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Selective, sensitive and rapid liquid chromatography–tandem mass spectrometry method for the determination of alfuzosin in human plasma

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Abstract

A selective, sensitive and rapid liquid chromatography–tandem mass spectrometry method for the determination of alfuzosin in plasma was developed. A PE Sciex API 2000 triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode, using TurboIonSpray with positive ionisation was used. Using prazosin as an internal standard, liquid–liquid extraction was followed by C_{18} reversed-phase liquid chromatography and tandem mass spectrometry. The mean recovery for alfuzosin was 82.9% with a lower limit of quantification set at 0.298 ng/ml, the calibration range being between 0.298 and 38.1 ng/ml. This assay method makes use of the increased sensitivity and selectivity of tandem mass spectrometric (MS–MS) detection to allow for a more rapid (extraction and chromatography) and selective method for the determination of alfuzosin in human plasma than has previously been described. The assay method was used to quantify alfuzosin in human plasma samples generated in a multiple-dose (5 mg bd.) study at steady state.
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1. Introduction

Alfuzosin, *N*-{3-[(4-amino-6,7-dimethoxy-2-quinazolinyl) methylamino] propyl} tetrahydro - 2 - furancarboxamide hydrochloride, is an antagonist of α_1 post-synaptic adrenergic receptors, showing some myorelaxant effects [1,2]. Alfuzosin is a basic compound with a pK_a value of 8.13 and is stable under normal conditions of temperature and light [3].

Several methods have been described for the determination of alfuzosin in plasma. The most widely used methods involve high-performance liquid chromatography (HPLC) with fluorimetric and luminescence detection, achieving lower limits of quantification (LLOQ) of around 1 ng/ml [3–7]. Carlucci et al. described a high-performance liquid chromatography method using a column-switching procedure without extraction to isolate the drug from the biological matrix. Their method was linear from 2 to 150 ng/ml [7]. Krstulovic et al. and Rouchouse et al. developed assay methods for the determination of the enantiomers of alfuzosin [4,5]. Guinebault et al. used a liquid–liquid extraction and large volume injection technique for the quantitation of alfuzosin in biological fluids [3].

The aim of this study was to develop and validate

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a more selective and sensitive assay method than previously described with minimal sample preparation and short chromatography time. This new method makes use of a tandem mass spectrometer in conjunction with liquid chromatography to increase the selectivity, which allows for more rapid chromatography and sample cleanup and is well suited for pharmacokinetic studies involving large numbers of samples.

2. Experimental

2.1. Materials and chemicals

A Supelco Discovery C_{18} 5 μm, 2.1×150 mm column (Supelco, Bellefonte, PA, USA) was used for separation at a flow-rate of 0.2 ml/min and injecting 10 μl onto the column. The mobile phase was delivered by a Perkin-Elmer series 200 Micropump (Perkin-Elmer, Foster City, CA, USA) and the samples injected by an Agilent Series 1100 autosampler (Agilent, Palo Alto, CA, USA). Detection was performed by an Applied Biosystems API-2000 detector (Applied Biosystems, Ontario, Canada) fitted with a TurboIonSpray source.

Methanol and acetonitrile (Burdick and Jackson, High Purity) were obtained from Baxter (USA). Anhydrous sodium carbonate and sodium bicarbonate were obtained from Fluka (Buchs, Switzerland). Formic acid (BDH, UK) was used without further purification. *tert*.-Butyl methyl ether was obtained from Aldrich (USA). Water was purified by a Millipore Elix 5 reverse osmosis and a Milli-Q (Millipore) Gradient A10 polishing system (Millipore, Bedford, MA, USA). Alfuzosin ($C_{19}H_{27}N_5O_4$) was supplied by Heumann. Prazosin was obtained from the FARMOVS-PAREXEL BSD pure substance reference library.

2.2. Preparation of standards and quality control samples

Calibration standards (STD) were prepared by dissolving alfuzosin in methanol to obtain a stock solution. By spiking an aliquot of this stock solution into a pool of blank human plasma and by serially diluting it with blank human plasma (1:1, v/v) seven times, a calibration standard range between 38.1 and 0.298 ng/ml was prepared. Similarly, quality control standards (QC) were prepared (using the same methodology) spanning a range between 32.3 and 0.391 ng/ml. Sufficient calibration standards and quality controls were prepared to validate the method and assay all the study samples. Aliquots of the standards and quality controls were stored together with the study samples at −20 °C until used for sample processing.

