Journal of Chromatography, 353 (1986) 361–369
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 729

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ALFUZOSIN IN BIOLOGICAL FLUIDS WITH FLUORIMETRIC DETECTION AND LARGE-VOLUME INJECTION

P. GUINEBAULT*, M. BROQUAIRE*, C. COLAFRANCESCHI and J. P. THÉNOT
Laboratoires d'Études et de Recherches Synthélabo, Department of Clinical Research, 92360 Meudon-La-Forêt (France)

SUMMARY

A high-performance liquid chromatographic method has been developed for the determination of alfuzosin, a new antagonist of α_1 post-synaptic adrenergic receptors, in blood, plasma or urine. With fluorimetric detection and the large volume injection technique, the limit of detection in plasma is 0.5–1 ng ml^{-1}, which is sensitive enough for pharmacokinetic studies in man. The calibration graph is linear between 1 and 200 ng ml^{-1} in blood plasma, with coefficients of variation of 6.2 and 1%, respectively. In urine, the linearity range is 0.05–10 μg ml^{-1}; at the lowest concentration, the coefficient of variation is about 10%. A constant plasma/blood concentration ratio (1.25 ± 0.05) allows the measurement of drug in either fluid. In blood or plasma, alfuzosin is stable at 37°C for 24 h and at −20°C for 6 months.

As expected for reversed-phase chromatography, the retention times of alfuzosin and a few of its analogues decrease inversely with the concentration of acetonitrile in the mobile phase. However, as this concentration reaches about 75%, the retention times increase sharply. This U-shaped curve may be explained by interactions of the amino groups with silanol groups of the stationary phase.

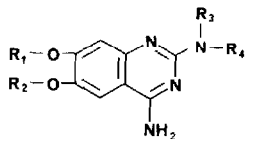
INTRODUCTION

Alfuzosin, N-{3-[(4-amino-6,7-dimethoxy-2-quinazolinyl)methylamino]propyl}tetrahydro-2-furancarboxamide hydrochloride (Fig. 1), is a new antagonist of α_1 post-synaptic adrenergic receptors, showing few myorelaxant effects[1,2]. Alfuzosin is a basic compound (pK_a = 8.13), stable at pH 1–13 and under normal conditions of temperature and light. Its highly fluorescent nature was used to develop a quantitative assay by high-performance liquid chromatography (HPLC) that would allow determinations of the drug in biological fluids (plasma, blood or urine) in the low nanogram range for clinical pharmacoketic studies.

Further, the influence of the composition of the mobile phase was investigated in order to optimize the analysis time in routine work and to gain an insight into the

* Present address: Laboratoires Lafon, 1, rue Georges Mederic, 94701 Maisons Alfort, France.

0021-9673/86/$03.50



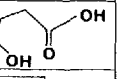
	R_1	R_2	R_3	R_4
ALFUZOSIN	CH_3	CH_3	CH_3	$-(CH_2)_3-NH-CO-$ 
A	CH_3	CH_3	CH_3	$-(CH_2)_3-NH_2$
B	CH_3	CH_3	H	H
C	CH_3	CH_3	H	CH_3
D	CH_3	CH_3	CH_3	$-(CH_2)_3-NH-CO-$ 
E	CH_3	OH	CH_3	$-(CH_2)_3-NH-CO-$ 
F	OH	CH_3	CH_3	$-(CH_2)_3-NH-CO-$ 
G	CH_3	CH_3	CH_3	$-(CH_2)_3-NH-CO-$ 
INTERNAL STANDARD	CH_3	CH_3	H	$-(CH_2)_3-N(CH_3)-CO-$ 

Fig. 1. Structures of alfuzosin, its internal standard and seven analogues.

chromatographic behaviour of alfuzosin and a few of its analogues for further work in metabolism studies.

EXPERIMENTAL

Standard and reagents

Alfuzosin and its internal standard, N-{-3-[(4-amino-6,7-dimethoxy-2-quinazolinyl)amino]propyl}-N-methyltetrahydro-2*H*-pyran-2-carboxamide hydrochloride (Fig. 1), were synthesized at the Chemistry Department of LERS (Bagneux, France). Analytical-reagent grade potassium dihydrogen phosphate and orthophosphoric acid and LiChrosolv acetonitrile, used for the mobile phase, were purchased from Merck (Darmstadt, F.R.G.). Analytical-reagent grade diethyl ether, used for extraction, was obtained from Carlo Erba (Milan, Italy).

