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Note

Simultaneous determination of diazepam and its metabolites N-desmethyldiazepam, oxydiazepam and oxazepam in plasma and urine of man and dog by means of high-performance liquid chromatography

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Benzodiazepine derivatives are widely used all over the world for a variety of clinical indications [1]. Recently, it has become obvious that several drug-associated events, such as drowsiness, hangover and other CNS depressions, are related to intrinsic pharmacological properties. Due to different pharmacokinetics and metabolism, a variety of active metabolites may be formed and these may be responsible for some of the adverse effects. Knowledge of the pharmacokinetic behaviour of diazepam, the most widely used benzodiazepine derivative, is limited by the analytical assay procedure. Due to extensive biotransformation and/or tissue distribution, parent drug and metabolites are present in only trace amounts in body fluids. Several attempts have been made to determine parent compound and metabolites by means of gas—liquid chromatography with electron capture detection [2—4], gas chromatography—mass spectrometry [5, 6], thin-layer chromatography [7, 8] and high-performance liquid chromatography (HPLC) [9—12]. Until now, none of these methods (except that of Kabra et al. [12]) has allowed the simultaneous determination of all the main metabolites of diazepam. Kabra's method [12] however, requires a large sample volume (2 ml).

The method described here affords the simultaneous determination of diazepam, N-desmethyldiazepam, oxydiazepam and oxazepam in 0.2 ml of sample. The method has been applied to pharmacokinetic studies in dog and man and also for routine monitoring in man, for which some results are presented.

MATERIALS AND METHODS

Apparatus

A Spectra Physics 3500 B high-performance liquid chromatograph was used, equipped with a spectrophotometric detector (Model 770). The detector was connected to a 1-mV recorder (BD7; Kipp & Zonen, Emmen, The Netherlands). A stainless-steel column, 10 cm × 4.6 mm I.D., commercially packed with LiChrosorb RP8, particle size 5 μm, (Chrompack, Middelburg, The Netherlands) was used. The injection loop was 100 μl size. Detection of the benzodiazepines and metabolites was effected at 230 nm; the detection limit is 30 ng/ml.

Solvent

The solvent was a mixture of water—methanol—acetonitrile (500:450:50, v/v) and the flow-rate was 1.6 ml/min, at a pressure of 250 atm.

Drugs

Diazepam, N-desmethyldiazepam, oxydiazepam (3-hydroxydiazepam), oxazepam, and flunitrazepam were obtained from Hoffmann-La Roche (Mijdrecht, The Netherlands).

Animals

Beagle dogs from the Central Animal Laboratory of the University of Nijmegen were used in this study. The benzodiazepines were administered at a dose of about 2 mg/kg. The dogs were kept under light anaesthesia (nitrous oxide, oxygen, halothane). Induction was achieved with pentobarbital (Nembutal®). A constant urine flow was achieved by continuous infusion of dextrose solution (5%, w/v).

Subjects

Ten subjects, all employees of the Department of Clinical Pharmacy, Nijmegen, participated in this study. Oxydiazepam and oxazepam were administered orally in doses ranging between 2 and 30 mg. Two volunteers took a mixture of oxazepam and oxydiazepam. The drugs were taken orally in the morning, 1.5 h after a standard breakfast [13]. Blood samples of 0.2 ml were collected at scheduled intervals by fingertip puncture (Microlance No. 433, Becton & Dickinson). Spontaneously voided urine was collected for 60 h.

Sample preparation

Plasma and urine. Ten microlitres of acetonitrile, containing 400 ng of flunitrazepam as internal standard, were added to 0.1 ml of plasma or urine and 2 ml of diethyl ether, and was mixed for 1 min. The mixture was then centrifuged for 5 min at 4000 rpm (2600 *g*) in a Heraus Christ centrifuge. The ether layer was transferred and evaporated to dryness. A 0.2-ml aliquot or the eluent was added to the residue and 0.1 ml was injected onto the column.

Deglucuronidation of the samples. Plasma or urine (0.2 ml) was incubated with 25 μl beta-glucuronidase (100,000 units/ml; Sigma, St. Louis, Mo. U.S.A.), 0.4 ml of KH_2PO_4 buffer (0.067 *M*) and one drop of 0.2 *M* acetic acid for 24 h.

