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Enantioselective determination of terazosin in human plasma by normal phase high-performance liquid chromatography–electrospray mass spectrometry

A. Paul Zavitsanos[1,*], Tanja Alebic-Kolbah[2]

[1]*Hewlett–Packard (Canada) Ltd., Chemical Analysis Division, 5150 Spectrum Way, Mississauga, Ontario, L4W 5G1, Canada*
[2]*Biovail Corp. Contract Research Division, Bioanalytical Laboratory, 460 Comstock Road, Toronto, Ontario, M1L 4S4, Canada*

Abstract

A sensitive and selective analytical method for the enantioselective determination of terazosin in human plasma has been developed. The chromatography is based on the normal-phase chiral separation utilizing the analogue prazosin as the internal standard. The detection involves the direct introduction of the normal-phase eluent into an electrospray source followed by mass-selective detection. No pre-column derivatization was required prior to analysis. The chiral stationary phase used was Chiralpak AD 100 mm×2.1 mm I.D. (10 μm particle size). The method was utilized to determine the concentrations of terazosin enantiomers in human subjects following a 5 mg single oral dose. Results were compared with those from an enantioselective HPLC–fluorescence method from the same subject plasma samples. The LC–MS results confirm with confidence that the two terazosin enantiomers have different elimination profiles. © 1998 Elsevier Science B.V.

Keywords: Enantiomer separation; Terazosin; Prazosin

1. Introduction

Terazosin (Fig. 1) is a selective α_1-adrenoreceptor antagonist used as an antihypertensive vasodilator [1].The saturated furan ring provides the molecule with one chiral center and therefore two enantiomers. There is presently a heightened regulatory interest in the pharmacokinetics of the individual enantiomers of chiral drugs [2]. A successful bioanalytical method for the enantiomers of a drug substance requires a simple rapid separation of the enantiomers and the sensitive detection of the separated components. Chiral chromatography with atmospheric pressure ionization mass spectrometry (API-MS) has provided an elegant solution to this challenge. Previous efforts have demonstrated an approach that allows direct introduction of the effluent from a highly effective normal phase chiral column into an atmospheric pressure chemical ionization (APCI) triple quadrupole instrument [3]. It had some advantages over previously successful approaches to the problem (references cited in [3]). This work is a step in the continuing effort to refine the technique, and to make it more applicable to a larger group of practitioners. While efforts with APCI triple quadrupole instruments have given promising results in this field [3], there is interest in seeing if the technique could be applied to a more general ionization mode and a less expensive and more generally available single stage quadrupole spectrometer.

There are no published methods for the determination of terazosin enantiomers in human plasma. The work by Patterson [4] describes a method for the achiral determination of terazosin with a lower limit

*Corresponding author.

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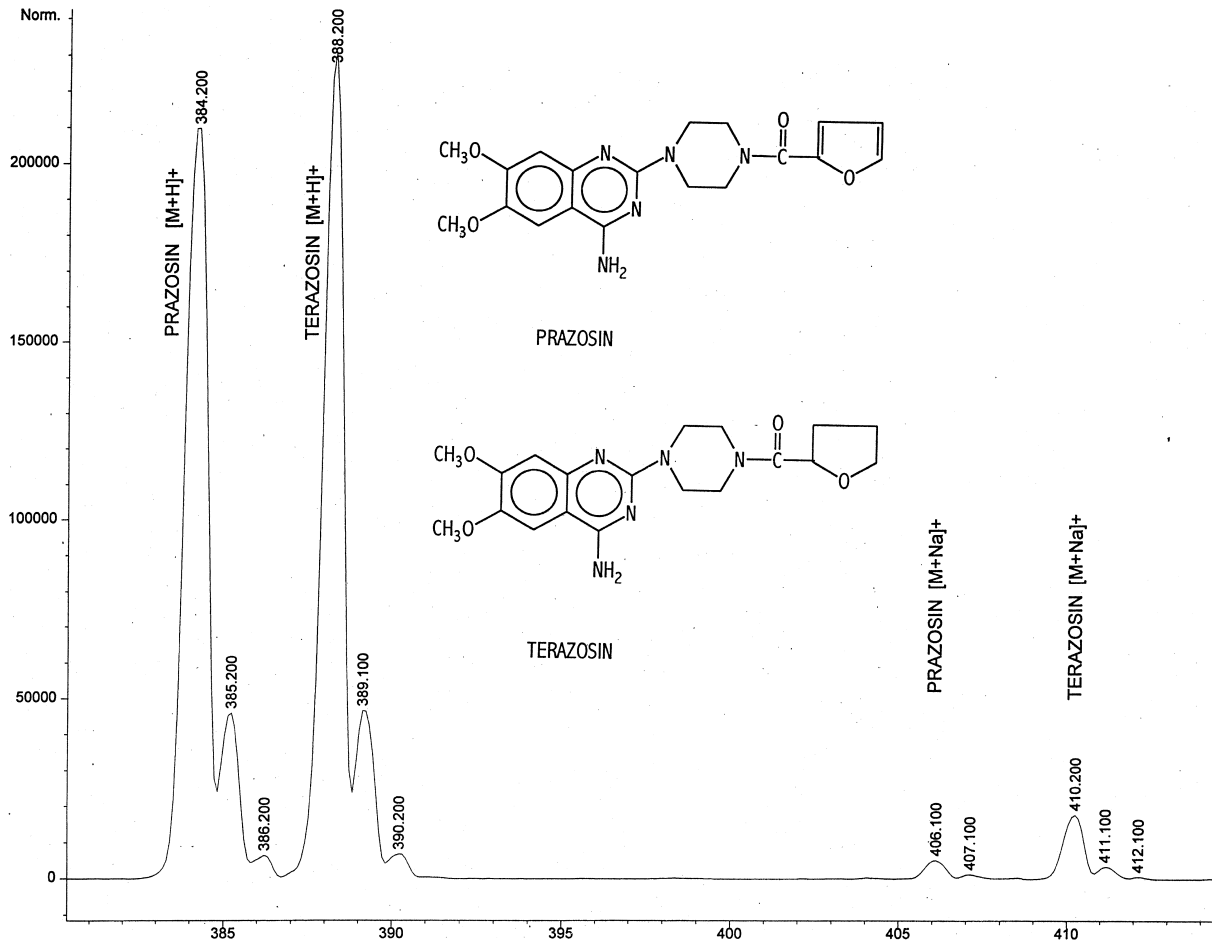

Fig. 1. Spectrum of the protonated molecule ion region for terazosin and prazosin (used as internal standard) and structures for both compounds.

of quantitation (LLOQ) of 1 ng/ml. The present paper describes a method for the bioanalysis of a chiral compound, terazosin, by electrospray ionization mass spectrometry (ESI-MS) and allows full and sensitive quantitation of both enantiomers, with an LLOQ of 62.5 pg/ml, in a human plasma matrix. There is no derivatization of the target compound required for the chiral separation. This work also describes a HPLC–fluorescence method for the enantioselective determination of terazosin. Both the ESI-MS and HPLC–fluorescence methods were applied to the same subject plasma samples and the experimental results obtained by ESI-MS are compared with those of the HPLC–fluorescence method.

