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COMPARATIVE DRUG METABOLISM OF DIAZEPAM IN HEPATOCYTES ISOLATED FROM MAN, RAT, MONKEY AND DOG

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Abstract—Diazepam (DZ) was used as a substrate in drug metabolism studies to characterise the differences in metabolite profiles in hepatocytes isolated from four species: Wistar rat, cynomolgus monkey, beagle dog and man. Hepatocytes were incubated with DZ (20 μM) for 180 min at 3 hr post isolation in culture, and the disappearance of parent compound and appearance of its metabolites determined. DZ disappearance was found to be monoexponential in rat, monkey and human cells, but that DZ disappearance in dog hepatocytes was best described by a two compartment process. There were considerable differences in both the rates of formation and the profiles of metabolites produced from DZ in each species. Drug metabolism of DZ was determined in five human hepatocyte preparations. The rates of formation of both the major metabolites, temezepam (TEM) and nordiazepam (NOR) were highly variable between samples, and oxazepam (OX) was detected in only three of the preparations. There was no evidence of further metabolism of these metabolites, and the profiles were comparable with *in vivo* findings. In a single case, human hepatocytes were cultured for five days, and DZ was used as substrate to characterise the changes in drug metabolising activities. There was a rapid loss in the production of OX in the initial 24 hr, and a complete loss of 3-hydroxylation activities in the succeeding 120 hr. *N*-demethylation activities, however, were well maintained, and the appearance of NOR declined to 47% of initial rate.

The hepatocytes of all species were found to produce NOR and TEM as metabolites; NOR representing the principal metabolite in the dog, monkey and human cells. In the dog, TEM was found only as a minor metabolite. OX was a significant metabolite in the monkey and a minor metabolite in the dog and human hepatocytes, but was not detected in rat cultures. The principal metabolite in rat cells was 4′-hydroxy diazepam, which was rapidly further metabolised to its glucuronide.

The drug metabolising activities of the hepatocyte cultures towards DZ were comparable with the drug metabolism of DZ found *in vivo* in each species. These findings substantiate hepatocytes as an *in vitro* model of hepatic metabolism.

Studies in this laboratory [1] and previously [2] have shown that a profile of hepatocellular metabolism can be established in cultured human hepatocytes. However, the effectiveness of human hepatocytes as a model of *in vivo* human hepatic metabolism is limited by the need to validate an *in vitro* to *in vivo* extrapolation in other species.

Rat hepatocytes have been used extensively in drug metabolism and toxicity studies, and in a number of instances have been shown to be an excellent *in vitro* model of *in vivo* metabolism [3–5]. However, rat cells show significant losses in their drug metabolising activities during short term culture [3, 6], and their monooxygenase activities are often unrelated to those observed in man, thereby reducing their facility as a model of human drug metabolism. Only rarely are hepatocytes from other species considered for their relative potential as a model of human drug metabolism either *in vitro* or *in vivo*, although Green *et al.* [7] have demonstrated that hepatocytes prepared from five species, including human, were able to reproduce overall patterns of amphetamine metabolism seen *in vivo*, and Gee *et al.* [8] have also shown differences in the metabolism of tolbutamide that reflect the hepatic profiles obtained *in vivo*.

The present studies were therefore designed to evaluate the use of hepatocytes isolated from rat, dog, cynomolgus monkey and man to investigate the accuracy with which species-related differences in drug metabolism could be reproduced *in vitro*. Diazepam (DZ), a benzodiazepine effective as an anxiolytic and muscle relaxant, was chosen as a model substrate. It is metabolised by a series of similar pathways in different species, but demonstrates differences in the rates of formation and disposition of these pathways and therefore has been shown to be a useful probe of species differences in drug metabolising activities both *in vivo* [9, 10] and *in vitro* [1].

MATERIALS AND METHODS

Materials. 4-(2 Hydroxyethyl)-1-piperazine-ethane sulphonic acid) (HEPES); ethylene glycol bis-(β-amino ethyl ether)-*N*,*N*′,*N*′-tetraacetic acid (EGTA), bovine pancreatic insulin; bovine serum albumin (BSA fraction V); hydrocortisone hemisuccinate and newborn calf serum (mycoplasma and virus screened); were obtained from Sigma Chemicals Ltd (Poole, Dorset, U.K.), Earle's balanced salt solution (EBSS) (10 × concentrate; calcium and magnesium free, without bicarbonate); sodium

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bicarbonate solution (7.5% w/v); penicillin (5000 IU.ml^{-1}) plus streptomycin (5000 μg.ml^{-1}); neomycin (10,000 μg.ml^{-1}) and NUNC multidish 6-well plates; were obtained from Gibco Europe Ltd (Cowley, Middx, U.K.).

Trypan blue and carbon monoxide were obtained from BDH Chemicals Ltd (Poole, Dorset, U.K.); collagenase from *Clostridium hystoliticum* was supplied by Boehringer Ltd (Lewes, Sussex, U.K.); Renex 690 by Atlas Chemicals (Surrey, U.K.); WME by Imperial Laboratories Ltd (Salisbury, Wilts, U.K.); collagen by Cooper Biomedical (Lorne Diagnostics, Bury St. Edmunds, U.K.); incubator by W. C. Heraeus Ltd (GmbH, Germany) and 65 μm nylon mesh by Henry Simon Ltd (Stockport, Cheshire, U.K.).

