the liver is the main organ involved in the metabolism of xenobiotics, isolated hepatocytes have been increasingly used to elucidate the metabolic behavior of various drugs [1].

For many years, our laboratory has been using the two-step collagenase perfusion technique described by Berry and Friend [2] for the isolation of rat and rabbit hepatocytes. Using this approach, identification and quantification of new metabolic pathways has been made possible in both rats [3, 4] and rabbits [5–7].

It is now well established that valuable indications concerning drug use in clinics can be drawn from this model. So far the majority of the information has been obtained from animals' liver including rat, rabbit and guinea-pig. However, problems could arise during the extrapolation of these results to the human situation.

For the past few years, various methods have been devised to isolate hepatocytes from adult human liver. These techniques, based upon the two-step collagenase perfusion, have been recently reviewed by Guguen-Guillouzo *et al.* [8, 9]. However, due to the size of the adult human liver, these different groups isolated cells either by perfusing a portion of the whole liver [9] or using biopsies [10–12]. Only one attempt on whole adult human liver has been reported [13].

In this study we have developed a technique that permits very large amounts of viable human hepatocytes to be obtained. This method is based on the perfusion of the whole human liver by a collagenase preparation according to a recirculated model. In order to assess the ability of freshly isolated human hepatocytes to metabolize drugs by Phase I and Phase II reactions, we chose to study the metabolism of a psychotropic molecule which belongs to the class

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of imidazo-1,4-benzodiazepines, midazolam (Ro 21-3981) [14–16]. Metabolism of this molecule, which is well known in man, has been studied in freshly isolated hepatocytes in suspension and compared to another model, the hepatic microsomal fraction. The inter-subject variability in terms of drug metabolism was also investigated.

MATERIALS AND METHODS

Chemicals and materials. Midazolam* (Ro 21-3981), ^{14}C-midazolam (32 μCi/mg), its metabolites Ro 21-6347 (1-hydroxy-midazolam), Ro 21-5975 (4-hydroxy-midazolam), Ro 21-0284 (1,4-dihydroxy-midazolam) and Ro 21-5259 (demethyl-midazolam) were kindly given by Hoffman–Laroche laboratories (Basel, Switzerland). Since the purity of the radio-labeled drug assessed by HPLC was found to be greater than 97%, the compound was used without any further purification.

Polyclonal antibodies directed against highly purified rabbit cytochrome P-450 LM_2, LM_{3c}, LM_4 and LM_6 were generous gifts from Dr. P. Maurel (INSERM U-128, Montpellier, France).

NADPH, β-D-glucuronidase (100,000 units/0.036 g) and Type IV collagenase were purchased from Sigma.

Tissue sources and human hepatocyte isolation. Human livers were obtained from donors' organs at the Marseilles Medical Center, la Timone. Patients' characteristics are reported in Table 1. No specific drug history was reported for any of these patients.

After an intensive *in situ* washing step with 6 to 10 l. of sterile Eurocollins medium at 4° through the portal vein and the aorta, the organs to be transplanted were removed. Under this sampling protocol, the ischemic period never exceeded a few seconds. The liver was still washed with 2 l. more of cold Eurocollins through the portal vein in order to improve the elimination of erythrocytes. The liver was then excised and washed immediately with 10 l. of oxygenated HEPES buffer (pH 7.4; 10 mM) at 4° containing 2.5% glucose. The flow rates of these washing steps ranged between 0.5 and 0.8 l/min. The organ was then transported in ice to the laboratory. This time lag never exceeded 15 min.

The perfusion of the whole human liver was performed by using a Travenol peristaltic pump (0.5–6 l/min), a recirculated waterbath at 38° and a membrane oxygenator. A canule was introduced into the portal vein and the perfused solutions were evacuated through the vena cava. The hepatic artery was ligaturated and gallbladder removed. Using HEPES buffer at a final concentration of 10 mM, the pH was easily maintained around 7.4–7.6 with only few additions of 2 N NaOH (2–6 ml depending on the size and the physiology of the organ). Over the perfusion period, oxygen consumption by the liver was important. A good oxygenation of both HEPES buffer and collagenase solution was achieved using a membrane oxygenator. The flow rate of the recirculating buffers ranged between 1.5 and 1.8 l/min. During the perfusion of the collagenase solution (0.05% in HEPES buffer; 10 mM; pH 7.5), aliquot parts of the liver effluent were collected and the hepatic enzyme, glutamo-oxalo-acetic transaminase, was immediately quantified using a Boehringer assay kit. Based upon different assays using both animal and human livers, the cellular damage in the organ correlated with an increase of the glutamo-oxalo-acetic transaminase concentration in the recirculating medium. The kinetics of the appearance of this enzyme in the perfusate was then recorded during the collagenase perfusion. Collagenase perfusion was stopped when circulating glutamo-oxalo acetic transaminase level was ten-fold higher than its initial 5th-min level.

