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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF TRIAZOLAM AND ITS METABOLITES IN HUMAN URINE

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SUMMARY

A high-performance liquid chromatographic method for the determination of triazolam and its main metabolites (1-hydroxymethyltriazolam and 4-hydroxytriazolam) in human urine has been developed. After hydrolysis with β-glucuronidase, the unchanged drug and its metabolites were extracted with a Sep-Pak C_{18} cartridge and further purified by a Sep-Pak silica cartridge. The extract was chromatographed on a reversed-phase column using a mobile phase consisting of methanol–10 m*M* phosphate buffer, pH 8 (65:35) and UV detection at 220 nm. The overall recoveries of triazolam, 1-hydroxymethyltriazolam and 4-hydroxytriazolam were ca. 88, 75 and 75%, respectively, and the detection limits of these compounds were 5 ng/ml, using a 10-ml specimen.

INTRODUCTION

Triazolam [8-chloro-6-(*o*-chlorophenyl)-1-methyl-4*H*-*s*-triazolo[4,3-*a*]-1,4-benzodiazepine] is a triazolobenzodiazepine derivative, which has recently been introduced as a short-acting hypnotic in Japan. This drug is extensively metabolized in humans, and the major urinary metabolites are 1-hydroxymethyltriazolam and 4-hydroxytriazolam [1, 2].

Several methods have been reported for the analysis of triazolam in biological fluids, such as radioimmunoassay [3], polarography [4], radioreceptor technique [5], high-performance liquid chromatography (HPLC) [6–9] and gas chromatography (GC) [10–12]. Almost all these methods, however, describe the determination of triazolam alone in serum or plasma. Recently the simultaneous determination of triazolam and its main metabolite, 1-hydroxymethyltriazolam, by capillary GC with electron-capture detection was reported [12], but its application to urine samples was not described.

In this paper, an HPLC method for the determination of triazolam and its

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major metabolites is described, and this method is applied to analysis of these compounds in human urine.

EXPERIMENTAL

Materials

Triazolam, 1-hydroxymethyltriazolam and 4-hydroxytriazolam were kindly supplied from Upjohn (Kalamazoo, MI, U.S.A.), and etizolam (internal standard) was from Yoshitomi Pharmaceutical Industries (Osaka, Japan).

Sep-Pak C_{18} and Sep-Pak silica cartridges were purchased from Waters Assoc. (Milford, MA, U.S.A.). Before use, the C_{18} cartridge was treated with 5 ml of methanol, 10 ml of dichloromethane–methanol (9:1, v/v), 5 ml of methanol and then 10 ml of water. The silica cartridge was washed successively with 20 ml each of dichloromethane, dichloromethane–methanol (9:1, v/v) and dichloromethane, and air-dried at room temperature.

β-Glucuronidase (Type IX) was obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals and reagents were analytical-reagent grade. The methanol used for the Sep-Pak silica cartridge step was prepared as follows: methanol was distilled and dried with Molecular Sieves 3A 1/16 (Wako Pure Chemical Industries, Osaka, Japan), and then water was added to give a concentration of 0.1% just before use.

Apparatus and chromatographic conditions

The high-performance liquid chromatograph consisted of a Waters Assoc. Model 510 pump, Model U6K injector, Model 481 UV detector set at 220 nm and 0.02 a.u.f.s. and Nippon Denshi Kagaku Model U-626 recorder. The column was Radial-Pak C_{18}, 100×8 mm I.D., 10 μm particle size (Waters Assoc.) and the column temperature was ambient. The mobile phase was methanol–10 m*M* phosphate buffer, pH 8.0 (65:35, v/v), and the flow-rate was 1.0 ml/min.

Preliminary experiments for the extraction by Sep-Pak C_{18} and silica cartridge

To examine the effect of the pH of the sample solution on the retention of triazolam and its metabolites on the Sep-Pak C_{18} cartridge, 5 μg of each compound were added to 5 ml of buffer solution: (1) 0.1 *M* acetate buffer, pH 5.0; (2) 0.1 *M* phosphate buffer, pH 7.0; (3) 0.1 *M* carbonate buffer, pH 9.0; or (4) 0.1 *M* carbonate buffer, pH 11.0. The buffer solution was poured into a Sep-Pak C_{18} cartridge, and the cartridge was eluted with 5 ml of methanol after washing with 5 ml of water. The methanol effluent was evaporated to dryness in vacuo after addition of 1 ml of internal standard solution (5 μg of etizolam in 1 ml of methanol). The residue was dissolved in 1 ml of the mobile phase, and 20-μl aliquots were applied to the HPLC system.

