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ANALYSIS OF LORAZEPAM AND ITS GLUCURONIDE METABOLITE BY ELECTRON-CAPTURE GAS—LIQUID CHROMATOGRAPHY

USE IN PHARMACOKINETIC STUDIES OF LORAZEPAM

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SUMMARY

This paper describes a rapid and sensitive method for analysis of lorazepam and its glucuronide metabolite in plasma and urine following therapeutic doses of lorazepam in humans. After addition of the structurally related benzodiazepine derivative, oxazepam, as the internal standard, 1-ml samples of plasma or urine are extracted twice at neutral pH with benzene (containing 1.5% isoamyl alcohol). The combined extracts are evaporated to dryness, reconstituted, and subjected to gas chromatographic analysis using a 3% OV-17 column and an electron-capture detector. Lorazepam glucuronide in urine is similarly analyzed following enzymatic cleavage with Glusulase. The sensitivity limits are 1—3 ng of lorazepam per ml of original sample, and the variability of identical samples is 5% or less. The applicability of the method to pharmacokinetic studies of lorazepam is demonstrated.

INTRODUCTION

Lorazepam (Fig. 1) is a 3-hydroxy-1,4-benzodiazepine derivative extensively used as a sedative and antianxiety agent in clinical practice [1, 2]. The major metabolic pathway of lorazepam in humans involves conjugation of the

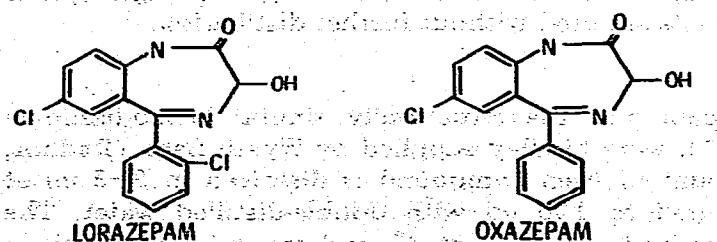

Fig. 1. Structural formulae of lorezepam and oxazepam.

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3-hydroxy substituent to glucuronic acid, yielding a water-soluble glucuronide metabolite that is excreted in urine [3—6]. Other minor metabolites have been identified, but none is of quantitative importance.

Several gas chromatographic methods are available for quantitation of lorazepam and lorazepam glucuronide in body fluids following therapeutic doses of lorazepam in humans. One approach involves acid hydrolysis of the benzodiazepine nucleus to the corresponding benzophenone derivative [4, 7]. This method is used successfully, but the extraction, derivatization, and clean-up steps are time-consuming and lead to considerable sample loss, due to aliquot taking. Quantitation of lorazepam is also possible using electron-capture gas—liquid chromatography without prior derivatization [8—10]. This report describes a rapid and sensitive method for assay of lorazepam and its glucuronide metabolite requiring minimal clean-up and no derivatization. The applicability of the method is illustrated in a study of the pharmacokinetics and bioavailability of lorazepam in a healthy human volunteer.

EXPERIMENTAL

Apparatus and chromatographic conditions

The analytic instrument is a Hewlett-Packard Model 5750 gas chromatograph equipped with a ^{63}Ni electron-capture detector (ECD). The detector is operated in the pulsed mode with a pulse interval of 150 μsec. The column is coiled glass, 6 ft. × 4 mm I.D. packed with 3% OV-17 on 80—100 mesh Chromosorb W HP (Supelco, Bellefonte, Pa., U.S.A.). The carrier gas is ultra-pure helium (Matheson Gas Products, Gloucester, Mass., U.S.A.) at a flow-rate of 50 ml/min. The purge gas is argon—methane (95:5), at a flow-rate of 80 ml/min. Operating temperatures are: injection port, 300°; column, 280°; detector, 320°. Before being connected to the detector, a new column is conditioned at 325° for 4 h with no carrier flow, followed by 48 h of conditioning with a carrier flow as described above.