The surrounding Glisson's capsule was disrupted and the digested pieces of the liver were then minced gently and resuspended in 10 vol. of a buffer containing 120 mM NaCl, 6.7 mM KCl, 10 mM HEPES (pH 7.4), 0.9 mM $CaCl_2$ and 2.5% glucose [17]. The resulting cell suspension was filtered through 250 μm then 150 μm nylon mesh and centrifuged

* Abbreviations used: MDZ, midazolam; 1-OH-MDZ, 1-hydroxy-midazolam; 4-OH-MDZ, 4-hydroxy-midazolam; di-OH-MDZ, 1,4-dihydroxy-midazolam; HPLC, high performance liquid chromatography; HEPES, *N*-2-hydroxyethyl piperazine-*N'*-ethane sulfonic acid.

Table 1. Patients and microsomal fraction characteristics

Donor number	Age (year)	Sex	Protein conc. (mg/ml)	Cyt P-450 conc. (nmol/mg)	Cyt b_5 conc. (nmol/mg)
HL-1	29	M	9.47	0.34	0.37
HL-2	30	M	8.11	0.46	0.58
HL-3	37	F	27.30	0.60	0.82
HL-4	40	M	6.97	0.53	0.62
HL-5	34	M	9.06	0.26	0.60
HL-6	20	F	6.23	0.15	0.49
HL-7	30	M	10.18	0.19	0.55
HL-8	20	M	10.19	0.47	0.69
HL-9	32	M	13.07	0.12	0.49
HL-10	46	M	12.87	0.16	0.37
HL-11	38	F	8.51	0.22	0.57
HL-12	21	M	22.43	0.32	0.54

Microsomal fractions were prepared from different human livers as described in the text. Protein and total cytochrome P-450 and cytochrome b_5 concentrations were quantified by the Bio-rad assay and by the method of Omura and Sato [20] respectively.

three times at 50 *g* in order to remove cell debris, damaged cells and non-parenchymal cells [8]. The addition of 1.0% bovine serum albumin ensured a better viability of the hepatocytes, especially during the washing steps. Cell viability estimated by the Trypan Blue exclusion test was higher than 85%.

A cell yield of between 10×10^9 and 50×10^9 hepatocytes was routinely achieved by perfusing the whole liver (1.2 to 1.6 kg), which corresponded approximately to 10×10^6–30×10^6 cells per gram of wet liver. This high yield of viable hepatocytes can be compared to that obtained by various authors [8].

Incubation conditions. Hepatocytes were suspended to a final cytocrit of 2.5×10^6 cells/ml and were incubated at 37° in Williams E medium in the absence or presence of 10% fetal calf serum. The pH was maintained at 7.4 by passing warm and humidified 95% O_2–5% CO_2 over the cell suspension. Throughout the incubation, the hepatocyte suspension was stirred by a Teflon paddle as described previously [5]. The experiment was initiated with the addition of radiolabeled midazolam.

At selected times, 0.5 ml of the cell suspension was layered on 400 µl of inert silicone oil (density 1.03) in 1.5 ml plastic Eppendorf tubes [3, 18]. The tubes were centrifuged at 15,000 *g* for 30 sec to separate the extracellular medium from the cell pellet. The cell pellet was extracted with 0.4 ml methyl alcohol, spun at 15,000 *g* for 10 min and analyzed for total ^{14}C by liquid scintillation counting. Because of the low amount of radioactivity in cell pellets (1000–6000 dpm) the intracellular metabolic behaviour of midazolam could not be investigated.

Extracellular radiolabel was analyzed by liquid scintillation counting. Portions of the medium were also analyzed by HPLC without further processing as described below.

High performance liquid chromatographic analysis of midazolam and its metabolites. A high performance liquid chromatograph (Hewlett-Packard 1084 B) was equipped with an automatic injector, variable wavelength spectrophotometer, and chromatographic terminal (Hewlett-Packard 79850 ALC). All analyses were performed on a reversed-phase C_{18} column (25 × 0.4 cm; pore size 10 µm) (Knauer, F.R.G.). Elution was carried out at 1.2 ml/min along a linear gradient of 50/50 (v/v) to 62/38 (v/v) methyl alcohol/distilled water over 30 min. Column temperature was maintained at 25°; absorbance was recorded at 254 nm. Eluent from the column was directed either to a LKB 2112 Redirac fraction collector (LKB instruments Rockville, MD) where 0.6 ml-timed fractions were collected, or to a radioactive flow detector (Radiomatic Instruments). Identical results were obtained using both methods.