Ten microlitres of acetronitrile containing the internal standard (400 ng flunitrazepam for plasma and 1000 ng for urine) were added together with 0.8 ml Na_2HPO_4 (0.067 *M*) buffer. The mixture was extracted with ether, centrifuged, evaporated to dryness and treated for injection onto the column as described above.

Recovery

The recovery of the extraction for diazepam is 96 ± 7%, for N-desmethyldiazepam 91 ± 7%, oxydiazepam 94 ± 7% and oxazepam 89 ± 6%. The calibration curves were linear for the concentration range 10 ng to 10 μg (r = 0.999). The sensitivity limit for all the derivatives was 30 ng/ml.

RESULTS

Chromatography

Fig. 1a shows a high-performance liquid chromatogram for a reference sample of the calibration curve extracted from human ACD plasma. Flunitrazepam (F) is used as internal standard. There is an excellent separation between diazepam (D) and its main metabolites, N-desmethyldiazepam (DD), oxydiazepam (OD) and oxazepam (OX). Fig. 1b gives an example of an extract of a plasma sample of a dog, 30 min after an intravenous bolus injection of 2.18

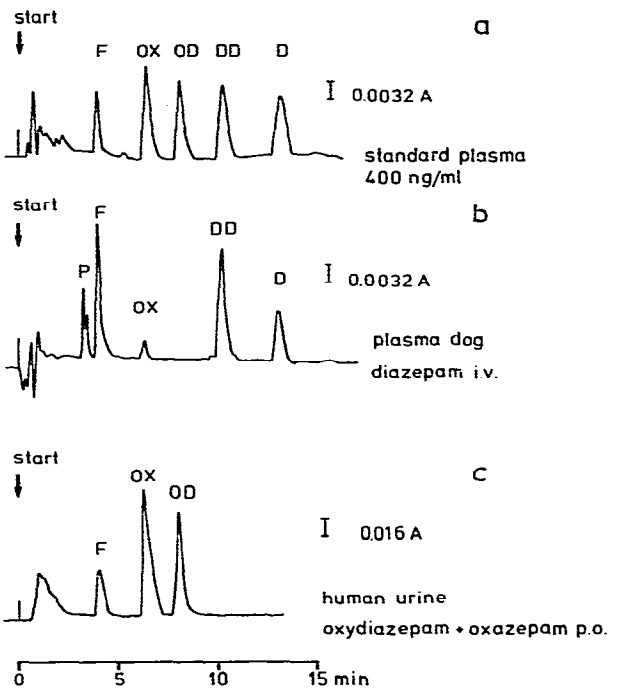

Fig. 1. HPLC chromatograms of diazepam (D) and its metabolites, N-desmethyldiazepam (DD), oxydiazepam (OD) and oxazepam (OX), obtained from: (a) reference sample; (b) dog plasma; (c) human urine. F is the internal standard flunitrazepam.

TABLE I

RELATIVE RETENTION TIMES OF DIAZEPAM AND ITS METABOLITES

Compound	Relative retention time (k')
Flunitrazepam	6.50
Oxazepam	10.67
Oxydiazepam	13.30
N-Desmethyldiazepam	17.00
Diazepam	21.80
Chlordiazepoxide	10.07
Pentobarbital	5.58

mg/kg was given. Note the pentobarbital (P) peak just before the flunitrazepam. Pentobarbital (Nembutal®) was used as the anaesthetic. No oxydiazepam could be detected and only small amounts of oxazepam.

Fig. 1c is an example of an extract of human urine containing relatively large amounts of oxazepam and oxydiazepam, after oral intake of both drugs simultaneously. No interfering compounds are seen. The relative retention times of diazepam and its metabolites are given in Table I. Chlordiazepoxide (Librium®) is also well separated from the other benzodiazepine derivatives, which may be important when measuring plasma concentrations following ingestion of a mixture of benzodiazepines [14].

Benzodiazepines in a beagle dog

Fig. 2 shows the structural formulae of diazepam and its main metabolites. Fig. 3 shows the pharmacokinetics of diazepam and its metabolites in a beagle dog after an intravenous dose of 2.18 mg/kg. Note that diazepam is rapidly eliminated ($t_{½}$ = 80 min) and converted mainly to N-desmethyldiazepam. This metabolite is relatively slowly eliminated ($t_{½}$ = 10 h) by hydroxylation at the C_3 position, which results in the formation of oxazepam (Fig. 2).

