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Direct high-performance liquid chromatographic determination of the enantiomers of alfuzosin in plasma on a second-generation α_1-acid glycoprotein chiral stationary phase

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SUMMARY

A direct liquid chromatographic method was developed for the determination of the enantiomers of alfuzosin in human plasma, without derivatization, on a chiral α_1-acid glycoprotein column. The influence of pH, of uncharged organic solvents and of a cationic modifier (tetrabutylammonium) of the mobile phase on retention and enantioselectivity was evaluated. The enantiomers and an internal standard, structurally related to alfuzosin, were extracted from plasma with dichloromethane–diethyl ether from alkaline solution, then separated with a mobile phase of 0.025 M phosphate buffer (pH 7.4) containing 0.025 M tetrabutylammonium bromide–acetonitrile (94:6, v/v). The limit of quantification for each isomer was 1 ng/ml. The method has been applied to the determination of the pharmacokinetic profile of alfuzosin enantiomers in healthy volunteers after intravenous administration of the racemate.

INTRODUCTION

Alfuzosin (XATRAL, Fig. 1), a quinazolinyl derivative, is a selective postsynaptic α_1-adrenoceptor antagonist, recently introduced in therapeutics for the treatment of benign prostate hypertrophy. The compound is a racemate and the R and S optical isomers have similar pharmacological activity. For the determination of the pharmacokinetic behaviour of each enantiomer after administration of the racemic mixture, it was desirable to determine the two enantiomers simultaneously in plasma.

Chiral high-performance liquid chromatographic (HPLC) resolution can be achieved by indirect or direct separations[1–4] but indirect chromatographic resolution based on the formation of diastereomers with various chiral reagents[5–12] has proved to be difficult with alfuzosin. An improved version of a chiral column containing α_1-acid glycoprotein (α_1-AGP) bound to silica was described by Hermansson[13]. In this paper, we report a sensitive, stereospecific HPLC method suitable for the determination of the enantiomers of alfuzosin in plasma on this α_1-AGP column. The effects of pH, uncharged organic solvents (acetonitrile, methanol, ethanol, 2-propa-
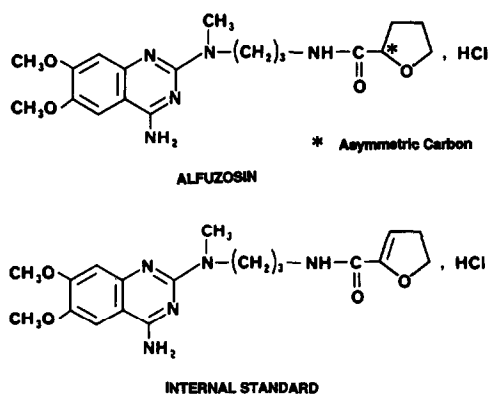
Fig. 1. Structures of alfuzosin and the internal standard.

nol) and of a cationic modifier, tetrabutylammonium, of the mobile phase on the retention and the enantioselectivity were evaluated. The assay procedure was applied to the determination of the pharmacokinetic profile of the enantiomers of alfuzosin in healthy volunteers after intravenous administration of the racemate.

EXPERIMENTAL

Materials

Racemic alfuzosin, its R and S enantiomers and an internal standard structurally related to alfuzosin (Fig. 1) were synthesized in the Chemistry Department of Synthélabo Recherche (L.E.R.S.). Anhydrous KH_2PO_4 and $K_2HPO_4 \cdot 3H_2O$ were of analytical-reagent grade from Merck (Darmstadt, F.R.G.), 1 M sodium hydroxide solution and 1 M hydrochloric acid were obtained from Prolabo (Paris, France), tetrabutylammonium bromide (TBA) from Aldrich (Strasbourg, France), HPLC-grade acetonitrile, UV-grade methanol and unstabilized diethyl ether (RPE grade) from Carlo Erba (Milan, Italy) and analytical-reagent grade dichloromethane from Prolabo. The water used for the preparation of the buffers was purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.).

Stock solutions of alfuzosin and the internal standard were prepared in methanol at a concentration of 100 ng/μl and diluted for calibration. These solutions were stable for at least 1 month when kept at 0–5°C.