The comparison shows that while there is excellent correlation between the plasma levels determined by the two techniques, the higher sensitivity of the ESI-MS technique allows enantioselective determination at lower plasma levels. Consequently, useful information about enantiomeric ratios could be determined from subject plasma samples taken at later time points post-dose.

2. Experimental

2.1. Reagents and standards preparation

Stock solutions were made by weighing reference standard *rac*-terazosin or prazosin (Fig. 1) (a

terazosin analogue used as internal standard) into volumetric glassware and making to volume with ethanol–2-propanol (95:5, v/v). A solution of prazosin internal standard working solution was made to a concentration of 200 ng/ml by dilution of an aliquot of the prazosin stock solution with water. The HPLC–fluorescence experiments used (+)-glaucine as the internal standard. A stock solution was made by weighing reference standard (+)-glaucine into volumetric glassware and making to volume with ethanol–2-propanol (95:5, v/v). A solution of (+)-glaucine internal standard working solution was made to a concentration of 100 ng/ml by dilution of an aliquot of the (+)-glaucine stock solution with water. A series of spiking solutions were derived by dilution of the stock *rac*-terazosin solution. Subsequently, a series of plasma standards were made by transferring small, known volumes of spiking solutions into known volumes of plasma. Plasma standards were prepared, in duplicate, in concentrations of 0.0625, 0.250, 0.500, 2.00, 4.00, 8.00, 32.0 and 64.0 ng/ml per enantiomer. In a similar manner, quality control (QC) samples were prepared with expected concentrations of 0.125, 0.500, 1.00 and 16.0 ng/ml per enantiomer in human plasma. All chemicals used were ACS grade or better. Milli-Q (Millipore, Bedford, MA, USA) water was used throughout. Blank plasma was derived from blood drawn into heparinized tubes from subjects in Biovail's clinic and was separated in the bioanalytical laboratory, pooled and stored at −20°C until use. Pharmacokinetic subject samples were obtained from two healthy male subjects dosed in Biovail's clinic according to an approved protocol sponsored by the Biovail Contract Research Division.

2.2. Extraction from plasma

The target compounds were extracted from plasma in the following manner for the LC–MS experiments: The plasma was thawed to room temperature and the tubes mixed on a rotatory mixer to render the thawed sample homogeneous. Exactly 1.00 ml of sample was transferred to a clean 16 mm×100 mm screw-top tube followed by 100 μl of prazosin internal standard working solution. After a brief (ca. 2 s) mixing period on a vortex mixer 1.0 ml of 0.9% (w/v) aqueous NaCl was added to each tube and then the sample was made alkaline by the addition of 100 μl of 2 *M* NaOH, then again mixed on the vortex mixer for another brief period (ca. 2 s). To each tube was added 5 ml of pentane–dichloromethane (50:50, v/v). The tubes were then capped and mixed on a rotating shaker for 20 min at 40 rpm. After centrifugation for 10 min at 500 *g* followed by flash freezing the lower aqueous layer in an acetone–dry ice bath, the organic layers were transfered to clean 16×100 mm culture tubes. The organic layers were evaporated to dryness under nitrogen gas at 15 p.s.i.g. in a TurboVap (Zymark, Hopkinton, MA, USA) evaporator (1 p.s.i.=6894.76 Pa). The tubes were then covered and stored dry, at −20°C until required for analysis. Just prior to analysis the residues were reconstituted in 70 μl of hexane–2-propanol (90:10, v/v) and strongly agitated for 15 s on a vortex mixer. The resulting solution was transfered to a conical vial, capped and 20 μl injected into the LC–MS unit.

The extraction for the HPLC–fluorescence experiment was identical with the exception of the volume and composition of the reconstituting solvent added to the sample residue, the choice of the internal standard as already noted above and the injection volume. The reconstituting solvent was the mobile phase used in the fluorescence work, and the volume added was 100 μl. The injection volume was 65 μl.

2.3. LC–MS

The LC–MS experiments were conducted with an HP 1100 LC–DAD–MS system (Hewlett–Packard Corporation, Palo-Alto CA, USA). The system components included a binary pump, mobile phase vacuum degassing unit, autosampler, UV–visible Diode Array Detection (DAD) system and HP 1100 mass spectrometric detector. The source was a nebulizer assisted electrospray unit incorporating a proprietary orthogonal spraying configuration.

The column was 100 mm×2.1 mm I.D., packed with Chiralpak AD (10 μm particle size) (Chiral Technologies, Exton PA, USA). The mobile phase was a hexane–2-propanol–diethylamine mixture. The pump was operated in isocratic mode with the hexane in pump A, and 2-propanol with 0.05% (v/v) diethylamine delivered by Pump B. The mobile

phase conditions for this set of LC–MS experiments used a constant ratio of 65% Pump A and 35% Pump B, at a flow of 0.15 ml/min, throughout the chromatographic run. A post-column solvent addition of 2-propanol–5 m*M* ammonium acetate (3:1, v/v) was used for reasons previously described [3]. The post-column addition pump was an HP 1050 Quaternary model and post-column solvent addition flow was set at 0.3 ml/min of premixed solvent.

The mass spectrometer was run in positive ion mode and tuned for unit mass resolution in the mass range utilized in these experiments. Fig. 1 shows the mass spectrum for terazosin and prazosin protonated molecule ions as well as the contributions from the sodium adducts of both molecules. No fragmentation of either molecule was observed under these conditions. The spectrum illustrates the mass resolution typically expected in this experiment. The exact source conditions were: drying gas temperature 310°C, drying gas flow 10 l/min., nebulizer pressure 60 p.s.i.g., quadrupole temperature 100°C and capillary voltage 4500 V. Nitrogen was used exclusively as drying gas. The ion for terazosin was m/z 388.2 and that for the internal standard prazosin was m/z 384.2, both ions had a dwell time of 280 ms per ion.