Retention times of standards have been evaluated ($n = 4$). They are: 9.47 ± 0.44 min (1,4-dihydroxymidazolam), 14.24 ± 0.68 min (4-hydroxymidazolam), 16.45 ± 0.69 min (1-hydroxymidazolam), 19.88 ± 0.83 min (demethylmidazolam) and 25.05 ± 0.89 min (midazolam).

Figure 1 represents an HPLC chromatogram of the separation of midazolam and its metabolites.

Human liver microsomes preparation. Pieces of liver were frozen and stored at −80°. The duration of the storage did not exceed eight months for experiments reported under "Results". We carried out liver microsome preparations from 18 human livers, named HL-1 to HL-18 respectively. Donors' characteristics, cytochromes P-450 and b_5 and protein contents for each liver preparation are reported in Table 1. Liver microsomes were prepared as described previously [19]. Protein concentration was assessed according to the Bio-rad dye reagent protocol. Total cytochromes P-450 and b_5 concentrations were spectrally evaluated according to Omura and Sato [20].

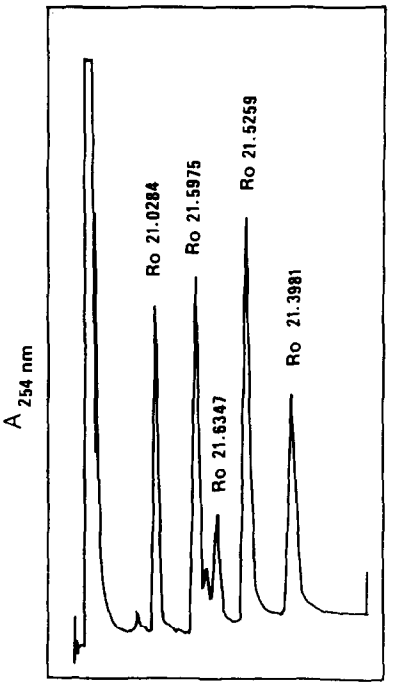

Fig. 1. High performance liquid chromatographic profile of unlabeled midazolam and its metabolites. Mixed standards of midazolam (Ro 21-3981), 1-hydroxy-midazolam (Ro 21-6347), 4-hydroxy-midazolam (Ro 21-5975), 1,4-dihydroxy-midazolam (Ro 21-0284) and demethyl-midazolam (Ro 21-5259) were resolved on a C_{18} reversed-phase column with a UV absorbance at 254 nm, as described in Materials and Methods.

Differential spectroscopic analysis of midazolam binding to human liver microsomes. Liver microsomes preparations were diluted to 1 mg/ml in 0.1 M potassium phosphate buffer (pH 7.4). All measurements were realized at 20° with a Uvikon 860 spectrophotometer. Midazolam (dissolved in pure ethyl alcohol) was added to the sample in order to achieve final concentrations ranging between 0.3 and 10 µg/ml (0.8 and 30 µM respectively). The final ethyl alcohol concentration never exceeded 1% and the same volume of solvent was added to the reference cuvette. After 3 min, the difference spectra were recorded between 525 and 350 nm.

Incubation of midazolam with microsomes. Human liver microsomes were diluted to 0.5–1.0 mg proteins/ml or to 0.5 nmol cytochrome P-450/ml in 0.1 M potassium phosphate buffer (pH 7.4) and midazolam (5–10 µg/ml; final concentration) was added to the suspension. After a 3 min pre-incu-

bation period at 37°, the reaction was initiated by adding 1 mM NADPH and proceeded thereafter at 37° for various durations of time (from 0 to 30 min). At selected times, the reaction was stopped by adding 0.2 vol. of ethyl alcohol. Precipitated proteins were removed by centrifugation (15,000 *g* for 5 min) and 100–200 μl of the supernatant fluid was analyzed by the HPLC method described above.

Incubation of cell extracts with β-D-glucuronidase. Hepatocytes were exposed for 90 min to 1.0 μg/ml ^{14}C-midazolam as described above. In these conditions, large amounts of a polar derivative were present in the extracellular compartment. Extracellular medium was incubated for 2 hr at 37° with 10,000 units of β-D-glucuronidase in sodium acetate buffer (0.3 M, pH 4.5) (final volume = 0.5 ml). At the end of the enzymatic reaction, the proteins were precipitated by addition of methyl alcohol (v/v) and removed by centrifugation. The supernatant fluid was directly analyzed by HPLC.

