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SIMPLIFIED DETERMINATION OF LORAZEPAM AND OXAZEPAM IN BIOLOGICAL FLUIDS BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

Lorazepam and oxazepam in plasma and urine were measued by gas chromatography—mass spectrometry. Oxazepam was used as an internal standard in the assay of lorazepam and vice versa. After removal of interfering substances with *n*-hexane, the drugs were extracted with benzene and converted to N_1,O_3-bistrimethylsilyl derivatives. Glucuronide forms of the drugs were extracted after hydrolysis with β-glucuronidase. A common fragment ion at *m/e* 429 was used to monitor the two drugs. The sensitivity was 2 ng/ml for both drugs, which was sufficient to determine plasma and urine concentrations after therapeutic doses to humans.

INTRODUCTION

Lorazepam and oxazepam are the 1,4-benzodiazepine class of tranquillizers bearing a hydroxy group at the C-3 position of the benzodiazepine ring. Several methods of determining these drugs in biological fluids have been reported and applied in clinical studies [1—7]. However, when we were faced with the necessity of measuring lorazepam in plasma in response to the request by clinicians, it was considered desirable to develop a simpler, less time-consuming assay to deal with a large number of samples. This paper describes a simplified method of determining lorazepam and oxazepam in plasma and urine. The method involves solvent extraction of the sample and gas chromatography—mass spectrometry of the silylated extract.

EXPERIMENTAL

Chemicals

Lorazepam and oxazepam tablets were obtained commercially (Wypax®, 0.5-mg tablet, Yamanouchi Pharmaceutical, Tokyo, Japan; Hilong®, 10-mg tablet, Banyu Pharmaceutical, Tokyo, Japan). Lorazepam standard was kindly supplied by Wyeth Japan Corporation (Tokyo, Japan). Oxazepam standard was obtained by extraction from commercial tablets with acetone and recrystallization from ethyl acetate—*n*-hexane; m.p. 200.5° (lit. 200—205° [8]); anal. calcd. for $C_{15}H_{11}ClN_2O_2$: C, 62.8, H, 3.87%; found: C, 62.2, H, 3.88%. Bis(trimethylsilyl)acetamide (BSA) and bovine liver β-glucuronidase (13,000 Fishman units per ml) were products of Tokyo Kasei Kogyo (Tokyo, Japan) and Tokyo Zoki (Tokyo, Japan), respectively. Other reagents used were commercial preparations and of analytical grade.

Gas chromatography—mass spectrometry

An Hitachi RMU-6MG combined gas chromatograph—mass spectrometer fitted with an accelerating voltage alternator was employed. Separation was accomplished using a glass column (1 m × 3 mm I.D.) packed with 3% OV-1 on Chromosorb W AW DMCS (80—100 mesh). The column temperature was maintained isothermally at 210°, while the flash and separator temperatures were held at 270°. The flow-rate of carrier gas helium was 30 ml/min. The ionization potential and trap current were 20 eV and 80 μA, respectively. The entrance and collector slits of the mass spectrometer were adjusted to 0.4 mm. The multiplier voltage supply was set at 1.2–2.0 kV.

Determination of unchanged drug in plasma and urine

To each plasma or urine sample (1 ml) either oxazepam (25 ng) or lorazepam (150 ng) was added as an internal standard. After addition of hydrochloric acid (0.1 *M*, 2 ml), the plasma sample was washed with *n*-hexane (4 ml). The aqueous layer was then saturated with sodium bicarbonate and washed again with *n*-hexane (4 ml). Urine sample was directly washed with *n*-hexane (4 ml) twice. Each aqueous layer was then extracted with benzene (4 ml). The benzene layer was evaporated to dryness under reduced pressure and a 40% solution of BSA in ethyl acetate (50 μl) was added to the residue. The mixture was kept in a water-bath at 60° for 30 min. After cooling, an aliquot (2 μl) was injected into the column for gas chromatography—mass spectrometry. A fragment ion at *m/e* 429 was used to monitor both lorazepam and oxazepam. The amount of the drug in each sample was calculated by measuring the peak height ratio and referring to the standard curve. A standard curve was prepared by subjecting the control plasma or urine samples, to which known amounts of the drugs had been added, to the above procedure. The peak height ratio of the drug and internal standard was plotted against the concentration of the corresponding drug.

