Journal of Chromatography, 277 (1983) 201—208
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1762

METHODS FOR THE DETERMINATION OF LORAZEPAM AND CHLORDIAZEPOXIDE AND METABOLITES IN BRAIN TISSUE

A COMPARISON WITH PLASMA CONCENTRATIONS IN THE RAT

RICHARD G. LISTER*,*

Department of Pharmacology, The School of Pharmacy, University of London, Brunswick Square, London WC1N 1AX (U.K.)

DARRELL R. ABERNETHY and DAVID J. GREENBLATT

Division of Clinical Pharmacology, Tufts-New England Medical Center, 171 Harrison Avenue, Boston, MA (U.S.A.)

and

SANDRA E. FILE

Department of Pharmacology, The School of Pharmacy, University of London, Brunswick Square, London WC1N 1AX (U.K.)

(First received August 9th, 1982; revised manuscript received April 13th, 1983)

SUMMARY

Rapid and sensitive methods are described for determining lorazepam and for determining chlordiazepoxide and its metabolites in brain tissue of the rat. Lorazepam was determined by means of solvent extraction and electron-capture gas—liquid chromatography and concentrations as low as 5 ng/g tissue could be measured. High-performance liquid chromatography with UV detection was used to determine chlordiazepoxide and its metabolites and was sensitive to 0.1 μg/g tissue. The methods were used to investigate the brain and plasma pharmacokinetics of these compounds in animals that had been chronically treated with lorazepam or chlordiazepoxide. In both experiments brain and plasma levels of all compounds assayed were found to correlate highly.

*Present address: National Institute on Alcohol Abuse and Alcoholism, 10/ACRF Building, 9000 Rockville Pike, Bethesda, MD 20205, U.S.A.

0378-4347/83/$03.00

INTRODUCTION

The plasma kinetics of many benzodiazepines have been extensively reported in both animals and man [1], although plasma levels of these compounds are not good predictors of their behavioural effects [2–6].

Although there are already many methods available for determining benzodiazepine concentrations in plasma [7—13], there is a need for routine methods for determining these compounds in brain tissue. Increased blood–brain permeability has been observed following electroconvulsive shock [14, 15], and similar pharmacokinetic changes may underlie other alterations to in vivo responses to drugs. Previous workers have investigated the brain pharmacokinetics of animals receiving either single low doses of [^{14}C]lorazepam or high doses of unlabelled compound [16]. Similarly, early studies on the metabolism of chlordiazepoxide used high doses of [^{14}C]chlordiazepoxide [17]. The purpose of this study was to develop rapid and sensitive methods for determining unlabelled lorazepam and for determining unlabelled chlordiazepoxide and its metabolites (Fig. 1) in brain tissue of the rat. In both studies plasma and brain kinetics of the drugs were to be compared in animals receiving once daily injections of the compounds for 5 or 10 days, using doses that are known to induce metabolising enzymes [6, 18]. Lorazepam has been determined in plasma using gas chromatography (GC) with either mass spectrometry [7], or electron-capture detection [13], and more recently using high-performance liquid chromatography (HPLC) [8]. Electron-capture GC was used in the present study since we have previously found this method to be reliable and sensitive for determinations of lorazepam in plasma and in urine [13].

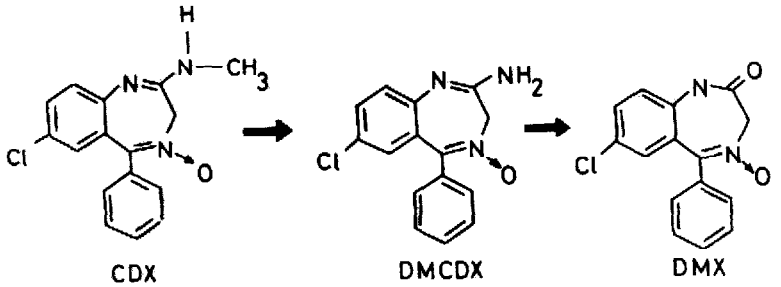

Fig. 1. The metabolic pathway of chlordiazepoxide in the rat, showing chlordiazepoxide (CDX) and its two principal metabolites, desmethylchlordiazepoxide (DMCDX) and demoxepam (DMX).

Methods for determining chlordiazepoxide and its metabolites in plasma have utilised reversed-phase HPLC [9—12], and this technique was therefore used in this study.