After acquisition the resultant single ion monitoring (SIM) chromatograms were integrated by the HP Chemstation software. The integration parameters were determined by the AutoIntegrate routine available in the software, and were used without

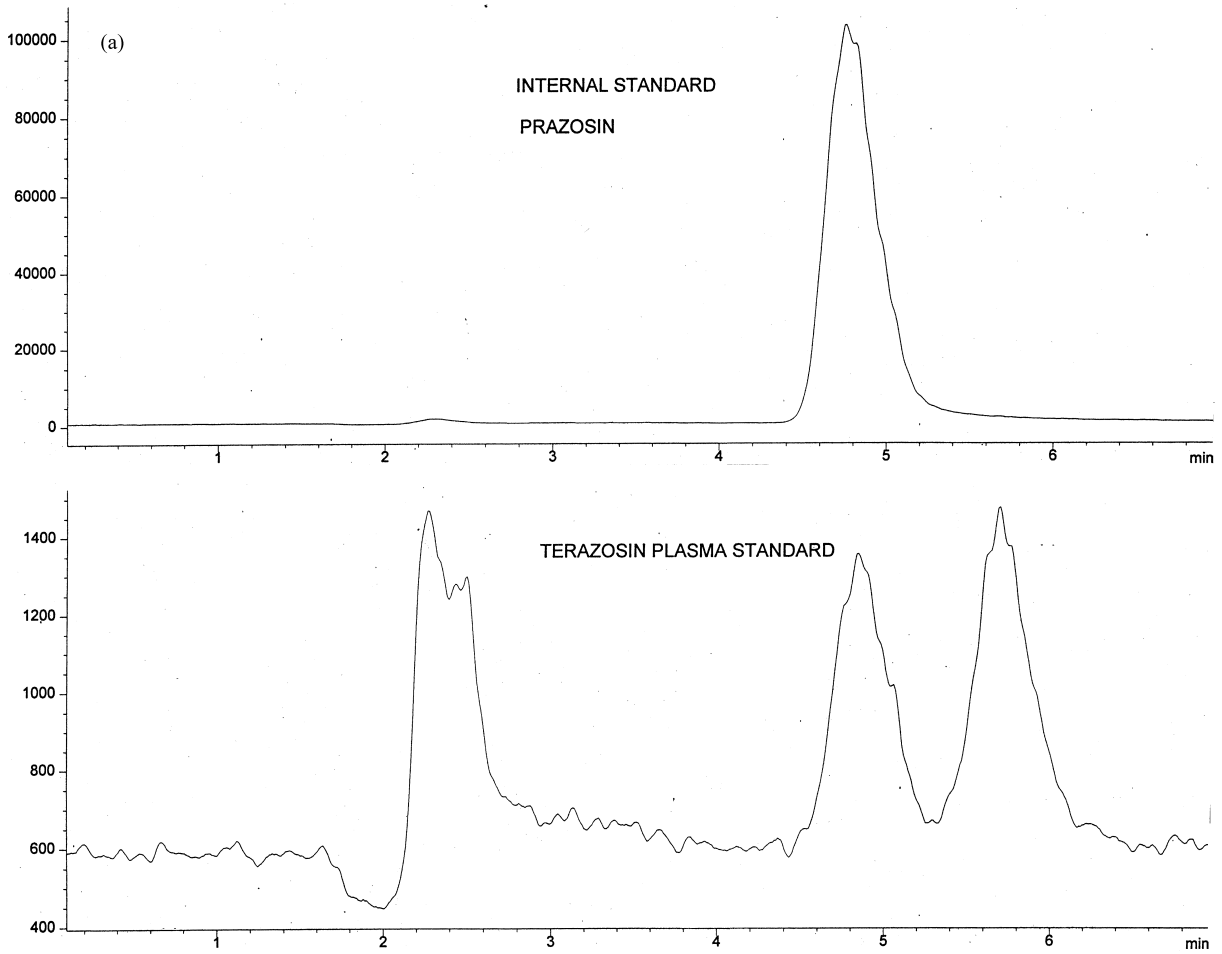

Fig. 2.

modification except for establishing a baseline point at 3.2 min. Baselines were set automatically and were not manually reviewed.

2.4. HPLC–fluorescence

The isocratic liquid chromatograph consisted of a Waters M510 pump (Milford, MA, USA), a Waters model 717 Plus autosampler, and a Perkin–Elmer (Beaconsfield, UK) LS-40 fluorescence detector with a 7 μl flowcell, connected to a Waters SAT/IN device. Data collection, data review and user interface was performed by a Waters Millennium system. The column was 250 mm×4.6 mm I.D. Chiralpak AD (10 μm particle size) maintained at 30°C by a Cool Pocket column oven (Keystone Scientific, Bellefonte, PA, USA). The mobile phase was composed of hexane–2-propanol–diethylamine (70:30:0.1, v/v/v). Typical flow rates were 1.0 ml/min with typical run times of 13 min. The excitation and emission wavelengths were 238 and 370 nm, respectively.