At the beginning of each working day, the column is primed by injection of 2 to 3 drug-free "blank" plasma extracts.

Reagents

The following reagents are used: pesticide grade certified benzene (Fisher Scientific, Fair Lawn, N.J., U.S.A.), certified isoamyl alcohol (Fisher), analytical reagent grade toluene (Mallinkrodt, St. Louis, Mo., U.S.A.), absolute ethanol (IMC Chemical Group, Terre Haute, Ind., U.S.A.), and reagent grade KH_2PO_4 (Mallinkrodt). Solvents are used without further distillation.

Reference standards

Pure samples of lorazepam and the structurally similar benzodiazepine derivative, oxazepam (Fig. 1), were kindley supplied by Wyeth Labs. (Radnor, Pa., U.S.A.). A 10-mg amount of each compound is dissolved in 2—3 ml of absolute ethanol, then diluted to 100 ml with double-distilled water. The stock solutions are stored in amber bottles at 4°, and are stable under these conditions for up to 1 year. Working standards, containing 0.25—1.0 μg/ml, are prepared as needed by appropriate dilution with double-distilled water.

Preparation of samples: intact lorazepam in plasma

Oxazepam serves as the internal standard. A 50-μl volume of stock solution (1.0 μg/ml) containing 50 ng of oxazepam is added to a series of conical 40-ml centrifuge tubes equipped with PTFE-lined screw-top caps. A 1-ml sample of "unknown" plasma is added to each of the tubes. Calibration standards are prepared by adding 12.5, 25, 37.5, and 50 ng of lorazepam to consecutive tubes. Drug-free control plasma, preferably taken from the experimental subject prior to lorazepam administration, is added to each of the calibration tubes and to one additional drug-free blank. Calibration standards are analyzed together with each set of unknowns.

Extraction procedure

A 6-ml volume of benzene (containing 1.5% isoamyl alcohol) is added to all tubes. The tubes are agitated gently in the upright position on a vortex mixer for 30 sec, then centrifuged at room temperature for 10 min at 400 *g* (Portable Refrigerated Centrifuge Model PR-2; head No. 269; International Equipment, Boston, Mass., U.S.A.). The organic layer is transferred to a conical 13-ml centrifuge tube. The procedure is repeated, and the combined organic extracts are evaporated to dryness at 40° under conditions of mild vacuum. Care is taken to ensure that the residue is rinsed from the sides of the tubes. The final dry residue is redissolved in 50 μl of toluene (containing 15% isoamyl alcohol), of which 1—3 μl is injected into the chromatograph.

Lorazepam and lorazepam glucuronide in urine

Analysis of intact lorazepam in 1 ml of urine proceeds exactly as described above for plasma.

Lorazepam glucuronide in urine is quantitated using the aqueous remainder following extraction of intact lorazepam. The aqueous phase is washed with 10 ml of ether, centrifuged, and the organic phase aspirated and discarded. The sample is heated to 40° under conditions of mild vacuum for 15 min to ensure removal of any remaining organic solvent. A quantity of 0.1 ml of each urine sample is transferred to another 40-ml conical centrifuge tube; 1.0 ml of 1 *M* KH_2PO_4 buffer (pH approximately 4.6) is added. Enzymatic cleavage of lorazepam glucuronide is achieved by addition of 30 μl of Glusulase (Endo Labs., Garden City, N.Y., U.S.A.), a commercial preparation of snail intestinal juice containing approximately 175,000 units of β-glucuronidase and 35,000 units of sulfatase per ml. The resulting mixture is agitated gently, then incubated for 12—18 h at 37° in a temperature-controlled incubation room. Following incubation, the pH of each tube is adjusted back to 7.0 by addition of approximately 0.65 ml of 1 *N* NaOH. A quantity of 100 ng of oxazepam, the internal standard, is added to each tube, and lorazepam (25, 50, 75, 100, 150 and 200 ng) is added to the calibration tubes. (Larger amounts of standards are used for analysis of lorazepam glucuronide in urine since high concentrations of this metabolite are present in most samples.) Extraction then proceeds as described for intact lorazepam in plasma, except that 8—10 ml of solvent are used for each extraction to avoid emulsion formation.