Stock solutions

Alfuzosin (or its internal standard) was dissolved in 0.01 *M* hydrochloric acid (100 ng μl^{-1}), then standard solutions were prepared by diluting this stock solution with water as required.

Equipment

The components of the chromatographic system were an LDC Constametric II G or a Micromeritics 750 pump, connected to an automatic injector (Micromeritics 725), equipped with a 500-μl loop, a spectrofluorimetric detector (Schoeffel FS 970 or Kontron SFM 23B) and a Perkin-Elmer 56 recorder.

Chromatographic conditions

The mobile phase [acetonitrile–0.02 *M* potassium dihydrogen phosphate (pH 2.5), 3:2] was pumped at a flow-rate of 1.00 ± 0.01 ml min^{-1} through a stainless-steel column (15 cm × 4.6 mm I.D.), packed in our laboratory[3] with Spherisorb ODS, 5 μm (batch 17/49) (Phase Separations, Queensferry, U.K.). For the Schoeffel detector the excitation wavelength was set at 314 nm and the emission was cut off by a KV 370 filter. For the Kontron SFM 23 detector the excitation and emission wavelengths were 334 and 378 nm, respectively.

Optimization of extraction pH

In order to determine the optimum extraction pH, plasma samples (1 ml), spiked with 50 ng of alfuzosin and 75 ng of internal standard, were extracted with diethyl ether (7 ml) at different pH varied by adding 1 ml of buffer. A 5-ml volume of the ether phase was evaporated to dryness at 37°C under a gentle stream of nitrogen. The residue was dissolved in 450 μl of the injection solution (see below) and injected into a 100-μl calibrated loop.

Extraction procedure

In a conical tube containing 50 ng of internal standard (10 μl of a 5-μg ml^{-1} solution), the plasma or blood sample (1 ml) was adjusted to pH between 9 and 12 by adding 1 ml of 0.1 *M* sodium hydroxide solution. Alfuzosin was extracted with diethyl ether (7 ml) in a shaker for 30 min. Following centrifugation (1000 *g* for 5 min at 4°C), 6.5 ml of the upper organic phase were transferred into a second tube and evaporated to dryness at 37°C under a gentle stream of nitrogen. The residue was then dissolved in 870 μl of the injection solvent [acetonitrile–0.02 *M* potassium dihydrogen phosphate (pH 2.5), 1:9][4,5] and injected on to the column by means of an automatic injector.

For the analysis of urine samples, 100 μl of urine were diluted to 1 ml with water, then alfuzosin was determined as described above for plasma samples and quantified by the internal standard technique, using the peak-height ratio method.

Plasma/blood concentration ratio

For the determination of the plasma/blood concentration ratio, blood was sampled from two healthy subjects 1 and 9 h after a single oral dose of alfuzosin (10 mg). A fraction of each sample was centrifuged at 1000 *g* for 20 min, then both plasma and blood fractions were stored at −20°C until taken for analysis.

RESULTS

Extraction

The maximum extraction yield was obtained at pH > 6 (Fig. 2); from pH 6 to 12 the recovery of alfuzosin was about 100%. Therefore, the plasma was adjusted to a pH between 9 and 12. In our experience, endogenous compounds are usually less extracted at high pH, thus giving a cleaner chromatogram. As the recovery of alfuzosin from alkalinized plasma was complete after extraction with diethyl ether, no other extraction solvent was investigated.