Diazepam, *N*-desmethyldiazepam, temazepam, oxazepam, 4′-hydroxy *N*-desmethyl diazepam and 4′-hydroxy diazepam were donated Roche Products Ltd (Welwyn Garden City, Herts, U.K.).

Preparation of hepatocytes. Liver was obtained from male Wistar rats (250–300 g) and male Beagle dogs (12–15 kg, 2–4 years) both supplied by Department of Laboratory Animal Science, SK&F; male cynomolgus monkeys (3.1–3.8 kg) were supplied by Huntingdon Research Centre (Huntingdon, Cambs, U.K.). Human liver was obtained as unmatched liver transplant material from Queen Elizabeth Medical Centre, Edgbaston, Birmingham, U.K.

Hepatocytes were isolated from dog, monkey and human tissue samples by end of lobe perfusion using the technique of Strom *et al.* [11]. The largest human liver sample or largest whole dog or monkey lobe was chosen and a selected portal vessel or vessels cannulated with a tight-fitting cannulae. The vessels chosen were those producing clearance of the maximum volume of the liver sample or lobe. Rat hepatocytes were isolated by whole liver perfusion, modified from that of Seglen [12]. The portal vein was cannulated *in situ* and, after clearing, the whole liver was removed into the perfusion chamber.

For all species perfusion medium was EBSS buffered at pH 7.4 with 10 mM $NaHCO_3$ and 15 mM HEPES. Perfusion was a 3-step procedure: (a) perfusion for 2 min with EBSS without calcium or magnesium containing 0.5 mM EGTA, to remove extracellular calcium, (b) perfusion for 3 min with EBSS without calcium or magnesium and without EGTA, to remove EGTA from the liver samples, (c) perfusion for 17–25 min with EBSS without magnesium but containing 1.7 mM Ca^{2+} ions as $CaCl_2.6H_2O$, containing 35 mg.100 ml^{-1} collagenase for rat liver, or 50 mg.100 ml^{-1} collagenase for monkey, dog and human livers. Collagenase activity was 0.17 U.mg^{-1} (Boehringer).

Cells isolated from human liver samples were generally of poorer quality than those of other species. The human liver samples were flushed with slightly hypertonic medium immediately after resection but perfusion took place at least 4.5 hr post-operatively. Neither the cell yields per gram tissue, nor the cell viabilities matched those obtained with fresh liver from dog and rat or from 1.5 hr-old liver tissue from monkey. Nevertheless human cells isolated from eight liver samples (perfused at intervals between 4.5 and 12.5 hours post resection) cultured sufficiently well to permit drug metabolism studies.

Isolated cells were filtered through nylon mesh, 65 μm (Henry Simon) and washed three times by centrifugation and resuspension in EBSS containing 1% w/v bovine serum albumin. Viability was assessed by Trypan-blue exclusion, and counted on an Improved Neubauer haemocytometer. Viabilities were better than 85% of good quality cells for dog, rat and monkey cells, and better than 65% viability for human hepatocytes.

Hepatocytes were plated onto 35 mm six-well plates coated with soluble calf skin collagen at an innoculation density of between 0.4 and 0.7 × 10^6 cells.well^{-1}. Cells were adhering to the plate within 2 hr post isolation and became confluent monolayers within the subsequent 24 hr. At confluency, cell densities varied considerably: rat, 605 × 10^3 cells.well^{-1}; dog, 245 × 10^3 cells.well^{-1}; monkey, 293 × 10^3 cells.well^{-1}; and human, 93.5 × 10^3 cells.well^{-1}.

Plates containing the cells were cultured in a 37° incubator (Heraeus) in a 5% CO_2:21% O_2 controlled atmosphere. Culture medium was Williams Medium E (WME) containing L-glutamine (4 mM): bovine insulin (1 μg.l^{-1}); hydrocortisone hemisuccinate (4.8 μg.l^{-1}); penicillin (100 × 10^3 IU.l^{-1});. streptomycin (100 mg.l^{-1}); neomycin (100 mg.l^{-1}) and 10% v/v newborn calf serum. 2 ml of medium was used for each well and medium was changed at 3 hr and every 24 hr thereafter.

Measurement of cytochrome P450. Cell culture medium was removed from six wells and the cell monolayer washed with isotonic saline. 1 ml of a solubilising buffer containing Renex 690 (in place of Emulgen 911; as described by Warner *et al.* [13] was added at room temperature to each well. The cell layer was scraped into the buffer and the contents of two wells combined. The solubilised cell mixture was then centrifuged at 1500 *g* for 2 min at room temperature (Sorvall RT 6000) to remove cell debris. The supernatant was decanted into a clean tube and total cytochrome P450 measured by the method of Omura and Sato [14] using a Hewlett-Packard 8450A diode-array spectrophotometer.

Incubation with drug substrates. Drug substrates were prepared in WME without phenol red containing the same additions as that in culture medium. Buffering differed from that used in culture and contained 8 mM $NaHCO_3$ plus 20 mM HEPES at pH 7.40 at 37° (pH 7.57 at 20°). Drug substrates under investigation were DZ, 20 μM; *N*-desmethyl diazepam (NOR), 6 μM, and Temazepam (TEM), 20 μM.