2.3. Extraction procedure

Plasma samples (500 μl) were pipetted into 10 ml amber glass ampoules. Sodium carbonate buffer (500 μl, 0.1 *M*, pH 10.5), internal standard solution (250 μl, 60 ng prazosin/ml water) and *tert*.-butyl methyl ether (5 ml) were added and the samples vortexed for 60 s. After centrifugation at 1300 *g* for 60 s, the aqueous phase was frozen in an alcohol freezing bath at −25 °C and the organic phase then decanted into 5 ml amber glass ampoules. Formic acid solution (250 μl, 2%) was added and the samples were vortexed for 60 s and centrifuged at 1300 *g* for 60 s. The aqueous phase was frozen in an alcohol freezing bath at −25 °C and the organic phase discarded. The residual organic phase that remained in the aqueous phase was evaporated under a gentle stream of nitrogen at 45 °C for 2 min. The final extract was transferred to an autosampler vial containing a micro glass insert, and 10 μl injected onto the HPLC column.

2.4. Liquid chromatography

Chromatography was carried out at ambient temperature with a mobile phase consisting of acetonitrile, methanol and aqueous formic acid (0.2%), (20:20:60, v/v) at a flow-rate of 0.2 ml/min. All chromatographic solvents were degassed with helium before use.

2.5. Mass spectrometry

Electrospray ionisation (ESI) was performed in the positive ion mode with nitrogen as the nebulizing, turbo spray and curtain gas with the optimum values set at 70, 90 and 50 (respective arbitrary

values). The heated nebulizer temperature was set at 400 °C. The instrument response was optimised for alfuzosin by infusing a solution of the drug dissolved in mobile phase at a constant flow. The same methodology was used to optimise the response of the instrument for the internal standard (I.S.). The pause time was set at 5 ms and the dwell time at 150 ms. The collision gas (N_2) was set at 3 (arbitrary value).

The Applied Biosystems API 2000 LC–MS–MS detector was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ion *m/z* 390.2 to the product ion *m/z* 71.2 for alfuzosin and also monitoring the transition of the protonated molecular ion *m/z* 384.2 to the product ion *m/z* 95.0 for the I.S. Presented in Fig. 1 is the product ion mass spectrum of protonated alfuzosin showing the [M+1] ion (*m/z* 390.2, molecular structure given) and the principal product ion at *m/z* 71.2 formed by collision-induced dissociation (CID). Presented in Fig. 2 is the product ion mass spectrum of protonated prazosin showing the [M+1] ion (*m/z* 384.2, molecular structure given) and the principal product ion at *m/z* 95 formed by CID. Plausible fragmentation patterns presented in Figs. 1 and 2 are suggested but not proven.

The instrument was interfaced with a computer running Applied Biosystems Analyst version 1.1 software.

2.6. Validation

The method was validated by analysing plasma quality control samples six times at six different alfuzosin concentrations, i.e. 32.3, 16.1, 8.06, 4.04, 0.783 and 0.391 ng/ml, to determine the accuracy and precision of the method. The quality control values were interpolated from a calibration curve containing eight different concentrations spanning the concentration range of 38.1 to 0.298 ng/ml. Calibration graphs were constructed using a Wagner regression of the drug peak-area ratios of the analyte to the internal standard versus nominal drug concentrations. Several regression types were tested and the Wagner regression [log transformed quadratic regression curve; $\ln Y = a(\ln X)^2 + b(\ln X) + C$] was found to be most suited for the specific range (Fig. 3). As we often optimise our systems for the lowest possible LLOQ we often find a curving at the higher