In order to examine the elution conditions of triazolam and its metabolites from the C_{18} cartridge, 5 ml of pH 9 buffer containing 300 ng of each compound were applied to a Sep-Pak C_{18} cartridge. The cartridge was washed with 5 ml of water, and then 5 ml each of water–methanol (8:2, v/v), water-methanol (7:3, v/v), water–methanol (5:5, v/v) and methanol were successively poured into the

cartridge to elute the drugs. Another C_{18} cartridge treated with drug-containing buffer was washed with 5 ml of water and eluted with 5 ml each of dichloromethane, dichloromethane–methanol (95:5, v/v), dichloromethane–methanol (90:10, v/v) and methanol. Each effluent was evaporated in vacuo after addition of 100 μl of the internal standard solution. Each residue was dissolved in 100 μl of the mobile phase, and 20-μl aliquots were injected into HPLC system for the determination of triazolam and its metabolites in each effluent.

To study the elution condition from the Sep-Pak silica cartridge, 5 ml of dichloromethane–methanol (99:1, v/v) containing 300 ng each of triazolam and its metabolites were poured into a silica cartridge. The cartridge was successively eluted with 20 ml each of dichloromethane, dichloromethane–methanol (99:1, v/v), dichloromethane–methanol (98:2, v/v), dichloromethane–methanol (95:5, v/v), dichloromethane–methanol (90:10, v/v) and dichloromethane–methanol (80:20, v/v), and the amounts of the drugs in each effluent were also determined by HPLC.

Extraction procedure

A 10-ml urine sample was adjusted to pH 5 with acetic acid, and then 2.5 ml of 0.5 *M* acetate buffer (pH 5.0) and 3000 U of β-glucuronidase were added. After incubation at 37 °C for 24 h, the mixture was alkalinized with ammonia and centrifuged at 1200 *g*. for 15 min. The supernatant was applied to a Sep-Pak C_{18} cartridge. The cartridge was washed with 5 ml of water, 5 ml of water–methanol (8:2, v/v) and 2 ml of water, and then eluted with 7 ml of dichloromethane–methanol (9:1, v/v). The eluate was evaporated to dryness in vacuo. The residue was dissolved in 5 ml of dichloromethane–methanol (99:1, v/v) and poured into a Sep-Pak silica cartridge. After washing with 20 ml of dichloromethane and 25 ml of dichloromethane–methanol (99:1, v/v), the silica cartridge was eluted with 20 ml of dichloromethane–methanol (9:1, v/v). The eluate was evaporated to dryness in vacuo. The residue was dissolved in 100 μl of the mobile phase, and 20-μl aliquots were injected into the liquid chromatogaph.

For the determination of triazolam and its metabolites, 100 μl of the internal standard solution were added to a urine sample after incubation with β-glucuronidase.

RESULTS AND DISCUSSION

Chromatographic conditions

Since 4-hydroxytriazolam appeared as a broad peak with acetonitrile–water mobile phases, methanol–water mobile phases were evaluated by examining the effects of pH, salt concentration and composition of mobile phase on the capacity factor, k', of triazolam and its main metabolites.

From peak shape and retention time, methanol–10 m*M* phosphate buffer, pH 8 (65:35, v/v) was selected as a mobile phase. Triazolam was then well separated from its metabolites and internal standard (Fig. 1).

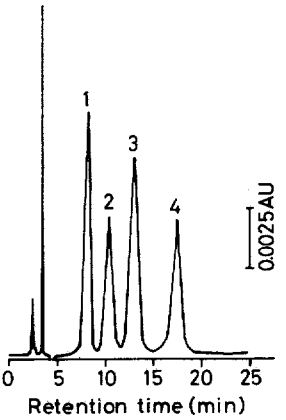

Fig. 1. Chromatogram of a mixture of authentic compounds. Peaks: 1 = 1-hydroxymethyltriazolam (30 ng); 2 = 4-hydroxytriazolam (30 ng); 3 = triazolam (30 ng); 4 = etizolam (100 ng, internal standard).

Calibration curve of standard solution

The calibration curves for triazolam, 1-hydroxymethyltriazolam and 4-hydroxytriazolam, obtained by plotting the ratio of the peak area of each compound to that of the internal standard (100 ng) against the amount of each drug injected, were linear in the range between 5 and 100 ng, and regression equations were as follows: y (the peak-area ratio) $= 0.02248x$ (the amount of compound injected, ng) $- 0.00085$ for triazolam; $y = 0.01534x + 0.00013$ for 1-hydroxymethyltriazolam; and $y = 0.01444x - 0.00885$ for 4-hydroxytriazolam. The detection limit of each compound at a signal-to-noise ratio of 3, with the detector sensitivity set at 0.02 full scale, was 2 ng.