The liquid chromatograph consisted of a Beckman Model 110 B constant-flow pump (Altex, San Ramon, CA, U.S.A.) and a JASCO 820 FP spectrofluorimeter (Japan Spectroscopic, Tokyo, Japan), operated at 265 nm for excitation and 400 nm for emission. The detector was coupled to an SP 4100 integrator–calculator (Spectra-Physics, San Jose, CA, U.S.A.) for the determination of peak height.

Chromatography of alfuzosin enantiomers was performed on a commercially available (ChromTech, Norsborg, Sweden) chiral-AGP column (100 mm × 4.0 mm I.D.). Chiral-AGP is a second-generation chiral separation column with α_1-acid glycoprotein AGP immobilized on porous, spherical, 5-μm silica particles.

Methods

The mobile phase buffer was prepared by dissolving 0.667 g of anhydrous KH_2PO_4, 4.587 g of $K_2HPO_4 \cdot 3H_2O$ and 8.060 g of TBA in 1 l of purified water. The standard conditions used for the determination of alfuzosin enantiomers in plasma were a flow-rate of 0.9 ml/min, ambient temperature and a mobile phase of 0.025 M K_2HPO_4–KH_2PO_4 buffer containing 0.025 M TBA–acetonitrile (94:6, v/v) (pH 7.4).

Extraction

To 1 ml of plasma in a 15-ml screw-capped Pyrex test-tube, fitted with a PTFE screw-cap, were added 40 ng of internal standard (20 μl of a 2 ng/μl solution in methanol), 1 ml of 0.1 M sodium hydroxide solution and 7 ml of extraction solvent (dichloromethane–diethyl ether, 3:4). The tube was shaken for 25 min on an agitator (Bioblock, Paris, France) then centrifuged at 1000 g for 5 min. The organic layer was transfered to tubes and evaporated to dryness at 30°C under a gentle stream of nitrogen. The residue was dissolved in 80 μl of the mobile phase and 20 μl were injected into the chiral-AGP column.

Quantitative yield

Five plasma samples were spiked with alfuzosin to give final concentrations of 25 ng/ml for each enantiomer and extracted according to the procedure described above, but without addition of internal standard. Exact volumes of the organic layer were sampled, and 30 ng of internal standard were added. The peak-height ratios of R and S isomers to the internal standard for the extracted samples were compared with those obtained after evaporation of solvent samples containing equivalent concentrations of alfuzosin and internal standard (unextracted samples).

Calibration and calculation

The retention times of R and S enantiomers were measured in duplicate, each isomer being injected separately. The capacity factors were calculated as $k' = (t_R - t_0)/t_0$, the separation factors, α, as k'_S/k'_R and the resolution, R_1, as $2\Delta t_R/(W_R + W_S)$, W_R and W_S being the peak width of the R and S isomers, respectively.

The concentrations of the R and S enantiomers in unknown samples were calculated by interpolation from calibration graphs prepared as follows: to 1-ml blank plasma samples, 2, 5, 10, 20, 50 or 100 ng of alfuzosin racemate and 40 ng of internal standard were added by using appropriate volumes of diluted solutions. The samples were analysed according to the procedure. Peak-height ratios (R enantiomer/internal standard and S enantiomer/internal standard) were used to generate a least-squares regression line, weighted by 1/concentration.

For assay validation, 1-ml plasma samples were spiked with 10 or 50 ng of alfuzosin racemate (5 or 25 ng of each isomer). Two analysts performed two assay series, containing five control samples for each concentration and calibration samples (1–50 ng for each isomer), on two separate days. Accuracy, precision, linearity and sensitivity of the assay, sensitivity or limit of quantification, minimum concentration that may be evaluated with a relative standard deviation (R.S.D.) < 10% and a residue (percentage difference from theoretical value) < 15%, were measured. Furthermore, quality control samples were used in daily routine analysis.

RESULTS AND DISCUSSION

The retention times of (R)- and (S)-alfuzosin and the enantioselectivity were strongly influenced by the charged and uncharged mobile phase additives the ionic strength and the pH. When the separation of enantiomers was achieved, the R enantiomer was always eluted before the S enantiomer.

