Journal of Chromatography, 421 (1987) 291–307
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3837

DETERMINATION OF DIAZEPAM AND ITS METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND THIN-LAYER CHROMATOGRAPHY

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(First received March 24th, 1987; revised manuscript received June 23rd, 1987)

SUMMARY

A sensitive, simple high-performance liquid chromatographic assay, capable of simultaneously measuring diazepam, its active metabolites oxazepam, temazepam and N-desmethyldiazepam and two phenyl hydroxylated metabolites, 4′-hydroxy-N-desmethyldiazepam and 4′-hydroxydiazepam, is described. The assay is easily modified to include separation of additional metabolite(s), e.g. oxazepam glucuronide(s). A thin-layer chromatographic assay, which resolves diazepam, the active metabolites and the two phenyl hydroxylated derivatives in one solvent system, is also reported. Application of these procedures to the quantitation of diazepam and its metabolites was shown, after delivery of diazepam (5 μg/ml or 16 μM) at a constant flow-rate (10 ml/min per liver) through the single-pass perfused rat liver preparation. Blood perfusion medium and bile were analysed for parent drug and metabolites before and after enzyme hydrolysis. These assay methods are found to be particularly pertinent and useful in providing a more comprehensive metabolic profile of diazepam metabolism, especially when aromatic hydroxylation pathways predominate.

INTRODUCTION

Numerous sensitive and selective high-performance liquid chromatographic (HPLC) methods that quantify diazepam (DZ) and its pharmacologically active metabolites N-desmethyldiazepam (nordiazepam), ND and oxazepam (OZ) [1–7] and the C_3-hydroxylation metabolite temazepam (TZ) [8–13] have been reported. The aromatic hydroxylated metabolites arising from diazepam [14], however, are seldom investigated by HPLC methodology. Thin-layer chromatographic (TLC) methods exist that resolve DZ from some of its active metabolites either directly [15,16] or after conversion of the benzodiazepines into their respective benzophenone derivatives [17,18]. Only one TLC report [19] documents the resolution of the aromatic hydroxylated metabolites, 4′-hydroxydiaze-

0378-4347/87/$03.50

pam (4′-OHDZ) and 4′-hydroxy-N-desmethyldiazepam (4′-OHND), after development in two solvent systems. To date, no single and reliable HPLC or TLC system separates DZ, ND, TZ and OZ from the hydroxylated metabolites.

DZ metabolism is highly species-dependent. In humans [16,20], dogs [20,21], and mice [15,22,23], the predominant metabolic species are ND, TZ, OZ and their conjugates. The rat, however, possesses a marked capacity for aromatic hydroxylation to form 4′-OHDZ, 4′-OHND, 4′-hydroxytemazepam (4′-OHTZ) and 4′-hydroxyoxazepam (4′-OHOZ) [14,24]. These metabolites are excreted into bile or urine as their conjugated forms [14,20,24]. Since the rat often serves as an animal model in the investigation of various aspects of benzodiazepine metabolism, tissue distribution and receptor binding [19,24–28], an analytical assay, capable of quantifying ring hydroxylated metabolites, in addition to the active metabolites OZ, TZ and ND, is desirable in considering mass balance relationships.

We report here a selective HPLC assay that quantifies DZ and five metabolites (ND, TZ, OZ, 4′-OHDZ and 4′-OHND) in blood perfusion medium prepared for liver perfusion studies in our laboratory. The procedure may be modified to procure separation of OZ and TZ conjugates in bile or other biological fluids, such as urine or plasma. This communication also reports a simple TLC method capable of separating these unconjugated compounds in a single solvent system. The correlated TLC and HPLC data, derived from experimental samples obtained from liver perfusion experiments, are presented.

EXPERIMENTAL

Reagents and materials

Methanol, chloroform (Caledon Labs, Georgetown, Canada) and ethyl acetate (Burdick & Jackson Labs, Muskegon, MI, U.S.A.) were of HPLC grade. Absolute ethyl alcohol was obtained from Consolidated Alcohol (Toronto, Canada). The N-ethyl analogue of DZ, 7-chloro-1-ethyl-5-phenyl-3*H*-1,4-benzodiazepin-2(1*H*)-one, served as internal standard. DZ, TZ, OZ, 4′-OHDZ, ND, 4′-OHND and the internal standard were kindly supplied by Dr. Ihor Bekersky (Hoffmann-La Roche, Nutley, NJ, U.S.A.). OZ glucuronide(s) were a gift from Dr. S.F. Sisenwine (Wyeth Labs., Philadephia, PA, U.S.A.). [2-^{14}C]DZ (specific activity 54 mCi/mmol; Amersham, Oakville, Canada) and Diazepam Injection U.S.P. (Hoffmann-La Roche, Etobicoke, Canada) were administered in the once-through perfused rat liver experiments.

Enzyme from *Helix pomatia* (600 000 β-glucuronidase units/g solid and 28 000 sulphatase units/g solid) and *Escherichia coli* (28 000 β-glucuronidase units/mg solid) were obtained from Sigma (St. Louis, MO, U.S.A.).

Experimental studies

Rat liver (male, Sprague–Dawley, 300–350 g, Charles River, St. Constant, Canada) were perfused with [2-^{14}C]DZ (0.023 μCi) and unlabelled DZ (16 μ*M*, 5 μg/ml) at a constant flow-rate of 10 ml/min per liver, in a non-recirculating fashion as previously described [29]. Perfusate consisted of 20% washed human red

blood cells, 1% bovine serum albumin (25% in Tyrode's buffer, Sigma), 300 mg/ml glucose (Dextrose Injection U.S.P., Travenol Canada, Mississauga, Canada) in Krebs Henseleit bicarbonate solution, buffered to pH 7.4. Inflow and outflow perfusate was sampled during the steady-state period, at which time parent drug and metabolite concentrations remained constant. Bile was collected and pooled during the first 30 min of perfusion and thereafter at 10-min intervals. Samples were stored at −20°C until assayed.

High-performance liquid chromatography

A Waters high-performance liquid chromatograph (Waters Scientific, Mississauga, Canada), equipped with two pumps (M-6000 and M-45), a fixed-wavelength UV detector (Model 440), a data module (Model 730), a WISP 710B automatic injector and system controller (Model 721), was used. Peaks were detected at 254 nm and 0.005 a.u.f.s. A C_{18} Corasil pre-column (37–50 μm, Waters Scientific) was used. Separation was achieved at ambient temperature.

