7. Atkinson HC and Begg EJ, Prediction of drug concentrations in human skim milk from plasma protein binding and acid-base characteristics. *Br J Clin Pharmacol* **25**: 495–503, 1988.
8. Allen JC, Sodium and potassium content and viability of mouse mammary gland tissue and acini. *J Dairy Sci* **71**: 633–642, 1988.
9. Syversen GB and Ratkje SK, Drug distribution within human milk phases. *J Pharm Sci* **74**: 1071–1074, 1985.
10. Brimblecombe RW, Duncan WAM, Durant GJ, Emmett JC, Ganellin CR and Parsons ME, Cimetidine—a non-thiourea H_2-receptor antagonist. *J Int Med Res* **3**: 86–92, 1975.
11. McKinney TD, Myers P and Speeg KV Jr, Cimetidine secretion by rabbit renal tubules *in vitro*. *Am J Physiol* **241**: F69–F76, 1981.
12. Cacini W, Keller MB and Grund VR, Accumulation of cimetidine by kidney cortex slices. *J Pharmacol Exp Ther* **221**: 342–346, 1982.
13. Takano M, Inui K, Okano T and Hori R, Cimetidine transport in rat renal brush border and basolateral membrane vesicles. *Life Sci* **37**: 1579–1585, 1985.
14. Gisclon L, Wong FM and Giacomini KM, Cimetidine transport in isolated luminal membrane vesicles from rabbit kidney. *Am J Physiol* **253**: F141–F150, 1987.
15. Berga SE and Neville MC, Sodium and potassium distribution in the lactating mouse mammary gland *in vivo*. *J Physiol (Lond)* **361**: 219–230, 1985.
16. Baumrucker CR, Cationic amino acid transport by bovine mammary tissue. *J Dairy Sci* **67**: 2500–2506, 1984.
17. Rosenberg LE, Downing SJ and Segal S, Extracellular space estimation in rat kidney slices using C^{14} saccharides and phlorizin. *Am J Physiol* **202**: 800–804, 1962.
18. Topper YJ, Oka T and Vonderhaar BK, Techniques for studying development of normal mammary epithelial cells in organ culture. *Methods Enzymol* **39**: 443–453, 1975.
19. Sivelle PC, Underwood AH and Jelly JA, The effects of histamine H_2 receptor antagonists on androgen action *in vivo* and dihydrotestosterone binding to the rat prostate androgen receptor *in vitro*. *Biochem Pharmacol* **31**: 677–684, 1982.
20. Carlson HE and Ippoliti AF, Cimetidine, an H_2-antihistamine, stimulates prolactin secretion in man. *J Clin Endocrinol Metab* **45**: 367–370, 1977.
21. Becu D and Libertun C, Serotoninergic involvement in the cimetidine-induced prolactin release. *Endocrinology* **113**: 1980–1984, 1983.

Biochemical Pharmacology, Vol. 39, No. 1, pp. 210–212, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + .00
Pergamon Press plc

Occurrence of 'natural' diazepam in human brain

(*Received* 26 *May* 1989; *accepted* 19 *July* 1989)

There is accumulating evidence that benzodiazepines (BZD), especially diazepam (D) and desmethyldiazepam, are naturally present in tissues of different animal species [1–3] as well as in plant products [2–4]. 'BZD-like' immunoreactivity has been detected even in human brain samples stored in paraffin since 1940 [1]. However in this experiment the material was not identified by specific chemical analysis. As the source of the BZD detected in the different biological samples has not been clarified, it might be speculated that it is of synthetic origin [5]. Therefore we have examined human brain material which has been kept in storage since at least 1959, a time prior to the first laboratory synthesis of these compounds. BZD were quantified by a specific gas chromatography (GC)/mass spectrometry (MS) technique in different parts of the stored brains.

Materials and methods

Slices of cerebrum, cerebellum and brainstem from three different human brains, which were stored in 10% formaldehyde solution, were examined.

Brain No. 1 was from a 31-year-old subject, whose death was due to an amyotrophic lateral sclerosis in February 1955. Brain No. 2 was obtained from a 27-year-old male schizophrenic patient whose death in July 1958 was attributable to an acute circulatory collapse. Brain No. 3 was taken from a 36-year-old male epileptic patient who died of pulmonary embolism in February 1959.