Immunoblot analysis (Western Blots). Appropriate quantities of microsomes from each patient, previously determined to give an immunoreactive response in a linear range, were subjected to electrophoresis on polyacrylamide gels. At completion of electrophoresis, the separated proteins were electrophoretically transferred to nitrocellulose paper, which was then immunoblotted with specific goat antibodies directed against rabbit cytochrome P-450 LM_{3c}. Specific content was estimated from densitometric analysis of the blot with a Shimadzu Dual-Wavelength Scanner. Details of the technique have been described elsewhere [19].

RESULTS

Metabolism of midazolam using freshly isolated human hepatocytes

The time course of the disappearance of midazolam and the appearance of the different metabolites in the extracellular compartment have been studied after exposure of freshly isolated human hepatocytes (HL-10) to 1 μg/ml ^{14}C-midazolam.

The metabolism of midazolam in the extracellular compartment was analyzed between 5 min and 2 hr after exposure of hepatocytes to the drug (Fig. 2A). The extracellular level of midazolam decreased by half of its initial value within 5 min and only very low amounts of unchanged drug were detectable after 45 min. As the level of extracellular midazolam declined, several metabolites, synthesized within the cells, appeared in the extracellular medium. Extracellular 1-hydroxy-midazolam level increased rapidly to reach a maximum value between the 30th and the 45th-min, then declined to very low values after a 2 hr incubation period. Two other midazolam metabolites have also been detected in the extracellular compartment. Their levels, however, remained low over the entire period of observation (0.045 and 0.013 μg/ml for 1,4-dihydroxy- and 4-hydroxy-midazolam respectively).

After a time lag of approximately 10 min, a highly polar derivative rapidly appeared in the extracellular compartment. This compound represented approximately 90% of total extracellular radioactivity after a 2 hr exposure.

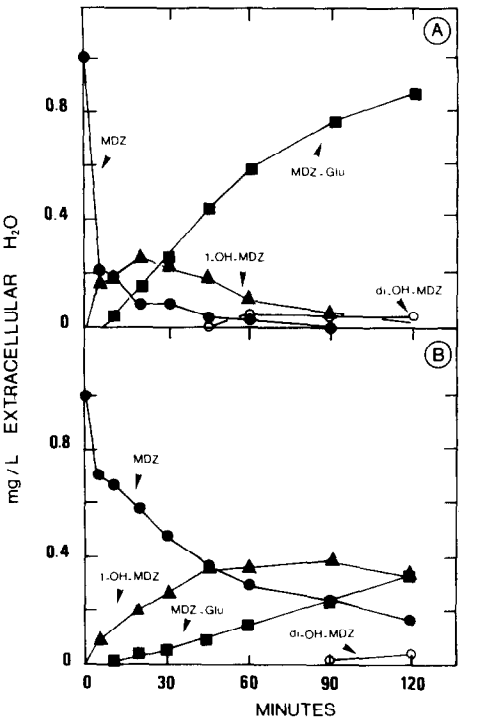

Fig. 2. Effect of fetal calf serum on midazolam metabolism by freshly isolated human hepatocytes. Hepatocytes (HL-10) were incubated with 1 μg ^{14}C-midazolam/ml in the absence (A) or the presence (B) of 10% fetal calf serum. At the indicated times, portions of the cell suspension were separated by centrifugation on silicone oil and total extracellular radiolabel was analyzed by HPLC as described in Materials and Methods.

As illustrated in Figs 3A and 3B this additional radioactive peak (referred as MDZ-Glu) did not co-elute with any of the available metabolites of midazolam. Figure 3C demonstrates that in the presence of β-D-glucuronidase, "MDZ-Glu" was completely converted into a product co-eluting with the 1-hydroxy-midazolam standard, confirming the identity of "MDZ-Glu" as a glucurono-conjugated derivative of the 1-hydroxylated metabolite of midazolam.

When 10% fetal calf serum was added to the incubation medium (Fig. 2B), the extracellular level of midazolam decreased more slowly and midazolam represented approximately 20% of total extracellular drug after a 2 hr exposure. Extracellular 1-hydroxy-midazolam achieved a steady-state level after a 1 hr incubation period. As previously observed for experiments without fetal calf serum, 4-hydroxy-midazolam and 1,4-dihydroxy-midazolam levels remained at a low level over the entire period of observation. "MDZ-Glu" appeared in the extracellular compartment after 10 min and its level increased slowly over 2 hr. 1-hydroxy-midazolam and MDZ-Glu represented the two main extracellular metabolites after a 2 hr incubation period.