Influence of uncharged modifier
Organic solvents of different character affect the enantioselectivity differently: their adsorption changes the conformation of the α_1-glycoprotein, and a "new" chiral phase is created by a simple adjustment of the mobile phase[14]. Table I summarizes the influence of different uncharged organic solvents in the mobile phase (4% methanol, ethanol, 2-propanol or acetonitrile) on the retention behaviour and the enantioselectivity. With alcohols the capacity factors decrease inversely with their hydrophobicity: the R and S enantiomers are eluted by 2-propanol ($\alpha = 1.03$) and not eluted by ethanol and methanol. The addition of alcohols reduces the retention, but these alcohols often reduce the chiral selectivity[15]. With 4% acetonitrile as organic solvent the capacity factors are higher than those obtained with 2-propanol, but the separation factor is better ($\alpha = 1.17$). In a series of experiments, other mobile phases with different percentage of modifiers were tested. The enantioselectivity was generally better with acetonitrile. Therefore, the resolution of alfuzosin enantiomers was optimized with acetonitrile as organic solvent.

TABLE I

INFLUENCE OF UNCHARGED MODIFIERS

Mobile phase, modifier–0.01 M KH_2PO_4 (pH 7.0) (4:96, v/v).

Modifier	k'_R	k'_S	α
2-Propanol	8.5	8.8	1.03
Acetonitrile	12.4	14.5	1.17
Methanol	Not eluted	Not eluted	—
Ethanol	Not eluted	Not eluted	—

Influence of a charged modifier (TBA) and of pH
Fig. 2 shows the effects of pH and a cationic modifier, TBA, of the mobile phase on the capacity factors (Fig. 2a) and on the resolution (Fig. 2b) of alfuzosin enantiomers with a mobile phase of 0.05 M buffer–acetonitrile (94:6, v/v), with 0.025 M TBA or without TBA.

The influence of pH is more drastic than the effect of the cationic modifier. At pH 7 the resolution is insufficient ($R = 0.8$) with or without TBA in the mobile phase. The capacity factor and the resolution improve with increase in pH. A slight change in pH affects the resolution. The latter increases from 0.8 to 1.3–1.4 when the pH increases 7.0 to 7.4. A pH of 7.4 was chosen for the separation of alfuzosin enantiomers.
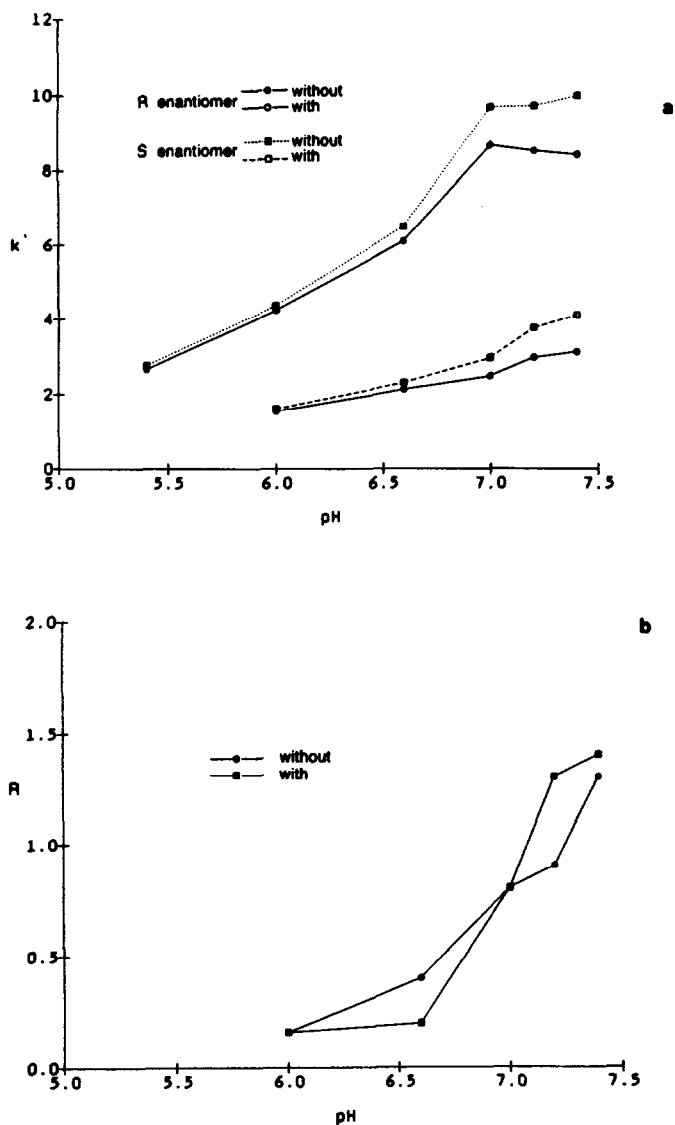
Fig. 2 Effect of pH on (a) the capacity factor (k') and (b) the resolution (R). Eluent, 0.05 M buffer–acetonitrile (94:6, v/v) with or without 0.025 M TBA.

