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High-performance liquid chromatographic method for the determination of alprazolam in plasma using the column-switching technique☆

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ABSTRACT

A reversed-phase high-performance liquid chromatographic method is described for the quantitative determination of alprazolam in the plasma of geriatric patients in the presence of 4-hydroxyalprazolam, α-hydroxyalprazolam, bromazepam, oxazepam, lorazepam, clobazam, desmethylclobazam, diazepam and desmethyldiazepam. The procedure is based on the enrichment of alprazolam on a PRP-1 pre-column, followed by the transfer of the compound in a forflush mode to the analytical column. Alprazolam can be quantified reliably down to a minimum concentration of 1 ng/ml of plasma.

INTRODUCTION

Age-related physiological changes may interfere with the absorption and elimination of many drugs and foreign compounds [1–3]. In the course of our work on pharmacokinetic changes in old age, alprazolam (Tafil) plasma levels were determined in an elderly cohort of patients (mean age 74.1 years, S.D. = 9.2 years) with chronic anxiety.

Alprazolam has been determined by gas chromatography [4–6] and high-performance liquid chromatography (HPLC) [7–12]. Published HPLC procedures [7–11] were tried for the measurement of alprazolam in plasma from geriatric patients, who are very frequently under long-term medication with further drugs. Because interfering substances were found in the plasma matrix under study [13] and because, for other reasons, alprazolam should be separated from other benzodiazepines (bromazepam, lorazepam, oxazepam, clobazam and diazepam), a new sensitive and selective HPLC assay was developed, using the column-switching technique. The method is suitable for pharmacokinetic studies.

EXPERIMENTAL

Reagents and solvents

Alprazolam, 4-hydroxyalprazolam, α-hydroxyalprazolam and triazolam were gifts from Upjohn (Heppenheim, Germany). HPLC-grade water, acetonitrile and methylene chloride were supplied by Promochem (Wesel, Germany). Extrelut columns and phosphoric acid (85%, p.a. quality) were obtained from Merck (Darmstadt, Germany).

Other benzodiazepines were gifts from Hoff-

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man-La Roche (Grenzach-Wyhlen, Germany; bromazepam, diazepam), Hoechst (Frankfurt/Main, Germany; clobazam, desmethylclobazam), Wyeth-Pharma (Münster/Westfalen, Germany; lorazepam) and Thomae (Biberach an der Riss, Germany; oxazepam). Desmethyldiazepam was purchased from Sigma (Deisenhofen, Germany).

Stock solutions of alprazolam (1 mg/ml), triazolam (1 mg/ml), 4-hydroxyalprazolam (500 μg/ml), α-hydroxyalprazolam (100 μg/ml), bromazepam (100 μg/ml), oxazepam (100 μg/ml), lorazepam (100 μg/ml), clobazam (1 mg/ml) and diazepam (100 μg/ml) were prepared in acetonitrile and stored at 4°C in the dark. Stock solutions of desmethylclobazam (50 μg/ml) and desmethyldiazepam (50 μg/ml) in acetonitrile were stored at room temperature in the dark. Standard solutions of alprazolam (1 μg/ml) and triazolam (10 μg/ml) were prepared by diluting aliquots of the stock solutions with water. The standard solutions were transferred to brown 4-ml vials, stored at 4°C and prepared freshly each week.

Test samples of 4-hydroxyalprazolam, α-hydroxyalprazolam, bromazepam, oxazepam, lorazepam, chlobazam, desmethylclobazam, diazepam and desmethyldiazepam were obtained by dilution of stock solutions with water or plasma.

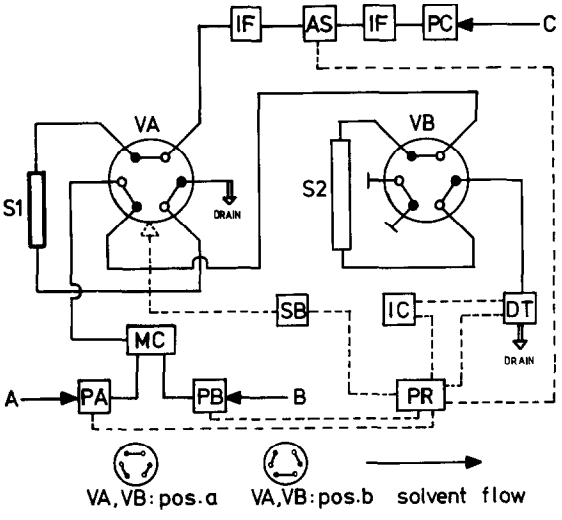

Fig. 1. Schematic representation of the HPLC system for the analysis of alprazolam and triazolam. For further details, see text.