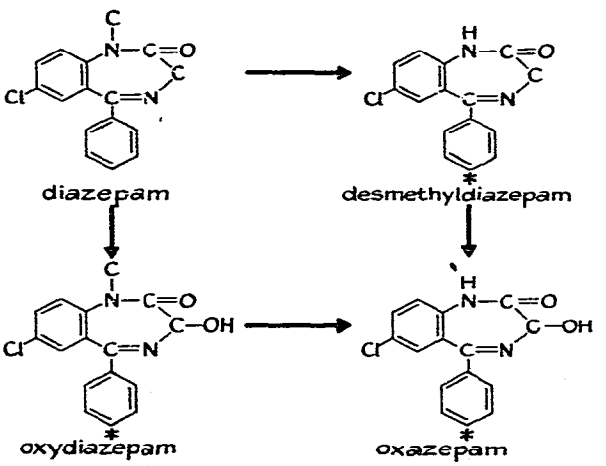

Fig. 2. Structural formulae of diazepam and its main metabolites, N-desmethyldiazepam, oxydiazepam and oxazepam. * = Position vulnerable for hydroxylation.

Diazepam may also be slowly hydroxylated at the C_3 position to give 3-hydroxydiazepam or oxydiazepam, which is present in very minute concentrations as the glucuronide. The N-demethylation of oxydiazepam is fast and results in oxazepam.

After deglucuronidation of the plasma samples only oxydiazepam and oxazepam were present as glucuronides. The formation of oxazepam glucuronide from oxazepam is shown in Fig. 4. Oxazepam, given as an intravenous bolus injection to a beagle dog, was eliminated slowly ($t_{1/2}$ = 3 h) as the free drug, and as the glucuronide with a $t_{1/2}$ of 8 h. The drug is mainly excreted as its glucuronide (24.5% in 7 h) and very little as the free drug (0.07%).