Metabolism of midazolam has also been studied using various human hepatocyte preparations. Experiments have been undertaken as reported in Fig. 2B, e.g. in the presence of 10% fetal calf serum

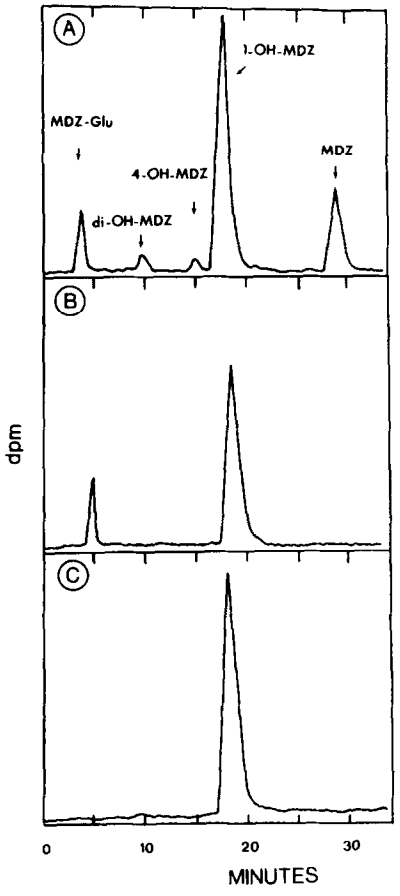

Fig. 3. (A) HPLC chromatogram of extracellular radiolabel after incubation of human hepatocytes for 30 min with 1 μg ^{14}C-midazolam/ml. (B) and (C) HPLC chromatograms of extracellular radiolabel after incubation of human hepatocytes for 90 min with 1 μg/ml ^{14}C-midazolam before (B) or after (C) a 2 hr incubation with β-D-glucuronidase.

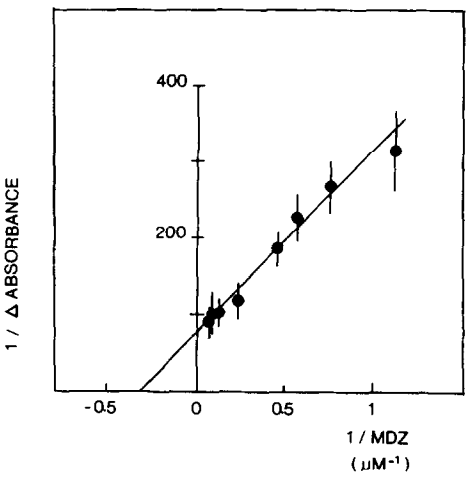

Fig. 4. Reciprocal plot of absorbance (390 nm minus 420 nm) vs midazolam concentration. Human liver microsomes (HL-2) were diluted to 1 mg/ml in 0.1 M potassium phosphate buffer and difference spectra were recorded after addition of various midazolam concentrations as described in Materials and Methods. Data are presented as the mean ± SD (bars) of three different experiments.

in the extracellular compartment. In Table 2 are reported the metabolic behaviour of midazolam after a 2 hr exposure of human hepatocytes from various liver specimens. Large inter-individual differences in midazolam metabolism could be observed among the various human hepatocytes. Hence, HL-15 hepatocytes only slowly metabolized midazolam. The 1-hydroxy-midazolam represented the major extracellular form after a 2 hr period of observation. On the other hand, HL-17 hepatocytes completely converted midazolam to 1-hydroxy-midazolam and then to its glucuronidated conjugate. After 2 hr, this latter derivative represented more than 95–99% of total extracellular radiolabel.

Differential spectroscopic analysis of the interaction between midazolam and microsomal cytochrome P-450

The binding of midazolam to human liver microsomes has been analyzed by differential spectroscopy. Typical difference spectra obtained by adding increasing amounts of midazolam to liver microsomes prepared from human liver HL-2 have been analyzed. The minima at 420 nm and the maxima at 390 nm were typical of a Type I binding spectrum resulting from the low to high spin transition accompanying the binding of midazolam to low spin cytochrome P-450. The reciprocal of the absorbance change (at 390 minus 420 nm) against midazolam concentration (Fig. 4) was linear over this concentration range as expected from a saturation process involving a single binding site (or binding sites with similar dissociation constants). From such a

Table 2. Metabolic behaviour of midazolam in various human hepatocyte preparations

Human liver	MDZ	1-OH-MDZ	4-OH-MDZ	di-OH-MDZ	MDZ-Glu
HL-10	0.162	0.324	0.016	0.033	0.325
HL-14	0.162	0.360	0.026	0.041	0.059
HL-15	0.256	0.286	0	0	0.123
HL-17	0	0	0	0	0.905
HL-18	0.016	0.429	0.021	0.066	0.107

Extracellular concentrations (μg/ml) of midazolam and its different metabolites after a 2 hr exposure of (2.5×10^6) hepatocytes/ml isolated from various human livers to 1.0 μg/ml ^{14}C-midazolam.