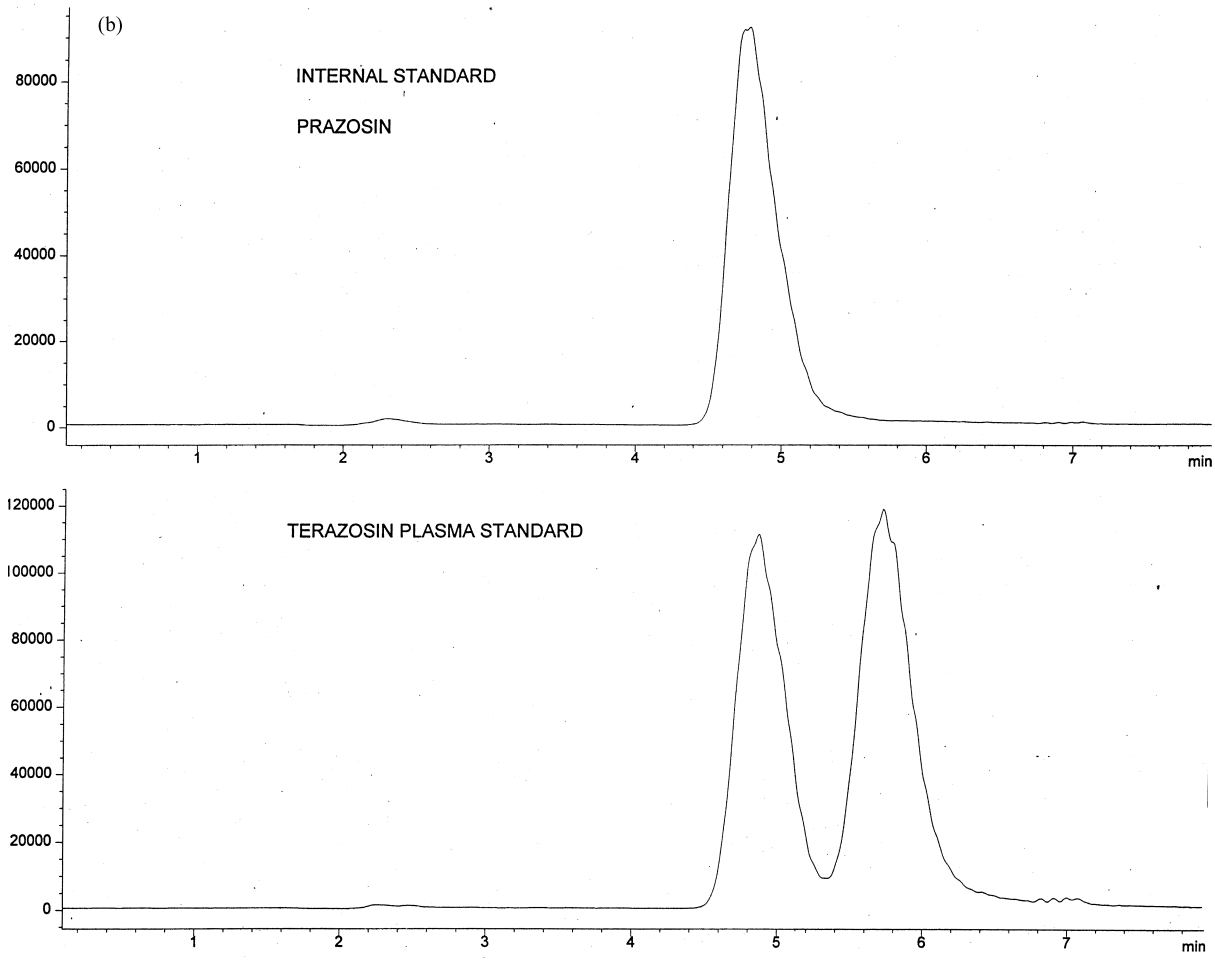

Fig. 2. (a) Mass chromatogram from an extracted plasma standard containing 125 pg/ml of each terazosin enantiomer. Terazosin was acquired at m/z 388.2, and the internal standard prazosin was acquired at m/z 384.2. (b) Mass chromatogram from an extracted plasma standard containing 16 ng/ml of each terazosin enantiomer. Terazosin was acquired at m/z 388.2, and the internal standard prazosin was acquired at m/z 384.2.

3. Results and discussion

Fig. 1 shows the mass/charge region of interest for terazosin and prazosin as produced by electrospray MS under the conditions described above. The spectrum is dominated by the protonated molecule ion $[M+H]^+$ for both molecules. There is also some minor contribution to the spectrum from the sodium adduct $[M+Na]^+$.

There are no other observed artifacts in the mass spectrum that can be attributed to the large ratio of hexane in the electrospray source. The spectrum signal was stable and promised good noise characteristics even in the presence of such a large excess of hexane. There was no evidence that the hexane content of the mobile phase in any way disturbed the expected ionization of these compounds under these conditions. These results are similar to those previously attained on APCI triple quadrupole instruments [3]. No source explosions were expected during this work and none occurred. The combination of water in the post-column effluent, electrospray ionization, and nitrogen as nebulizer and source gas seems to have further minimized concerns with the explosion hazard. The conditions for the onset of explosion in the source have not been determined in this work and practitioners are cautioned that some experimental conditions may cause conditions favorable to source explosions. The use of air as nebulizer gas in APCI, in particular, should always be treated with caution

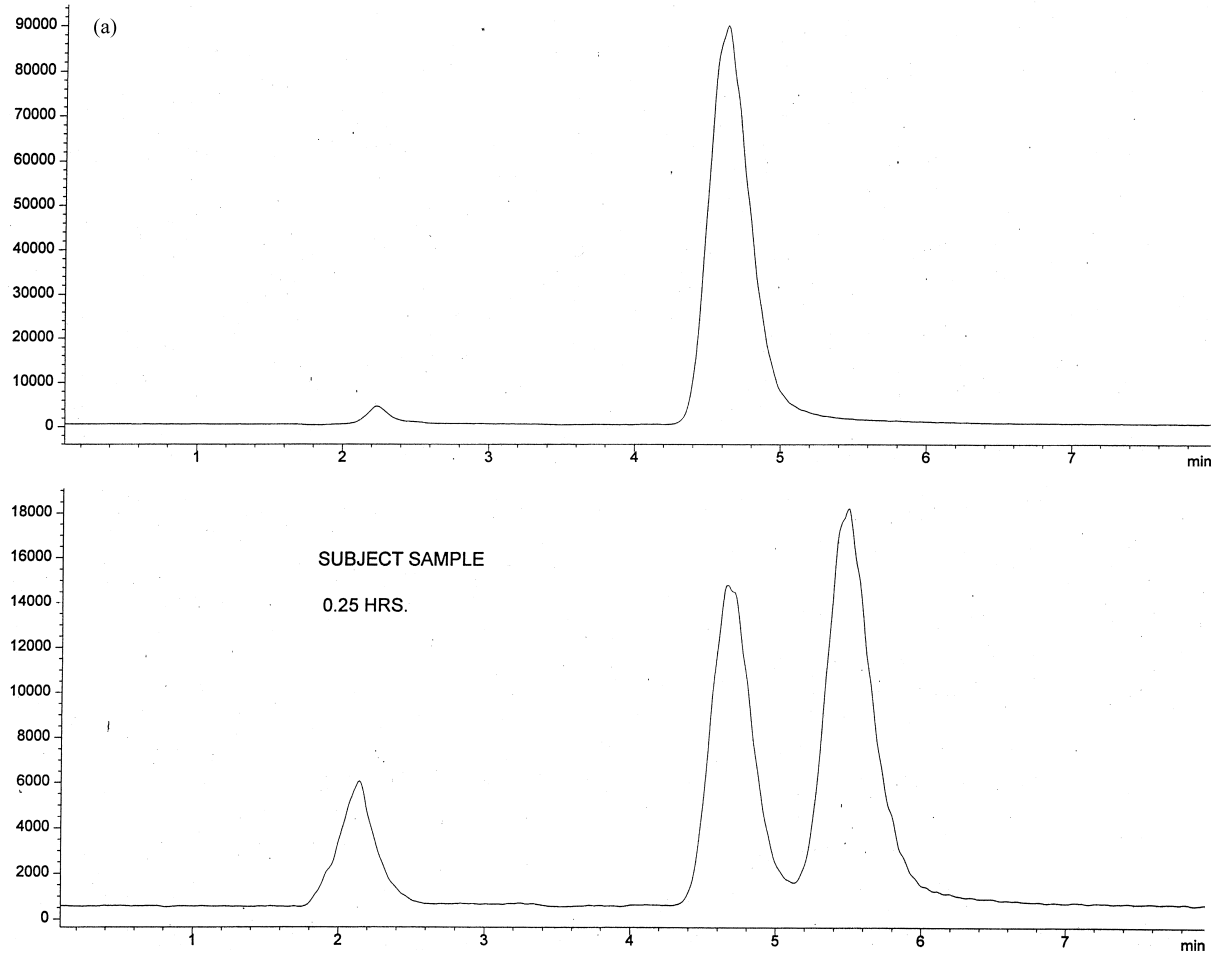

Fig. 3.

