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Studies on the pharmacokinetics and metabolism of N_4-carbamoyl-1,3,4,5-dihydro-diazepam (Uxepam®) in rats, dogs and man

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Abstract: The pharmacokinetics and metabolism of Uxepam®, a new minor tranquillizer, have been investigated in rats [2], dogs and man. For the experiments in rats [2] separation of metabolites of 2-^{14}C-uxepam was achieved by thin-layer chromatography. In the experiments on dogs and man, Extrelut microcolumns were used for pre-separation. Recovery was 95% ± 12.77 (S.D.) determined by radioactive tracer experiments. The compounds were separated and determined by reversed-phase HPLC with UV detection at 240 nm. The limit of detection for uxepam was 10 ng ml^{-1}. The metabolites were identified by mass spectrometry.

The main metabolites in the rat were desmethyl-uxepam, 5-hydroxy-phenyl-desmethyl-uxepam and diazepam. Desmethyl-carbamoyl-dihydro-diazepam, diazepam and desmethyl-diazepam were found in human plasma.

In dogs only one metabolite, desmethyl-uxepam, was detected in plasma. Enterohepatic recycling was observed in dogs and in humans.

Keywords: *Uxepam; pharmacokinetics; metabolism; HPLC.*

Introduction

7-Chloro-1,3,4,5-tetrahydro-1-methyl-4-carbamoyl-5-phenyl 2h-1,4,-benzodiazepine-2-one (Uxepam®) [1] is a new minor tranquillizer which is a good anxiolytic agent with a slight muscle relaxant effect. A study of its metabolism *in vivo* was required.

According to results of earlier experiments [2] uxepam is metabolized in rats by N^1-demethylation to 7-chloro-1,3,4-5-tetrahydro-4-carbamoyl-5-phenyl-2H-1,4-benzodiazepine-2-one (desmethyl-uxepam) or by hydrolysis of the carbamoyl group followed by oxidation and decarboxylation to form diazepam. No data are available on the details of those reactions. An oxidative decarboxylation is also possible.

Although dihydro-diazepam could not be detected as a metabolite in rats, the detection of two other metabolites suggested that dihydro-diazepam was present in blood as an intermediate. Because of the possibility of the appearance of dihydro- and tetrahydro-diazepines with similar substituents, the method had to be capable of

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separating these compounds. Although numerous reports of methods suitable for the separation of benzodiazepine derivatives for pharmacokinetic studies have been published [3–6], none describe methods to separate dihydro- and tetrahydro-compounds. Therefore it was necessary to develop a method for use in human pharmacokinetics and metabolism studies, where the drug and its metabolites can be determined simultaneously in plasma. Since most published methods for separation by HPLC were conducted at pH 4.5–8.0, i.e. on the unprotonated compound, it was decided to investigate the protonation equilibria of the compounds and to use Extrelut extraction and reversed-phase HPLC. Since retention times alone were insufficient for the identification of metabolites, mass spectrometry was used.

Materials and Methods

Chemicals

Reference substances were uxepam, desmethyl-uxepam diazepam, desmethyl-diazepam, ^{14}C-oxazepam, methyloxazepam, 2^{14}C-uxepam and 2-^{14}C-dihydro-diazepam. All solvents were of analytical grade. The compounds were prepared at the Central Research Institute for Chemistry, The Hungarian Academy of Sciences, Budapest, Hungary. Extrelut adsorbent was obtained from Merck (Darmstadt, FRG).

Experiments in human volunteers

The human clinical protocol was approved by the local committee on ethics of the National Institute for Neurology and Psychiatry. Six male volunteers, judged to be healthy by physical and laboratory tests, were used in this study. Data on the subjects are shown in Table 1. After an overnight fast the ambulatory volunteers received two 20-mg Uxepam tablets as a single oral dose. No food was allowed until 4 h after administration of the drug.

Blood samples were taken through a heparin cannula at the following time intervals: 0.5, 0.75, 1, 2, 3, 4, 6, 12, 24, 48 and 72 h after drug administration. Sodium citrate (3.8% v/v) was added to the blood sample (1:9, v/v) to prevent coagulation. The blood samples were immediately centrifuged at 3000*g* for 15 min; the plasma obtained was kept deep frozen until it was analysed.