EXPERIMENTAL

Apparatus and chromatographic conditions

Gas chromatograph. The analytical instrument was a Model 5830A or 5840A Hewlett-Packard gas chromatograph equipped with a 15-mCi ^{63}Ni electron-

capture detector. The column was coiled glass, 183 cm × 2 mm I.D., packed with 3% OV-25 on Chromosorb W HP (80—100 mesh, Supelco, Bellefonte, PA, U.S.A.). The carrier gas was argon—methane (95:5) at a flow-rate of 30 ml/min. The operating temperatures were: injection port 310°C, column 255°C, and detector 310°C. The column was primed daily by an injection of 2—3 μl of an azolectin solution in benzene (1 mg/ml).

HPLC. A Waters Assoc. (Milford, MA, U.S.A.) high-performance liquid chromatograph consisting of a Model 6000A pump for delivery of mobile phase, a WISP Model 610B automatic sample processor and a 30 cm × 3.9 mm stainless steel C-18 μBondapak (10 μm) reversed-phase column was used. The chromatograph was linked to a Model 440 UV detector operated at 254 nm.

The mobile phase for the chromatography was prepared by mixing 450 ml methanol—acetonitrile (50:50), 550 ml water and 1 ml 1 *M* sodium acetate. The flow-rate was 2.0 ml/min. The organic and aqueous components of the mobile phase were filtered prior to use.

Drugs

Lorazepam (Wyeth, U.K.) was dissolved in a vehicle of 2% benzyl alcohol, 18% polyethylene glycol and 80% propylene glycol to concentrations of 2 and 4 mg/ml. This was then further diluted with water to give concentrations of 0.25 and 0.50 mg/ml. Chlordiazepoxide hydrochloride (Roche, U.K.) was dissolved in water to give a concentration equivalent to 25 mg/ml chlordiazepoxide free base. Injection volumes were 2.0 ml/kg.

Standards and solvents

Pure samples of lorazepam and oxazepam were kindly supplied by Wyeth (Radnor, PA, U.S.A.). Standard solutions were prepared by dissolving 10 mg of each compound in 2—3 ml absolute alcohol and diluting to 100 ml with distilled water. Working standards were prepared by appropriate dilution with distilled water.

Chlordiazepoxide (CDX) hydrochloride, demoxepam (DMX), desmethylchlordiazepoxide (DMCDX) and chlorodesmethyldiazepam (RO 5-3027) were all kindly donated by Dr. W.E. Scott of Hoffmann-La Roche (Nutley, NJ, U.S.A.). Standard solutions were prepared by dissolving these compounds in methanol. Solutions of CDX, DMX and RO 5-3027 are stable when stored in amber bottles at 4°C for up to four months but DMCDX is unstable and was prepared fresh for each determination.

Organic solvents were liquid-chromatography grade and were obtained from commercial sources.

Drug administration

Experiment 1. Twenty-one male hooded Lister rats (Olac. Bicester), weighing 350—400 g, received a daily intraperitoneal (i.p.) injection of 0.5 mg/kg lorazepam for 5 days. A further 21 animals received a similar 5-day treatment with 1.0 mg/kg lorazepam. On the 5th day groups of animals were killed at intervals of 30, 90 and 240 min after administration of the drug. Immediately after death blood samples were collected in heparinised tubes and the animals' brains were removed and stored at −20°C. Plasma was separated by centrifugation and also stored at −20°C.

Plasma concentrations of lorazepam were determined as described previously [13].

Experiment 2. Twenty-eight male hooded rats (350—400 g) received once daily i.p. injections of 50 mg/kg CDX for 10 days. On the 10th day groups of animals were killed at intervals of 30, 120, 240 and 360 min after the final injection. Plasma and brain samples were collected and stored as in Experiment 1.

Plasma concentrations of CDX and its metabolites DMCDX and DMX were determined as previously described [12].

Determination of lorazepam in brain

Oxazepam was used as the internal standard since it has pK_a values similar to those of lorazepam [19] and also undergoes the same thermal rearrangement on the chromatographic column [20]. A series of round-bottomed stoppered tubes was prepared containing 75 ng oxazepam (75 μl of 1.0 μg/ml stock solution). Each brain minus the cerebellum was bisected along the midline. Half-brains were weighed, homogenized in 3.5 ml 0.4 *M* perchloric acid and the homogenate transferred to one of the tubes. (Each half-brain weighed about 0.6 g.) The pH was adjusted to approx. 9 by addition of 2 *M* sodium carbonate solution; 4 ml benzene—dichloromethane (80:20) were added, the tubes vortexed for 1 min and then centrifuged at 400 *g* for 10 min.