Extraction and purification by Sep-Pak C_{18} and Sep-Pak silica cartridge

Triazolam and its metabolites were quantitatively recovered into the methanol effluent from the Sep-Pak C_{18} cartridge treated with pH 7.0–11.0 sample solution, as shown in Table I. This fact indicated that the drugs were completely retained in the cartridge in the range pH 7–11. Therefore urine samples were alkalinized with ammonia before application to a Sep-Pak C_{18} cartridge.

TABLE I

EFFECT OF pH OF SAMPLE SOLUTION ON THE RETENTION OF TRIAZOLAM AND ITS METABOLITES IN SEP-PAK C_{18} CARTRIDGE

Results are the mean ± S.D. ($n = 5$); 5 μg of each compound were added to 5 ml of buffer solution.

Compound	Recovery (%)			
	pH 5.0	pH 7.0	pH 9.0	pH 11.0
Triazolam	99.7 ± 0.1	96.8 ± 3.2	98.0 ± 1.0	98.9 ± 1.7
1-Hydroxymethyltriazolam	90.7 ± 0.1	98.6 ± 0.7	99.2 ± 0.3	95.6 ± 1.1
4-Hydroxytriazolam	99.2 ± 0.3	98.9 ± 1.9	100.4 ± 0.1	99.0 ± 1.4

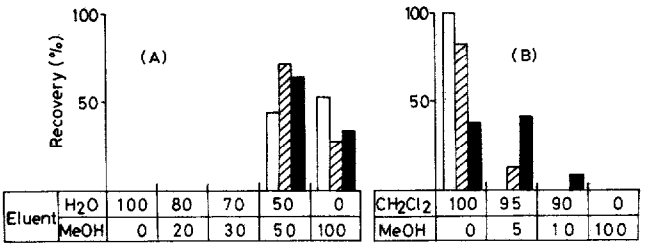

Fig. 2. Elution profiles of triazolam and its metabolites by water-methanol (A) and dichloromethane-methanol (B) from Sep-Pak C_{18} cartridge. □ = Triazolam; ▨ = 1-hydroxymethyltriazolam; ■ = 4-hydroxytriazolam.

In order to remove interfering components from urine, the C_{18} cartridge that retained triazolam and its metabolites was washed with a water–methanol mixture. No drugs were eluted from the cartridge until the concentration of methanol was increased to 30% (Fig. 2). However, the water–methanol (5:5, v/v) and methanol effluents of blank urine still showed interference on the chromatograms, and the dichloromethane–methanol mixture was evaluated as the eluting solution. When the concentration of methanol in the eluting solution was increased to 10%, most of the triazolam and its metabolites in the C_{18} cartridge were eluted as shown in Fig.2.

From these facts, triazolam and its metabolites retained on a Sep-Pak C_{18} cartridge were eluted with 7 ml of dichloromethane–methanol (9:1, v/v) after washing with 5 ml of water, 5 ml of water–methanol (8:2, v/v) and 2 ml of water. Blank urine without hydrolysis did not show any interference after this extraction, but an interference peak appeared at 11.2 min after hydrolysis with β-glucuronidase. Therefore, a purification step with a Sep-Pak silica cartridge was carried out. Neither triazolam nor its metabolites were eluted from the silica cartidge with 20 ml each of dichloromethane and dichloromethane–methanol (99:1, v/v) (Fig. 3), while the endogenous component with a retention time of 11.2 min was removed with dichloromethane–methanol (99:1, v/v).

The elution profiles of triazolam and its metabolites were markedly influenced by the water content of the methanol used for elution. When distilled methanol was used, the drugs were eluted together with the endogenous component by dichloromethane–methanol (99:1, v/v). On the other hand, the drugs were not eluted even with dichloromethane–methanol (90:10, v/v) when methanol dried with

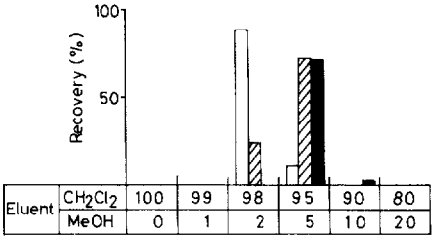

Fig. 3. Elution profiles of triazolam and its metabolites by dichloromethane-methanol from Sep-Pak silica cartridge. □ = Triazolam; ▨ = 1-hydroxymethyltriazolam; ■ = 4-hydroxytriazolam.