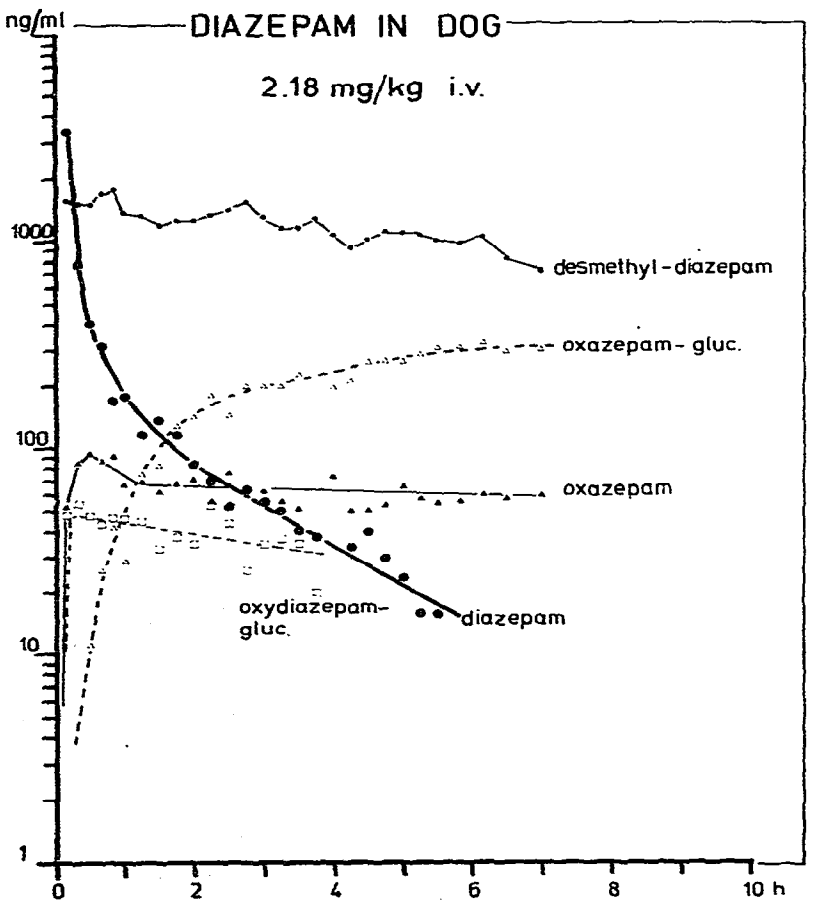

Fig. 3. Pharmacokinetics of diazepam in a beagle dog. Diazepam is rapidly eliminated ($t_{1/2}$ = 80 min) and converted to N-desmethyldiazepam ($t_{1/2}$ = 10 h), oxydiazepam glucuronide, oxazepam and oxazepam glucuronide.

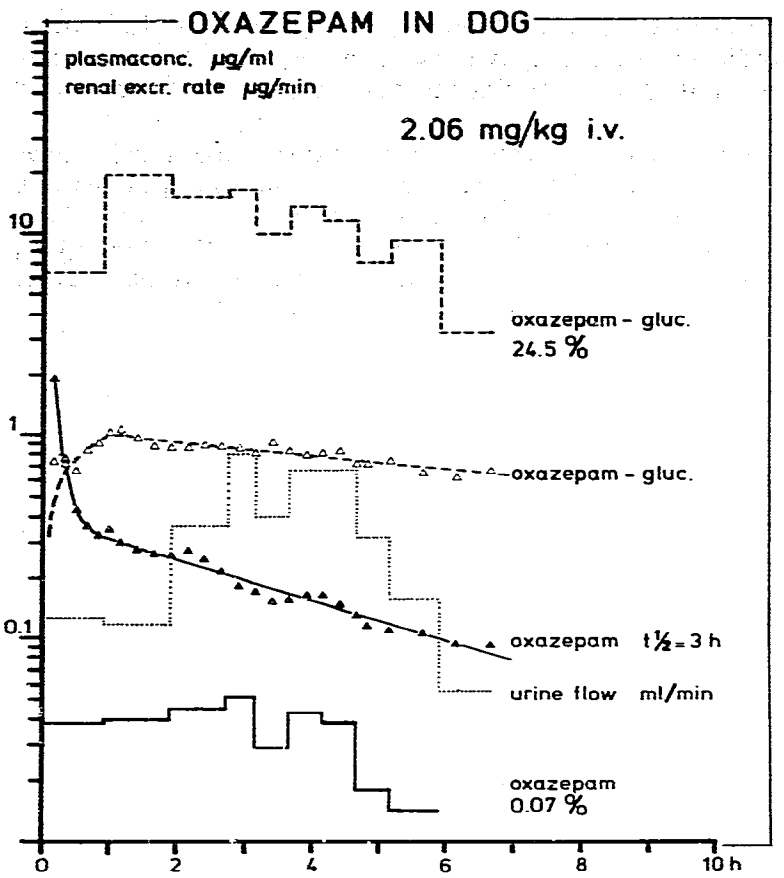

Fig. 4. Pharmacokinetics of oxazepam in a beagle dog. Oxazepam is eliminated by glucuronidation and renal excretion of free drug and glucuronide.

Oxydiazepam and oxazepam in man

The method of extraction and HPLC analysis of benzodiazepines can also be applied to the pharmacokinetic study of diazepam and its metabolites in man. Fig. 5 shows the pharmacokinetics of oxydiazepam and its metabolite oxazepam after an oral dose of 17.8 mg of oxydiazepam (Temazepam®). The renal excretion rate of oxydiazepam glucuronide has a $t_{1/2}$ of 10 h, the metabolite, oxazepam glucuronide, has a $t_{1/2}$ of 12.5 h. Less than 1% of the free drugs is excreted by the kidneys.