Chromatographic conditions for blood perfusate samples: system A

For perfusate samples, a 5-μm Ultrasphere ODS column (15 cm×4.6 mm I.D., Beckman Instruments, Mississauga, Canada) provided the separation. A 0.005 *M* potassium dihydrogenphosphate solution was adjusted to pH 3.04 with HPLC-grade phosphoric acid (85%, Fisher Scientific). The mobile phase for system A, methanol–0.005 *M* potassium phosphate buffer (50:50, v/v), was delivered at 1 ml/min. Solvent conditions were constant for the first 4 min. From 4 to 25 min, the methanol concentration was increased from 50 to 53% by means of an exponential gradient (Waters System controller curve 9). The concentration was maintained at 53% for 10 min, then increased linearly to 55.5% over the next 10 min (Fig. 1a). A 10-min delay between injections re-established initial column conditions (50% methanol).

Blood perfusate samples (1 ml) were mixed with 2 μg of internal standard (60 μg/ml methanol solution) in 12-ml glass test-tubes prior to extraction with 5 ml of ethyl acetate. The tube contents were shaken for 20 min on a tilt-type mixer, then centrifuged (3000 *g* for 10 min). For quantitative transfer of the (top) organic layer, the (bottom) aqueous layer was frozen in acetone–dry ice, and the organic phase was poured into a 20-ml glass scintillation vial. With the aqueous layer frozen, tubes were rinsed twice with 2 ml of ethyl acetate. The total extractant (9 ml) was evaporated to dryness at 37°C under a stream of nitrogen. The residue was reconstituted with 250 μl of mobile phase and centrifuged (9650 *g* for 1 min), and 25–50 μl of the supernatant were injected onto the column.

Samples were also treated with enzyme. Blood perfusate (1 ml) was incubated with 2000 U β-glucuronidase derived from *E. coli* in 1 ml of 0.075 *M* potassium dihydrogenphosphate (pH 6.8), or *Helix pomatia* in 1 ml 0.1 *M* sodium acetate (pH 5.0) for 24 h at 37°C. The internal standard was added prior to extraction. Sets of standards (0.05–5.0 μg/ml of perfusate) were subjected to an identical extraction procedure.

Since the experimental application of this assay procedure involved the use of radiolabelled DZ, two quantitation methods were possible. Unknown concentra-

tions of the unlabelled parent drug DZ and metabolites (ND, TZ, OZ, 4′-OHDZ, 4′-OHND) in perfusate, with (enzyme plus 1 ml of buffer) or without enzyme incubation, were quantitated using the calibration curves (area of standard/internal standard versus amount spiked). Labelled DZ and metabolites were quantitated by radio-elution. Eluate HPLC fractions were collected, and 10 ml of scintillation cocktail (HP/b, Beckman) were added prior to liquid scintillation counting (LSC, Model LS 6800, Beckman). Values were corrected for absolute recovery from the extraction procedure. Recoveries were determined by the ratio of peak areas for known concentrations in extracted perfusate to that for the same concentration of compound in methanolic solution. Quantitation by both methods (unlabelled from calibration curve, and labelled by radio-elution after correction for recovery) yielded excellent correspondence.

Chromatographic conditions for bile samples: system B

Bile analyses (system B) were performed on a 5-μm ODS-3 RAC II column (100 mm×4.6 mm I.D., Whatman, Clifton, NJ, U.S.A.). The mobile phase, methanol–0.005 *M* potassium dihydrogenphosphate buffer (35:65, v/v), was delivered at 1 ml/min for 26 min. The concentration of methanol was increased linearly to 55% from 26 to 58 min. Re-injections were delayed for 10 min to reinstate initial conditions (Fig. 2a).

Bile samples were routinely analysed with and without enzyme hydrolysis. Samples not subjected to enzyme hydrolysis were diluted 1:5 (v/v) with water, and 8 μl were injected onto the column. For enzyme hydrolysis, 500 U β-glucuronidase (*Helix pomatia*), dissolved in 500 μl of 0.2% cold sodium chloride, and 500 μl of 0.1 *M* sodium acetate buffer (pH 5.0), were added to 40 μl of diluted bile (1:20 dilution with water). Samples were incubated for 24 h at 37°C, then extracted with 5 ml of ethyl acetate. The aqueous layer was frozen with dry ice, and the organic layer was transferred to a scintillation vial, along with two 1-ml rinses, as described previously. Samples were dried under nitrogen at 37°C. The residue was reconstituted with 250 μl of mobile phase, and 100 μl were injected onto the column. Sets of standards containing various amounts of DZ, TZ, ND, OZ, 4′-OHND (5–50 μg/ml of bile) and 4′-OHDZ (0.5–15 mg/ml of bile) were similarly processed for preparation of calibration curves.

The concentrations of unlabelled DZ and metabolites in untreated bile were generally below the sensitivity limits of the assay and were therefore quantified by HPLC radio-elution. These concentrations increased after enzyme hydrolysis, permitting quantitation both by radio-elution, with correction for absolute recovery from the extraction procedure, and by reference to the respective calibration curves.

Thin-layer chromatography

One-dimensional TLC was performed on precoated silica gel plates (5×20 cm, 250 μm, Analtech, Newark, DE, U.S.A.). Plates were pre-spotted with authentic compounds. The developing solvent system consisted of chloroform–ethyl acetate–ethanol–ammonium hydroxide (81:5.5:11:2, v.v). The following R_F values

were observed: DZ, 0.97; ND, 0.80; TZ, 0.89; OZ, 0.62; 4′-OHDZ, 0.72; 4′-OHND, 0.48. The developed regions were visualized under UV light. Co-migration of labelled and unlabelled compounds was verified by comparing the radioactivity profile of experimental samples, plated and scraped at 0.5-cm intervals, with the development pattern of the authentic unlabelled compounds. The six radioactive bands coincided with the UV-visualised bands associated with unlabelled standards.

Perfusate samples (1 ml), with or without enzyme incubation, were subjected to the extraction procedure, as outlined for HPLC analysis. Dried residue was reconstituted in 200 μl of ethyl acetate, and 100 μl were plated. For quantitation by LSC, all values from perfusate samples were corrected for absolute recovery, as determined by the HPLC procedure. It was further assumed that no radioactivity was lost to the thin-layer plate.