The cell monolayers in 12 wells (two plates) were washed with isotonic saline at 37°. One millilitre of WME containing the substrate was added to each well. The plates were held at 37° in the CO_2 incubator for 180 min and 1 ml samples were taken from successive wells at 15 min intervals between 0 and 180 min. These samples were placed in capped, polystyrene tubes and stored at −80° until analysis.

Determination of diazepam and its metabolites. The concentration of DZ and its metabolites was determined by HPLC using a Hewlett-Packard (Winnersh, Berks, U.K.) 1090A chromatograph fitted with a filter photometric detector (230 nm) and auto-

injector. The separation was performed on a Waters C-18 μBondapak 30 cm × 46 mm column with prefrit filter, employing a gradient; methanol (65% to 78% v/v) and ammonium acetate 0.01 M pH 6.00 (adjusted with CF_3COOH; 35% to 22% v/v). Oven temperature was held at 50° and retention time for DZ as the least polar compound was 9.5 min. The system was controlled by a Hewlett-Packard-85 microcomputer running resident Hewlett-Packard-HPLC software, hard disc and flexible disc as methods cache and recorded with a Hewlett-Packard 3392A integrator.

Authentic standards of DZ, NOR, TEM, OX, 4′-OH DZ and 4′-OH NOR (Roche) were formulated in water and determined in the same manner as WME samples. The identity of metabolites produced during incubation with hepatocytes was confirmed by liquid chromatography/mass spectrometry and by diode-array analysis. Limit of quantitation was 0.1 μM. Incubated samples were analysed within 14 days of experimentation; metabolites were stable at −80° in WME for at least 90 days.

Normalisation of semilog plots. For comparative purposes, the data sets of the disappearance of DZ in each species were normalised to 500×10^3 cells.well^{-1}. Datum points, from each data set, for the ordinate axis (time), were reduced by the fraction representing the normalisation. For example:

Number of cells.well^{-1} in monkey hepatocyte incubations = 293×10^3,

$$\text{fraction representing the normalisation} = \frac{293}{500},$$

therefore, new ordinate scale = 0 min to

$$\left(\frac{293}{500} \times 180\right) \text{min}.$$

Datum points plotted for human, dog and monkey were 15 min intervals from 0 to 180 min. Datum points for rat cells were 0 to 90 min. For clarity, points from 90 to 180 min were not included.

RESULTS

The scheme of metabolism of DZ in rat, dog, monkey and human hepatocytes is shown in Fig. 1.

Clearance of diazepam in rat, dog, cynomolgus and human hepatocytes. Figure 2 is a semi-log plot of DZ metabolism in the four species investigated. For comparative purposes the plots were normalised for 0.5×10^6 cells. The results are shown for a typical experiment repeated at least once. The disappearance of DZ in rat, human and monkey cells was found to be monoexponential, but the kinetics of its disappearance in dog hepatocytes was best described by a two-compartment process. The plots also show that the clearances for DZ in cultured hepatocytes differ significantly between the species studied. The relative clearances of DZ are in the sequence: monkey > rat > human, with dog cells showing a mixed profile, with the α-phase elimination initially greater than monkey, but with the β-phase elimination somewhat greater than human hepatocytes, but lower than in rat cells. The sequence in the later stages of incubation was therefore: monkey > rat > dog > human. This sequence is reflected in the cytochrome P450 content of the hepatocyte preparations (Table 1) with monkey cells containing the highest levels of cytochrome P450, and human the lowest. Again, the sequence was: monkey > rat > dog > human.

Additional studies were undertaken to establish the biphasic mechanism of DZ in dog hepatocytes.

There are reports of an *in vitro* inhibition of DZ metabolism by NOR, TEM and OX in the rat [15], so the possibility of a product inhibition of DZ metabolism was investigated in a further study in dog cells. Inhibition studies with TEM or OX were not undertaken, but NOR was introduced to the dog hepatocytes in the medium at concentrations 3 μM, 6 μM, 9 μM, 12 μM and 15 μM in the presence of DZ (20 μM). There was no evidence that the clearance of DZ by the hepatocytes was affected by the addition of extracellular NOR. However, total amounts of both NOR and OX were found to partition between cells : medium in the ratio 30 : 70, indicating that the cell-associated concentration of these metabolites was more than 200-fold that in the medium. Assuming an approximate cell monolayer volume of 10 μl.10^6 cells^{-1}, NOR attained a cell-associated concentration of 1700 μM compared to an extracellular concentration of 8.2 μM, and OX attained 490 μM compared with 1.1 μM, Fig. 3. The partition-ratio was independent of the extracellular concentration, and at no concentration was there an increased inhibition of DZ clearance. The extracellular/cell-associated partition is evident (Fig. 3) as the metabolism of DZ in the dog cells reaches steady state at 90 min.

Metabolism of diazepam in human hepatocytes. Figures 4 and 5 compare the drug metabolism of DZ in human hepatocytes in suspensions (H3, H4, H5) and as cultured cells (H7 and H8). Activities were adjusted to nmol.0.5×10^6 cells^{-1}, and show large differences in the rates of metabolism between cell preparations. These differences are intrinsic. They are not related to the interval between removal of the liver sample at resection and perfusion into isolated hepatocytes. They are also unrelated to the quality of the hepatocytes after preparation. The cells from preparations H5, H7 and H8 were separated using a Percoll density gradient, with no apparent differences or raised metabolic activities, compared with H3 and H4 cells. The rates of disappearance of DZ.10^6 human cells^{-1} were always lower than those found in the other species, perhaps reflecting the lower levels of total P450 in human cells (Table 1).