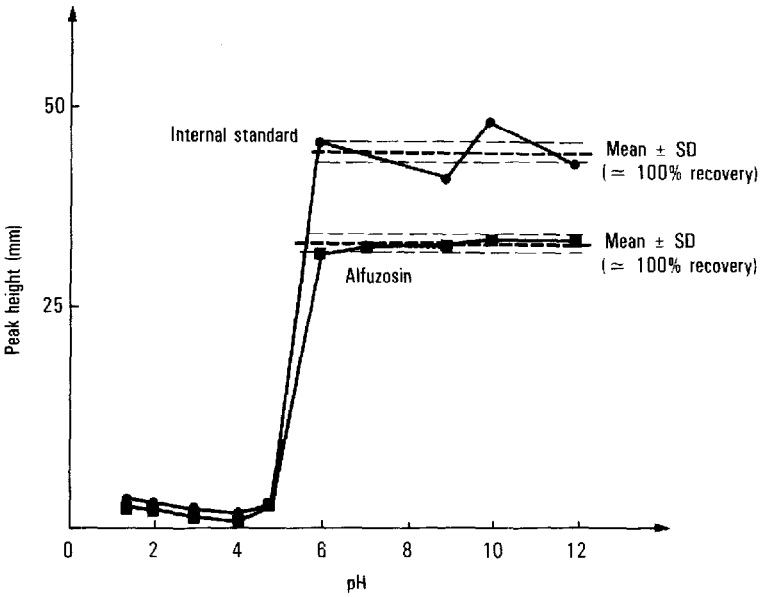


Fig. 2. Effect of pH on the extraction of alfuzosin and its internal standard.

Quantitation

Chromatograms obtained from blood, either blank or spiked with alfuzosin and its internal standard, and from a patient sample are shown in Fig. 3A, B and C, respectively. Fig. 4 shows the chromatogram of a spiked urine sample. The peaks

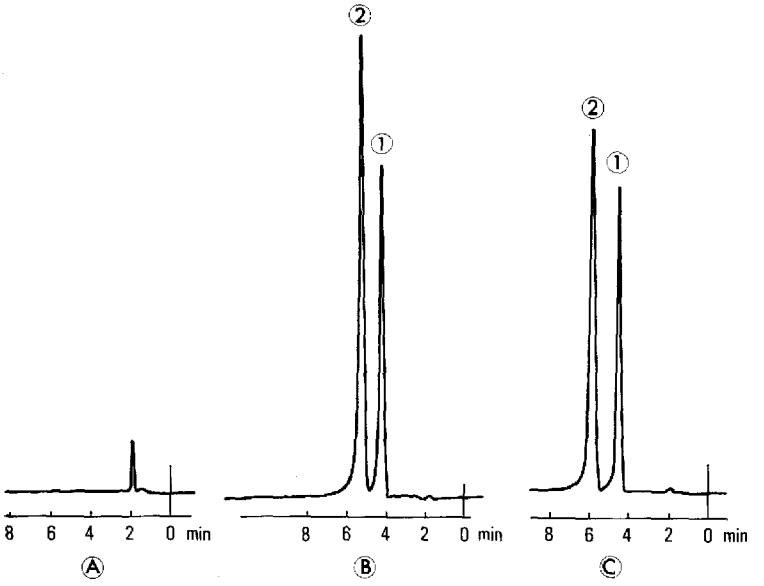


Fig. 3. Typical chromatograms of human blood extracts for alfuzosin determinations. (A) Blank blood; (B) spiked blood (25 ng ml^{-1} of alfuzosin); (C) blood sample following oral administration of 5 mg of alfuzosin. 1 = Alfuzosin; 2 = internal standard. For chromatographic conditions, see text.