Instrumental and chromatographic conditions

A schematic representation of the column-switching set-up is given in Fig. 1. The eluent C, 1 l of water plus 20 ml of acetonitrile plus 50 μl of phosphoric acid (pH 3.2), degassed with a Model ERC-3510 degasser (ERC, Alteglofsheim, Germany) was delivered by a single-piston pump PC (Model 410; Kontron, Eching, Germany) at a flow-rate of 0.3 ml/min, and passed through a column inlet filter IF (Type 7302, 2-μm frit; Rheodyne, Cotati, CA, USA) into the injection valve of the autosampler AS (Model MSI 660, fitted with a 250-μl sample loop; Kontron).

The sample solution (250 μl) was injected via AS into the chromatographic system. The eluent C transported the injected sample via a further column inlet filter IF onto the pre-column S1 (MPLC cartridge PRP-1, particle size 10 μm; 3 cm × 2.1 mm I.D.; Kontron), which was connected by means of a Brownlee cartridge holder system to the automatic valve VA (Type 7000, fitted with a pneumatic actuator and a 12-V D.C. solenoid valve; Rheodyne).

Enrichment of the alprazolam and triazolam from the injected sample solution was achieved on column S1. Two pumps (PA and PB, single-piston pumps, Models 414T; Kontron) were employed to form a mobile phase A–B (40:60, v/v). Eluent A was degassed acetonitrile and eluent B was identical with eluent C. The flow-rate of mobile phase A–B was 1 ml/min. The two eluents A and B were mixed in a mixing chamber MC (Kontron) and carried the enriched alprazolam and triazolam from column S1 to the analytical column S2 (MPLC cartridge RP-8 Spheri-5, particle size 5 μm, 10 cm × 4.6 mm I.D.; Kontron). The column S2 was likewise connected by means of a Brownlee cartridge holder system to the valve VB (Type 7000, manually switchable; Rheodyne).

Alprazolam and triazolam were detected with a UV detector DT (Model Uvikon 720 LC, 2.8-μl cell; Kontron) at a wavelength of 230 nm (rec. scale 1.000; time constant = 4). An integrator IC (attenuation = 32, chart speed = 0.5 cm/min; Model SP 4270, Spectra-Physics, Darmstadt, Germany) and an AT computer (equipped with

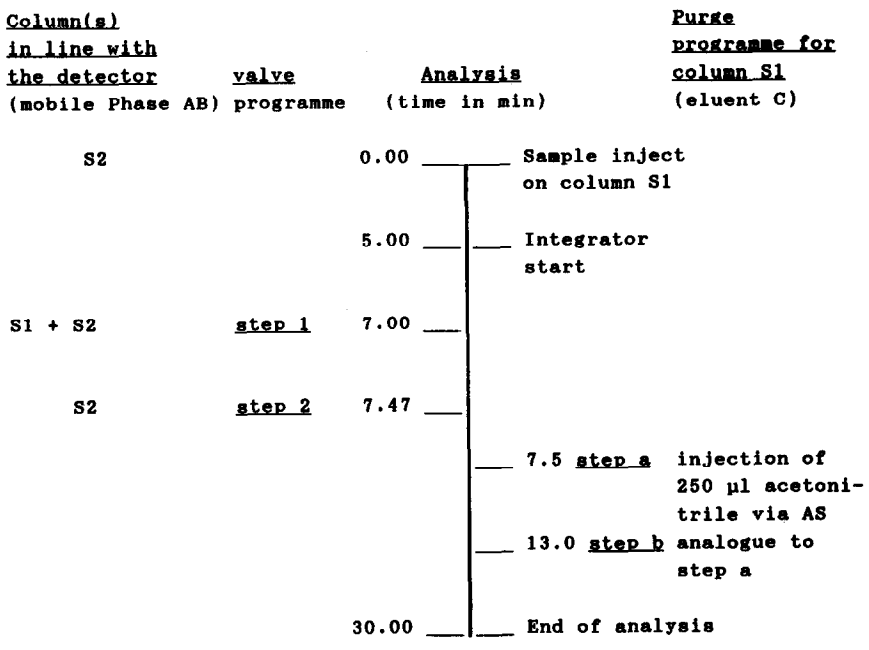

Fig. 2. Schematic representation of the analysis programme (for abbreviations see text). VB = position a; PA = 0.4 ml/min; PB = 0.6 ml/min; PC = 0.3 ml/min. Step 1: 7.00–7.47 min; VA = position b. Step 2: 7.47–30.00 min; VA = position a.

the WINer/286 package from Spectra-Physics) were used for recording and evaluating the resulting chromatograms. The external standard method was chosen for calibration.