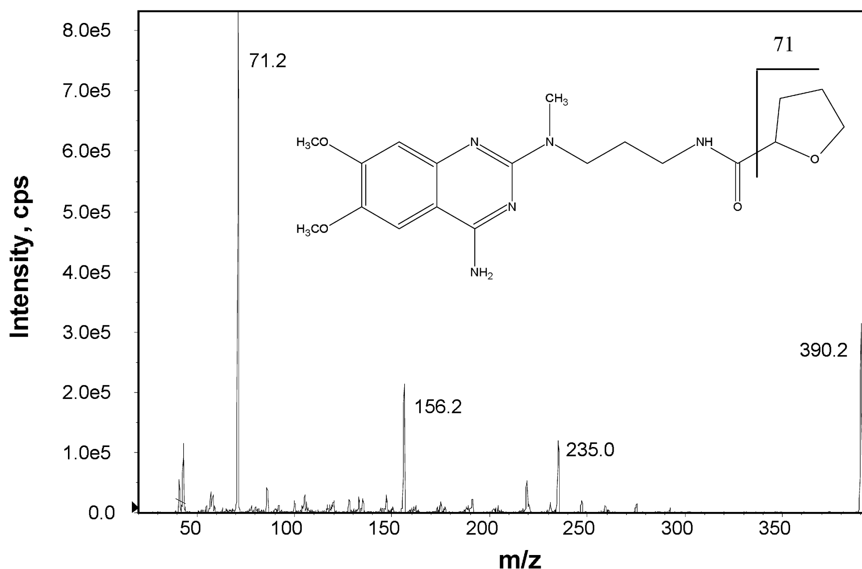

Fig. 1. Product ion mass spectrum of protonated alfuzosin showing the [M+1] ion (*m/z* 390.2, molecular structure given) and the principal product ion at *m/z* 71.2 formed by CID.

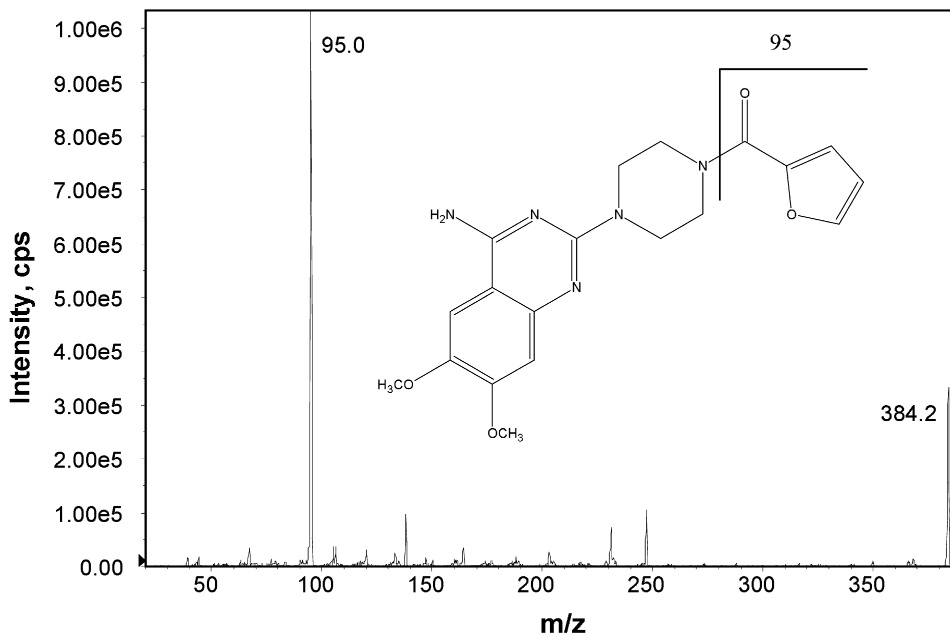

Fig. 2. Product ion mass spectrum of protonated prazosin showing the [M+1] ion (m/z 384.2, molecular structure given) and the principal product ion at m/z 95.0 formed by CID.

and lower concentration ranges on the MS systems thereby making an atypical regression like the Wagner regression more suitable than other simpler regressions like linear or weighted linear.

The matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionisation) was investigated using the procedure described by Matuszewski et al. [8].

Absolute recoveries of the analyte were determined in triplicate in normal plasma by extracting drug-free plasma samples spiked with alfuzosin. Recoveries were calculated by comparison of the analyte peak-areas of the extracted samples with those of the non-extracted system performance verification standard mixtures (prepared in the injection vehicle) representing 100% recovery.