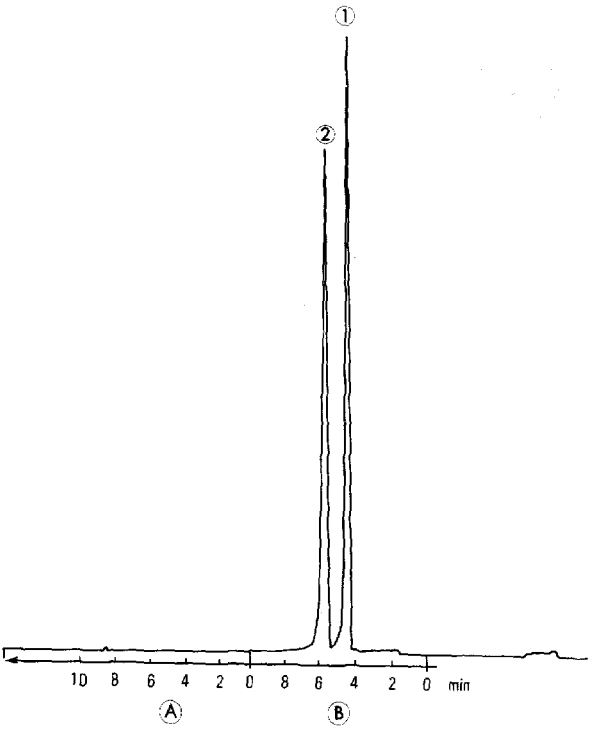


Fig. 4. Typical chromatograms of human urine for alfuzosin determinations. (A) Blank urine; (B) spiked urine (0.5 μg ml^{-1} of alfuzosin). 1 = Alfuzosin; 2 = internal standard. For chromatographic conditions, see text.

corresponding to alfuzosin and its internal standard, under the conditions previously described, were well resolved and no endogenous compounds, extracted from biological samples, interfered with these peaks. The retention times were 4.4 and 5.6 min for alfuzosin and its internal standard, respectively, with reduced heights equivalent to a theoretical plate of 5.9 and 4.6, respectively (mean particle diameter of the stationary phase = 5.7 μm). Calibration graphs obtained after extraction from blood (or plasma) were linear between 0.5 and 100 ng ml^{-1} (r = 0.9999) and between 0.05 and 10 μg ml^{-1} after urine extraction (r = 0.999). Four calibration graphs obtained with the SFM 23 detector showed that the method was linear up to 200 ng ml^{-1}.

The precision of the method was determined by analysing blood (or plasma) and urine samples spiked with the alfuzosin used for the preparation of the daily calibration graph. These results are shown in Table I. The coefficient of variation ranged from 6.2% for 1 ng ml^{-1} to 0.7% for 100 ng ml^{-1} blood. The extreme value was 18.6% at 0.5 ng ml^{-1}. For urine the coefficients of variation were 10% and 0.8% at 0.05 and 5 μg ml^{-1}, respectively.

Plasma/blood concentration ratio

The concentrations in blood and plasma were measured and the mean plasma/blood concentration ratio was found to be 1.25 ± 0.05, which indicates that 69

TABLE I

REPRODUCTIBILITY OVER EIGHTEEN MONTHS OF THE HPLC METHOD FOR THE MEASUREMENT OF ALFUZOSIN IN BIOLOGICAL FLUIDS AT DIFFERENT CONCENTRATIONS

Sample	*Alfuzosin concentration (ng ml^{-1})*	*Number of measurements*	*Concentrations obtained*		*Coefficient of variation (%)*
			Mean (ng ml^{-1})	*Standard error (ng ml^{-1})*	
Blood	0.5	8	0.55	0.036	18.6
	1.0	8	1.04	0.023	6.2
	2.5	12	2.47	0.036	6.0
	5.0	13	4.96	0.047	3.4
	10	13	9.93	0.091	3.3
	25	13	24.7	0.184	2.7
	50	9	50.3	0.274	1.6
	75	4	75.6	0.915	0.6
	100	4	99.5	0.359	0.7
	200	4	200	2.06	1.0
Urine	50	8	50	5	10.2
	500	6	496	18	3.6
	5000	7	5020	41	0.8

± 7% of the blood alfuzosin was in the plasma (Table II). Hence the alfuzosin measurement can be performed with either plasma or blood samples.

Stability of alfuzosin

In order to determine the stability of alfuzosin and its internal standard in biological samples, plasma or blood standards (1 ml), spiked with 50 ng of alfuzosin and 75 ng of internal standard, were incubated at 37°C for various periods up to 24 h and then extracted with diethyl ether. The residue was treated and chromatographed as described above. The results indicated that plasma or blood alfuzosin samples were stable at 37°C for 24 h. Spiked blood samples, adjusted to pH 9–12

TABLE II

PLASMA/BLOOD CONCENTRATION RATIO (C_p/C_s) OF ALFUZOSIN IN HEALTHY VOLUNTEERS

Subject	*Sampling time (h)*	*Plasma concentration (ng ml^{-1})*	*Blood concentration (ng ml^{-1})*	$\frac{C_p}{C_s}$
1	9.30	17	14	1.21
	18.00	1.9	1.5	1.27
2	10.30	7.8	5.7	1.37
	18.00	3.6	3.1	1.16
			Mean	1.25 ± 0.05 (coefficient of variation, 7.2%)

with 0.1 *M* sodium hydroxide, were extracted with diethyl ether for 30, 60, 90, 120 and 150 min. No degradation of either alfuzosin or its internal standard occured within 120 min (which is four times the normal extraction time), but a 14% loss of the two products was observed after shaking for 150 min.