Slices of different areas of brains No. 1 and 2 were stored separately in formaldehyde solution. The individual tissue slices were homogenized thoroughly in this solution using an ultraturrax mixer for 1 min prior to extraction. Tissues of brain No. 3 were stored together in formaldehyde solution and were separated prior to analysis. Slices of cerebellum and brainstem were removed from the solution and homogenized in distilled water. One section of the cerebrum was homogenized in distilled water, whereas the remainder was homogenized in the original formaldehyde solution. In addition, the formaldehyde solution was analysed separately to examine whether BZD had been extracted during storage. A sample from each part of each brain (7.5 to 10 g wet weight tissue) was analysed at least in duplicate.

Deuterated D (d_5-D, Sigma Chemical Co., Poole, U.K., 5 ng) was added to the homogenized samples as the internal standard. The biological samples were extracted and subsequently purified by HPLC according to the method described in detail recently [3]. The HPLC fraction, collected at a retention time corresponding to that analysed for D was quantified by GC/MS in negative ion chemical ionization mode (for details see Ref. 3). Concentrations of D were calculated from the peak height ratios of D to d_5-D recorded from the tracings in the selected ion monitoring (see Fig. 1).

To test the stability of D under the influence of formaldehyde, a 10% formaldehyde solution was spiked with aliquots of 100 ng D and stored at 80° for one week to simulate storage at 10°–15° for over 30 years.

Results and discussion

All brain material examined by GC/MS contained D. The concentrations observed ranged from 0.15 to 0.34 ng/g wet weight tissue (see Table 1).

Controls of 20 ml distilled water were processed through the whole work-up procedure and contained maximally 0.005 ng/ml D. The original solution in which tissues of brain No. 3 were stored contained a concentration of

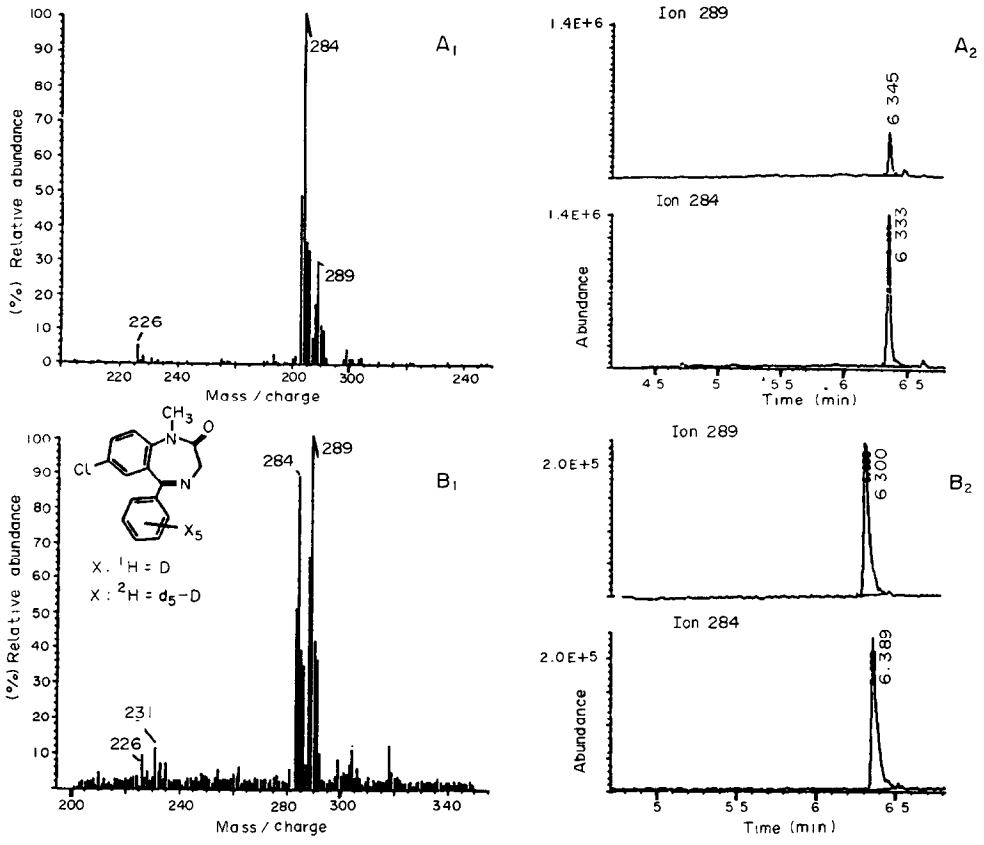

Fig. 1. Partial mass spectra in negative chemical ionization mode and GC/MS tracings by selected ion monitoring. (A_1 and A_2) biological sample: cerebrum of brain No. 1. (B_1 and B_2) reference compounds: diazepam (D) and d_5-D.