RESULTS

The chromatogram of an aqueous test mixture of DZ, its metabolites and internal standard demonstrates excellent resolution of all compounds in system A (Fig. 1a). The order of elution and corresponding retention times under these conditions are: 4′-OHND, 10 min; 4′-OHDZ, 12 min; OZ, 15.5 min; TZ, 18.9 min; ND, 24 min; DZ, 30 min; internal standard, 40 min. The extracted perfusate blank (drug-free) from samples with (Fig. 1c) or without enzyme (Fig. 1b) was shown to be devoid of interfering endogenous substances, except for a large peak eluting at 9 min just prior to 4′-OHND (Fig. 1b and c). This interference was inconsequential for both incubated and non-incubated samples; the amount of unconjugated 4′-OHND appearing in the perfusate in rat liver perfusion studies was below the sensitivity limit of the assay for unlabelled material, so that quantitation of this species was routinely achieved by radio-elution.

The separation of the parent compound, five metabolites and the OZ glucuronide(s) in system B is illustrated in Fig. 2a. This system provided a substantial increase in the selectivity factor of the two more polar hydroxylated metabolites, allowing for further separation of the OZ glucuronides. OZ glucuronide was resolved into two peaks, corresponding to the $S(+)$- and $R(-)$-diastereomers [30]. All compounds were adequately resolved: 4′-OHND, 16.5 min; OZ glucuronide, 18–20 min; 4′-OHDZ, 25.5 min; OZ, 40 min; TZ, 48 min; ND, 52 min; DZ, 58 min.

Chromatography of a blank bile sample, with (Fig. 2c) or without (Fig. 2b) enzyme incubation, revealed a profile with negligible interference. Experimental bile samples without enzyme hydrolysis demonstrated numerous radioactive peaks; those co-eluting with OZ glucuronide, 4′-OHND, 4′-OHDZ, ND and DZ, however, accounted for only 2–3% of the total radioactivity of the sample. The radio-chromatogram of an enzyme-hydrolysed, extracted bile specimen (Fig. 2c) demonstrated increased radioactivity co-eluting especially with 4′-OHDZ, and less with 4′-OHND, TZ and ND. The absence of OZ and DZ from hydrolysed bile samples can be explained by the different dilution factors and HPLC injection volumes used in unhydrolysed (Fig. 2b) and hydrolysed (Fig. 2c) samples; chromatograms represent 1.6 and 0.8 μl of bile, respectively. Three other compounds,

eluting at 7, 36 and 42 min, were consistently present in experimental samples, whereas OZ (retention time 39–40 min) was absent. The possibility of these unknowns arising from degradation of known compounds under incubation con-

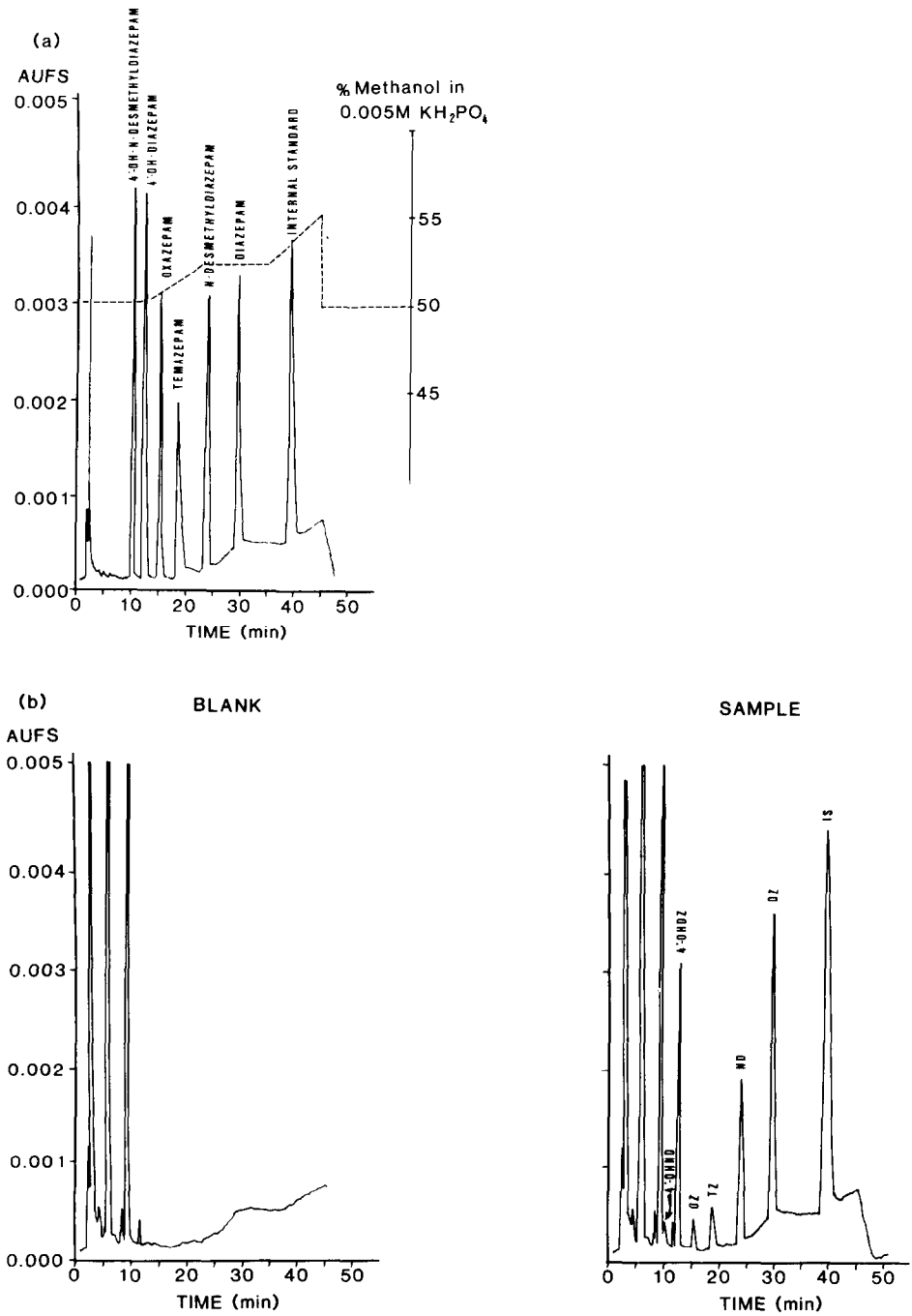

Fig. 1.