Determination of glucuronide conjugate in plasma and urine

To each plasma or urine sample (1 ml) oxazepam (25 ng) or lorazepam (150 ng) was added as an internal standard. After addition of acetate buffer (0.5 *M*,

2 ml, pH 5.0) and β-glucuronidase (1000 U), the mixture was incubated at 37° for 24 h. The plasma mixture was then acidified with hydrochloric acid (1 *M*, 0.2 ml) and washed with *n*-hexane (4 ml). The aqueous layer was saturated with sodium bicarbonate and washed again with *n*-hexane (4 ml). Urine mixture was washed twice with *n*-hexane (4 ml). The subsequent procedure was followed as described for the unchanged drug. The quantity of the glucuronide conjugate was calculated by subtracting the drug concentration determined before hydrolysis from that after hydrolysis.

Extraction recoveries

Control plasma samples (1 ml) containing lorazepam (30 ng) or oxazepam (300 ng) were carried through the above procedure without addition of the internal standard. Oxazepam (25 ng) or lorazepam (150 ng), dissolved in benzene, was added to the benzene extract and the benzene solution was evaporated to dryness under reduced pressure. The subsequent procedure was carried out as described above. Recoveries were calculated by comparing the peak height ratios with those obtained when each compound and internal standard, dissolved in benzene, were processed without the extraction procedure.

Human studies

After overnight fasting, three male subjects received 1.5 mg of lorazepam as three 0.5-mg tablets. Another three subjects received a 10-mg tablet of oxazepam. Blood samples were obtained from the antecubital vein with heparinized syringes and centrifuged at 980 *g* for 15 min. Urine samples were collected for 24 h. The plasma and urine samples were stored at −20° until required for assay.

RESULTS AND DISCUSSION

1,4-Benzodiazepine drugs usually contain electronegative groups so that analysis can be done by electron-capture gas chromatography [9]. Earlier methods depended on the acid-catalyzed hydrolysis of the 1,4-benzodiazepine nucleus to the corresponding aminobenzophenone derivative before chromatography [10]. Knowles and co-workers [1, 2] applied this principle to lorazepam and oxazepam and established an assay method which was sensitive enough to determine drug levels as low as 10 ng/ml (lorazepam) or 20 ng/ml (oxazepam). Plasma concentrations of these drugs in man have been determined by this method. Determination of lorazepam and oxazepam as benzophenones is not necessarily free from drawbacks. Theoretically, the parent drug and certain metabolites [11, 12] may be hydrolyzed to the same benzophenone, which impairs the specificity of the assay. Practically, the lengthy hydrolysis procedure is inconvenient for analyzing a large number of samples.

More recently, methods have been developed in which the intact benzodiazepine molecules are directly subjected to gas chromatography after suitable clean-up [13, 14]. This modification greatly simplified the previous method which required hydrolysis. Lorazepam and oxazepam presented a special

problem in the application of this modification because of the presence of a hydroxyl group at the C-3 of the benzodiazepine nucleus. They undergo thermal rearrangement to quinazoline derivatives in the gas chromatographic column with the result that the reproducibility of the assay is not good [4]. Two approaches have been made to solve this problem. One is an attempt to make the rearrangement quantitative by selecting the solvent in which the drug is injected into the column. De Groot et al. [4] used toluene for this purpose and determined without hydrolysis the lorazepam level in human plasma after an overdose. The sensitivity, however, seemed insufficient to measure drug concentrations occurring after therapeutic doses, although recently Greenblatt et al. [7] have reported better sensitivity with practically the same method. The other approach is to derivatize these drugs into forms that are unable to rearrange. De Silva et al. [15], in their comprehensive and informative study, worked out several general methods for determining a wide range of 1,4-benzodiazepines including lorazepam and oxazepam. These two drugs were determined apparently as their monotrimethylsilyl (TMS) derivatives. Still, the procedure seemed to require considerable sample purification, consisting of extraction and back extraction at different pH's before derivatization. Vessman et al. [6] derivatized oxazepam into its dimethyl form by the procedure known as extractive alkylation and established a method which is capable of determining oxazepam levels as low as 1 ng/ml. The method reported in this paper falls into the same category as these two methods in that the drugs are derivatized to avoid thermal rearrangement. The characteristic of the present method lies in its greater simplicity, as compared with the above methods, without sacrificing sensitivity.