The organic phase was transferred to a tube containing 1.5 ml 4 *M* hydrochloric acid. The tubes were vortexed, centrifuged and the organic phase discarded. The aqueous phase was washed with a further 4 ml of benzene—dichloromethane. After discarding the organic phase the pH of the aqueous phase was adjusted to approx. 9 by addition of 2 *M* sodium carbonate solution; 2.0 ml benzene–dichloromethane were added. After mixing and centrifugation, the organic phase was removed, evaporated to dryness at 40°C under conditions of mild vacuum and reconstituted with 175 μl toluene (containing 15% isoamyl alcohol); 6 μl were injected into the chromatograph.

Standard curve. A series of tubes containing 75 ng oxazepam and 12.5, 25, 50, 75, 100 or 150 ng lorazepam were prepared. Drug-free control brain samples were homogenized in 3.5 ml 0.4 *M* perchloric acid and added to each of the tubes. A standard curve was obtained by taking these tubes through the extraction process. Standards were assayed with each set of unknowns. Since the chromatographic peaks corresponding to both oxazepam and lorazepam are Gaussian, the standard curve was obtained by plotting lorazepam concentration against lorazepam to oxazepam peak height ratio.

Determination of chlordiazepoxide and metabolites in brain

A series of tubes containing 10 μg of the internal standard RO 5-3027 was prepared (0.1 ml of a solution containing 100 μg/ml evaporated to dryness). Brains were weighed, homogenized, centrifuged and made alkaline as described for the lorazepam determination. After addition of the sodium carbonate solution, 4 ml benzene—isoamyl alcohol (98.5:1.5) were added and the tubes vortexed for 1 min and centrifuged. The organic phase was transferred to a pointed centrifuge tube and evaporated to dryness under mildly reduced

pressure. The residue was reconstituted in 200 μl HPLC-grade methanol and transferred to an autoinjection tube; 25—30 μl were injected into the chromatograph.

Standard curve. A standard curve was obtained in a similar manner to Experiment 1. Standard tubes contained 10 μg RO 5-3027 and 0.1, 0.25, 0.5, 1.0, 2.0, 5.0 and 10.0 μg CDX, DMX and DMCDX. Standards were again assayed with each set of unknowns.

Statistics

Brain/plasma concentration ratios were analysed using analysis of variance with time (and dose in the lorazepam experiment) as the independent factor(s).

RESULTS AND DISCUSSION

Experiment 1

Under the described conditions, the retention times of oxazepam and lorazepam are 4.9 and 6.4 min, respectively (see Fig. 2). Plots of peak height ratio against lorazepam concentration were linear over the concentration range studied and yielded correlation coefficients of 0.99 or greater.

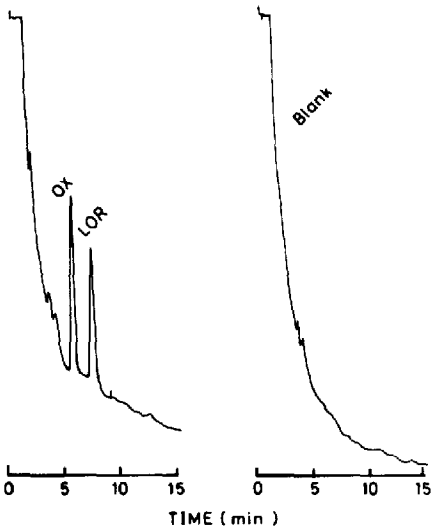

Fig. 2. A chromatogram of a drug-free control brain extract (right) and a sample to which 75 ng of oxazepam (OX) and 50 ng lorazepam (LOR) had been added (left).

Since the pK_a values of lorazepam are 1.3 and 11.5 and of oxazepam are 1.7 and 11.6 [19] it is important that the aqueous phase is not too basic before the extraction into benzene—dichloromethane. Concentrations of 5—10 ng/g tissue could be determined using this method and recoveries of 50% were obtained. Coefficients of variation for identical samples (n = 5) were 9.6% at 25 ng and 8.6% at 100 ng/g tissue.

The mean concentrations of lorazepam in brain tissue and in plasma, together with the brain/plasma concentration ratios are given in Table I. At

TABLE I

CONCENTRATIONS OF LORAZEPAM IN BRAIN AND IN PLASMA AS A FUNCTION OF TIME AFTER I.P. ADMINISTRATION OF 0.5 AND 1.0 mg/kg

Figures are means ± S.E.M.