Clinical pharmacokinetic study

A healthy 23-year-old female volunteer participated after giving written informed consent. Single 4-mg doses of lorazepam were administered on three occasions in a cross-over study, with at least one week elapsing between trials. Modes of administration were: 5-min intravenous infusion, deltoid intramuscular injection, and oral ingestion in the fasting state with 100 ml of water. Multiple venous blood samples were drawn during 48 h, and all urine collected in divided samples for 72 h, following each dose. Concentrations of intact lorazepam in plasma, and of lorazepam and lorazepam glucuronide in urine, were quantitated as described above.

Plasma lorazepam concentrations following each mode of administration were analyzed by iterative weighted non-linear least-squares regression analysis as described in detail elsewhere [3, 11, 12]. The following pharmacokinetic variables were determined following intravenous injection of lorazepam: distribution half-life, elimination half-life, total volume of distribution, and total clearance. Following oral and intramuscular dosage, the following variables were determined: lag time prior to the start of absorption, absorption half-life, and elimination half-life. The systemic availability (completeness of absorption) of oral and intramuscular lorazepam was determined from the

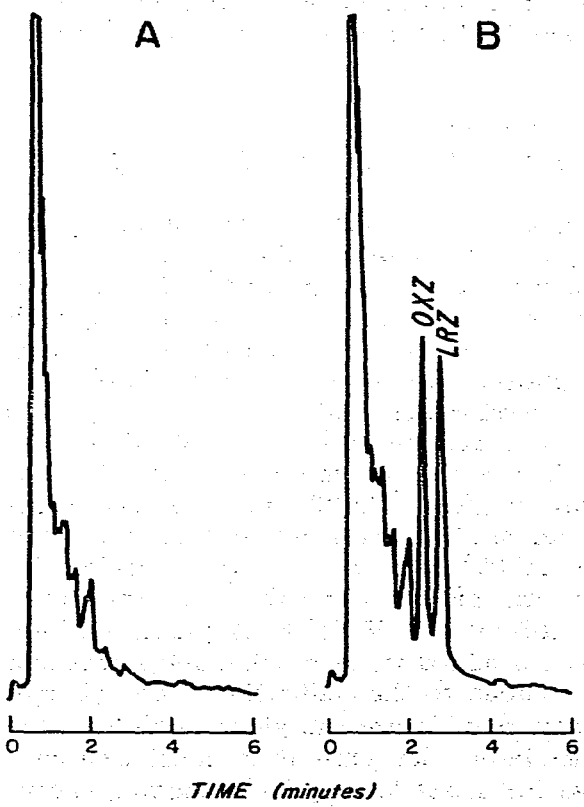

Fig. 2. A, Chromatogram of a drug-free control plasma extract. B, The same sample to which was added 50 ng/ml of oxazepam (OXZ) and 25 ng/ml of lorazepam (LRZ).

total area under the plasma concentration curve (AUC) calculated from the pharmacokinetic function, and upon 72-h urinary excretion of lorazepam glucuronide. AUC and 72-h excretion after the two extravascular modes of administration were compared with those observed following intravenous injection of the same dose [11, 13, 14].

RESULTS

Evaluation of the method

Under the described conditions, the retention times of oxazepam and lorazepam are 2.4 and 2.7 min, respectively (Fig. 2). The chromatographic peaks probably do not correspond to the intact compounds, but rather to quinazoline carboxaldehyde derivatives formed by on-column rearrangement [10, 15–18].

The relation between plasma lorazepam concentration and the lorazepam-to-oxazepam peak height ratio is linear up to at least 50 ng/ml. Analysis of 34 standard curves constructed on different days over a period of 6 months indicated that the correlation of peak height ratio and lorazepam concentration is always 0.99 or greater. The day-to-day coefficient of variation in the slope of the calibration curves was 8.6%.