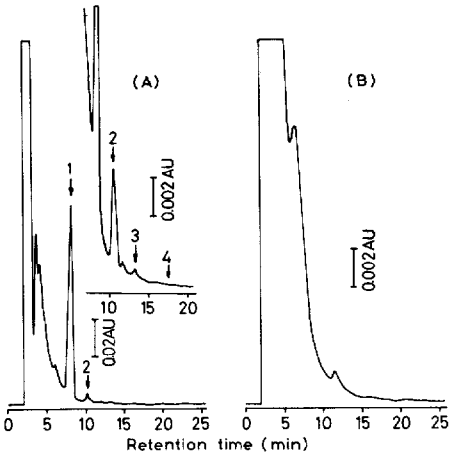

Fig. 4. Typical chromatograms of the extract from a urine sample (A) and from blank urine (B). Arrows show the retention times of authentic compounds. Peaks 1 = 1-hydroxymethyltriazolam; 2 = 4-hydroxytriazolam; 3 = triazolam; 4 = etizolam (internal standard).

molecular sieves was used. Control of the water content in methanol was essential to obtain reproducible results, hence the methanol used for the silica cartridge was prepared to contain 0.1% water, as described in *Materials*. Etizolam behaved similarly to triazolam in both cartridges.

From the above results of preliminary experiments, extraction procedures for urine samples were performed as mentioned in Experimental, and no interferences were observed on the HPLC chromatogram of an extract from blank urine (Fig. 4).

Recoveries, linearity, accuracy and sensitivity

The recoveries of 300 ng each of triazolam, 1-hydroxymethyltriazolam and 4-hydroxytriazolam added to 10 ml of blank urine were 88.5 ± 5.5, 75.0 ± 4.8 and 74.6 ± 7.0% ($n = 5$), respectively.

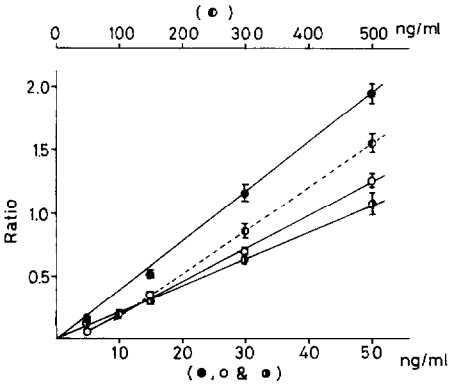

Fig. 5. Calibration curves of triazolam (●), 1-hydroxymethyltriazolam (○ and ◐) and 4-hydroxytriazolam (◑) obtained after extraction from urine. Solid lines were obtained using 10 ml of urine, and the dashed line was obtained using 1 ml of urine. Each point represents the mean ± S.D. ($n = 5$).

TABLE II

CONTENT OF TRIAZOLAM AND ITS METABOLITES IN THE SAMPLE URINE

Sample No. 1 was the 8-h urine from a healthy volunteer who received a 0.25-mg tablet of triazolam and sample No. 2 was the 11-h urine after administration of a 0.5-mg tablet. Results are mean ± S.D. ($n=5$).

Compound	Sample No. 1		Sample No. 2	
	Concentration (ng/ml)	C.V. (%)	Concentration (ng/ml)	C.V. (%)
Triazolam	Trace		10.2 ± 1.0	10.1
1-Hydroxymethyltriazolam	281 ± 20	7.1	491 ± 8.3	1.7
4-Hydroxytriazolam	39.6 ± 2.6	6.6	45.8 ± 1.6	3.4

In order to evaluate the linearity of this method, known amounts of each compound were added to 10 ml of blank urine (5–50 ng/ml), and these standard samples were analysed by the described procedure. For 1-hydroxymethyltriazolam, 1-ml urine samples (50–500 ng/ml) were also employed. The ratio of the peak area of each compound to that of the internal standard was plotted against the known concentrations of the drugs. The satisfactory results were obtained over the concentration ranges studied as shown in Fig. 5.

The accuracy of the method was investigated by assaying five control urine samples containing 30 ng/ml standards on different days. Concentrations of triazolam, 1-hydroxymethyltriazolam and 4-hydroxytriazolam found in the spiked samples were 30.2 ± 1.8 ng/ml (coefficient of variation 6.0%), 29.9 ± 1.9 ng/ml (6.2%) and 29.9 ± 2.1 (7.0%) ng/ml, respectively. The sensitivity limit for urine determination was 5 ng/ml with a 10-ml specimen.

Application

The 8-h urine from a healthy volunteer who received a 0.25-mg tablet of triazolam and the 11-h urine after administration of a 0.5-mg tablet were analysed by the present method. On each chromatogram of the extracts, two peaks with the same retention times as 1-hydroxymethyltriazolam and 4-hydroxytriazolam, respectively, were apparently observed, and a very small peak was detected at the same retention time as triazolam (Fig. 4). The peaks corresponding to the metabolites, however, were not observed when urine samples were analysed without hydrolysis by β-glucuronidase.

Based on the calibration curves in Fig. 5, the concentrations of the metabolites and the reproducibility of this method were determined (Table II).

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