The addition of TBA to the mobile phase enhances the resolution. In particular, it achieves enantioselectivity after 7 min (k'_S = 4.1 at pH 7.4). Without TBA, the capactiy factors are too high. Under the same conditions at pH 7.4, k'_R decreases from 8.4 to 3.1 when TBA is added to the mobile phase, whereas the resolution is slighty improved from 1.3 to 1.4. Generally, the retention of the cationic solutes is significantly decreased by addition of a cationic modifier, but the enantioselectivity is almost unaffected[14].
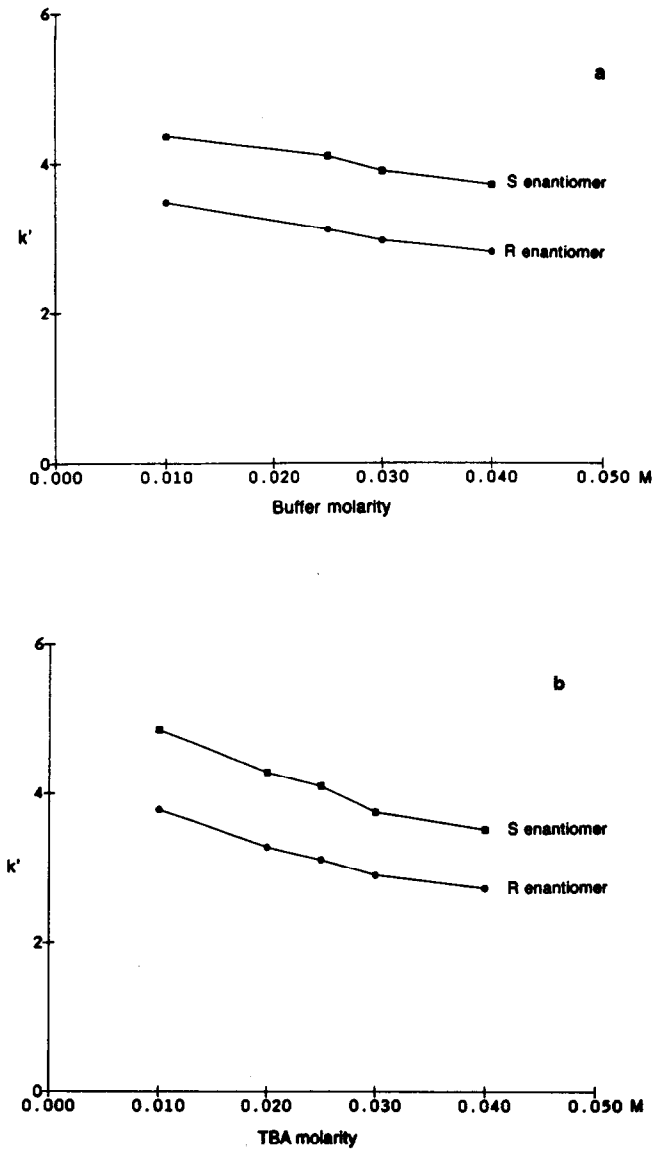
Fig. 3. (a) Effect of buffer molarity on the capacity factor (k'). TBA concentration 0.025 M. (b) Effect of TBA concentration on the capacity factor (k'). Final buffer molarity, 0.05 M. Eluent, phosphate buffer (pH 7.4) containing TBA–acetonitrile (94:6, v/v).