when combined with normal phase eluents such as hexane. Nitrogen is the recommended gas in this particular spectrometer. Fig. 2a,b and Fig. 3a,b show typical chromatograms attained with the method described above. The separation between enantiomers is adequate and, while prazosin is not separated from the first eluting enantiomer of terazosin, the internal standard does not contribute to the terazosin signal. Given the condition that the spectrometer was a single stage quadrupole there was a concern that the four m/z-unit difference between the internal standard and terazosin would cause difficulties if the three compounds were not separated in time. Moreover, although prazosin is an ideal internal standard for the chiral terazosin assay, it could not be used in the HPLC–fluorescence experiments due to co-elution with the first eluting enantiomer of terazosin (4.8 min) on Chiralpak AD. In the LC–MS method and under these conditions there is no mass interference from the internal standard and it does not interfere with the determination of terazosin even at 62.5 pg/ml. The LC–MS method can take advantage of the superior internal standard and suffers no negative effects to the results. As can be seen from Fig. 2a and Fig. 3b the sensitivity is more than adequate for both the calibrated range and the

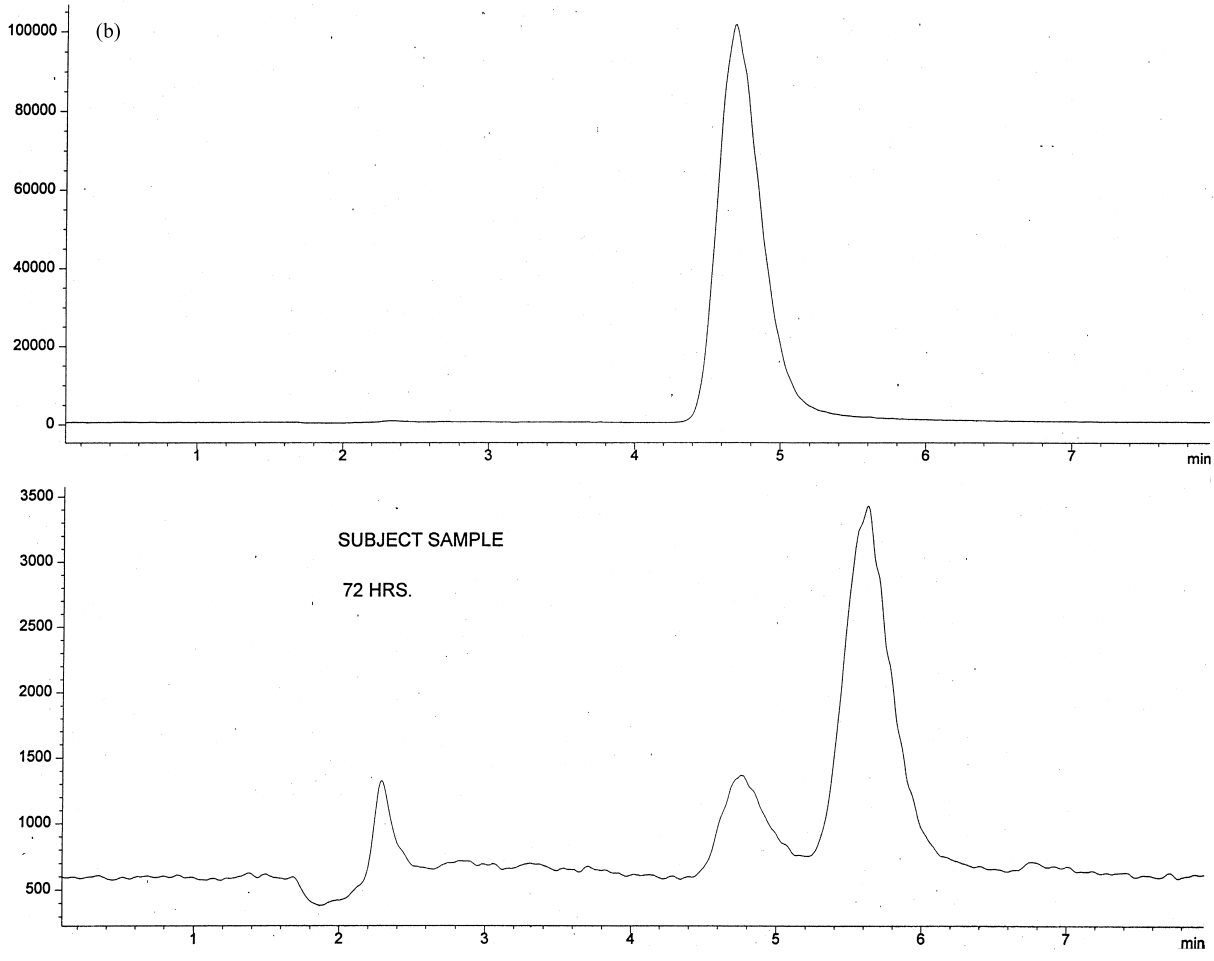

Fig. 3. (a) Mass chromatogram from an extracted subject sample drawn at 0.25 h post dose. Terazosin was acquired at m/z 388.2, and the internal standard prazosin was acquired at m/z 384.2. (b) Mass chromatogram from an extracted subject sample drawn at 72 h post dose. Terazosin was acquired at m/z 388.2, and the internal standard prazosin was acquired at m/z 384.2.

concentrations present in vivo at the extremes of the pharmacokinetic curve. In addition the sensitivity is far greater than that provided by the fluorescence method used here for this compound. There are no published chromatographic methods for the determination of terazosin enantiomers in human plasma. The chiral HPLC–fluorescence technique described in this article shows similar sensitivity, at an LLOQ of 500 pg/ml per enantiomer, to a previously published achiral method [4]. The work by Patterson [4] showed an LLOQ of 1 ng/ml in an achiral assay. The sensitivity advantage offered by LC–MS adds information that was not available with the HPLC–fluorescence method alone. The zero concentration sample showed no interference from either endogenous compounds or from the internal standard. Blank samples also confirmed this result. The LC–MS method proved to be very selective and rapid with a sample to sample run time of 8 min.