The sensitivity limit of the method is 1—3 ng of lorazepam per ml of original sample. Coefficients of variation for identical samples were: at 25 ng/ml,

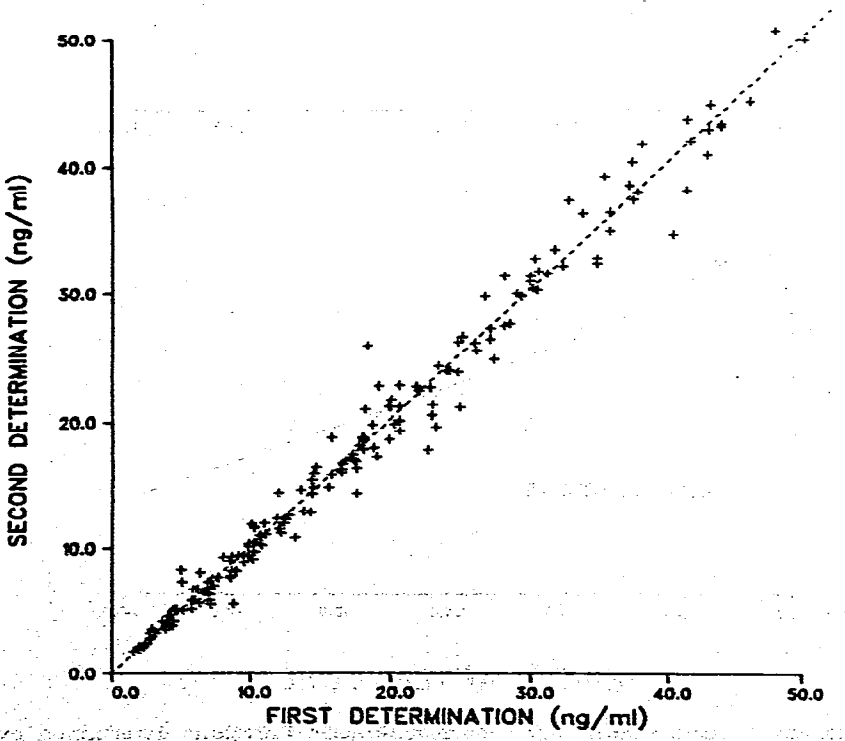

Fig. 3. Relation between lorazepam concentrations measured in a series of 171 duplicate plasma samples. The overall correlation coefficient is 0.99.