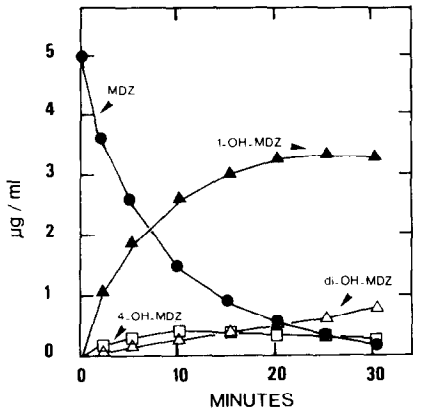

Fig. 5. Kinetics of midazolam consumption and metabolites production by human liver microsomes HL-4. Microsomes (0.5 mg/ml) were incubated at 37° in the presence of 5 µg ^{14}C-midazolam/ml and 1 mM NADPH. At different intervals of time after initiation of the reaction, aliquots of the reaction mixture were analyzed by HPLC to quantify the drug and its metabolites.

plot, an apparent dissociation constant, K_s(app.) of 3.3 µM (around 1 µg/ml) could be estimated.

Metabolism of midazolam by human liver microsomes

The time dependence of midazolam consumption over a 30 min incubation period of human liver microsomes HL-4 (0.5 mg/ml) with 5 µg/ml ^{14}C-midazolam is depicted in Fig. 5. Midazolam was rapidly metabolized and, after a 30 min exposure, the unchanged drug represented only 9.8 ± 6.4% (n = 3) (0.49 ± 0.32 µg/ml) of the total radiolabel. Over this observation period, 1-hydroxy-midazolam was recovered as the main metabolite (3.16 ± 0.11 µg/ml). Only low amounts of the other derivatives, 4-hydroxy-midazolam and 1,4-dihydroxy-midazolam, were detected (0.25 ± 0.11 and 0.66 ± 0.11 µg/ml respectively).

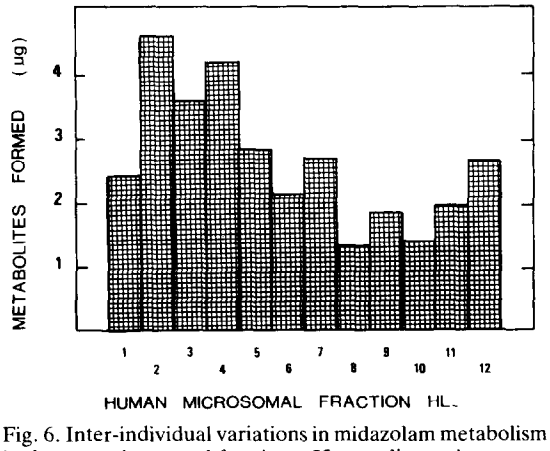

Fig. 6. Inter-individual variations in midazolam metabolism by human microsomal fractions. Human liver microsomes (HL-1 to HL-12 respectively) at a final total cytochrome P-450 concentration of 0.5 nmol/ml were exposed for 10 min with 10 µg/ml ^{14}C-midazolam and total metabolites formed were determined by HPLC.

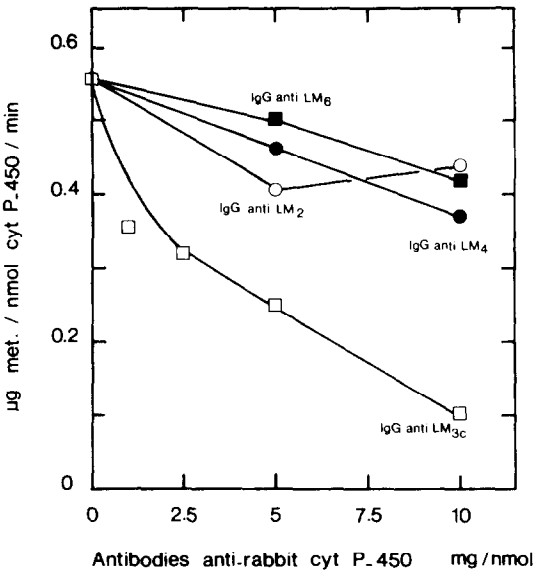

Fig. 7. Immunoinhibition of midazolam metabolism in human liver microsomes. Human liver microsomes (HL-4) were diluted to a final concentration of 0.5 nmol/ml (according to Omura and Sato [20]) and incubated for 20 min at room temperature in the absence or the presence of increasing amounts of antibodies against various forms of rabbit cytochrome P-450. The reaction was then initiated at 37° by the addition of 10 µg/ml ^{14}C-midazolam and 1 mM NADPH successively. After a 5 min period of incubation, the reaction was quenched by the addition of methyl alcohol, proteins were removed by centrifugation and the clear supernatant fluid was analyzed by HPLC.