Blood samples, spiked with 2 and 60 ng ml^{-1} of alfuzosin were frozen at −20°C and analysed periodically during a 6 month period. No degradation of the product was observed during this period, allowing storage of alfuzosin samples at −20°C up to six months prior to analysis.

Effect of the mobile phase

The influence of the composition of the mobile phase on the retention times of alfuzosin and its analogues was investigated in order to optimize their chromatographic separation. The results of these studies can be summarized as follows: the retention time of alfuzosin and related compounds decreased with pH and inversely with the molarity of the aqueous fraction of the mobile phase.

An unusual dependence of the retention time (t_R) of alfuzosin and its analogues on the percentage of acetonitrile in the mobile phase was observed (Fig. 5). It was characterized by a U-shaped curve, in which the retention times for all compounds

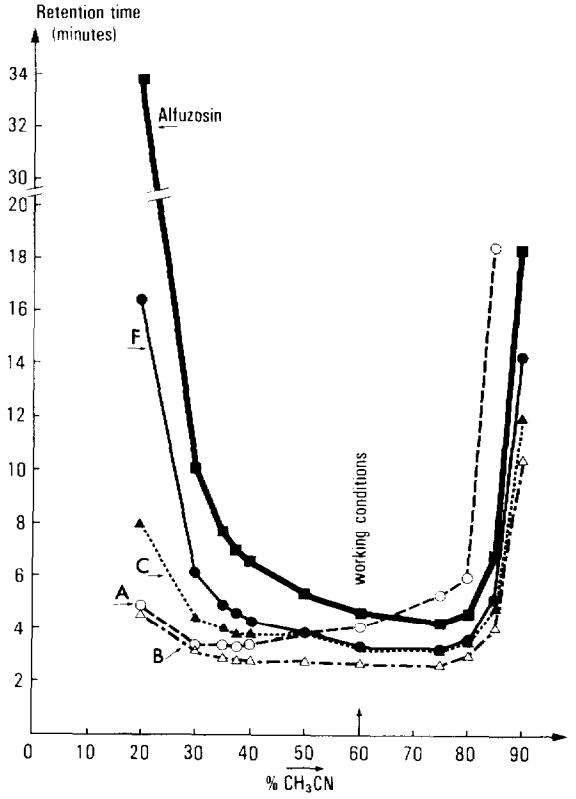


Fig. 5. Retention times of alfuzosin and some analogues *versus* the percentage of acetonitrile in the mobile phase (the aqueous part of the mobile phase was 0.02 *M* potassium dihydrogen phosphate at pH 2.5).

increased at low and high percentages of acetonitrile. The retention times decreased with increasing concentration of acetonitrile until it reached about 75% and then increased sharply with further increase acetonitrile concentration. For compound A (Fig. 1), which is more polar than the others, this phenomenon was observed with a lower concentration of acetonitrile (about 37.5%).

Fig. 5 indicates that with 60% of acetonitrile in the mobile phase, the time necessary to separate the compounds listed in Fig. 1 is minimal. The retention time of alfuzosin decreased slightly until the acetonitrile concentration reached 70%. However, under these conditions, compound A was eluted after alfuzosin. As variations in the mobile phase composition around 60% of acetonitrile have little effect on the retention time of alfuzosin, this concentration was chosen for routine analysis.