Plasma samples were washed with *n*-hexane at an acid pH and, after neutralization, again washed with *n*-hexane, while urine samples were washed directly with *n*-hexane. This procedure removed interfering endogenous materials without loss of the drugs. Subsequently, the drugs were extracted with benzene. The extraction recoveries of lorazepam and oxazepam added to plasma at a concentration of 30 ng/ml and 300 ng/ml were 87.8 ± 0.6% and 76.8 ± 1.8% (mean ± S.E.M., n = 6), respectively. The drugs extracted were derivatized into the N_1,O_3-bis-TMS forms on treatment with BSA and subjected to gas chromatography—mass spectrometry. The introduction of two TMS groups under the conditions employed was confirmed from the mass spectra of the two derivatives (Fig. 1), which showed corresponding molecular ions at *m/e* 464 (lorazepam) and *m/e* 430 (oxazepam), respectively. Both spectra had an intense fragment ion at *m/e* 429, which was used to monitor the two drugs. The bis-TMS derivatives were preferred over the O_3-mono-TMS derivatives because of less adsorption onto the column and less tailing of the chromatographic peaks. The chromatograms obtained from human plasma samples are shown in Fig. 2. The peaks of oxazepam and lorazepam appeared at retention times of 1.7 min and 2.7 min, respectively, and were well separated from endogenous peaks. Two endogenous peaks were observed in drug-free control plasma at 3.3 min and 11.0 min, the latter being identified as cholesterol from its mass spectrum. When the plasma samples were directly extracted with benzene, additional endogenous peaks appeared within 3 min and disturbed

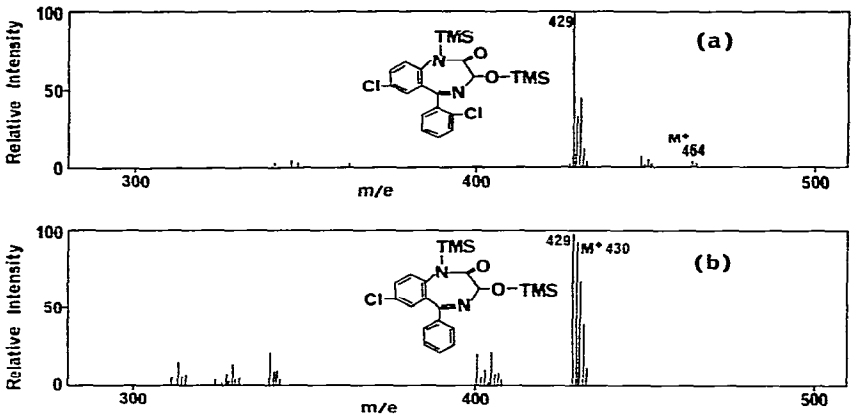

Fig. 1. Mass spectra of TMS derivatives of lorazepam (a) and oxazepam (b).

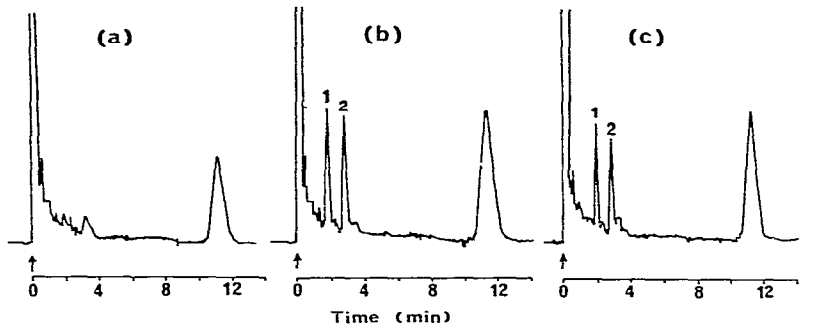

Fig. 2. Chromatograms of lorazepam (2) and internal standard (1, oxazepam) extracted from plasma: (a) control plasma; (b) control plasma to which lorazepam (25 ng/ml) and internal standard (25 ng/ml) were added; (c) plasma obtained 2 h after administration of lorazepam (1.5 mg) to a subject.

the assay. Washing with *n*-hexane removed these interfering materials and also reduced the cholesterol peak significantly, so that continuous assay of the samples was done readily. The chromatograms obtained from urine samples did not show endogenous peaks regardless of simplified washing procedure. Standard curves prepared for determining plasma levels of the drugs are shown in Fig. 3. Similar standard curves were obtained for urine samples. The lowest level of the two drugs measurable was 2 ng/ml for both plasma and urine.

Free and glucuronide forms of lorazepam were measured by the present method after three volunteer subjects had received an oral dose of 1.5 mg. The results are illustrated in Fig. 4 and Table I. Average plasma concentrations of the free drug reached a peak level of 27.7 ng/ml at 2 h and declined with a half-life of about 11 h. The concentration of the conjugated drug in plasma peaked at 3 h and was lower than that of the free drug. Urinary excretion was mostly in the form of the conjugate, which amounted to 69% of the dose in 24 h. The unchanged drug accounted for less than 1% of the dose. These data generally agreed with those reported previously [7].