In order to evaluate the inter-individual variabilities in midazolam metabolism, we also studied the metabolism of this drug by human liver microsomal preparations (HL-1 to HL-12). We incubated 10 µg/ml ^{14}C-midazolam with 0.5 nmol cytochrome P-450/ml for 10 min. Results are illustrated in Fig. 6 and they demonstrated large inter-individual variabilities in terms of midazolam metabolism.

Identification of the human cytochrome P-450 isozyme involved in midazolam metabolism

Microsomes from human liver HL-4 were diluted to a final concentration of 0.5 µM cytochrome P-450 and incubated for 20 min at room temperature in the absence or the presence of increasing concentrations of different antibodies directed to various forms of rabbit cytochromes P-450, including P-450 LM_2, LM_{3c}, LM_4 and LM_6 isozymes. The reaction was then initiated by the addition of 5 µg/ml ^{14}C-midazolam and 1 mM NADPH and quenched after a 5 min incubation at 37°. The data obtained are illustrated in Fig. 7. It was clear that under these experimental conditions, only antibodies directed against rabbit cytochrome P-450 LM_{3c} inhibited midazolam metabolism by approximately 75%.

Moreover, there was a linear relation (r = 0.795) between midozolam oxidase activity and the microsomal content in a cytochrome P-450 isozyme immunoquantified with antibodies directed against rabbit cytochrome P-450 LM_{3c} isozyme.

DISCUSSION

Isolated hepatocytes from different species have been routinely and increasingly used over the past decade for pharmacological and toxicological studies. Since hepatocytes express most of the functional activities of the intact liver, they are therefore suitable for investigating xenobiotic metabolism. The major drawback of this approach was that most of these studies have been carried out with rodents and that results had to be extrapolated to humans.

In this study we describe a new perfusion method that permits large amounts of hepatocytes to be obtained from a whole adult human liver. Due to the size of the human liver (1.2–1.6 kg) some adjustments had to be realized in order to achieve an optimal enzymatic dissociation of the organ. The main changes, as compared to animal liver perfusion, concerned the perfusion rate and the adjustment of both the temperature and the pH of the recirculating buffers. Three successful experiments were made on both dog and pig livers before starting to isolate hepatocytes from a whole human liver.

It is now well recognized that isolated adult hepatocyte suspensions are a heterogeneous cell population regarding centrilobular and periportal hepatocytes as well as their degree of ploidy [21]. As reviewed by different authors [8, 21], these various sub-populations expressed different metabolic abilities. In this paper we developed a technique to enable the almost complete dissociation of a whole adult human liver and the isolation of a 10×10^9 to 50×10^9 hepatocytes. Methods based on the perfusion of a portion of the whole liver or large biopsies only permitted the hepatocyte isolation from well perfused parts of the liver [8]. The recirculating perfusion of the whole liver allowing a 70–90% dissociation of the organ would permit the obtainment of a more representative hepatocyte population relative to the entire liver.

Obtaining a large number of hepatocytes also enabled a simultaneous study of transport and metabolic processes of various drugs under identical and/or different experimental conditions, in both suspensions and primary cultures. Data obtained with other compounds such vincaalkaloids and mitoxantrone, two anticancer drugs, and using the same liver preparations, have already been described [22, 23].

As a single liver perfusion results in a large number of cells, we are devising a protocol for freezing hepatocytes that may metabolize various xenobiotics under suspension conditions after thawing, but also attach to a support and survive for several days in primary-culture. This approach also gives the opportunity to store hepatocytes which expressed specific metabolic functions. At present, this part of the work is under further investigation.

In this report we have checked some metabolic activities of hepatocytes by using a substance probe, midazolam. This compound was particularly interesting since (i) its biotransformation processes involved Phase I and Phase II reactions [15, 24], (ii) all metabolites were already known and available, and (iii) its pharmacokinetics as well as that of its 1-hydroxylated derivative were intensively studied in our laboratory*. Heizmann *et al.* [24] reported that in the liver, midazolam was metabolized in 1-hydroxy-, 4-hydroxy- and 1,4-dihydroxy-midazolam which were subsequently glucuronidated before renal elimination. In freshly isolated human hepatocytes, midazolam was rapidly cleared from the extracellular medium, accumulated within the cells and metabolized into different derivatives which were rapidly recovered in the extracellular compartment. As reported by various authors, 1-hydroxy-midazolam and "MDZ-Glu" are, in humans, the main metabolites recovered in plasma and urine respectively [24–27]. The hepatocyte model also permits the identification and quantification of the Phase II reactions, consisting for midazolam in the conjugation of glucuronic acid to the 1-hydroxylated derivative of midazolam. After a 2 hr exposure of hepatocytes to 1 μg/ml midazolam, 90–95% of extracellular drug were recovered as the glucuronidated metabolite. This is in agreement with the *in vivo* studies of Heizmann *et al.* [24] who reported that 90% of the radioactivity, presumably as a glucuronic form, appeared in the 24-hr urine after an oral administration of ^{14}C-midazolam to humans.