In freshly isolated cells NOR and TEM were both produced as major metabolites in each of the human preparations. The principal metabolite formed was usually NOR, but in preparation H8, NOR and TEM were produced at nearly identical rates. In this respect human hepatocytes differed from the metabolism found in the cells of other species. TEM was formed as a significant metabolite in both the monkey and the rat, but only in the rat was the rate of formation of TEM comparable with the rate of formation of NOR, and in this case there was significant further metabolism on both metabolites. Similarly to the monkey, preparations H3, H5 and H8 produced OX as a minor metabolite, and in general

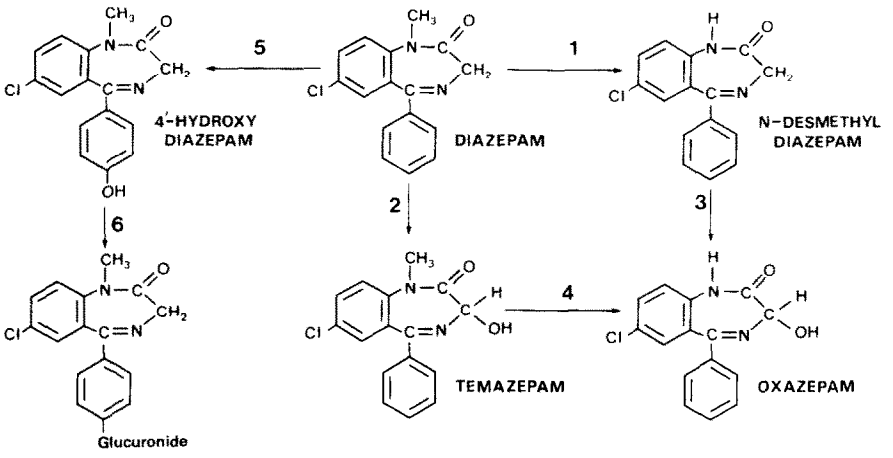

Fig. 1. Scheme of metabolism of DZ in hepatocytes isolated from rat, monkey, dog and human livers. Pathways 1 and 4 are *N*-demethylation activities. Pathways 2 and 3 are 3-hydroxylation activities. *N*-desmethyl DZ is found in rat, monkey, dog and human cells. Temazepam is found in rat, dog, monkey and human cells. Oxazepam is found in dog, monkey and human cells. In rat hepatocytes DZ undergoes a 4'-hydroxylation of the C4 aromatic ring by pathway 5, and subsequent glucuronidation, by pathway 6. There was no glucuronidation of other metabolites.

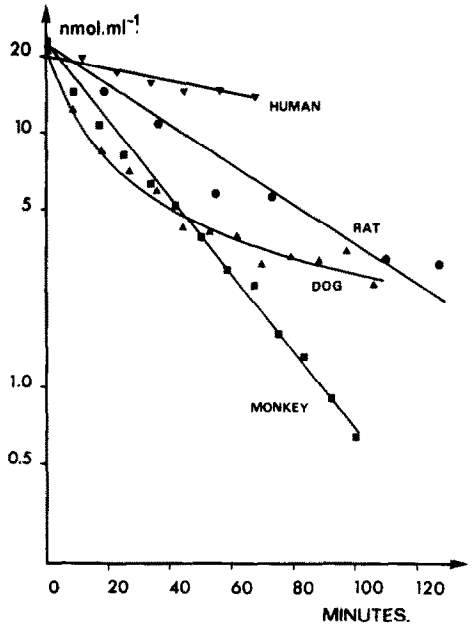

Fig. 2. Comparative semilog plots of the disappearance of DZ in rat, monkey, dog and human hepatocytes. Disappearance was monoexponential in rat, monkey and human cells, with disappearance resembling a two-compartment process in dog hepatocytes. The ordinate axis (time) was normalised to the metabolism in 500 × 10^3 cells.

human cells showed much greater similarity with the monkey than the other species.

Hepatocytes prepared from H8 were cultured for five days to investigate the loss of drug metabolising functions during this period. Cells were challenged with 20 μM DZ at 3, 24 and 120 hr in culture.

Table 1. Comparison of the total cytochrome P450 content of hepatocytes isolated from dog, rat, human and monkey liver, cultured for 3 hr in WME, supplemented with hydrocortisone and insulin

Species	Number of preparations	Cytochrome P450 (pmol.10^6 hepatocytes^{-1})
Cynomolgus	2*	430.2
Rat	6	257.6 ± 37.3
Dog	4	184.3 ± 23.5
Human	4	140.1 ± 45.7

Values are mean ± 1 SD.
* Mean of two determinations.

Figure 5 shows there was clearly a differential loss in the *N*-demethylation and the 3-hydroxylation metabolising activities during culture. The loss in 3-hydroxylation activity was greater, the activity being unmeasurable by 120 hr whereas there was a loss of approximately 50% of demethylation activity in the same period.