Separation of alfuzosin analogues

From the results shown in Fig. 5, it was obvious that the chromatographic system developed for the routine determination of alfuzosin is not convenient for the separation of the compounds listed in Fig. 1. By increasing the polarity of the stationary phase (using Spherisorb-hexyl, 5 μm), these compounds can be resolved with

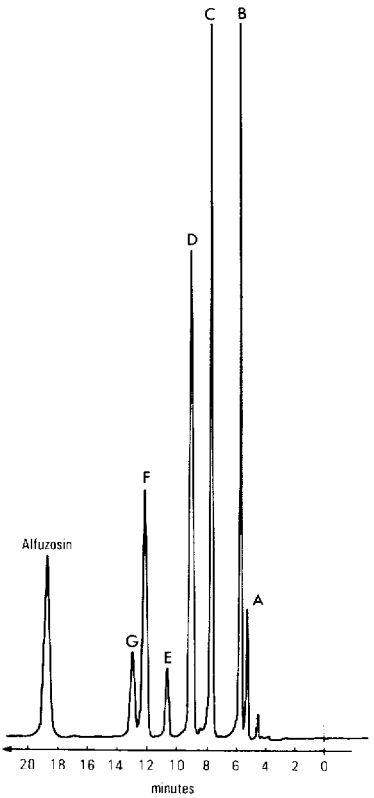


Fig. 6. Separation of alfuzosin and seven of its analogues on a stainless-steel column (150 × 4.6 mm I.D.) packed with Spherisorb-hexyl (5 μm) as the stationary phase. Flow-rate, 1 ml min^{-1}; mobile phase, acetonitrile–0.02 *M* potassium dihydrogen phosphate (pH 2.5), 1:4; fluorimetric detector, $\lambda_{exc.}$ = 314 nm, emission cut off with a KV 370 filter.

the mobile phase acetonitrile–0.02 *M* potassium dihydrogen phosphate (pH 2.5), 1:4 (Fig. 6).

DISCUSSION

In the method described, the injection was performed automatically, and with this particular injector about 37% of the sample was lost, decreasing the sensitivity by this factor. In order to obtain the highest sensitivity, either more than 2 ml of plasma (or blood) should be extracted or all of the extract should be injected (manually or with an automatic injector of a different type). This would change the sensitivity to 0.5 ng ml^{-1}, the coefficient of variation being 18.6% at this level. The large-volume injection technique[4,5] is particularly useful, as at least 400 μl are needed to dissolve the extract. Smaller volumes tend to give less precise results.

The observed increased in retention time with increasing acetonitrile concentration may be explained by a dual interaction phenomenon, hydrophobic interaction with the bonded stationary phase and hydrogen bonding between the amine and the silanol groups. When the mobile phase is much more polar than the stationary phase, true reversed-phase chromatography is observed, but as the relative polarity of the mobile phase decreases (with respect to the stationary phase), interactions between amino groups of the solute and silanol groups prevail, thus producing a normal-phase behaviour.

Similar results have been observed with N,N-diethylaniline on a Zorbax CN column[6], with crown ethers[7,8], with chloramphenicol intermediates[9], with aromatic amines[10,11] and with various protonated amines[12,13].

REFERENCES

1 I. Cavero, F. Lefevre-Borg and P. Manoury, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, 43 (1984) 3, abstract No. 2627.
2 I. Cavero, F. Lefevre-Borg and P. Manoury, *Br. J. Pharmacol.*, 81, Suppl. (1984) 13 P.
3 M. Broquaire, *J. Chromatogr.*, 170 (1979) 43.
4 M. Broquaire and P. R. Guinebault, *J. Liq. Chromatogr.*, 4 (1981) 2039.
5 P. R. Guinebault and M. Broquaire, *J. Chromatogr.*, 217 (1981) 509.
6 L. C. Sander and L. R. Field, *Anal. Chem.*, 52 (1980) 2009.
7 A. Nahum and C. Horváth, *J. Chromatogr.*, 203 (1981) 53.
8 K. E. Bij, C. Horváth, W. R. Melander and A. Nahum, *J. Chromatogr.*, 203 (1981) 65.
9 Z. Varga-Puchony and G. Vigh, *J. Chromatogr.*, 257 (1983) 380.
10 E. Papp and G. Vigh, *J. Chromatogr.*, 259 (1983) 49.
11 E. Papp and G. Vigh, *J. Chromatogr.*, 282 (1983) 59.
12 N. El Tayar, H. Van de Waterbend and B. Testa, *J. Chromatogr.*, 320 (1985) 293.
13 N. El Tayar, H. Van de Waterbend and B. Testa, *J. Chromatogr.*, 320 (1985) 305.