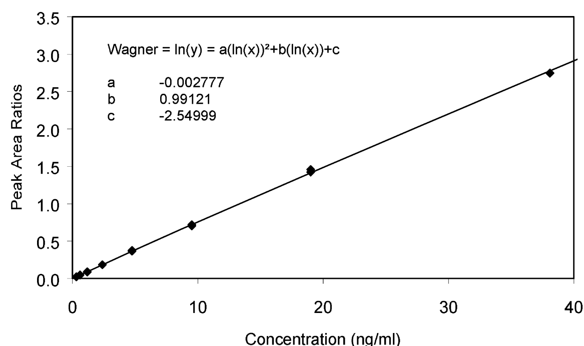

Fig. 3. Calibration curve based on peak-area ratios as obtained from the intra-batch validation.

3. Results and discussion

The mean absolute recoveries of alfuzosin determined in triplicate at 16.1, 4.04 and 0.783 ng/ml were 81.3, 82.7 and 84.8%, respectively. The mean absolute recovery of prazosin was 77.1%.

No significant matrix effect for alfuzosin was observed for 10 different plasma pools tested. The peak areas of the 10 reconstituted samples had a coefficient of variation of 3.6% for alfuzosin and 2.8% for the I.S., indicating that the extracts were ‘‘clean’’ with no co-eluting compounds influencing the ionisation of the analyte and I.S.

The much higher selectivity of MS–MS detection

allowed the development of a very specific and rapid method for the determination of alfuzosin in plasma.

The LLOQ, defined as that concentration of alfuzosin which can still be determined with acceptable precision (C.V.% <20) and accuracy (bias <20%), was found to be 0.298 ng/ml (concentration of the lowest calibration standard) with a signal-to-noise ratio of 20. Results of the intra-batch and inter-batch validation assays presented in Table 1 indicate a valid calibration range of 0.298–38.1 ng/ml for alfuzosin. The calibration parameters for 16 batches are summarised in Table 2, indicating a reproducible and reliable method. The quality control values found during the processing of 16 batches of study samples over a period of 16 days are summarised in Table 3 and attest to the excellent inter-day performance of the assay method.

On-instrument stability was inferred from stability samples that were prepared and included in the first intra-day validation batch. No significant degradation

Table 3
Summary of the back-calculated quality control standard concentrations of alfuzosin (16 batches) showing the repeatability of the method (inter-day variation)

Nominal (ng/ml)	0.391	0.783	4.04	8.06	16.1
Mean	0.407	0.808	3.986	8.243	15.897
C.V.%	5.8	6.4	5.9	6.9	4.5
N	31	32	31	32	32
% Nom	104	103.2	98.7	102.3	98.7

Table 1
Summary of intra- and inter-batch quality control results

Validation batch	Nominal replicates	32.3 ng/ml	16.1 ng/ml	8.06 ng/ml	4.04 ng/ml	0.783 ng/ml	0.391 ng/ml
Intra-batch validation	1	32.78	15.45	8.09	4.15	0.81	0.39
	2	32.52	16.98	8.32	4.29	0.78	0.38
	3	31.74	16.38	8.11	4.13	0.79	0.42
	4	31.76	17.15	8.41	4.18	0.80	0.40
	5	32.53	17.53	8.58	4.33	0.81	0.37
	6	35.07	16.61	8.15	3.73	0.76	0.38
Inter-batch validation 1	1	31.33	15.64	7.70	4.10	0.74	0.40
	2	31.97	15.71	7.82	3.87	0.78	0.42
	3	32.45	16.27	8.10	4.26	0.81	0.38
	4	31.89	16.06	8.24	4.14	0.77	0.36
	5	32.51	15.80	7.98	4.05	0.80	0.40
	6	33.37	16.01	8.10	4.16	0.78	0.43
Inter-batch validation 2	1	33.99	15.96	7.44	4.16	0.83	0.40
	2	32.16	15.32	8.22	4.18	0.87	0.45
	3	34.79	16.23	8.07	4.09	0.82	0.43
	4	32.20	16.27	8.33	4.01	0.84	0.42
	5	33.95	16.78	8.69	4.10	0.85	0.42
	6	32.22	15.52	7.88	3.97	0.83	0.43
	Mean	32.73	16.20	8.12	4.11	0.80	0.41
	% Nom	101.3	100.6	100.8	101.6	102.5	103.6
	C.V.%	3.2	3.7	3.6	3.4	4.0	5.9

Table 2
Summary of the calibration parameters as obtained from 16 batches

	r	r^2	a	b	c
Mean	0.9994420	0.9988843	−0.0167805	1.0144447	−2.9108783
SD	0.00019136	0.00038251	0.008068818	0.02945442	0.12543491
C.V.%	0.0	0.0	−48.1	2.9	−4.3

could be detected in the samples (cooled at 5 °C on the autosampler while awaiting injection) left on the autosampler for at least 28 h.