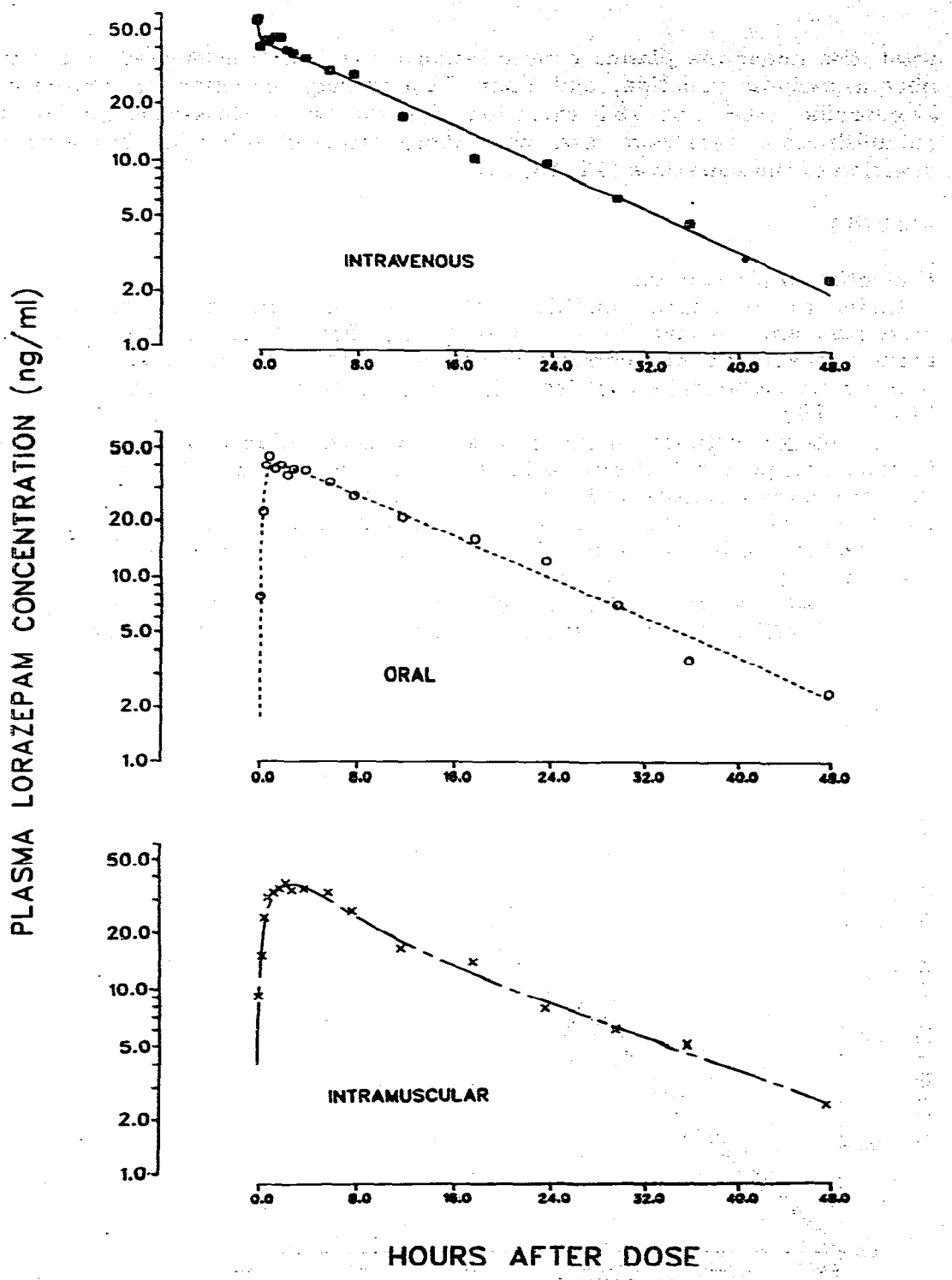

Fig. 4. Plasma lorazepam concentrations, and pharmacokinetic functions determined by least-squares regression analysis, following single 4-mg doses of lorazepam administered by three different routes to a healthy volunteer.

2.8% (n = 10); at 12.5 ng/ml, 4.5% (n = 8); at 6.25 ng/ml, 4.3% (n = 11); at 2.5 ng/ml, 5.1% (n = 10). Residue analysis indicated that extraction of both oxazepam and lorazepam is more than 95% complete.

A series of 171 plasma samples from pharmacokinetic studies, each analyzed in duplicate, was assessed to determine the replicability of identical samples (Fig. 3). The correlation coefficient between duplicate samples was 0.99, with an overall mean deviation of 5.2%.

Pharmacokinetic study

Fig. 4 shows plasma lorazepam concentrations, together with pharmacokinetic functions, following intravenous, oral, and intramuscular administration of lorazepam to the volunteer subject. Disappearance of lorazepam from plasma following intravenous infusion proceeded with two exponential phases, with an apparent elimination half-life during the terminal or "beta" phase of 10.6 h (Table I). The total volume of distribution was 1.62 l/kg, indicating reasonably extensive drug distribution. Following oral administration, a short lag time elapsed prior to the start of absorption, after which absorption proceeded as an apparent first-order process with a half-life of 14.3 min. A peak concentration of 44.4 ng/ml was measured in the sample drawn 1.0 h after the dose; elimination thereafter proceeded with a half-life of 10.8 h. After intramuscular injection, no lag time was observed, but the absorption phase had a somewhat longer half-life of 73.2 min. A peak level of 36.2 ng/ml was measured in the 2.5-h sample. The apparent elimination half-life was 13.9 h, slightly longer than following intravenous and oral administration. Based