Fig. 4a,b show the calibration curves for the first and second eluting enantiomers of terazosin, respectively. The calibration data was examined for fit to a large number of models by the TableCurve 2D program (Jandel). Both enantiomers were best fitted by a similar, well defined relationship between area ratio and concentration. Calibration curves for the two enantiomers of terazosin were fitted to the relationship $y=a+bx^c$ weighted 1/concentration where y is the area ratio and x is the concentration. All but two (2) of sixteen (16) standard calibration

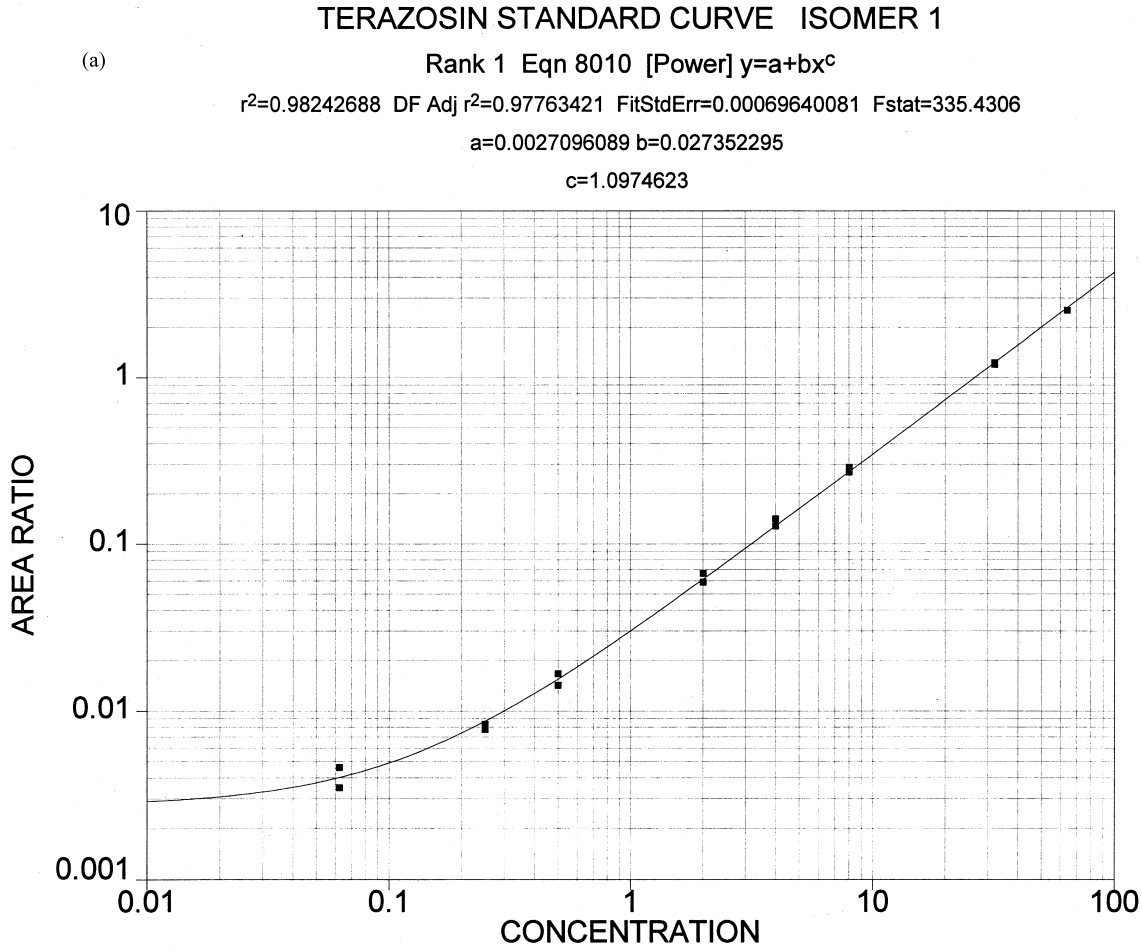

Fig. 4.