0.05 ng/ml and thus it was obvious that formaldehyde was able to extract a certain amount of D. No decomposition of D was observed by qualitative and quantitative HPLC analysis after incubation for one week in a formaldehyde solution. The partial mass spectrum of the biological extract from the cerebrum of brain No. 1 was compared with that of the reference compound D and internal standard d_5-D. All typical mass peaks in the molecular ion region (MW 284 and 289 for D and d_5-D respectively) could be identified in the brain extracts (see Fig. 1).

Table 1. Concentrations of diazepam in different human brains (ng/g wet weight), quantified by gas chromatography/mass spectrometry using a stable isotope dilution technique

Brain No. (year) Tissue	1 (1955)	2 (1958)	3 (1959)
Cerebrum	0.23	0.17	0.24 0.18*
Cerebellum	0.31	0.19	0.15*
Brainstem	n.m.	n.m.	0.34*

All values represent means of at least two measurements.
n.m. not measured.
* Homogenized in distilled water.

The present data illustrate that in addition to the detection of BZD in brain material of different species [1–3] which might have been exposed to industrial BZD in their food, the BZD agonist D could be detected in human brain tissues which had been stored before the first BZD were synthesized and patented in July 1959 (chlordiazepoxide [6]) and in 1963 (Diazepam [7]). As laboratory contamination was rigorously excluded, our results provide strong evidence that environmental pollution by synthetic BZD and subsequent intake can be virtually excluded. Thus, as indicated by the detection of 'BZD-like' material in old human brains [1], the quantification of D in such old material by a specific and sensitive GC/MS technique suggests that the human brain contains natural occurring BZD. The origin of these BZD and how biosynthesis occurs is still unclear, however it was demonstrated in different studies [2–4] that the compounds could also be extracted from different edible plant materials and thus might be incorporated via the food chain.

The concentrations observed in all old brain samples (see Table 1) was higher than that determined in the brain of a 22-year-old accident victim (0.06 ng/g; see also Ref. 3). This could be attributable to firstly a better extraction of BZD from different parts of the old brains which had been stored in a formaldehyde solution, compared to the fresh brain which had been homogenized *in toto* and extracted subsequently. Secondly, as old brain tissues could only be obtained from patients who suffered from various CNS disorders, it may be speculated that these pathological conditions could have influenced the concentration of D detected. However the concentration of this BZD deter-

mined is much lower than that required for therapeutic effects in the CSF [8] and thus its clinical significance in modifying CNS functions remains to be demonstrated.

Acknowledgement—The authors would like to thank Mr F. Schönberger for his excellent technical assistance and Dr A. Gross for carefully going through the manuscript. This work was supported by the Robert-Bosch Foundation, Stuttgart, F.R.G.

*Dr Margarete Fischer-Bosch-Institut für Klinische Pharmakologie
Auerbachstr. 112
7000 Stuttgart 50
and
†Max-Planck-Institut für Psychiatrie
8000 München 40
Federal Republic of Germany

E. UNSELD*
C. FISCHER*
E. ROTHEMUND†
U. KLOTZ*‡

‡ Author to whom correspondence should be addressed.