TABLE I

PHARMACOKINETIC VARIABLES FOR LORAZEPAM

Variable	Route of administration		
	Intravenous	Oral	Intramuscular
Distribution half-life (min)	5.1	—	—
Elimination half-life (h)	10.6	10.8	13.9
Total volume of distribution (l/kg)	1.62	—	—
Total clearance (ml/min/kg)	1.77	—	—
Lag time prior to start of absorption (min)	—	11.3	0
Peak plasma concentration (ng/ml)	—	44.4	36.2
Time of peak concentration (h after dose)	—	1.0	2.5
Apparent absorption half-life (min)	—	14.3	73.2
72-h excretion of lorazepam glucuronide (% of dose)	94.6	82.4	72.2
Systemic availability (%)			
Based on AUC	—	100	93.4
Based on urinary excretion of lorazepam glucuronide	—	82.4	72.2

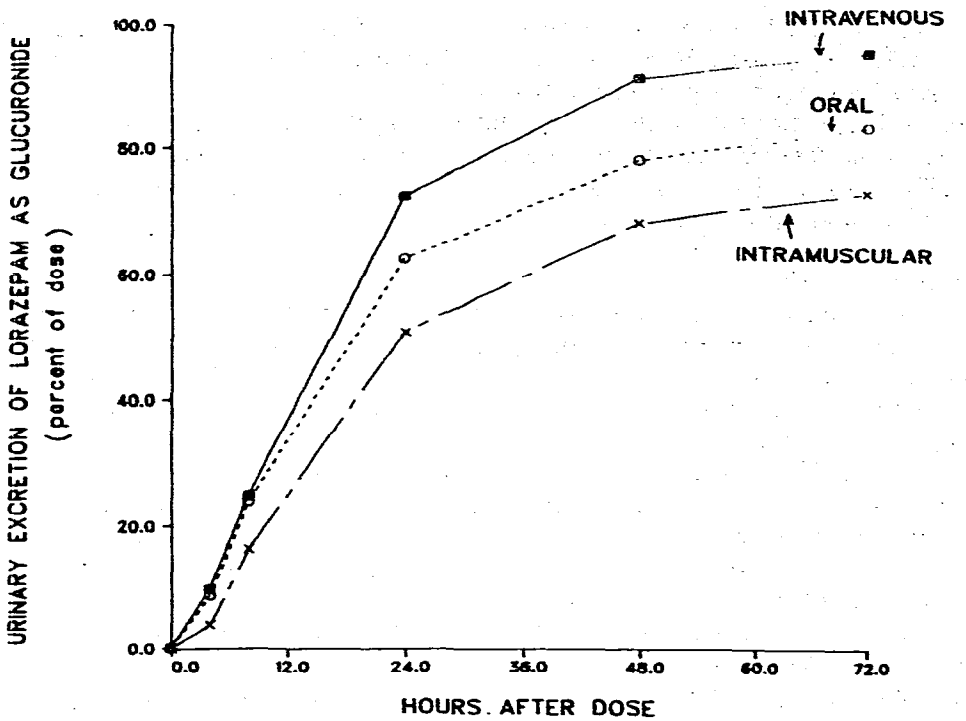

Fig. 5. Urinary excretion of lorazepam glucuronide during 72 h after single 4-mg doses administered by three different routes to the healthy volunteer subject.

on AUC, the systemic availability of oral lorazepam was 100%, and 93.4% following intramuscular injection (Table I). Based upon 72-h urinary excretion of lorazepam glucuronide following each mode of administration, the systemic availability of oral lorazepam was 82%, and that of intramuscular lorazepam 72% (Fig. 5). Urinary excretion of intact lorazepam accounted for less than 0.4% of the dose following all three routes of administration.