This is in contrast to drug-metabolising activities found in long-term cultured monkey hepatocytes, in which there is a selectively greater loss of demethylation activity, and a specific maintenance of 3-hydroxylation pathways for, at least six days (in press).

In common with both the dog and monkey, but in contrast to rat cells, human hepatocytes showed evidence neither of glucuronidation, nor of 4'-hydroxylation metabolism.

Metabolism of diazepam in non-human species. Profiles of the metabolism of DZ in cultured parenchymal hepatocytes isolated from dog, rat and monkey livers are presented in Fig. 6. There are clear differences between the species in both rates and routes of drug metabolism of DZ, and significant

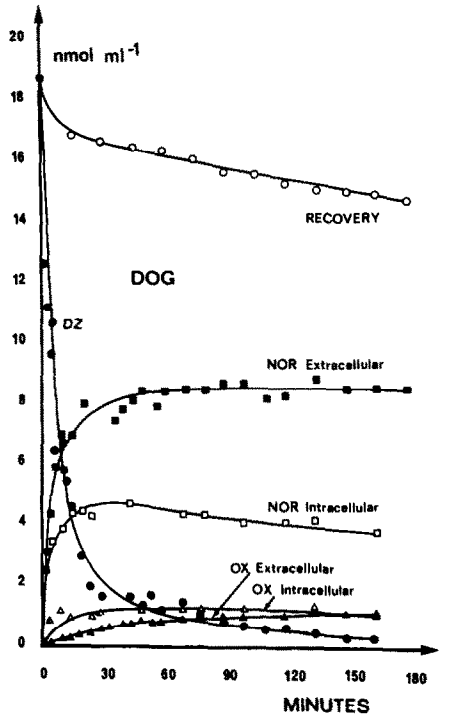

Fig. 3. Profiles of DZ metabolism in the cell-associated (intracellular) and extracellular compartments in dog hepatocyte cultures. Extracellular metabolites were measured in medium removed from the cell monolayer. Cell-associated metabolites were measured in extracts of 1 ml of 30% methanol:70% water, into which the cell monolayer was homogenised and the cell debris removed by centrifugation. The volume of the monolayer for 245×10^3 cells.well^{-1} was assumed to be 2.45 μl (volume occupied by 10^6 cells = 10 μl). The hepatocytes exceeded a 200-fold accumulation of OX and NOR. At 90 min, in an intracellular volume of 2.45 μl and an extracellular volume of 1 ml, the concentration of: cell-associated NOR = 1700 μM; extracellular NOR = 8.2 μM; cell-associated OX = 490 μM; extracellular OX = 1.1 μM; extracellular DZ = 0.85 μM. DZ was not detected in the intracellular compartment and TEM was not detected in either compartment.

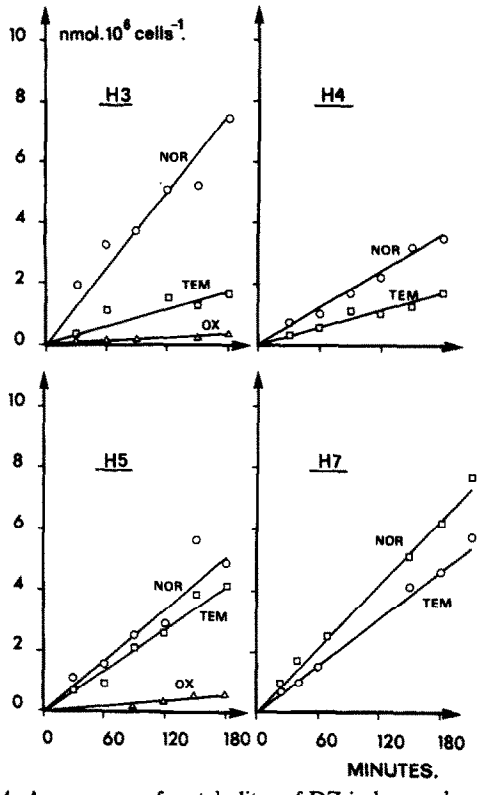

Fig. 4. Appearance of metabolites of DZ in human hepatocytes prepared from four human liver samples, H3, H4, H5 and H7. Plots are normalised 10^6 cells^{-1}. Cell numbers were $<0.5 \times 10^6$ cells.ml^{-1} and disappearance of DZ was less than 20% of initial substrate (20 μM) in all preparations. OX was detected only in preparations H3, H5 (and H8, Fig. 5). Metabolites were determined in cell suspensions for H3, H4 and H5; and in three-hour cell culture for H7. Each point represents a single estimation.

differences in the secondary metabolism of the principal metabolites of DZ, TEM and NOR.

Metabolism in rat hepatocytes. In several respects the metabolism of DZ in rat hepatocytes (Fig. 6a) differed considerably from dog, monkey and human cells. Rat cells were able to completely eliminate DZ from the medium with TEM and NOR produced as major metabolites. TEM and NOR were formed rapidly in the early period of incubation, with subsequent secondary metabolism of both. None of the other species showed further metabolism of NOR, and rat cultures differed substantially in this respect. The profile of appearance of TEM resembled that found in the monkey hepatocytes. Both NOR and TEM were extensively further metabolised by pathways other than those to OX, since OX was undetectable in the medium, although neither of these pathways were by conjugation with glucuronide.