REFERENCES

1. Sangameswaran L, Fales HM, Friedrich P and de Blas AL, Purification of a benzodiazepine from bovine brain and detection of benzodiazepine-like immunoreactivity in human brain. *Proc Natl Acad Sci USA* **83**: 9236–9240, 1986.
2. Wildmann J, Möhler H, Vetter W, Ranadaler U, Schmidt K and Maurer R, Diazepam and *N*-desmethyldiazepam are found in rat brain and adrenals and may be of plant origin. *J Neural Transm* **70**: 383–389, 1987.
3. Unseld E, Krishna DR, Fischer C and Klotz U, Detection of desmethyldiazepam and diazepam in brain of different species and plants. *Biochem Pharmacol* **38**: 2473–2478, 1989.
4. Wildmann J, Vetter W, Ranadaler UB, Schmidt K, Maurer R and Möhler H, Occurrence of pharmacologically active BZD in trace amounts in wheat and potato. *Biochem Pharmacol* **37**: 3549–3559, 1988.
5. Unseld E and Klotz U, Benzodiazepines: Are they of natural origin? *Pharmac Research* **6**: 1–3, 1989.
6. Chlordiazepoxide, US patent No. 2893992, July 7, 1959.
7. Diazepam, US patent No. 3109843, 1963.
8. Greenblatt DJ, Ochs HR, Lloyd BL, Entry of diazepam and its major metabolite into the cerebrospinal fluid. *Psychopharmac* **70**: 89–93, 1980.

Biochemical Pharmacology, Vol. 39, No. 1, pp. 212–216, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + .00

Comparative inhibitory effects of mefloquine and primaquine on hepatic drug-metabolizing enzymes

(*Received* 17 *February* 1989; *accepted* 31 *July* 1989)

Mefloquine (MQ) and primaquine (PQ) are quinoline derivatives used mainly as antimalarial drugs. Several studies have shown that PQ is a potent inhibitor of many enzymes *in vitro* including those dependent on the hepatic cytochrome P-450 [1–5]. In an *in vivo* study in man, PQ has been reported to be a potent inhibitor of antipyrine metabolism [6]. However, little is known about the effect of MQ on hepatic drug metabolism, except in a recent study by Riviere and Back [7], who have demonstrated that both MQ and PQ could inhibit the metabolism of aminopyrine and ethinylestradiol *in vitro* as well as the metabolism of tolbutamide *in vivo*.

In the present communication, we have investigated and compared the inhibitory effects of these two antimalarial drugs on a wide range of hepatic drug-metabolizing enzymes *in vitro* and studied the nature of their inhibition on these enzymes. Their inhibitory effects on the *in vivo* metabolism of hexobarbital and zoxazolamine were also tested.

Methods

Male Wistar rats (150–200 g), obtained from the National Laboratory Animal Center of Mahidol University, were housed under standardized conditions of light (6:00 a.m. to 6:00 p.m.) and temperature (25–28°). All animals were untreated and allowed at least 5 days to become acclimated to the housing conditions prior to use in experiments. They were given free access to food (standard rat chow; Purina Laboratory Chow, Zuellig Pte. Ltd, Singapore) and tap water *ad lib.* until 14–16 hr before killing, during which they were allowed access to water only. Animals were killed by heart puncture under light ether anesthesia between 7.00 and 9.00 a.m. Livers were quickly removed and washed with ice-cold buffered KCl (1.15% KCl in 0.1 M Na^+/K^+ phosphate buffer, pH 7.4) to remove the blood. The liver was blotted dry with filter paper and weighed. All subsequent procedures were performed below 4°. After weighing, the liver was teased with scissors and then homogenized with 2–7 volumes of buffered KCl in a Potter–Elvehjem homogenizer using a Teflon pestle. The homogenate was centrifuged at 9000 *g* in a refrigerated centrifuge for 15 min using Beckman Centrifuge Model J-21B with JA-21 rotor. After centrifugation, the supernatant was carefully aspirated off with Pasteur pipette. Three milliliters of this post-mitochondrial fraction were further centrifuged at 105,000 *g* for 60 min in a Beckman Model L5-65 refrigerated ultracentrifuge, whereas the remaining portion (about 9.0 ml) was used as the enzyme source in the *in vitro* hepatic drug-metabolizing enzyme assays. After ultracentrifugation the soluble supernatant fraction was carefully removed with a Pasteur pipette and the microsomal pellet was rinsed and resuspended in 3.0 ml ice-cold 0.1 M Na^+/K^+ phosphate buffer, pH 7.4. This microsomal suspension was used for the determination of microsomal protein content.

The activities of aminopyrine *N*-demethylase, aniline hydroxylase, hexobarbital oxidase, and *p*-nitroanisole *O*-demethylase were measured in the presence of various cofactors according to the methods described in detail in previous publications [8, 9]. *p*-Chloro-*N*-methylaniline *N*-