The plasma concentrations of oxydiazepam and metabolite show a much shorter $t_{1/2}$ of about 3 h, perhaps indicating an alpha phase in the plasma elimination curve. We have to remember that in plasma, oxydiazepam is measured

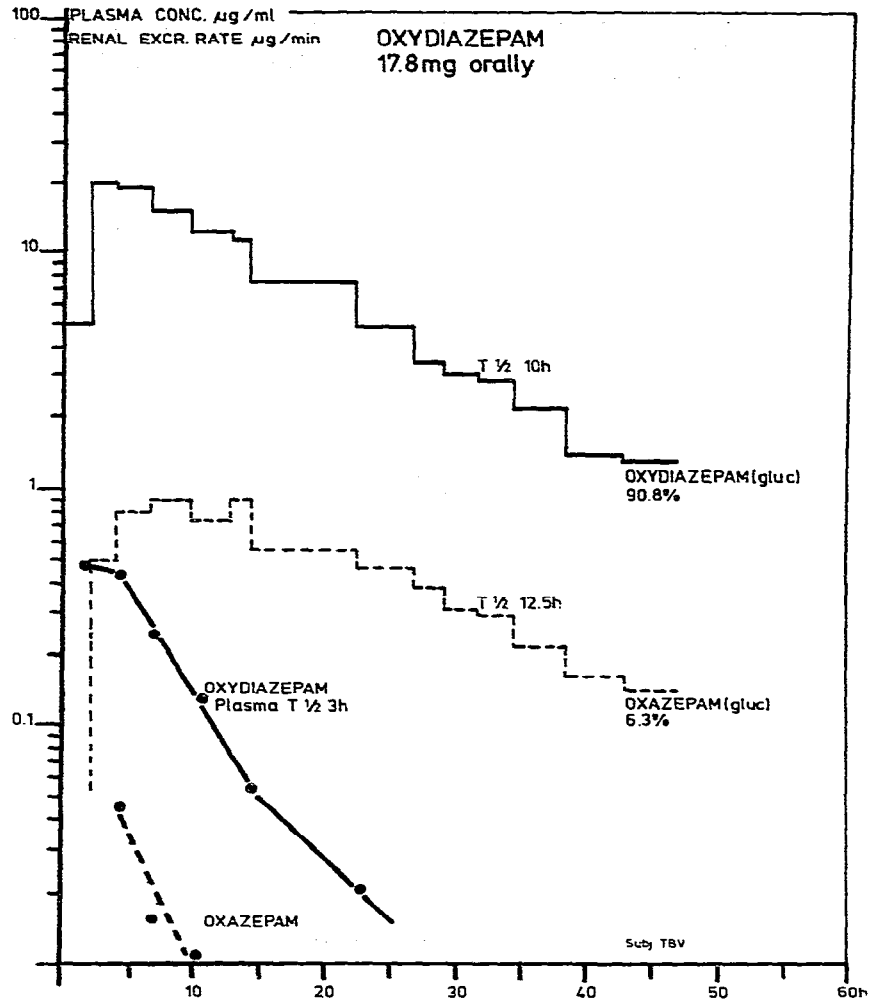

Fig. 5. Pharmacokinetics of oxydiazepam in man after an oral dose of 17.8 mg. The plasma elimination curve, which shows a much shorter $t_{1/2}$ than that of the renal excretion rate of both drugs, shows only the alpha phase of the elimination curve.

as the free compound while in urine the glucuronide is measured. Oxazepam, as parent drug, is excreted almost entirely as glucuronide, with a $t_{1/2}$ of 11.5 h (Fig. 6 and Table II). The renal excretion rates of oxydiazepam glucuronide and oxazepam glucuronide are independent of urinary pH and urine flow. The renal clearance constant varies between 20 and 60 ml/min. Table II summarizes the pharmacokinetic parameters of oxydiazepam and oxazepam in man.