Uniquely, in these studies rat hepatocytes were found to metabolise DZ to a phenyl 4′-hydroxylated product, 4′-hydroxy DZ, that was subsequently rapidly glucuroniated (pathways 5 and 6, Fig. 1). This pathway represented nearly 50% of the initial disappearance of DZ and showed that rat hepatocytes were also able to hydroxylate DZ at a site away from the diazepine ring. Authentic standards for both 4′-hydroxy-DZ and 4′-hydroxy-NOR were obtained and the appearance of 4′-hydroxy-DZ verified by retention times. A metabolite corresponding to 4′-hydroxy-NOR was not observed, indicating that the observed rapid secondary metabolism of NOR was not by this pathway. Also, there were no other metabolites with a similar HPLC retention to those of either of the 4′-hydroxy metabolites of DZ or NOR, suggesting that the disappearance of TEM in rat cells was also not by the formation of 4′-hydroxy TEM.

Metabolism in cynomolgus hepatocytes. Drug metabolism of DZ in hepatocytes isolated from the monkey is shown in Fig. 6(b) and was found to be predominantly by the *N*-demethylation of DZ to NOR. In common with human and rat hepatocytes but in contrast with dog hepatocytes, the 3-hydroxylation pathway contributed a much greater pro-

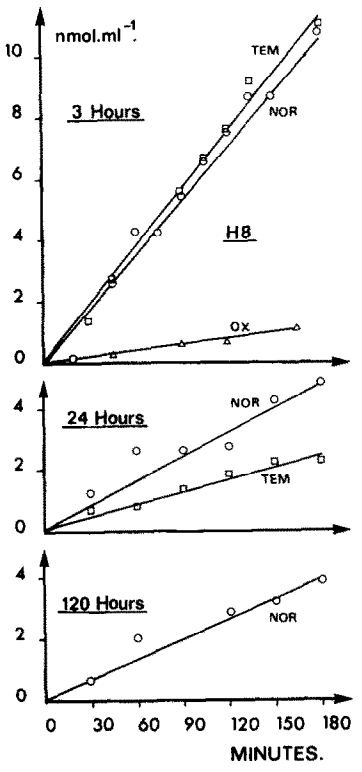

Fig. 5. Profiles of the drug metabolism of 20 μM DZ by human hepatocytes prepared from H8, and cultured for five days in WME. Drug metabolising activities were determined at 3, 24 and 120 hr post isolation. There was a reduction in both *N*-demethylation and 3-hydroxylation activities in the initial 24 hr of culture, and an abolition in the formation of OX. At 120 hr 3-hydroxylation activities were also abolished, and only NOR was produced. Each point represents a single estimation.

portion of the disappearance of DZ, with significant transitional appearance of TEM. In the monkey, TEM was further metabolised by two pathways (data not shown): (a) to OX by *N*-demethylation and (b) by an unidentified pathway that was neither glucuronidation nor C-5 phenyl hydroxylation, pathway 6 in Fig. 1; a pathway found *in vivo* in several species. Only a minor proportion of TEM was metabolised to OX, representing approximately 25% of the total disappearance of TEM. In common with dog hepatocytes, this pathway appeared to be the principal route by which OX was formed in monkey cells. There was no evidence of further metabolism of NOR, so that the 3-hydroxylation of NOR was probably not significant to the appearance of OX.

Metabolism in dog hepatocytes. The metabolism of DZ in dog hepatocytes is presented in Fig. 6c. The demethylation product, NOR, was found to be the principal metabolite in the dog. There was a rapid initial production of NOR, reaching steady state at about 120 min of incubation, although DZ was not fully eliminated from the medium at this time; the extracellular concentration of DZ reducing to about 3 μM. In common with human and monkey hepatocyte cultures, further metabolism of NOR was undetectable in the dog. Rates of formation of both TEM and OX in dog cells were low, and no further metabolism of either of these metabolites was detected. Final extracellular concentrations of NOR in dog cultures exceeded 10 μM yet the appearance of OX remained at low levels, indicating that 3-hydroxylation of NOR to OX is not a significant pathway. This was substantiated in studies (not shown) in dog hepatocytes where it was shown there was a much greater affinity of the 3-hydroxylation enzyme(s) for DZ than for NOR despite the similarities of these substrates. There was no evidence of formation of glucuronides of either TEM or OX.

DISCUSSION

It is inappropriate to represent metabolism and disposition of drugs in liver parenchymal cell cultures as directly equivalent to that obtained *in vivo*. In a closed culture system, the accumulation of a given metabolite will depend on both the rate of its formation and the rate of its removal by subsequent metabolism. In contrast, the extent of accumulation of the same metabolite in the plasma will depend upon a larger number of factors including the rate of its formation, the partition of the metabolite between the plasma and bile, the volume of distribution of the metabolite and the rate by which the metabolite is eliminated by excretion or further metabolism. Thus, the appearance and accumulation of a metabolite in the plasma, bile or urine will depend upon the relative rates of these processes. Therefore, a direct correlation of the accumulation of a metabolite in the hepatocyte system with the extent of accumulation in any one body fluid such as plasma, bile or urine is not necessarily to be expected. Thus studies in simple hepatocyte systems enable the experimenter to focus on the biotransformation processes occurring on a molecule in the absence of other species differences such as variation in protein binding [16] of DZ or variation in the excretion of metabolite via the bile or urine [17].