A switchbox SB (Model S112; ERC, Alteglofsheim, Germany) was installed between the valve VA and the programmer PR so that the valve VA could be switched by the programmer PR (Model 200; Kontron).

The HPLC system was operated at ambient temperature.

Analytical procedure

The analysis programme, carried out by the programmer PR, is given in Fig. 2. To guarantee the chromatographic efficiency of column S1 and the reproducibility of the column-switching processes, the column S1 was purged with two 250-µl volumes of acetonitrile after sample transfer to column S2.

Preparation of standard plasma samples

Venous blood (containing neither alprazolam nor triazolam) was centrifuged for 10 min at 3000 *g* in a heparinized test-tube. Samples of 3 ml of the separated plasma were treated in 10 ml ground-glass test-tubes first with 15, 30, 60 or 150 µl of the alprazolam standard solution.

Each of the spiked plasma samples was finally treated with 30 µl of the triazolam standard solution, mixed for 1 min and allowed to stand for 15 min at room temperature.

The concentrations of alprazolam, calculated per 3 ml of plasma, were 5, 10, 20 and 50 ng/ml, respectively. The concentration of triazolam, calculated in the same way, was 100 ng/ml.

The spiked plasma sample was transferred directly onto the 3-ml Extrelut column. For 10 min the sample was allowed to soak, before elution with 15 ml of methylene chloride was started. In contrast to the recommended work-up procedure for 3-ml Extrelut columns an additional elution step with 5 ml of methylene chloride was performed.

The solvent was removed from the obtained eluate on a rotary evaporator at 30°C maximum water-bath temperature, and 1 ml of acetonitrile–water (5:95, v/v) was added to the residue. After

standing for 15 min at room temperature, the sample solution was transferred to an Eppendorf micro-test-tube, centrifuged for 2 min (Eppendorf centrifuge, Model 3200, 14 000 *g*), and 800 μl of the supernatant were placed in a 2-ml autosampler vial.

Patients' plasma samples

Patients' plasma was isolated as described for the standard plasma samples. It was stored at −20°C until the time of assay and then prepared in the same way as the spiked standard plasma samples.

Quality control samples

To control the chromatographic system a quality control sample was used, which was prepared by dilution of a mixture of the standard solutions of alprazolam and triazolam with water to a concentration of 100 ng/ml.

RESULTS AND DISCUSSION

The mobile phase (eluents B and C)

In an acidic medium, a ring-opening reaction of the alprazolam molecule occurs [14], which possibly is detrimental to quantitative analyses. Under our chromatographic conditions, however, no difference was found in the peak areas of defined amounts of alprazolam when analysed in an acidic eluent under standard conditions, compared with analyses in which the eluents B and C were pure water.

The use of pure water as eluent resulted in a reduction of the resolution between the signals of triazolam and alprazolam (*ca.* 20%) as well as in an enhanced trapping of water impurities on the precolumn S1. Therefore, acidic eluents B and C were preferred.

Calibration graph

An internal standard and an external standard method were used in parallel: peak-area ratios of alprazolam and triazolam, as well as the actual peak area of alprazolam in relation to the corresponding concentrations, were used to construct calibration graphs. A linear relationship was obtained in both cases, covering the concentration range 5–50 ng/ml alprazolam. Regression analysis of data gave correlation coefficients of $r = 0.998$ (internal standard method) and $r = 0.999$ (external standard method). Unknowns were quantified routinely by the external standard method.

The results were controlled by the internal standard method occasionally and found to be identical. Therefore, the peak area of triazolam was used to prove the external standard method, but was not used for routine quantification because of possible interferences (see below).

Precision and recovery

The efficiency of the method is shown in Table I, in which precision and recovery data are given.