Experiments in dogs

Six healthy 18-months old beagle dogs (11–13 kg) were used. Uxepam (10 mg) in tablet form and as injectable solution was administered by mouth and intravenously with

Table 1
Data on experimental subjects

Subject code	Sex	Age (years)	Weight (kg)	Oral dose (mg)
N. L.	M	22	66	2 × 20
K. A.	M	22	83	2 × 20
U. L.	M	21	77	2 × 20
K. I.	M	22	60	2 × 20
P. B.	M	21	68	2 × 20
T. P.	M	21	63	2 × 20

'cross-over' intervals of 2 weeks. The animals were fasted for 12 h prior to the experiment. Blood samples were collected before and 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10 and 24 h after the oral and intravenously administration of 10 mg of Uxepam. Blood samples were collected as described above for humans.

Extraction procedure

Chromatography columns (150 × 5 mm i.d.) were packed with 500 mg of Extrelut. A 1 ml sample of plasma diluted with 0.5 ml of 0.01 M phosphate buffer (pH 9.5) was applied to the top of the column. Metabolites were eluted first with 4 ml of hexane and then with 4 ml of ethyl acetate. The solutions collected separately were washed with 1 ml of water. Ethyl acetate was evaporated from the ethyl acetate extract in a dry stream of nitrogen and to the residue the hexane solution was added; the hexane was then removed by evaporation. The final residue was dissolved in 50 μl of methanol.

In independent experiments 2-^{14}C-uxepam, 2-^{14}C-dihydro-diazepam and ^{3}H-diazepam were added to human plasma to determine the recovery. Protein concentration in extracts was determined according to the method of Lowry *et al.* [7].

Chromatographic conditions

A Knauer HPLC pump with a Rheodyne 7120 injection valve (20 μl loop) coupled with a Perkin–Elmer Sigma 10 data system were used. A 20 μl portion was injected on the 250 × 4.6 mm i.d. column packed with RP-18 LiChrosorb (Merck). The mobile phase was 0.01 M phosphate buffer (pH 3.8)–methanol (45:55, v/v). The compounds were detected at 240 nm.

Quantitation was based on the use of solutions of an external standard injected on the column. The calibration curves were linear using data obtained from the known concentrations of the standard solutions and the peak areas. The calibration was checked daily by injecting standard mixtures.

Identification of the metabolites

The compounds were isolated by micropreparative HPLC by collecting the peaks manually. The solvent was evaporated to one-quarter of the original volume; the pH was adjusted to pH 9 with 0.01 M NaOH and the solution was re-extracted with ether. The ether solution was evaporated and the residue was analysed by mass spectrometry with an AEI model MS-902 double focussing instrument.

Results and Discussion

Uxepam and its respective biotransformation products could be extracted from blood plasma with solvents in good yield, but the extracts obtained contained much endogenous material and the chromatogram was not clear. The elimination of proteins from biological materials and extraction of benzodiazepines was made relatively simple by the use of Extrelut [8, 9]; this material was also used successfully to remove proteins from plasma samples and to extract the metabolites.

Preliminary examination with authentic materials showed that dihydro-diazepam was partly acetylated if the ethyl acetate solution was evaporated. To avoid this undesirable reaction, dihydro-diazepam and the main part of diazepam were first extracted with hexane; the other compounds were then extracted with ethyl acetate. The ethyl acetate extract was evaporated first and the hexane solution was added to its residue. Elution of

4 ml of hexane followed by 4 ml of ethyl acetate and washing each solution with 1 ml of water gave the best recovery. After extraction of 1 ml plasma containing 20–50 ng of radioactive materials the samples contained 96% of 2-^{14}C-uxepam, 94% of 2-^{14}C-dihydro-diazepam and 95% of ^{3}H-diazepam. The protein content of the extracts was less than 5 μg. Dihydro-diazepam and diazepam were eluted by hexane. Ethyl acetate extracted the other compounds.

The sensitivity of the method was 10 ng ml^{-1} (limit of quantitation) for the different compounds. The recovery of the three derivatives, determined by using radiolabelled drugs, was 95% ± 12.77 (S.D.). The calibration curves obtained by adding known amounts of the drug to blank plasma were linear in the concentration range of interest (20–300 ng ml^{-1}). The linear regression curve for data used for calibration for uxepam could be described by the equation $y = 1.8801x + 0.4888$; the correlation coefficient was 0.9802 ($n = 12$).