In the rat [17] and monkey [18], DZ and its metabolites are predominantly excreted in the bile, whereas the principal route of excretion in the dog [19] and man is in the urine. However, in all cases DZ is eliminated predominantly through metabolism.

Despite these theoretical limitations, cultures of parenchymal cells clearly show utility as an *in vitro* drug metabolising system. In the case of DZ there are specific similarities that exist between the plasma metabolite profiles of DZ and the hepatocyte metabolite profiles obtained in the rat, monkey, dog and human, that help to substantiate hepatocytes as a model of hepatic metabolism *in vivo*.

The plasma profiles of DZ, NOR and OX reported in man indicate several similarities with the profiles found in hepatocytes, *in vitro*. Boxenbaum [20] and Klotz *et al.* [9] reported relatively long half-lives for DZ in human plasma, of 36 and 45 hr respectively, with low clearance values. The clearance in human hepatocytes was the slowest obtained in the four species. The principal metabolite, both *in vitro* and *in vivo* was NOR. Boxenbaum reported a half-life of 45 hr for NOR, with very slow further metabolism to OX, and then to OX-glucuronide, the pre-

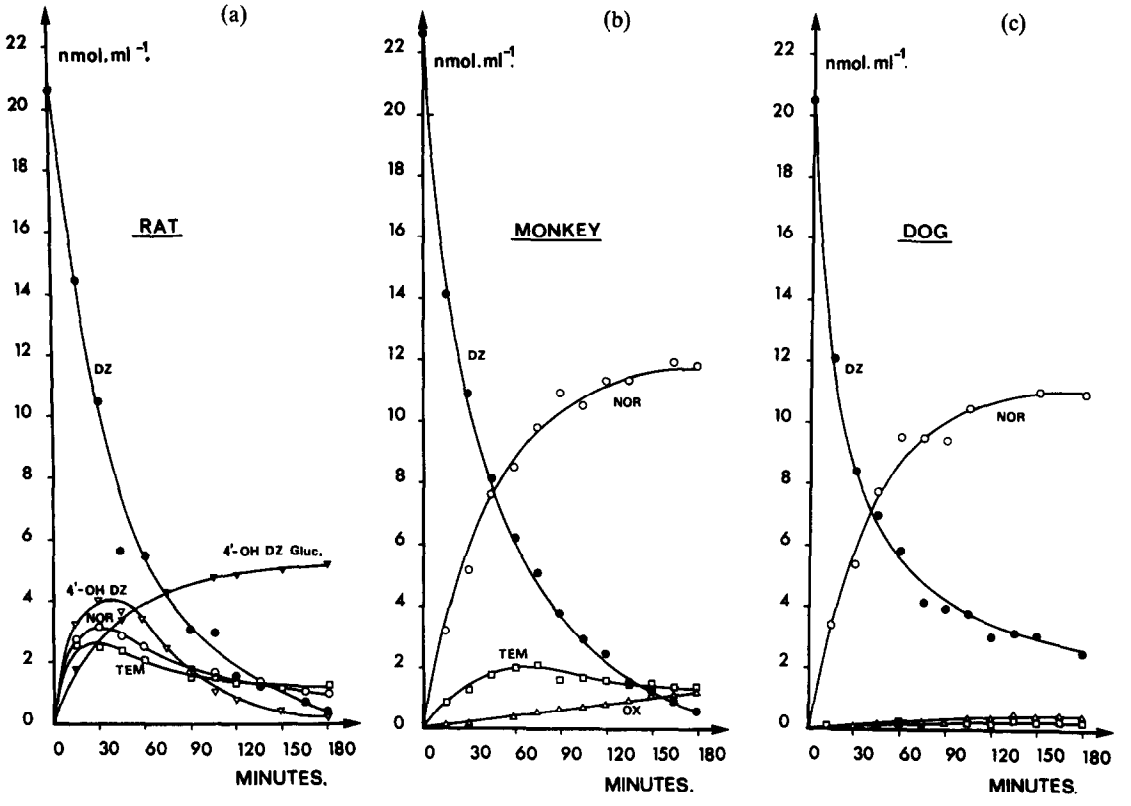

Fig. 6. Profiles of the drug metabolism of 20 μM diazepam in hepatocytes isolated from rat (a), cynomolgus monkey (b) and dog (c) hepatocytes. Hepatocytes were cultured in WME for 3 hr in six-well plates. Drug metabolising activities were measured for 180 min at 15-min intervals. Each experiment was repeated with essentially similar results.

dominant urinary product. However, the glucuronide of OX was detected neither in plasma, nor in the hepatocyte cultures.

The disposition of TEM *in vitro* showed a poorer resemblance to the plasma profile *in vivo*. Vree *et al.* [21] and Guentert [22] reported TEM as achieving generally low plasma levels, compared with NOR, with a short half-life. All human hepatocyte preparations produced TEM as a major metabolite, and in cells from H8 at an equivalent rate to the appearance of NOR, reducing the similarities between *in vitro* and *in vivo*. However, Guentert [22] reported very widely differing interindividual values for steady-state human plasma concentrations of DZ, NOR, TEM and OX. Specifically, the NOR:TEM ratio varied between 2:1 and 13:1 in plasma.