	Time (min)	Brain concn. (ng/g)	Plasma concn. (ng/ml)	Brain/plasma
0.5 mg/kg lorazepam	30	189 ± 9	79 ± 3	2.39 ± 0.07
	90	94 ± 8	23 ± 2	4.26 ± 0.39
	240	16.1 ± 4.2	3.6 ± 1.0	4.53 ± 0.80
1.0 mg/kg lorazepam	30	306 ± 16	176 ± 16	1.78 ± 0.13
	90	163 ± 17	43 ± 3	3.75 ± 0.28
	240	26 ± 4	6.8 ± 0.7	3.75 ± 0.60

any given time after the administration of 0.5 or 1.0 mg/kg lorazepam the brain tissue/plasma (B/P) ratios showed little variation. At all time points the ratio was greater than 1.0 indicating a localization of lorazepam in brain tissue. The B/P ratio varied significantly with time [$F(2,36) = 12.9, p < 0.001$], reflecting an equilibration delay at 30 min after each dose. At this time there was also a

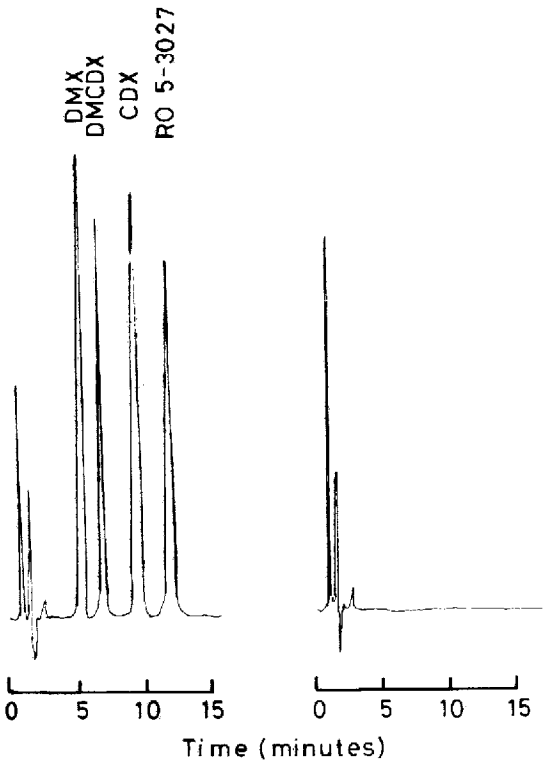

Fig. 3. A chromatogram of a drug-free control brain extract (right) and a sample to which chlordiazepoxide (CDX), desmethylchlordiazepoxide (DMCDX) and demoxepam (DMX), 5 μg of each, and the internal standard chlorodesmethyldiazepam (RO 5-3027), 10 μg have been added (left).

significant difference between the B/P ratios for the two doses ($p < 0.01$), but there were no significant differences between doses at the other times when samples were taken. For both doses plasma and brain half-lives were just less than 1 h.

There was a high correlation between the brain and plasma concentrations of lorazepam ($r = 0.88, p < 0.001$).

Experiment 2

The retention times for DMX, DMCDX, CDX and chlorodesmethyldiazepam are approximately 5.5, 7, 9.5 and 12 min, respectively (see Fig. 3). Plots of peak area or height ratio (relative to the internal standard) against concentration were linear for each compound up to concentrations of at least 10 μg/g brain tissue and gave correlation coefficients of greater than 0.99. Recoveries of 75—85% were obtained and concentrations as low as 0.1 μg/g brain tissue could

TABLE II

REPLICABILITY OF IDENTICAL SAMPLES

Brain concentration (μg/g)	Coefficient of variation* ($n = 6$)		
	Chlordiazepoxide	Desmethylchlordiazepoxide	Demoxepam
0.25	12.0	11.6	8.9
2.0	2.7	9.1	3.2

*Standard deviation divided by mean, in per cent.

TABLE III

CONCENTRATIONS OF CHLORDIAZEPOXIDE AND METABOLITES IN PLASMA AND IN BRAIN AS A FUNCTION OF TIME AFTER I.P. ADMINISTRATION OF 50 mg/kg

Figures are means ± S.E.M.