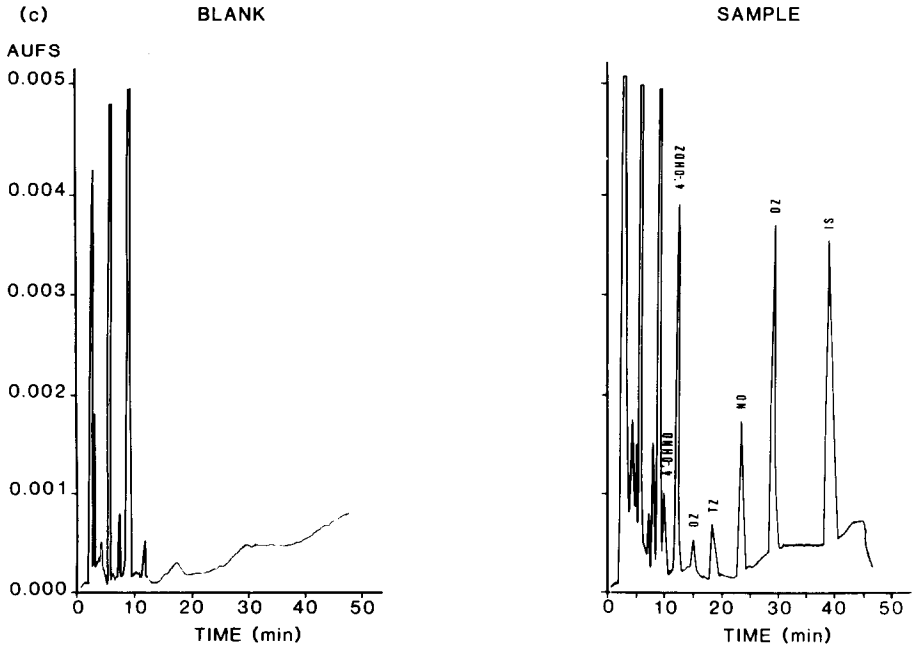

Fig. 1. (a) System A for the separation of diazepam and five of its metabolites and internal standard (N-ethyl analogue of diazepam) from an aqueous test mixture. A solvent gradient varied the concentration of methanol in phosphate buffer (0.005 *M* potassium dihydrogenphosphate pH 3.04) from 50 to 55.5% (dashed line). (b) Chromatogram of extracted blank and outflow perfusate obtained from perfusion experiments. Rat livers were perfused once-through with [2-^{14}C]diazepam (0.023 μCi) and unlabelled diazepam (16 μM) at a constant flow-rate of 10 ml/min per liver for 60 min. (c) Chromatogram of extracted blank and outflow perfusate after incubation for 24 h with β-glucuronidase from *Helix pomatia* (2000 U/ml of sample). Experimental conditions in b.

ditions may be excluded, since these unidentified peaks did not appear when standards were subjected to the procedure.

Evaluation of the assay procedures

Standard curves (system A) (ratio of peak area of each component to that of internal standard versus amount) were linear over a 50-fold variation in concentration with correlation coefficients of > 0.999. The limits of detection for system A were 3–4 ng for each compound. The sensitivity was at least 10 ng/ml for DZ, ND, OZ, TZ and 4'-OHND, concentrations that are within the experimental and clinical range found in human serum after oral administration of DZ [16]. The sensitivity of 4'-OHDZ was somewhat compromised (50 ng/ml) because elution of an adjacent endogenous substance interfered with peak integration. Under the present experimental conditions for rat liver perfusion, however, the area of this metabolite peak greatly exceeded the area of the interfering substance.

Within-day precision for perfusate samples (system A) was tested at concentrations of 0.08, 1.0 and 8 μg/ml ($n=6$, Table I). The coefficients of variation (C.V.) ranged from 2.1 to 7.6%. The relative recoveries of each coumpound were in the range 93–109%. Inter-day precision was determined by comparing slopes

of calibration curves prepared on consecutive days. The maximum C.V. obtained was 3.6%.

The absolute recovery and corresponding precision data for system A are shown in Table II. Recoveries of metabolites 4′-OHND, 4′-OHDZ, OZ and ND were