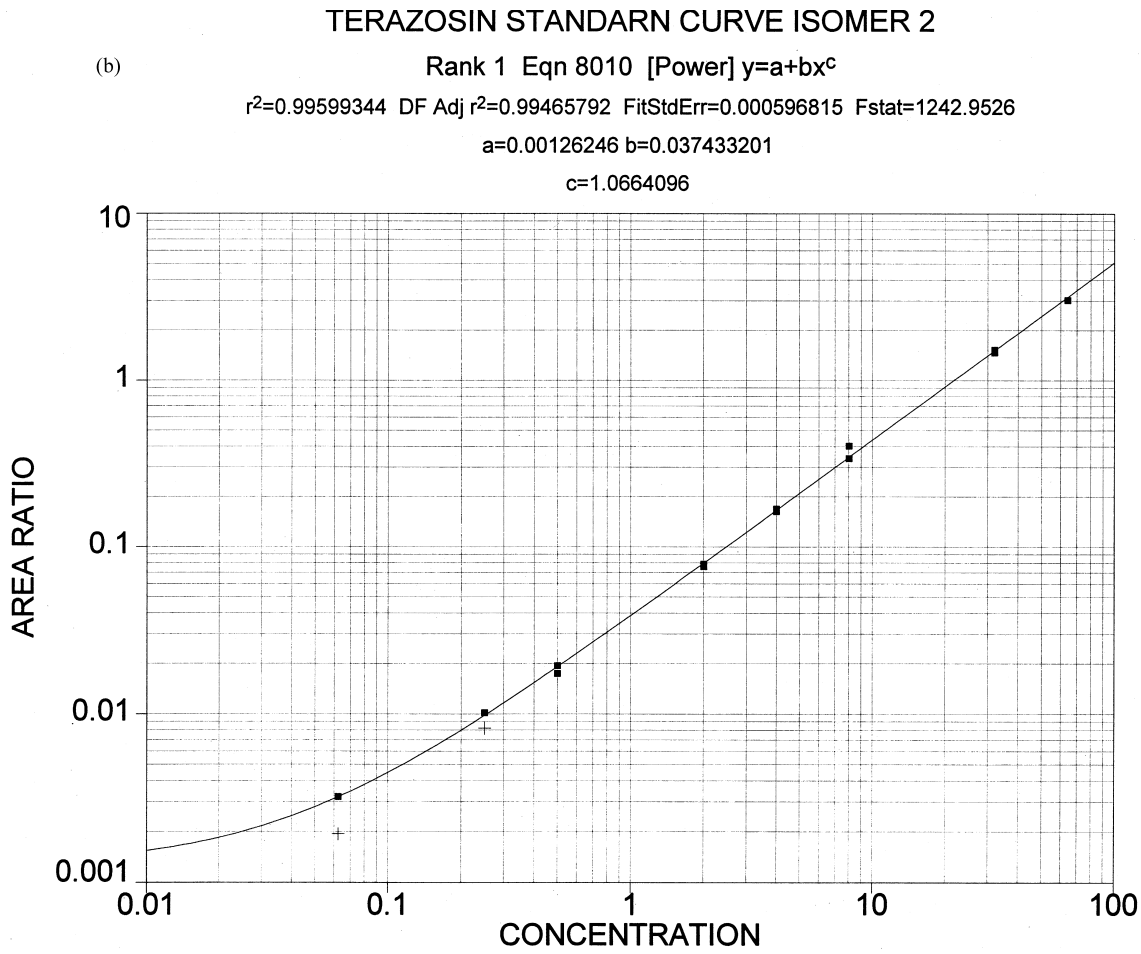

Fig. 4. (a) Standard curve for the first eluting enantiomer of terazosin. (b) Standard curve for the second eluting enantiomer of terazosin. + indicates points removed from the regression calculation.

points were used in the regression analysis and the residuals from back calculated values were less than ±15% across the concentration range. The positive deviation from linearity in the lowest concentration standard was expected and not unusual in low level ESI-MS data. The calibration curve was free from the negative deviations from linearity at the highest concentrations as frequently encountered in electrospray methodology. Quality control samples (QC) were within ±15% of expected values throughout the concentration range. The regression analysis confirms a limit of quantitation (LOQ) of 62.5 pg/ml per enantiomer.

Fig. 5a,b show the comparison of plasma levels for both enantiomers of terazosin from the same plasma samples as determined by the HPLC–fluorescence and LC–MS methods. The data analysis show an excellent correlation between the two techniques. Using the data from the HPLC–fluorescence and LC–MS experiments from subject 2, the first eluting enantiomer displayed $r^2=0.997$, with the slope of the regression line of 0.957, and a y-intercept value of −0.726. The second eluting enantiomer displayed $r^2=0.996$ with the slope of the regression line of 0.921, and a y-intercept value of 0.405. The LC–MS approach combines good agreement with other, more

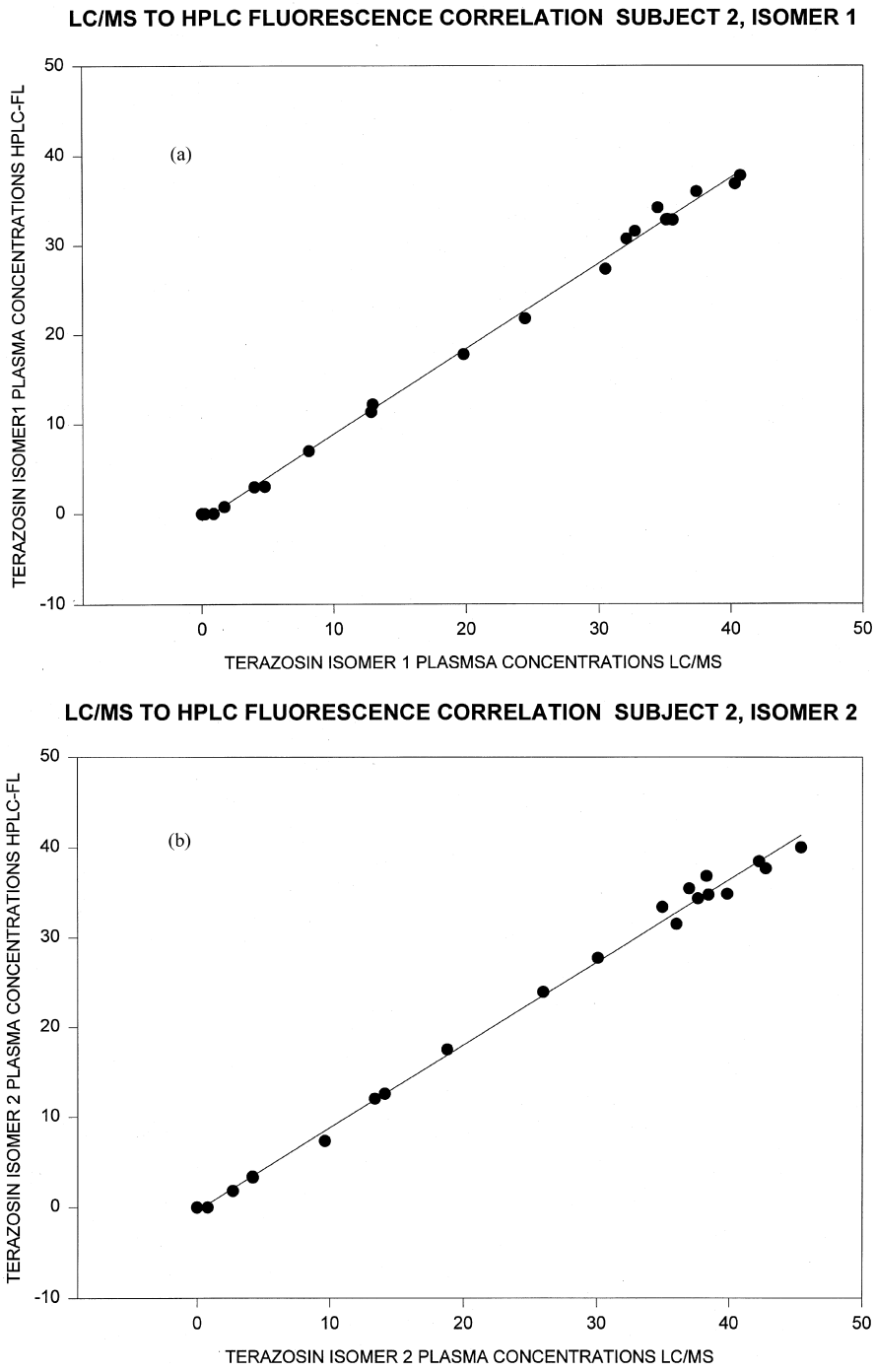

Fig. 5. (a) Correlation between subject sample results from LC–MS analysis with results of the same plasma samples from HPLC–fluorescence analysis for the first eluting enantiomer of terazosin. (b) Correlation between subject sample results from LC–MS analysis with results of the same plasma samples from HPLC–fluorescence analysis for the second eluting enantiomer of terazosin.