UV spectra showed that at pH 3–4 the dihydro-compounds (desmethyl-uxepam and dihydrodiazepam) are completely protonated whereas diazepam and desmethyl-diazepam are in the unprotonated form. Uxepam is not protonated at pH 3–4. Therefore the HPLC experiments were done at pH 3–4. Values for the capacity factor of the protonated compounds were very sensitive to change in buffer concentration, pH, temperature, and the type of buffer anion. This makes it probable that the protonated compounds migrate as ion pairs with the buffer anion. The value of the selectivity factor α for diazepam/dihydrodiazepam was 1.5 at pH 3.7 and 1.0 at pH 4.6. Separations were good at pH 3.5–3.8 and endogenous plasma peaks did not interfere with determination.

The plasma kinetics of uxepam and its metabolites in man are illustrated in log

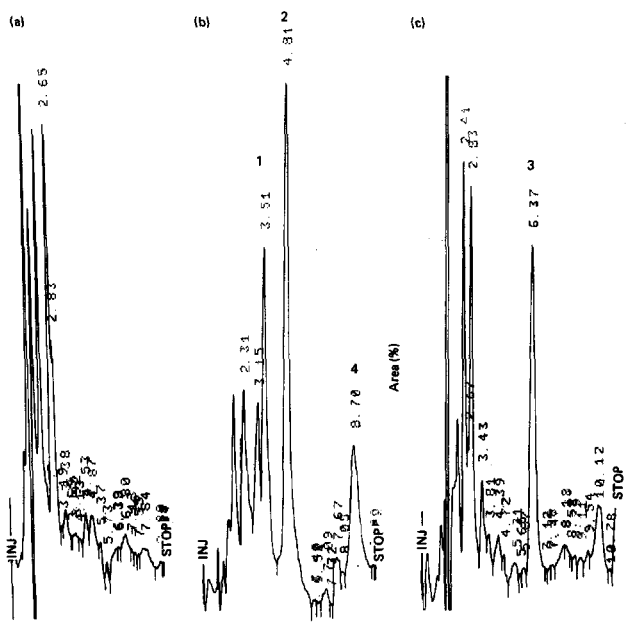

Figure 1
Chromatograms of human plasma extract: (a) human blank plasma; (b) 1 h after oral administration of 40 mg uxepam. (c) 48 h after oral administration. (1) Desmethyl-uxepam, (2) uxepam, (3) desmethyl-diazepam, (4) diazepam.

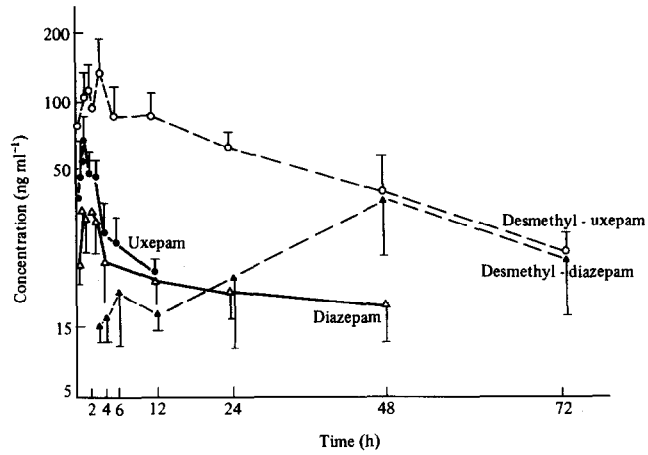

Figure 2
Log concentration–time curves of Uxepam and its metabolites, desmethyl-uxepam, diazepam and desmethyl-diazepam in human plasma, after oral administration of two 20-mg Uxepam tablets.

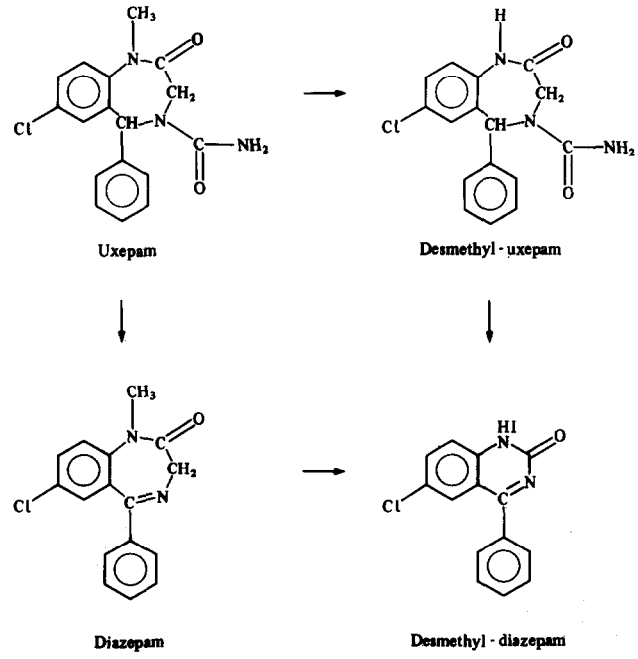

Figure 3
Metabolic pathway of uxepam in humans.

concentration–time curves (Fig. 2). The curves represent the mean concentrations (±S.D.) of the four compounds measured independently in six healthy volunteers.