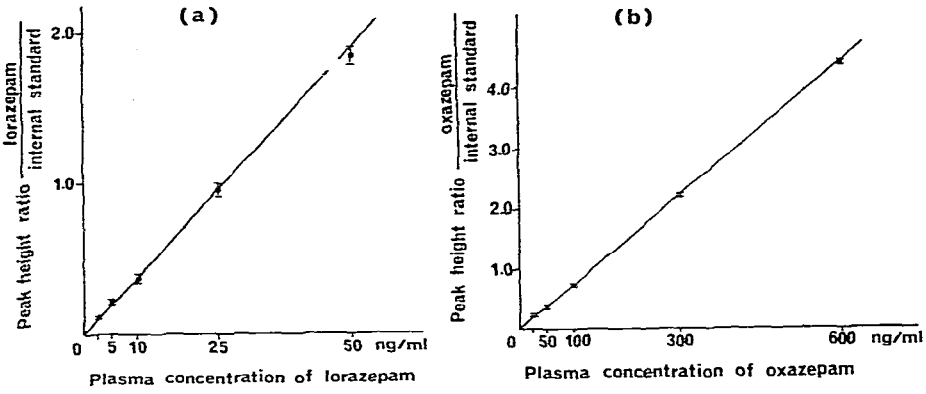

Fig. 3. Standard curves of lorazepam (a) and oxazepam (b) (mean ± S.E.M., $n = 3$).

Similarly, plasma and urine levels of oxazepam were measured after an oral dose of 10 mg was given to another three subjects. The maximum plasma concentration of the free drug was 210.8 ng/ml, which was reached at 3 h. Elimination half-life was about 4 h. The concentration of conjugated oxazepam in plasma was lower than that of the free drug until the 5th hour (Fig. 5). As with lorazepam, the majority of the dose was excreted in urine as the conjugate (Table I). These studies demonstrated the usefulness of the present method in monitoring the lorazepam and oxazepam levels in clinical practice.

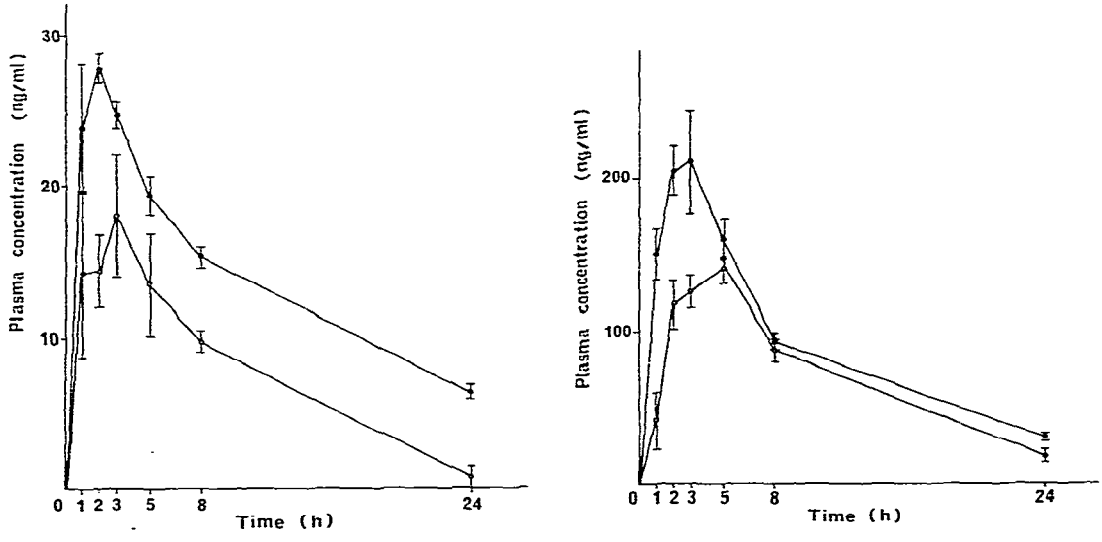

Fig. 4. Plasma concentration of unchanged drug (•) and conjugated drug (○) after oral administration of 1.5 mg lorazepam to three subjects (mean ± S.E.M.).

Fig. 5. Plasma concentration of unchanged drug (•) and conjugated drug (○) after oral administration of 10 mg oxazepam to three subjects (mean ± S.E.M.).

TABLE I

URINARY EXCRETION OF UNCHANGED AND CONJUGATED DRUG AFTER ORAL ADMINISTRATION OF 1.5 mg LORAZEPAM AND 10 mg OXAZEPAM TO THREE SUBJECTS

Time (h)	Recoveries of dose (mean ± S.E.M. %)		
	Drug	Lorazepam	Oxazepam
0—8	Unchanged	0.10 ± 0.02	5.2 ± 0.5
	Conjugated	26.1 ± 3.6	41.7 ± 9.0
0—24	Unchanged	0.59 ± 0.24	7.7 ± 0.5
	Conjugated	68.3 ± 5.2	67.3 ± 1.7

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