Influence of ionic strength

Fig. 3 illustrates the effects of buffer and TBA molarity on the capacity factors of the two isomers. The latter decreases in parallel with the buffer and TBA molarity, but these effects are not drastic; the enantioselectivity is almost unaffected. Under the analytical conditions [phosphate buffer (pH 7.4) containing 0.025 M TBA–aceto-nitrile (94:6; v/v)], k'_S decreases from 4.4 to 3.7 when the buffer molarity increases

from 0.01 to 0.04 M, the TBA molarity being constant (0.025 M). When the total molarity of the mobile phase is kept constant (0.05 M), k'_s decreases from 4.8 to 3.5 as the TBA molarity increases from 0.01 to 0.04 M.

The final composition of the mobile phase, established through the influence of these different parameters was 0.025 M KH_2PO_4–K_2HPO_4 buffer (pH 7.4) containing 0.025 M TBA–acetonitrile (94:6, v/v). The retention times of (R)- and (S)-alfuzosin and the internal standard were 5.32, 6.58 and 9.75 min, respectively, at a flow-rate of 0.9 ml/min. The values for the separation factor (α) and resolution were 1.35 and 1.45, respectively.

Assay validation

R and S enantiomer plasma control samples (5 and 25 ng/ml) were assayed four times by two analysts on two different days. The precision and accuracy of the method for these two concentrations are shown in Table II. The analytical recoveries of alfuzosin enantiomers in 20 plasma samples spiked with 5 and 25 ng/ml were identical for the two enantiomers, averaging 106% (5 ng/ml) and 105% (25 ng/ml). The R.S.D. was identical for both isomers, *ca.* 5 and 3% at 5 and 25 ng/ml, respectively.

TABLE II

SENSITIVITY LIMITS, PRECISION AND ACCURACY OF THE METHOD

Enantiomer	Sensitivity limit (ng/ml)	Accuracy and reproducibility (n = 20)			
		Theoretical concentration (ng/ml)	Mean observed concentration (ng/ml)	Difference from theoretical value (%)	Relative standard deviation (%)
R	1	5	5.28	+5.6	4.9
		25	26.12	+4.5	3.2
S	1	5	5.32	+6.4	5.1
		25	26.20	+4.8	2.9

Calibration graphs, weighted by the inverse of the concentration, were linear from 1 to 50 ng/ml for each isomer. The sensitivity limit, equal to the lowest point of the calibration (R.S.D. < 10%; difference from the theoretical values < 15%) was 1 ng/ml with fluorimetric detection. The quantitative yield of alfuzosin enantiomers in human plasma was 97.7 ± 2.3% (n = 5). Alfuzosin and its enantiomers were stable under our assay conditions.

The interference with the HPLC assay of other drugs that might be administered with alfuzosin in human therapy was investigated. The retention times of these compounds, listed in Table III, indicate that they would not interfere with the alfuzosin enantiomers. However, zolpidem was found to be eluted near the internal standard.

Routine analysis

The method was used in routine analysis for the determination of alfuzosin enantiomers in biological fluids, and tested by the assay of control samples (10 and 50 ng/ml of alfuzosin, *i.e.*, 5 and 25 ng/ml for each isomer) over several days. The mean

TABLE III

DRUGS TESTED FOR POSSIBLE INTERFERENCE

Compound	Retention time (min)[a]	Compound	Retention time (min)[a]
Metoprolol	—	Enalapril	—
Betaxolol	—	Captopril	—
Propranolol	—	Furosemide	2.0
Diazepam	—	Zolpidem	9.16
(R)-Alfuzosin	5.5	Piroxicam	—
(S)-Alfuzosin	6.8	Warfarin	—
Internal standard	10.01		

[a] — Not eluted and/or not detected.

values found were 10.6 and 52.0 ng/ml (n = 36) with R.S.D. 4.6 and 3.0%, respectively.