DISCUSSION

This report describes a rapid and sensitive method for quantitation of lorazepam in plasma, and of lorazepam and its glucuronide metabolite in urine. The method utilizes direct extraction of lorazepam together with the internal standard, evaporation of the organic solvent, and injection of the redissolved residue directly into the chromatograph. Blank samples of plasma and urine are consistently free of contaminants in the areas corresponding to retention times of oxazepam amd lorazepam. Therefore, extensive clean-up procedures are unnecessary. Since chromatographic peaks corresponding to both compounds are Gaussian, peak heights rather than peak areas were used to quantitate detector response [19], thereby making an electronic integrator unnecessary. Studies utilizing gas chromatography combined with mass spectroscopy indicate that the chromatographic peaks correspond to quinazoline carboxaldehyde derivatives of oxazepam and lorazepam, formed by on-column

rearrangement and loss of a molecule of water [10, 15—18]. This thermal rearrangement does not influence the reliability of the method. Standard curves are always linear and have similar slopes from day to day. Furthermore, peak height ratios following repeated injection of the same sample do not vary by more than 2%. It is evident that the same technique can be used for quantitation of oxazepam, simply by reversing the roles of oxazepam and lorazepam.

The use of this method obviates the need for acid hydrolysis of the benzodiazepine derivatives to corresponding benzophenones. Although acid hydrolysis allows good sensitivity and specificity, it involves multiple steps, is time-consuming, and results in sample losses due to aliquot taking. In our experience, the hydrolysis method also requires redistillation of solvents. A detailed comparison of intact-drug *versus* acid hydrolysis techniques for quantitation of nitrazepam, another benzodiazepine derivative, is reported by Kangas [20].

Several aspects of the procedure require further mention. An appropriate choice of internal standard is critical for reliable and reproducible quantitation of lorazepam. Howard et al. [8] for example, utilize flunitrazepam as an internal standard for analysis of lorazepam, but coefficients of variation for identical samples exceed 10%. Our use of desmethyldiazepam as an internal standard for lorazepam analysis also yielded unacceptably large variability. Only oxazepam has served as an acceptable internal standard in our experience, presumably because its characteristics of extraction and of on-column rearrangement are similar to those of lorazepam. Other 3-hydroxy benzodiazepines having N-1 alkyl substitutions (such as temazepam and 3-hydroxyprazepam) are less suitable than oxazepam since they do not rearrange on-column [16]. Addition of isoamyl alcohol to the extracting solvent also appears critical, since it greatly reduces problems of inconsistent and poor recovery, and/or adsorption of the compounds onto glassware. Finally, not all brands of disposable plastic syringes are suitable for pharmacokinetic studies of lorazepam. The rubber plungers contained in Monoject syringes (Sherwood Medical Ind., St. Louis, Mo., U.S.A.) are contaminated with a large and as yet uncharacterized electron-capturing substance having a retention time similar to that of oxazepam. The contaminant is transmitted to all biological fluids collected in these syringes, and complicates quantitation of detector response to oxazepam and lorazepam. Syringes produced by Becton Dickinson & Co. (Rutherford, N.J., U.S.A.), contain much smaller quantities of this contaminant and are suitable for use in pharmacokinetic studies.

Our method is readily applicable to studies of the pharmacokinetics and bioavailability of lorazepam in humans. Consistent with previous reports [3—7, 12, 21, 22] we observed an elimination half-life of lorazepam in the range of 10—15 h. Lorazepam clearance was accomplished mainly by conjugation to glucuronic acid, followed by urinary excretion of lorazepam glucuronide [3—7, 12 21, 22]. More than 90% of an intravenous dose of lorazepam was recovered in the urine as the glucuronide metabolite. Absorption of oral lorazepam was rapid and nearly complete; intramuscular lorazepam absorption was less rapid and slightly less complete. Further studies are needed to es-

tablish within- and between-subject variability in the pharmacokinetics and bioavailability of lorazepam.

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