Due to the high specificity of MS–MS detection, no interfering or late-eluting peaks were found when chromatographing blank plasma extracts from six different sources.

Various liquid–liquid extraction procedures were tested which included different organic solvents [a mixture of dichloromethane and diethyl ether (3:4, v/v), diethyl ether and *tert*.-butyl methyl ether], buffers [sodium hydroxide solutions (0.1 and 1 *M*) and carbonate buffers (0.1 *M*; pH 9, 9.5, 10, 10.5 and 11)] and back-extracting solutions [formic acid and acetic acid solutions (1 and 2%)]. The best results were obtained with *tert*.-butyl methyl ether, carbonate buffer (0.1 *M*, pH 10.5) and 2% formic acid solution. Average recoveries above 77% were found for both analytes.

Mobile phase solutions with varying concentrations of formic acid were tested for optimum ionisation of the analytes and it was found that 0.2% formic acid gave the best result. The best resolution and chromatographic peak shape were obtained with a mobile phase consisting of acetonitrile, methanol and 0.2% formic acid (20:20:60, v/v).

Prazosin is structurally related to alfuzosin and was tested as a possible internal standard. Ionisation, retention and extraction characteristics were found to be similar to that of alfuzosin. The retention times for alfuzosin (I) and prazosin (II) were 2.5 and 2.6 min, respectively (Fig. 4). The total chromatography run time of 4 min made it possible to analyse a large number of samples in a batch. Fig. 5 shows a representative chromatogram obtained of an alfuzosin (I) calibration standard at a concentration of 0.298 ng/ml in plasma (the LLOQ) and of a study sample (II) at the late elimination phase (24 h) of the pharmacokinetic profile for the analyte.

This assay method was employed to analyse plasma samples containing alfuzosin obtained during a multiple oral dose study of 5 mg alfuzosin in 40 healthy volunteers. Concentration vs. time profiles were constructed for up to 24 h after the last dose (Fig. 6).

4. Conclusion

A rapid, sensitive and highly selective method for the determination of alfuzosin in plasma was developed, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. This newly developed assay method was

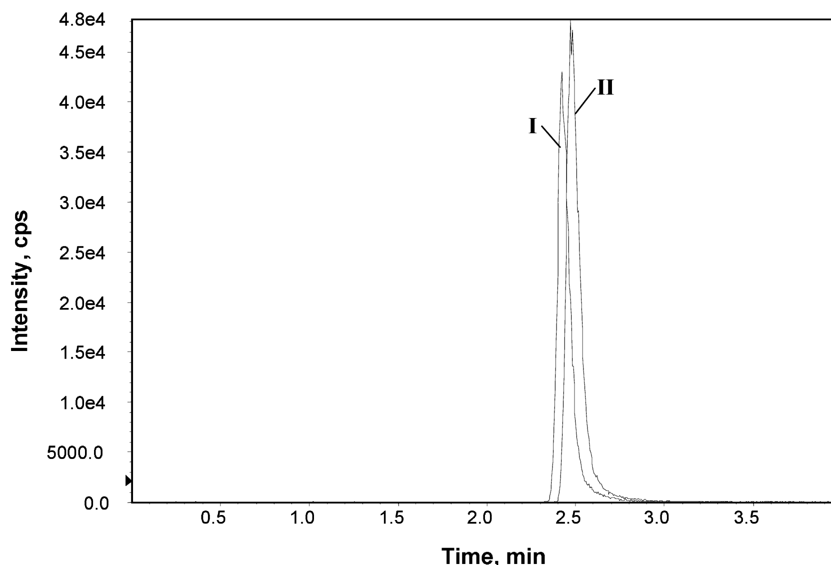

Fig. 4. Representative chromatogram illustrating the retention times of alfuzosin (I) and of the internal standard, prazosin (II).