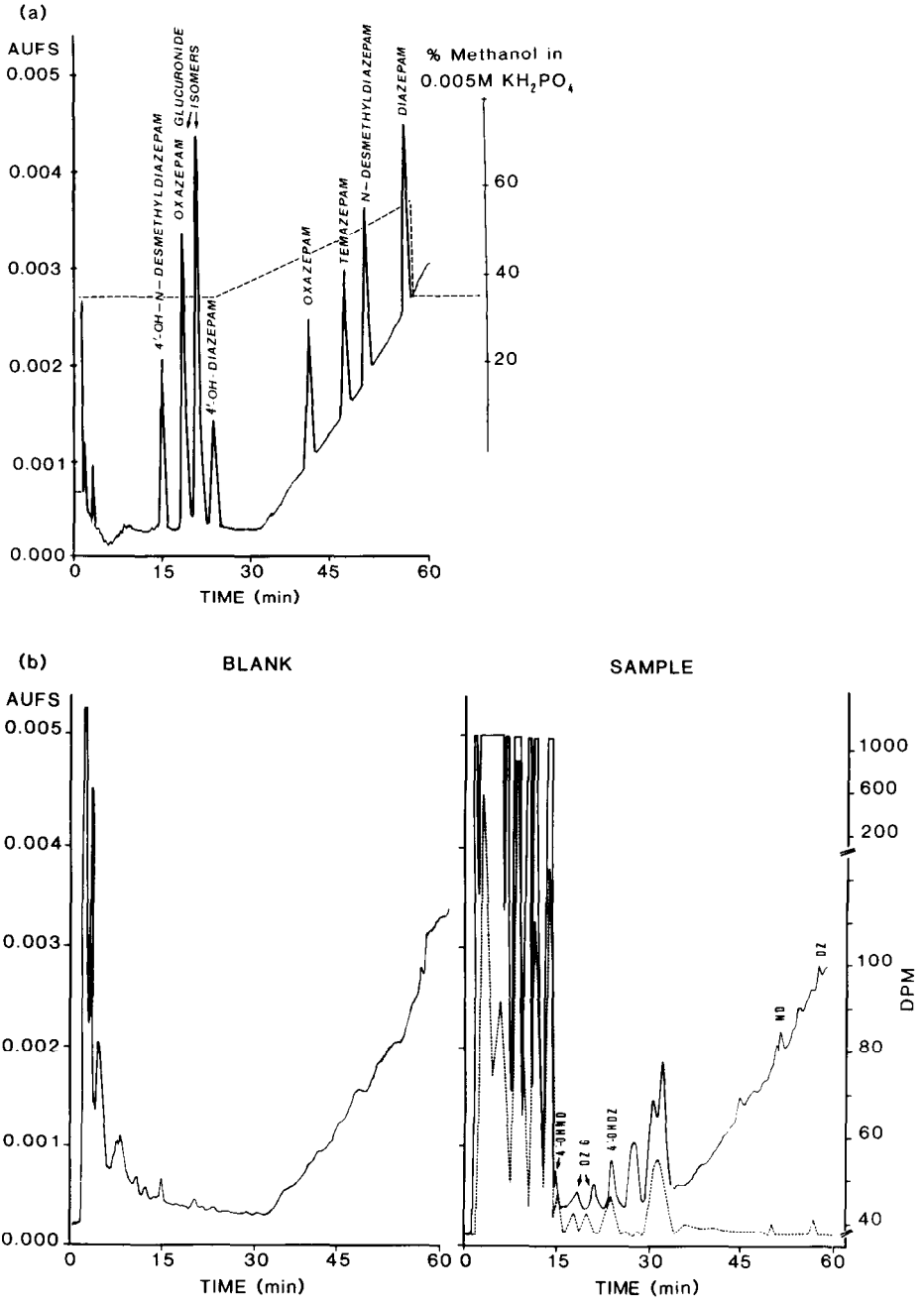

Fig. 2.

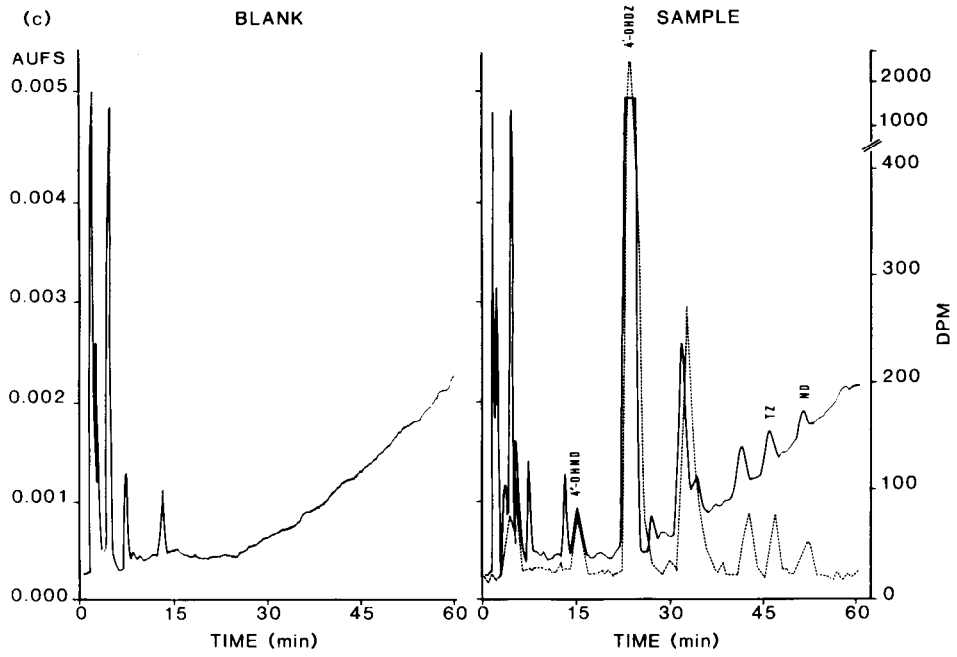

Fig. 2. (a) System B for the separation of an aqueous mixture of diazepam and six metabolites including oxazepam glucuronide, resolved as diastereomers. The solvent gradient (dashed line) varied the concentration of methanol in phosphate buffer from 35 to 55%. (b) Blank bile and experimental unhydrolysed bile sample from perfusion studies as described in Fig. 1b. The radioactivity elution profile (dotted line) is superimposed on the chromatogram (solid line) after correction for the collection delay (1 min). The unresolved peaks eluting at/before 15 min comprise 90–95% of the radioactivity of the sample. (c) Extracted blank bile and experimental bile sample after incubation with β-glucuronidase enzyme from *Helix pomatia* for 24 h. The superimposed radioactive profile (dotted line) was corrected for collection delay (1 min). The radioactive peak (unidentified) eluting at 36 min accounted for 12–15% of the radioactivity from the sample.

similar (89–91%). The average recovery of DZ was 85% whereas that for TZ was 83.8%. There was no perceivable trend in the values over the concentration range studied. Recoveries from the incubation–extraction phosphate buffer procedure (Table II) were less efficient for the most polar metabolite (4′-OHND) but more efficient for TZ (92%). As expected for basic compounds, addition of sodium acetate buffer (pH 5.0) decreased the recovery of all except 4′-OHDZ.

Linearity of detector response (system B) towards OZ glucuronide was evaluated by injecting various amounts (11–330 ng) of aqueous standards. Peak areas for both diastereomers were summed and found to be linear with a correlation coefficient of 0.997. The detection limit was 6 ng with a sensitivity limit of 4 μg/ml of bile. The detection limit for all other compounds (system B) was 2 ng, with sensitivity limits of 2 μg/ml of bile. The sensitivity of the extracted bile assay was at least 5 μg/ml of bile for OZ, TZ, ND, DZ, 4′-OHND and 4′-OHDZ.

Calibration curves (peak area of standards versus amount added) constructed for extracted (enzyme-treated) bile (system B) were linear over a ten-fold range

TABLE I

INTRA-ASSAY VARIABILITY FOR COMPOUNDS EXTRACTED FROM PERFUSATE BY THE HPLC PROCEDURE — SYSTEM A ($n=6$)

Compound	Concentration spiked (μg/ml)	Concentration recovered (μg/ml)	C.V. (%)
4′-Hydroxynordiazepam	0.087	0.081	3.4
	1.162	1.206	6.5
	9.296	9.272	4.8
4′-Hydroxydiazepam	1.166	1.089	5.4
	9.331	9.623	4.6
Oxazepam	0.067	0.072	7.6
	0.893	0.839	4.5
	7.147	7.148	5.9
Temazepam	0.076	0.082	4.2
	1.015	1.041	2.1
	8.122	8.043	7.2
Nordiazepam	0.076	0.076	5.0
	1.00	1.038	2.5
	8.05	8.125	6.0
Diazepam	0.082	0.081	4.3
	1.10	1.20	3.3
	8.55	8.91	5.5

TABLE II

ABSOLUTE RECOVERIES OF DIAZEPAM AND METABOLITES IN PERFUSATE AFTER THE EXTRACTION PROCEDURE WITH ETHYL ACETATE AS DETERMINED FROM HPLC — SYSTEM A

Values in parentheses are coefficients of variation (%); $n=6$.