Both the recovery, which represents a measure of the accuracy at a given selectivity, and the good precision clearly demonstrate that the column-switching technique can be used for the determination of therapeutic levels of alprazolam. Furthermore, this assay can be used in pharmacokinetic studies in humans.

The absolute recovery was calculated by comparing the peak areas for alprazolam extracted from spiked plasma samples with those of equal amounts injected directly into the chromatographic system. For the concentration range 5–50

TABLE I

PRECISION AND RECOVERY FOR ALPRAZOLAM (EXTERNAL STANDARD METHOD)

Actual plasma concentration (ng/ml)	Measured plasma concentration (mean ± S.D.) (ng/ml)	R.S.D. (%)	Relative error[a] (%)
Within-day (n = 3)			
10.00	10.01 ± 0.47	4.66	100.10
20.00	20.45 ± 0.74	3.61	102.24
50.00	49.58 ± 0.82	1.65	99.17
Between-day (n = 5)			
10.00	9.63 ± 0.72	7.51	96.27
20.00	20.83 ± 0.81	3.90	104.14
50.00	50.77 ± 2.54	4.99	101.53

[a] (Concentration found/actual concentration) × 100.

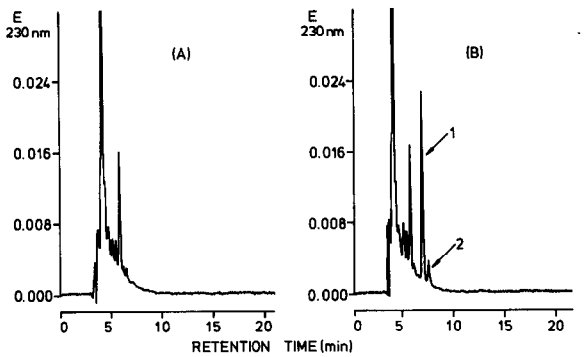

Fig. 3. Chromatograms of patient's plasma samples before (A) and after (B) oral administration of alprazolam (single dose of 2 × 0.5 mg of Tafil). Peaks: 1 = triazolam (internal standard and extraction marker; 6.99 min); 2 = alprazolam (7.57 min, 6.3 ng/ml).

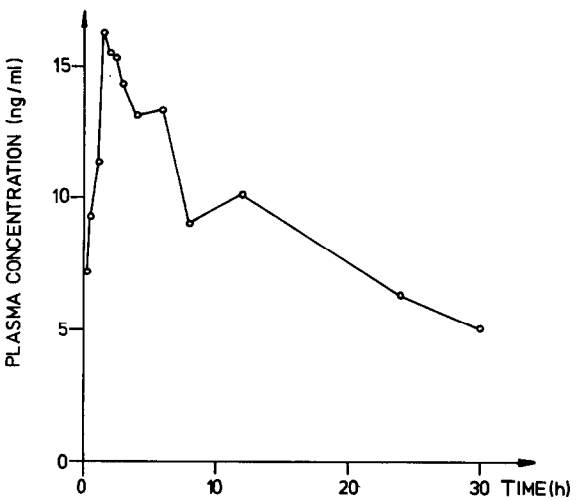

Fig. 4. Profile of alprazolam plasma concentrations *versus* time after a single oral dose of 2 × 0.5 mg of Tafil.

ng/ml the recovery was found to 85.6% (relative standard deviation, R.S.D. = 5.2%, n = 20).

Limit of quantitation, selectivity and practical application of the method

Our method provides a reliable determination of alprazolam down to a minimum concentration of 1 ng/ml in plasma (R.S.D. = 8.9%; n = 5).

The quantification of alprazolam and triazolam was not disturbed by the metabolites α-hydroxyalprazolam and 4-hydroxyalprazolam, or by the benzodiazepines bromazepam, oxazepam, lorazepam and desmethylclobazam, which eluted at times between 4 and 6 min or between 12 and 14 min (diazepam, desmethyldiazepam). Clobazam (6.83 min) interfered with triazolam (6.99 min). Therefore, in the presence of clobazam, a quantification with triazolam as the internal standard is not recommended. In the absence of clobazam, triazolam was useful to validate the results of the external standard method.

Figs. 3 and 4 show typical chromatograms obtained from a patient, together with the individual pharmacokinetic curve. The patient was under long-term medication, which was started before this pharmacokinetic study. Digimerck, Augmentan, Fluimucil, Euglucon and Liquemin were administered and did not interfere with the quantification of alprazolam.

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