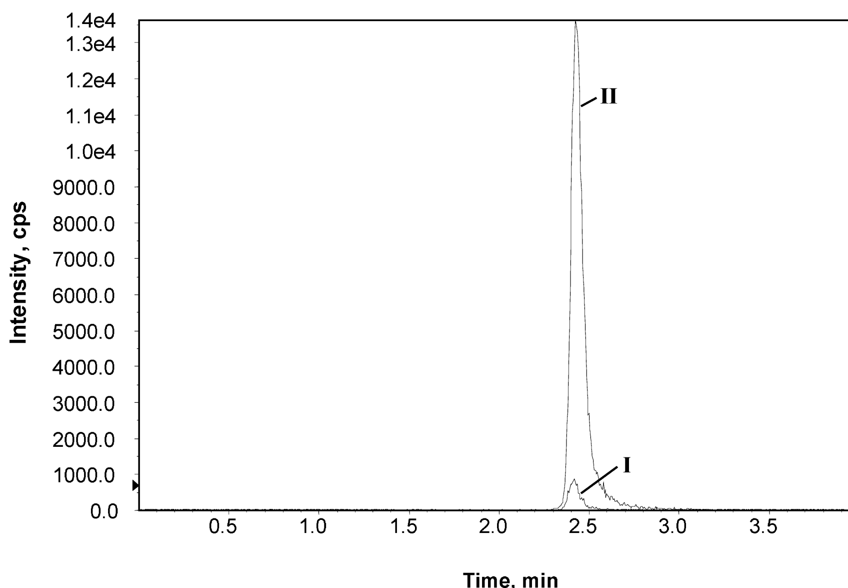

Fig. 5. High-performance liquid chromatograms of the calibration standard at the limit of quantification (I) containing 0.298 ng/ml alfuzosin and of a study sample (II) at the late elimination phase (24 h after dose) of the pharmacokinetic profile for the analyte.

used in a clinical study in which 40 healthy volunteers were each given 5 mg multiple oral doses of alfuzosin. The assay method is more selective than previously described methods and allows for a much higher sample throughput due to the short chromatography time (4 min) and simple sample preparation. Robust LC–MS–MS instrument performance was observed, with only slight variations in the instrument response within batches. It was not necessary to clean the ion source during the entire

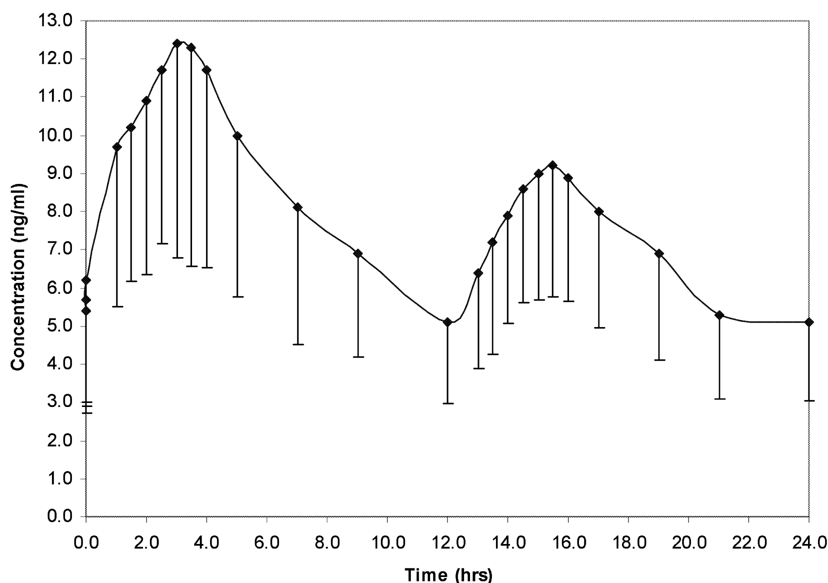

Fig. 6. Alfuzosin plasma concentrations vs. time profile as obtained after a multiple 5 mg oral dose of alfuzosin (average of 40 subjects).

study and a single analytical column was used to chromatograph more than about 2400 extracts without significant deterioration of the column performance. This attests to the clean nature of the final extracts injected onto the column. This method is an excellent analytical option for rapid quantification of alfuzosin in human plasma.

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