Perfusate concentration (μg/ml)	Mean recovery of compounds tested (%)					
	4′-OHND	4′-OHDZ	OZ	TZ	ND	DZ
0.1	87.3 (1.7)	–	90.7 (1.6)	84.8 (4.1)	89.5 (2.8)	84.5 (1.7)
1.0	92.6 (1.5)	91.0 (2.9)	90.6 (1.3)	82.0 (3.8)	89.2 (2.2)	87.2 (5.1)
7.5	93.3 (5.1)	88.5 (8.0)	89.2 (4.3)	84.5 (7.6)	89.6 (4.3)	85.3 (5.3)
Incubated standards (1 ml of 0.075 M potassium dihydrogenphosphate added (pH 6.8))						
0.1-9.0	80.2 (2.7)	88.9 (3.4)	82.5 (2.3)	92.5 (6.3)	86.0 (3.6)	87.7 (3.3)
Incubated standards (1 ml of 0.1 M sodium acetate added (pH 5.2))						
0.1-9.0	84.8 (6.6)	89.1 (6.5)	81.2 (4.9)	75.5 (6.1)	86.8 (7.3)	81.7 (7.6)

in concentration (correlation coefficients >0.99; data not shown). Absolute recoveries from the extraction method were consistently above 85% (C.V.=1.2-4.1%) except for 4′-OHND, whose mean recovery was 78% (C.V.=7.4%) (Table III). Within-day precision varied between 1.2 and 5.0%

TABLE III

ABSOLUTE RECOVERIES OF DIAZEPAM AND METABOLITES FROM EXTRACTED BILE AFTER ENZYME INCUBATION — SYSTEM B ($n=8$)

Compound	Concentration (μg/ml)	Mean recovery* (%)	C.V. (%)
4'-Hydroxynordiazepam	5- 100	78.0	7.4
4'-Hydroxydiazepam	140-1330	99.2	1.2
Oxazepam	5- 100	85.6	4.3
Temazepam	10- 100	85.9	4.1
Nordiazepam	10- 100	89.1	2.2
Diazepam	10- 100	87.8	3.8

*Recoveries were averaged over the range of concentrations used.

TABLE IV

WITHIN-DAY PRECISION OF HPLC ASSAY FOR DIAZEPAM AND METABOLITES EXTRACTED FROM HYDROLYSED BILE — SYSTEM B ($n=6$)

Compound	Concentration (μg/ml)	C.V. (%)
4'-Hydroxynordiazepam	6.25	5.0
4'-Hydroxydiazepam	1000	1.2
Temazepam	18.5	4.3
Nordiazepam	10.0	4.4

(Table IV). OZ and DZ were not included because their concentrations were negligible in extracted bile.

Application of the methods

Application to an experimental situation is demonstrated by analysis of perfusate and bile obtained from in situ rat livers perfused once-through with ^{14}C-labelled and unlabelled DZ. At the input concentration of 5 μg/ml (16 μM), 4'-OHDZ was the major metabolite in unhydrolysed perfusate, with an average concentration of 600 ng/ml. OZ and TZ (50–60 ng/ml) and 4'-OHND (20–25 ng/ml) were minor metabolites, whereas ND was present in greater concentrations (ca. 150 ng/ml). The perfusate efflux profiles of unconjugated species, 4'-OHND, 4'-OHDZ, OZ, TZ, ND and DZ (Fig. 3), demonstrate attainment of steady state after 40–60 min at a constant DZ influx rate of 50 μg/min.

Enzyme hydrolysis of perfusate samples yielded a 20% increase in OZ concentrations but no detectable change in TZ concentrations. A 30–40% increase in 4'-OHDZ concentrations and four-fold elevation in 4'-OHND perfusate levels occurred. β-Glucuronidase from *Helix pomatia* was consistently more effective in deconjugating 4'-OHDZ and 4'-OHND glucuronides than enzyme from *E. coli*, whereas OZ glucuronides were hydrolysed equally well by both enzymes. The

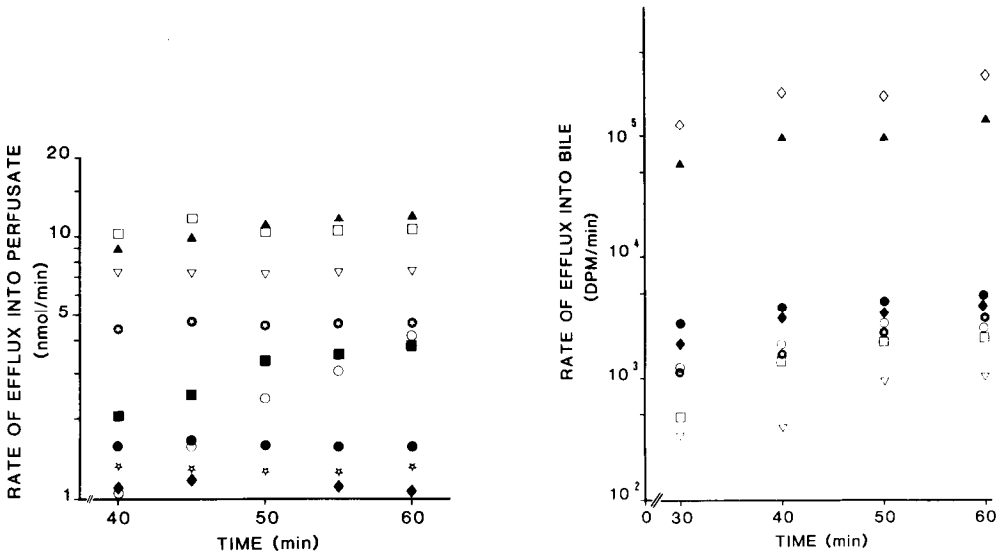

Fig. 3. Perfusate metabolic profile obtained from rat liver perfusion study as described in Fig. 1b. The efflux of diazepam and its metabolites into perfusate during the final 20-min period of the study are expressed in nmol/min. Key: (♦) 4′-OHND, (■) 4′-OHND conjugate, (□) 4′-OHDZ, (▲) 4′-OHDZ conjugate, (☆) OZ, (○) OZ conjugate, (●) TZ, (⊛) ND, (▽) DZ.