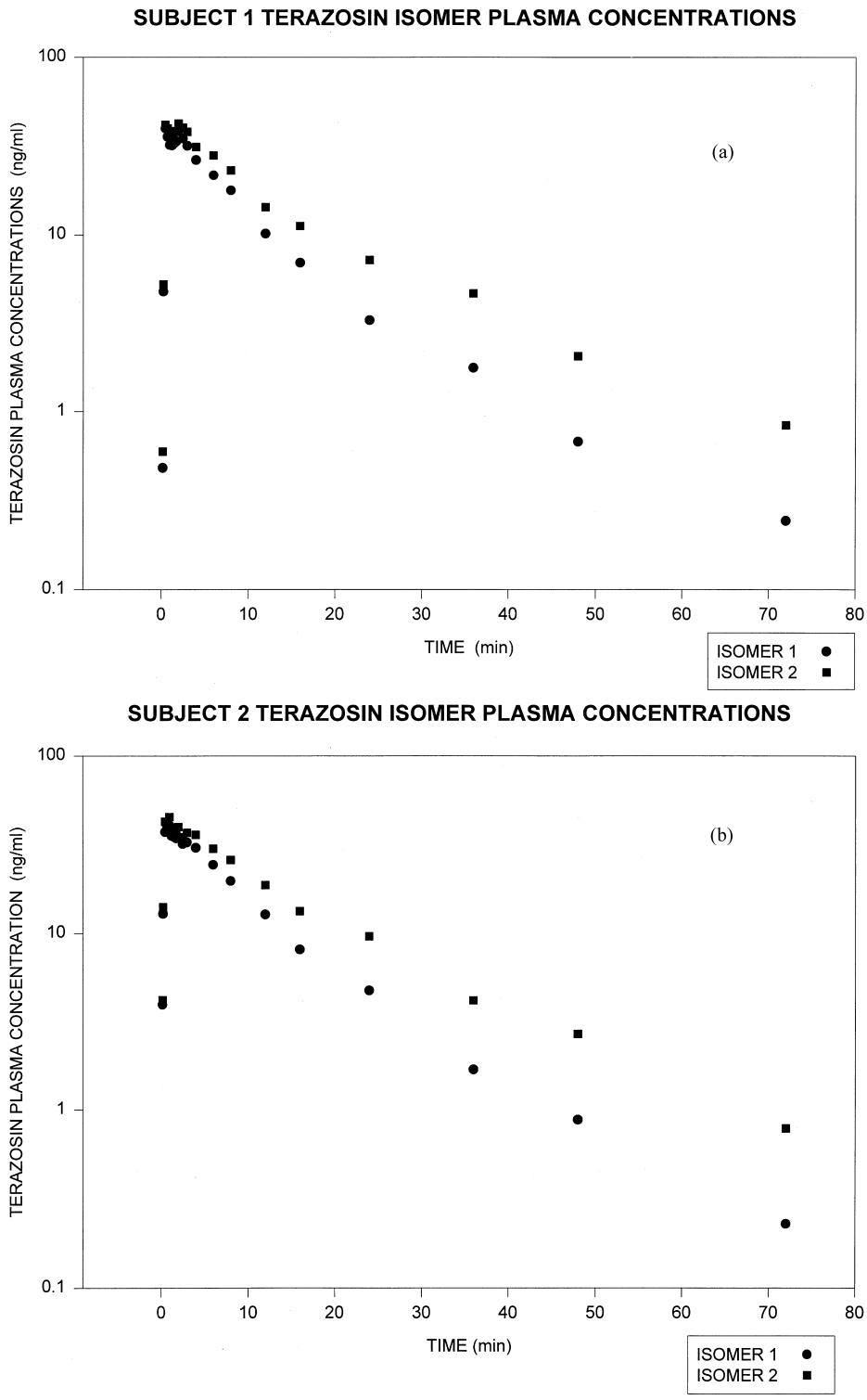

Fig. 6. (a) Pharmacokinetic curve for the enantiomers of terazosin from subject 1 as determined by LC–MS. (b) Pharmacokinetic curve for the enantiomers of terazosin from subject 2 as determined by LC–MS.

commonly used methods for terazosin but extends the reliable quantitation range downward by an order of magnitude.

The utility of such an enhancement of the analytical range can be seen in Fig. 6a,b. These figures display the pharmacokinetic profiles of the terazosin enantiomers determined from the plasma samples, by the above LC–MS technique, of two healthy, male subjects dosed with single, 5 mg oral doses of terazosin under fasting conditions. The profiles indicate an obvious difference in the concentration of the two enantiomers during the elimination phase. With the sensitivity afforded by the more commonly used HPLC–fluorescence technique it would be difficult to assert, based on quantitative data, that there was in fact a difference in the levels of the two terazosin enantiomers at the end of the 72 h study. The fluorescence technique simply did not have the sensitivity to measure the compounds at the levels and time points where the differences in the two enantiomers were the most pronounced. In fact the HPLC–fluorescence results for the same samples could not give reliable quantitative chiral data beyond the 48 h time point. The LC–MS approach has ample sensitivity to follow the profiles to time points where there can be no doubt about the relative ratios of the two enantiomers following a single 5 mg oral dose.

4. Conclusions

This paper details a direct approach for the determination of the enantiomers of terazosin in human plasma by the highly selective, and sensitive method of LC–MS. The separation was achieved on a chiral stationary phase and required no column switching or pre-column derivatization. The normal phase column eluent was directly introduced into an electrospray MS instrument. The hazards of using such eluents in API environments was minimized by the post-column addition of water containing solutions and the rigorous use of nitrogen as nebulizing and solvent drying gas. The application of the technique to the determination of terazosin enantiomers allowed quantitation in human subject plasma samples at low oral dose levels and at time points that clearly demonstrated a marked difference in the relative ratios of the two enantiomers during the elimination phase.

Future efforts will continue to apply this technique to new chiral compounds in a continued effort to understand their metabolism. The excellent chromatographic properties of the packings used in the normal-phase system described above suggest that supercritical fluid chromatography may offer some interesting areas for study and possibly unique contributions to the area chiral compound analysis with mass spectrometric detection.

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