		Brain (μg/g)	Plasma (μg/ml)	Brain/plasma
30 Minutes	Chlordiazepoxide	25.3 ± 4.0	15.3 ± 1.7	1.62 ± 0.13
	Desmethylchlordiazepoxide	3.9 ± 0.5	1.6 ± 0.2	2.43 ± 0.13
	Demoxepam	0.5 ± 0.1	4.0 ± 0.3	0.13 ± 0.01
120 Minutes	Chlordiazepoxide	13.5 ± 2.9	8.2 ± 1.6	1.61 ± 0.05
	Desmethylchlordiazepoxide	6.5 ± 1.2	1.7 ± 0.4	4.23 ± 0.58
	Demoxepam	0.8 ± 0.1	5.2 ± 0.7	0.15 ± 0.03
240 Minutes	Chlordiazepoxide	6.2 ± 0.9	3.9 ± 0.6	1.68 ± 0.20
	Desmethylchlordiazepoxide	4.9 ± 0.6	1.2 ± 0.2	4.54 ± 0.60
	Demoxepam	0.6 ± 0.1	4.0 ± 0.4	0.16 ± 0.02
360 Minutes	Chlordiazepoxide	3.9 ± 1.1	2.4 ± 0.8	1.80 ± 0.22
	Desmethylchlordiazepoxide	4.5 ± 1.0	0.8 ± 0.2	5.91 ± 0.61
	Demoxepam	0.5 ± 0.2	3.3 ± 0.9	0.18 ± 0.02

be determined for all compounds. Coefficients of variation for identical samples are shown in Table II. The mean brain and plasma concentrations of CDX and its metabolites are given in Table III.

The B/P ratios for CDX and DMX showed little variation, although the ratio for DMCDX increased with time [$F(3,24) = 7.44$, $p < 0.002$]. There were significant correlations between plasma and brain concentrations of CDX, DMX and DMCDX ($r = 0.98$, 0.74 and 0.66, respectively, $p < 0.001$ for each compound).

Plots of log plasma concentration or log brain concentration against time for CDX were linear ($r > 0.9996$). Plasma and brain half-lives calculated from the gradients were 103 and 107 min, respectively.

The methods described for determining lorazepam and for determining CDX and its metabolites in brain tissue were rapid, clean and sensitive and will be useful to workers needing to follow the brain pharmacokinetics of benzodiazepines in the investigation of drug interactions.

ACKNOWLEDGEMENTS

R.G.L. is supported by a School of Pharmacy postgraduate award. S.E.F. is a Wellcome Trust senior lecturer. This work was supported in part by Grants MH-34223 and AM-32050 from the United States Public Health Service.

REFERENCES

1 D.J. Greenblatt and R.I. Shader, Benzodiazepines in Clinical Practice, Raven Press, New York, 1974.
2 J.D. Christensen, Acta Pharmacol. Toxicol., 33 (1973) 262.
3 M. Divoll, D.J. Greenblatt, Y. Lacasse and R.I. Shader, Psychopharmacology, 73 (1981) 381.
4 E.H. Ellinwood, M. Linnoila, M.E. Easler and D.W. Molter, Clin. Pharmacol. Ther., 30 (1981) 534.
5 S.E. File, Psychopharmacology, 73 (1981) 240.
6 S.E. File, Psychopharmacology, 77 (1982) 284.
7 S. Higuchi, H. Urabe and Y. Shiobara, J. Chromatogr., 164 (1979) 55.
8 L.M. Walmsley and L.F. Chasseaud, J. Chromatogr., 226 (1981) 155.
9 V. Ascalone, J. Chromatogr., 181 (1980) 141.
10 T.B. Vree, A.M. Baars, Y.A. Hekster and E. van der Kleijn, J. Chromatogr., 224 (1981) 519.
11 G.L. Lensmeyer, C. Rajani and M.A. Evenson, Clin. Chem., 28 (1982) 2274.
12 M. Divoll, D.J. Greenblatt and R.I. Shader, Pharmacology, 24 (1982) 261.
13 D.J. Greenblatt, K. Franke and R.I. Shader, J. Chromatogr., 146 (1978) 311.
14 S.H. Preskorn, G.H. Irwin, S. Simpson, D. Friesen, J. Rinne and G. Jerkovich, Science, 213 (1981) 469.
15 C.L. Davies and D.J. Nutt, Br. J. Pharmacol., 78 (1983) 114P.
16 R.T. Schillings, S.F. Sisenwine and H.W. Ruelius, Drug Metab. Dispos., 5 (1977) 425.
17 D.R. Hoogland, T.S. Miya and W.F. Bousquet, Toxicol. Appl. Pharmacol., 9 (1966) 116.
18 S.E. File, Eur. J. Pharmacol., 81 (1982) 637.
19 J. Barrett, W.F. Smyth and I.E. Davidson, J. Pharm. Pharmacol., 25 (1973) 387.
20 A. Frigerio, K.M. Baker and G. Belvedere, Anal. Chem., 45 (1973) 1846.