Fig. 4. Biliary metabolic profile obtained from rat liver perfusion study as described in Fig. 1b. Bile was pooled over the first collection interval (30 min) and over subsequent intervals (10 min). The efflux rates (dpm/ml) of total radioactivity, diazepam and its metabolites into bile were averaged over the collection interval. ND and 4′-OHND values represent the combined conjugated and unconjugated species obtained from hydrolysed bile samples (see text). Key: (♦) 4′-OHND, (□) 4′-OHDZ, (▲) 4′-OHDZ conjugate, (○) OZ glucuronide, (●) TZ conjugate, (⊛) ND, (▽) DZ, (◇) total radioactivity.

presumed conjugates of OZ, 4′-OHND and 4′-OHDZ approach steady-state conditions more slowly, as expected for secondary and tertiary metabolites (Fig. 3).

The complex metabolic scheme of DZ metabolism in the rat is exhibited in bile (system B). At least five radiolabelled peaks (90% of total radioactivity), presumably polar conjugates, appeared before 15 min, followed by small amounts of 4′-OHND, the OZ glucuronides and 4′-OHDZ. Two fused unidentified peaks (30–36 min), representing 5% of total radioactivity, had the same retention times as metabolites found after TZ administration to the rat liver (unpublished data), suggesting that they may be TZ metabolites. DZ and ND were present in trace amounts (Fig. 2b). After enzyme hydrolysis of bile, marked increases in 4′-OHDZ and the unknown peak(s) at 30–36 min were seen (Fig. 2c).

The biliary radioactive and metabolic profile followed throughout the study period (Fig. 4) demonstrates the approach to steady-state conditions of unconjugated and conjugated species. The efflux rate (dpm/min) of total radioactivity and individual compounds was averaged over the collection interval. The conjugates of 4′-OHDZ, OZ and TZ appeared constant after 30 min of perfusion, whereas the efflux rate of unconjugated species of 4′-OHDZ and DZ showed a

TABLE V

WITHIN-DAY PRECISION OF TLC ASSAY FOR DIAZEPAM AND ITS METABOLITES EXTRACTED FROM PERFUSATE ($n=6$)

Compound	Concentration (dpm/ml)	C.V. (%)
4'-Hydroxynordiazepam	925	8.4
	1040	9.0
4'-Hydroxydiazepam	5000	1.9
	6000	1.5
Oxazepam	1560	5.3
	1225	3.8
Temazepam	800	4.8
	900	4.0
Nordiazepam	2960	2.9
	2850	2.5
Diazepam	2455	1.2
	2500	1.1

more gradual approach to a steady state. The efflux rate of ND and 4'-OHND represent the total amount after enzyme hydrolysis; ND values were always greater after enzyme hydrolysis, although conjugation of ND has not been a reported metabolic pathway and unconjugated 4'-OHND levels were below the sensitivity limit of the assay (Fig. 2c).

Thin-layer chromatography

The reproducibility of the method was demonstrated in two series ($n=6$) of perfusate analyses. The concentrations of DZ and five metabolites varied between 1.0 and 9.0% (Table V). Fig. 5 shows the correlation obtained when perfusate samples were subjected to both TLC and HPLC analysis. The regression line obtained from all points corresponding to DZ, TZ, 4'-OHND and 4'-OHDZ exhibited a strong correlation ($r^2=0.999$, slope = 1.002). Individual species were similarly correlated: DZ ($r^2=0.99$), TZ ($r^2=0.93$), 4'-OHDZ ($r^2=0.92$), ND ($r^2=0.99$) and 4'-OHND ($r^2=0.90$). OZ concentrations (not shown) were ca. 10% higher after TLC analysis, possibly owing to a minor unidentified metabolite co-migrating in this region. The lack of authentic compounds precluded an evaluation of the extent to which this had occurred. The radioactive profile of plates scraped at 0.5-cm intervals was examined in an attempt to identify unknown radioactive components. No spurious peaks were found; however, their presence could have been masked by proximity to known metabolites.

TLC of bile (unhydrolysed) revealed that 95% of the radioactivity of the sample remained at the origin, identical with the unconjugated fraction recovered after HPLC analysis. For testing possible interference in the TLC system from the two unknown peaks, detected at 36 and 42 min from HPLC (Fig. 2c), HPLC radioeluate fractions of these compounds from bile were collected, dried under nitrogen, then plated and developed in the TLC system. Radioactive bands co-