In agreement with the findings in rats, demethylation seems to be one of the first metabolic steps of uxepam in man. Desmethyl-uxepam appeared in blood in higher concentration than did the intact drug (Figs 1 and 2); the amount of this metabolite diminished at a lower rate than that of uxepam.

The peak plasma level of desmethyl-uxepam was 112 ng ml^{-1}. This was attained 2 h after administration; desmethyl-uxepam could still be detected at 72 h. Figure 3 shows the metabolic pathway suggested by results after administration of uxepam in man. For dogs neither diazepam nor desmethyl-diazepam was found in plasma after oral or intravenous administration of 10 mg uxepam, as can be seen clearly on the chromatogram (Fig. 4). Figure 5 represents the log concentration–time curves of uxepam in dog plasma samples after oral (p.o.) and intravenous administration.

Dihydro-diazepam (DHD) was not detected as a metabolite. Diazepam, desmethyl-diazepam and desmethyl-uxepam could be detected in significant amounts (Fig. 2). Desmethyl-diazepam can be derived from both diazepam and desmethyl-uxepam.

The metabolism of diazepam in man was elucidated by Schwartz *et al.* [10], who found desmethyl-diazepam in blood in addition to traces of methyl-oxazepam and oxazepam. Although diazepam is a metabolite of uxepam its hydroxylated metabolites could not be detected. This fact is not in disagreement with earlier work [11] since these metabolites are reported to be present as glucuronide conjugates and could have been extracted only after deconjugation by enzymatic hydrolysis. An unknown peak was detected on the chromatograms of each plasma sample taken at 0.75 h, but it was not identical with any of these hydroxylated products. In the log concentration–time curves for both man and dogs a second peak appeared after C_{max} which indicated enterohepatic recycling. Other authors have observed this phenomenon after administration of other benzodiazepine derivatives.

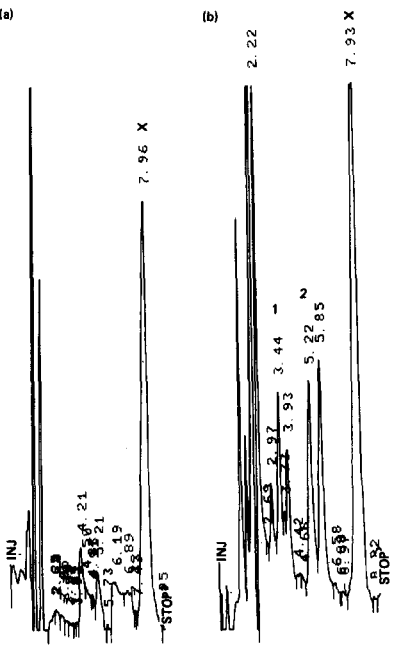

Figure 4
Chromatogram of dog plasma extracts. (a) Dog blank plasma; (b) 2 h after intravenous administration of 10 mg uxepam injection. (1) Desmethyl-uxepam, (2) Uxepam x. Unknown peaks from plasma extract.

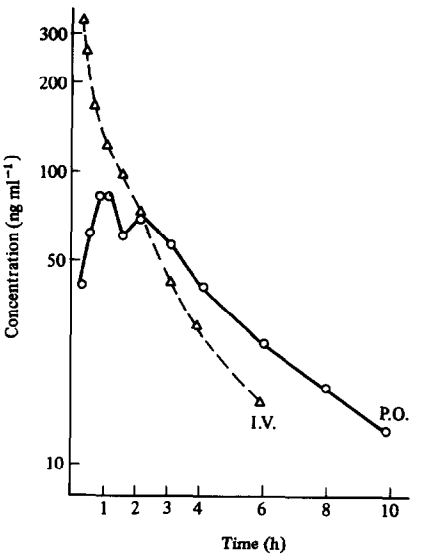

Figure 5
Log concentration–time curves of Uxepam in dogs after intravenous and oral administration of 10 mg Uxepam.

The method was found to be useful for the simultaneous separation and determination of tetrahydro- and dihydro-benzodiazepines and could be applied to the analysis of human and animal plasma in pharmacokinetic experiments.

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