Pharmacokinetic study

The method was applied to the preliminary evaluation of the pharmacokinetic profile of the R and S enantiomers in six healthy volunteers after a single intravenous dose of 5 mg of alfuzosin. Chromatograms of 1 ml of blank plasma extract and 1 ml of plasma extract, obtained from a healthy volunteer 20 min after intravenous administration, are shown Fig. 4. Blank plasma samples were free from endogenous contaminants at the retention times corresponding to alfuzosin enantiomers and the in-
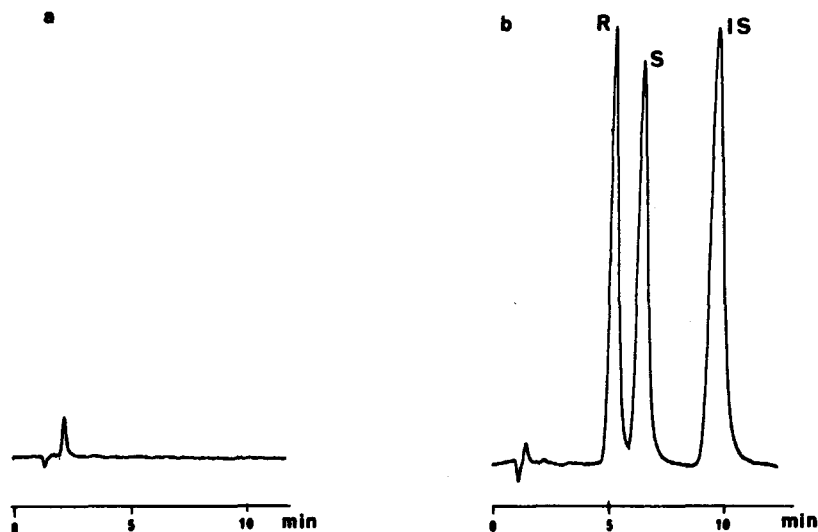

Fig. 4. Typical chromatograms of (a) blank plasma extract and (b) plasma extract obtained from a healthy volunteer, 20 min following a single intravenous administration of 5 mg of alfuzosin. Chromatographic conditions: column, 100 × 4 mm I.D., chiral-AGP, 5 μm; mobile phase, 0.025 M KH_2PO_4–K_2HPO_4 containing 0.025 M TBA (pH 7.4)–acetonitrile (94:6, v/v); flow-rate, 0.9 ml/min; detection, λ_{ex} = 265 nm, λ_{em} = 400 nm.

TABLE IV

MEAN PHARMACOKINETIC PARAMETERS ($n = 6$) OF R AND S ENANTIOMERS OBTAINED IN SIX HEALTHY VOLUNTEERS AFTER INTRAVENOUS ADMINISTRATION OF 5 mg OF ALFUZOSIN BY PERFUSION OVER A 30-min PERIOD

$t_{1/2}$ (β) = Terminal half-life; AUC = area under curve; Cl = systematic clearance; Vd = distribution volume.

	$t_{1/2}$ (β) (h)		AUC (ng/ml/h)		Cl (1/h/kg)		Vd (1/kg)	
	R	S	R	S	R	S	R	S
Mean	3.6	2.5	76.9	54.5	0.465	0.649	2.35	2.33
Standard error	0.3	0.1	7.7	4.3	0.027	0.037	0.10	0.12

ternal standard. The mean plasma concentration *versus* time curves for (R)- and (S)-alfuzosin after intravenous administration of the racemate shows that the pharmacokinetic profiles of the R and S enantiomers are different.

The pharmacokinetic parameters (terminal half-live, area under curve, clearance, distribution volume) were calculated with the PHARM program[16] and are reported in Table IV. The R and S isomers are eliminated from the central compartment with terminal half-lives of 3.6 ± 0.6 and 2.5 ± 0.3 h, respectively. In the same way, the area under the time *versus* concentration curve for the R isomer was larger than that for the S isomer. There was no difference in the distribution volume.

CONCLUSIONS

As the amine function of alfuzosin is far from the chiral centre, the two chiral centres of diastereomers formed with chiral reagents are too far apart to allow the resolution of the diastereomers by reversed-phase chromatography[1]. Direct resolution of alfuzosin enantiomers has been achieved on a new chiral AGP column. The present HPLC method is rapid, sensitive (1 ng/ml for each isomer) and was found to be suitable for the simultaneous determination of alfuzosin enantiomers in plasma after intravenous administration of the racemate (5 mg) to healthy volunteers.

The column performance decreased after the injection of 250 extracted plasma samples. As the mobile phase contained a small amount of organic solvent, no precolumn could be used to protect the column. However, the efficiency can be restored by repacking the first few millimeters of the stationary phase and then each column allows the analysis of *ca.* 500 samples.

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