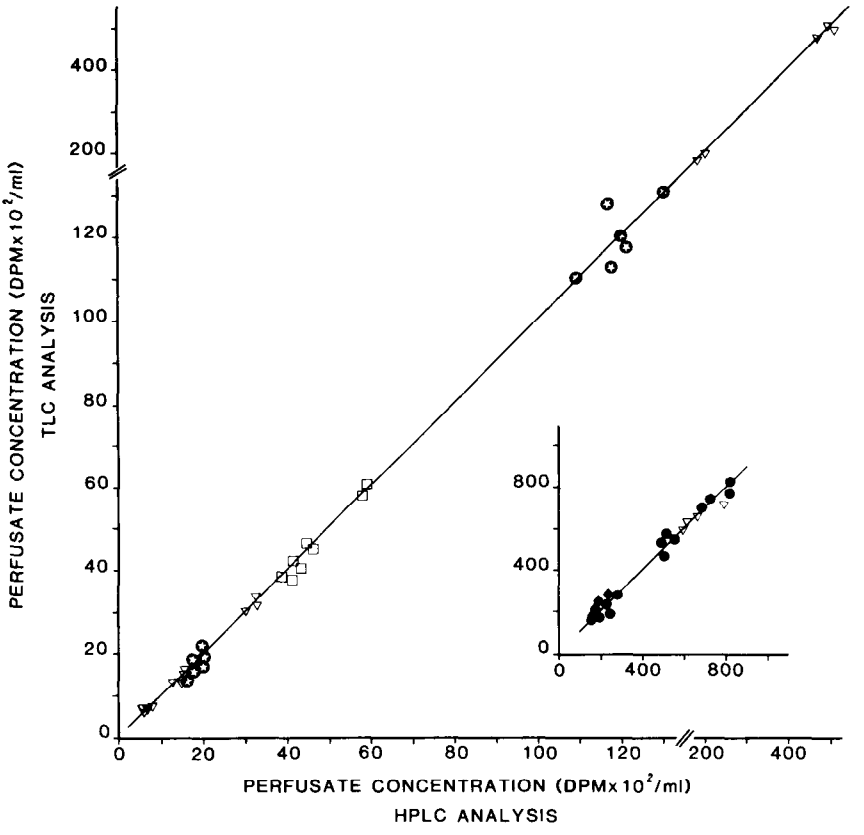

Fig. 5. Perfusate concentrations (dpm/ml) of diazepam and its metabolites, following rat liver perfusion studies (described in Fig. 1b), determined by HPLC and TLC. Regression line: $r^2 = 0.999$; slope = 1.002. Inset shows enlargement of origin and axes; scales are dpm/ml. Key: (♦) 4′-OHND, (□) 4′-OHDZ, (●) TZ, (⊛) ND, (▽) DZ.

migrated with 4′-OHDZ and OZ, respectively. Owing to this interference, bile samples were subjected only to HPLC analysis.

DISCUSSION

The HPLC method (system A) successfully separated DZ from ND, TZ and OZ from two of the ring hydroxylated metabolites 4′-OHDZ and 4′-OHND, major metabolites in the rat. System B provided additional separation of OZ glucuronides but at the expense of a longer analysis (60 min). It is applicable when OZ glucuronides and the unidentified metabolites found in rat bile are present.

Resolution of the three more polar metabolites in system A was optimized by lowering the pH of the mobile phase below 3.1. The additional separation of the optical isomeric glucuronides of OZ from 4′-OHND and 4′-OHDZ (system B) was optimal at pH 3.04. With an increase in pH above 4, the chromatogram showed two poorly resolved peaks which co-eluted with 4′-OHDZ. A pH value of less than

3 altered the elution order, with 4′-OHDZ eluting prior to and unresolved from the glucuronides.

The choice of ethyl acetate as extractant from the neutral perfusate medium (pH 7.4) optimized the recovery of all compounds (>85%). The pH of the perfusate is midway between the pK_a values for protonated and unprotonated forms of all species. Since the parent drug, internal standard and metabolites vary considerably with respect to polarity, the use of a less polar extractant such as ethyl acetate–hexane or methylene chloride increased the recovery of DZ and ND while compromising that of the phenyl hydroxylated derivatives. Ethyl acetate was preferable also because it forms the top layer of an aqueous–organic mixture and can be quantitatively transferred using the dry-ice method.

Applicability of the HPLC procedure is aptly demonstrated by the experimental results. A steady-state perfusion of DZ (5 μg/ml of perfusate) yielded a metabolic profile wherein 4′-OHDZ was the major primary metabolite, with the combined perfusate and biliary efflux rate of this species (conjugated and unconjugated) accounting for 40–50% of the total input rate. Two other primary metabolites, ND and TZ, constituted 4.5 and 2.7%, respectively, of the input rate, and the total efflux rate of the secondary metabolites, 4′-OHND and OZ, achieved 5.7 and 2.3%, respectively, of the steady-state input rate. In addition to the six known compounds, the extracted bile assay consistently resolved two unidentified peaks, suggesting the presence of additional secondary metabolties: 4′-OHTZ and 4′OHOZ have been documented in bile [24], and further tertiary metabolites arising from the metabolism of OZ have been reported [32].

A previous HPLC method has reported separation of ring hydroxylated metabolites, namely 4′-OHND, 4′-OHOZ and 4′-OHTZ, from OZ, TZ, ND and DZ after radiolabelled DZ administration to rats [24]. However, the mobile phase, sensitivity, recovery and internal standard were not described. Moreover, the separation, reported to be achieved between 4.9 and 12.2 min, is questionable. A TLC method from the same report also claimed separation of the same seven compounds with very close R_F values (0.09–0.45). Most importantly, the retention time and R_F of the major metabolite, 4′-OHDZ, are unknown. Another TLC method, which afforded separation of DZ, TZ, OZ, ND, 4′-OHDZ and 4′-OHND, required two solvent systems [19]. The recovery from the extractant (ethyl acetate) was only reported for DZ. Incomplete recovery of the metabolites would cause underestimation of the extent of metabolite formation.

Our present HPLC method is quantitative and offers a decided analytical advantage over previous reports, in that six rather than three metabolites are assayed in biological fluids, including the major metabolite in the rat (4′-OHDZ). The sensitivity is as low as those in all but one of the previously published methods [8]. The presence of the unidentified radioactive peaks in the hydrolysed and unhydrolysed bile samples suggests that the method (system B) could be easily adapted to the analysis of additional metabolites.

Our TLC procedure is also an improvement over existing methods in that resolution of six compounds is possible in one solvent system. Basification with ammonium hydroxide proved critical to the separation. Since the proportion of ammonium hydroxide used was barely miscible, the solvent system was prepared

immediately before use. The immiscibility proved troublesome, resulting in curvature of the migration bands. However, reproducibility was good and a strong correlation was obtained between the perfusate HPLC and TLC analyses. This TLC method is applicable to the experimental situation wherein DZ is metabolized to the phenyl hydroxylated derivatives of DZ and ND, in addition to the more commonly measured OZ, TZ and ND. The potential interference by unknowns (HPLC peaks at 30–36 and 42 min) in bile was tested and shown to interfere with 4′-OHDZ and OZ. Therefore, the use of this method to analyse rat bile, which contains additional hydroxylated metabolites [24], would require modification of the procedure.

CONCLUSION

The present assay permits the quantitation of DZ and at least five of its metabolites, ND, TZ, OZ, 4′-OHDZ and 4′-OHND, in both bile and blood perfusate. The concurrent separation of OZ glucuronide and possibly other conjugates obviates the need for enzyme hydrolysis in biological fluids such as bile and urine, where OZ and TZ conjugates may be present in significant amounts. The assay procedure accounts for 60–70% of the input of DZ (5 μg/ml at 10 ml/min) in the once-through rat liver preparation, confirming that aromatic hydroxylation, rather than N-demethylation and oxidation at C-3, is the major metabolic pathway in the rat. The method is simple to perform and is particularly suited to the complex metabolism encountered in the rat liver.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council of Canada, Operating Grant MA-9104. K.S.P. is a recipient of the Faculty Development Award (DG-263), M.R.C., Canada.

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