The low levels of OX found in the human cultures, were less comparable to those found in human plasma. *In vivo*, circulating levels of OX are similar to those for TEM. This is not so *in vitro*, where levels of OX were always very low compared with TEM, or absent.

In the dog, Schwartz *et al.* [19] reported the *in vivo* clearance of DZ was rapid, with simultaneous rapid production of NOR as the principal metabolite in the plasma. The metabolism profile of DZ *in vitro* was similar to that in dog plasma. The initial clearance of DZ compared to the other species was rapid. Moreover, NOR was found to persist as the principal plasma metabolite throughout the period of complete elimination of DZ *in vivo* in the dog, and *in vitro*, NOR was found to persist, without evidence of further metabolism.

The *in vitro* and *in vivo* disposition of NOR, OX and TEM were also similar for the dog. The ratio of NOR to OX in 36-hr pooled plasma was found by Schwartz [19] to be about 7:1 and TEM was undetectable. *In vitro*, the ratio of NOR to OX was in favour of NOR. In the early period of metabolism in culture, when the conditions approximated to initial-rate, the ratio of appearance of NOR:OX was about 20:1. TEM was barely detectable. Neither NOR nor OX were further metabolised *in vitro*. Similarly, these metabolites were the only detectable products found in plasma, with no evidence of further hepatic metabolism.

Metabolite profiles determined in cynomolgus monkey blood by Coutinho *et al.* [8] showed there were similarities with the metabolism in hepatocytes. Both *in vitro* and *in vivo*, DZ was rapidly cleared and NOR was the principal metabolite. TEM was present as a significant product in both blood and hepatocytes, and no other metabolites were found in either blood or cell culture.

In vitro TEM was extensively further metabolised by an unidentified route and there was an accumulation of OX. The further metabolism may have been to the benzophenone which, *in vivo*, is found in dogs

and rabbits [23, 24] but standards were unavailable for confirmation. Coutinho *et al.* [18] did not measure urinary profiles in the monkey, so comparison of urinary profiles with *in vitro* hepatocyte profiles cannot be made. However, the metabolism of DZ *in vitro* in the monkey are similar to those found in plasma.

Plasma metabolites determined by Bell *et al.* [25] in the rat were found, at steady state, to be 4′-hydroxy DZ, TEM and NOR, in approximately equimolar ratios. Similar *in vitro* ratios were obtained in rat hepatocytes. The same metabolites were found to attain near equimolar levels at 30 min of incubation in hepatocytes. Bell did not measure deconjugated metabolites *in vivo*, so the presence of 4′-hydroxy-DZ glucuronide, which was found *in vitro*, was not established. Nevertheless, the similarities between *in vitro* and *in vivo* rat metabolites are evident. An additional similarity is that OX was detectable neither *in vitro*, nor as a plasma metabolite, though its glucuronide is the major urinary metabolite in the rat, representing 38% of the urinary output from an oral dose [26].

Trennery and Waring [26] reported extensive glucuronidation of metabolites of DZ in the urinary profile from the rat, yet there was no evidence of conjugation other than 4′-hydroxy DZ, found in the present studies in hepatocytes. The implication is that the rat undertakes extensive extrahepatic conjugation *in vivo*.

These findings highlight one of the potential uses of hepatocytes. Hepatocytes may be unrepresentative of the complete profile of *in vivo* drug metabolism, but they can be used to clearly distinguish between hepatic and extrahepatic metabolism.

The mechanism underlying the complex disappearance kinetics of DZ observed in the dog hepatocytes compared with other species remains unclear. There is, however, evidence that DZ metabolism in the dog *in vivo* shows complex kinetics, compared with other species. For example, DZ elimination from the plasma was shown to exhibit a triphasic profile *in vivo* [10, 27], whereas a biphasic profile is more common in other species [9]. The complex clearance would therefore appear to be specific to dog hepatocytes. Inhibition of DZ metabolism by NOR, TEM and OX has been reported [15] in perfused liver preparations in the rat. However, in our experiments with dog hepatocytes there was no difference obtained in the rates of nonlinear clearance of DZ when NOR was introduced as an inhibitor (data not shown). This would appear to preclude a simple product inhibition by NOR. However, when extracellular and cell-associated metabolites were determined separately in dog hepatocytes, cell-associated NOR and OX were both found to exceed a 200-fold higher concentration compared with extracellular concentrations of these metabolites. The accumulation of either metabolite at high concentrations inside the cell may contribute to the biphasic kinetic profile observed in the cells.

In summary, these studies have shown that the profiles of metabolism of DZ in rat, dog, monkey and human hepatocytes are quite dissimilar. The metabolite profiles clearly show significant changes with time, and therefore if the metabolite profile is measured at only one time-point the family of metabolites that are produced from a substrate may well fail to represent an adequate model of the relative rates of drug metabolism obtained *in vivo*. It is therefore recommended that in an *in vitro* hepatocyte investigation of a drug substrate a kinetic profile of the metabolism should be incorporated.

There were clear similarities between the metabolites found *in vitro* and the metabolites found *in vivo* in each species. The findings substantiate the utility of hepatocytes as an *in vitro* model of *in vivo* hepatic metabolism.

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