It is now well known that human hepatocytes in primary-culture or in suspension represent a suitable model for drug metabolism studies. In a recent report, Le Bigot *et al.* [28] clearly demonstrated that cultured hepatocytes retained their *in vivo* specific drug metabolizing activities, including inter-species polymorphism, using ketotifen as a model substrate. The applicability of isolated hepatocytes to the study of interspecies difference in hepatic xenobiotic metabolism was also demonstrated by Green *et al.* [29] for amphetamine and by Chenery *et al.* [20] for diazepam.

In conclusion, the data presented in this study and from other studies [28–30] indicate that isolated or cultured hepatocytes are an excellent system for studying qualitative and quantitative species differences in drug metabolism. Clearly, many further studies will be required to fully establish the relationships between metabolism *in vivo* with metabolism in the hepatocyte model.

Midazolam is also rapidly metabolized by microsomal fractions prepared from different human livers. The cytochrome P-450 mono-oxygenases which are located in the endoplasmic reticulum may exist in many forms characterized by their substrate specificities, electrophoretic, chromatographic and immunological properties [31]. The mono-oxygenases are inducible by environmental chemicals such as phenobarbital [21], macrolide antibiotics [18, 32], steroids, and other exogenous compounds. These enzymes are also regulated by various endogenous factors such as hormones, age, sex and nutritional conditions [33].

From the data presented in this paper we can postulate that the human cytochrome P-450 HLp, homologous (81% homology on the basis of nucleic acid sequences) to the LM_{3c} form in rabbits [32] was predominantly involved in the metabolism of midazolam by human liver microsomes. This con-

* Unpublished observations.

clusion was drawn from the following observations: (i) Among a library of liver microsomal fractions prepared from untreated or inducer-treated rabbits, only those obtained from animals treated with macrolide antibiotics—specific inducers of the cytochrome P-450 LM_{3c} in rabbits—exhibited a Type I binding spectrum upon midazolam addition and significantly metabolized midazolam to its 1-hydroxylated derivative [34]. (ii) When human liver microsomes were analyzed by immunoblotting with anti-cytochrome P-450 LM_{3c} antibodies, each of the 12 specimens (HL-1 to HL-12) reacted with the antibody and produced a single band [35, 36]. (iii) Anti-cytochrome P-450 LM_{3c} antibodies strongly inhibited the metabolism of midazolam in human liver microsomes whereas anti-rabbit cytochrome P-450 LM_2, LM_4, or LM_6 antibodies only slightly decreased midazolam metabolism. (iv) A linear correlation existed between midazolam oxidase activity and the specific content in cytochrome P-450 immunoquantified by anti-rabbit cytochrome P-450 LM_{3c} antibodies.

Using this sub-cellular model we quantified the relative affinity of midazolam for this enzyme(s) and evaluated the inter-individual variabilities in drug metabolism. However, due to the lack of conjugation processes in the microsomal fractions (in the absence of UDP-glucuronic acid), the glucuronidated derivative of 1-hydroxy-midazolam was the main metabolite in hepatocytes while the 1-hydroxy-midazolam represented more than 70% of total radiolabel in microsomal preparations. Nevertheless this latter model was of utmost interest to (i) discriminate the enzymatic process (cytochrome P-450 isozyme) involved in midazolam metabolism, (ii) evaluate the affinity of the drug for the enzyme complex(es) and (iii) demonstrate the inter-individual variabilities.

In conclusion the development of a methodology allowing the perfusion and enzymatic dissociation of a whole adult human liver permits very large amounts of hepatocytes to be obtained. This abundant cellular material, which was shown to conserve both cellular integrity and Phases I and II enzyme processes, can be used for several purposes such as (i) studies in freshly isolated hepatocytes, (ii) studies on short- and/or long-term cultures, and (iii) constitution of a human hepatocyte library stored in liquid nitrogen. These *in vitro* tools can be, at a preclinical stage, very powerful in determining the qualitative and quantitative importance of transport and biotransformation processes of a class of drugs, using human material.

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