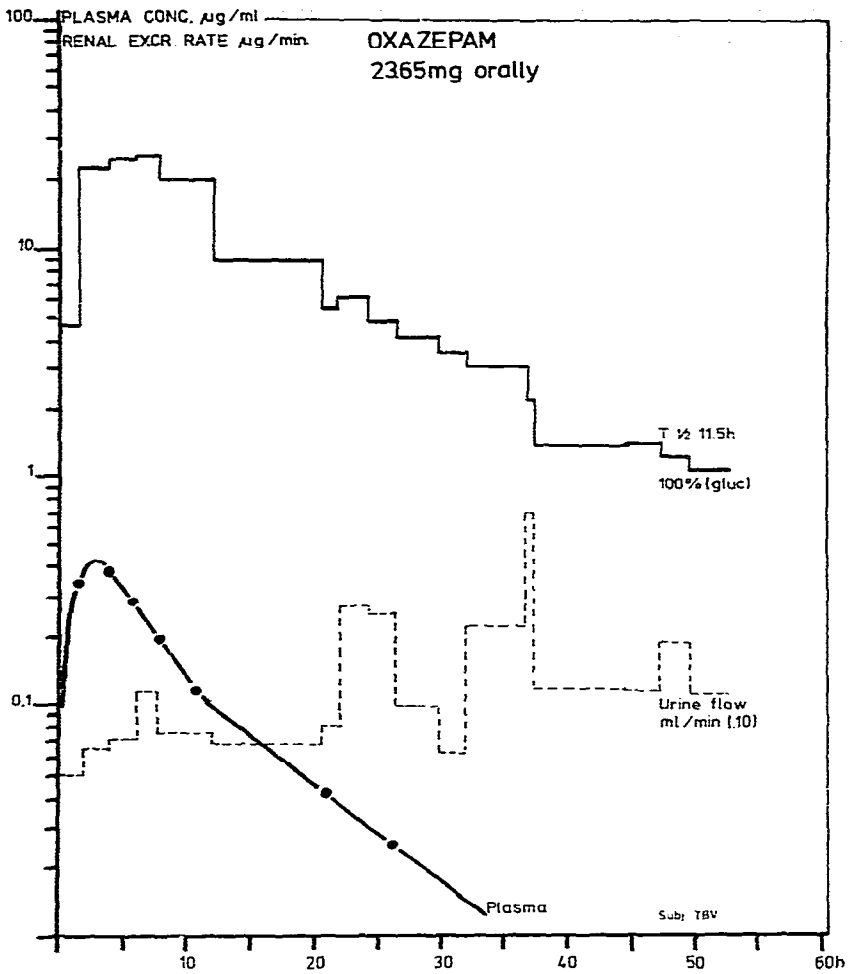

Fig. 6. Pharmacokinetics of oxazepam in man after an oral dose of 23.6 mg. The alpha and beta phases of the plasma elimination curve can be observed. Oxazepam is excreted almost solely as the glucuronide.

TABLE II

PHARMACOKINETIC PARAMETERS OF OXAZEPAM AND OXYDIAZEPAM IN MAN

Compound	*n*	Percentage excreted as glucuronide	Percentage of metabolite excreted as glucuronide	$t_{½}$ (h)	$t_{½}$ of metabolite (h)
Oxydiazepam	5	86.6 ± 9.6	5.8 ± 3.8	7.7 ± 1.7	9.7 ± 2.4
Oxazepam	10	98.3 ± 10.7		7.5 ± 2.1	

DISCUSSION

The method described in this communication permits the study of the pharmacokinetics of diazepam and its metabolites. The small amount of blood (0.2 ml plasma) required, together with the sensitivity limit of 30 ng/ml allows the convenient use of volunteers as subjects in these experiments. Also volunteers are able to collect blood samples of 0.2 ml in a fashion which greatly simplifies and facilitates the experimental design [12]. There are few papers describing the pharmacokinetics of oxydiazepam [15, 16] and oxazepam [15, 17], probably due to difficulties encountered in analysis.

The glucuronidation of oxazepam and subsequent renal excretion of oxazepam glucuronide is the main pathway for elimination of the drug. Similar behaviour is found for lorazepam [18—20], which indicates that glucuronidation of benzodiazepines only takes place at the C_3-hydroxyl group.

Pharmacokinetic processes in man are slower than in the dog. The half-life of diazepam in man is about 40 h [21, 22] and of N-desmethyldiazepam about 60 h [21, 22]. These two drugs have been thoroughly investigated, and that accumulation of the parent drug and metabolite occurs at a consumption rate of diazepam of 5—30 mg/day has been known for about ten years [17]. In analogy to dogs, oxydiazepam and oxazepam may be present in man with a $t_{½}$ of 7—10 h from the first dose of diazepam, but, as can be seen from Fig. 3, at a plasma concentration ten times less than that of N-desmethyldiazepam.

This HPLC method is superior to others, as it allows the determination of the main metabolites of diazepam and requires only a small volume of blood. Therefore it can be used in routine patient monitoring in hospitals and as a tool to establish the clinical importance of oxazepam and oxydiazepam.

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