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Measurement of brimonidine concentrations in human plasma by a highly sensitive gas chromatography/mass spectrometric assay

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Abstract

Brimonidine is an α_2-adrenergic agonist that is efficacious in lowering intraocular pressure in humans. A highly sensitive and selective gas chromatography/mass spectrometry (GC/MS) assay is described for quantitation of brimonidine in human plasma following ocular installation. Brimonidine in 1 ml of plasma was extracted together with tetradeuterated brimonidine (internal standard) and clonidine (carrier) by solvent extraction. After solvent evaporation, 3,5-bis(trifluoromethyl)benzoyl derivatives were formed and injected onto a GC/MS appartus under negative chemical ionization conditions. The ions monitored for derivatized brimonidine and tetradeuterated brimonidine were m/z 691 [M − HBr] and m/z 694 [M − DBr], respectively. Calibration curves were linear from 2 to 1000 pg ml^{-1} ($r^2 = 0.981$–0.996). The method was specific for brimonidine relative to endogenous compounds in plasma. The inter-day relative standard deviation for analysis of quality controls was 12% or less, and the inter-day assay accuracy ranged from 97 to 104% of nominals. The GC/MS assay showed adequate sensitivity for analysis of human samples from volunteers ocularly dosed with 0.5% brimonidine tartrate solution. Overall, the GC/MS assay showed excellent precision and accuracy, and a minimum quantifiable concentration of 2 pg ml^{-1}.

Keywords: Assay; Brimonidine; Chemical ionization; Gas chromatography/mass spectrometry; Picogram; Plasma concentrations

1. Introduction

Brimonidine tartrate (AGN 190342-LF or UK-14,304-18) (Scheme 1) is an effective ocular hypotensive agent which is currently undergoing clinical evaluation. Brimonidine possesses the 2-aminoimidazoline moiety in common with clonidine and is a relatively selective α_2-adrenoceptor agonist. A 35 µl eyedrop of 0.5% brimonidine tartrate solution is equivalent to 0.23 mg base (0.0038 mg kg^{-1} for a 60 kg person) after topical instillation to each eye. Oral systemic hypotensive doses ($\approx$1 mg kg^{-1}) in animal toxicity studies produced concentrations in the ng ml^{-1} range. Therefore, at very low ocular hypotensive doses, a more sensitive and selective bioanalytical method was sought for measuring plasma brimonidine concentrations in the pg ml^{-1} range to support clinical and pharmacokinetic studies after ocular dosing. Monitoring of plasma concentrations was also needed to elucidate relationships between plasma concentrations and potential systemic effects.

For a brimonidine assay requiring high sensitivity, negative chemical ionization gas chro-

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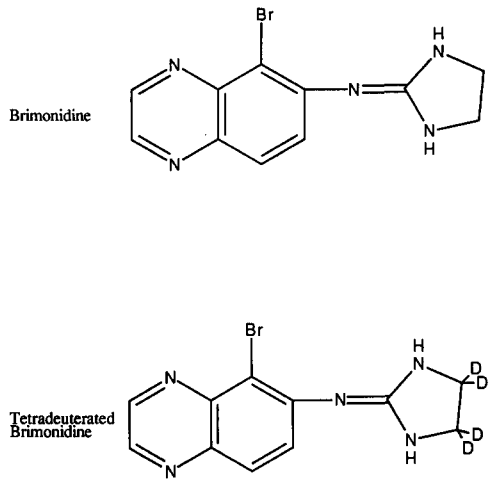

Scheme 1.

matography/mass spectrometry (GC/MS) or GC-electron capture detection methods were considered, since they have been used to analyze plasma concentrations of clonidine [1–4]. *tert*-Butyldimethylsilyl, heptafluorobutyryl and 3,5-bis(trifluoromethyl)benzoyl (TFMB) derivatives of clonidine were formed for GC [1] or GC/MS analysis in the electron impact [4] or chemical ionization mode [2,3]. To suit our requirements for ocular dosing, a detection limit (≈ 1 pg ml^{-1}) lower than that reported for oral dosing of analogs [1] was needed to characterize the systemic exposure and elimination kinetics of brimonidine. The purpose of this study was to develop a negative ion chemical ionization GC/MS method and investigate its suitability for measuring low pg ml^{-1} concentrations of brimonidine in plasma observed after an ophthalmic dose administration.

2. Experimental

2.1. Chemicals

Brimonidine tartrate (purity >99%) was supplied by Pfizer (Connecticut, USA). The internal standard, brimonidine-d_4 (isotopic purity, ≈99%) was synthesized by the Allergan Chemistry Department, using a known procedure involving reaction of 5-bromo-6-isothiocyanatoquinoxaline with 1,1,2,2-tetradeuteroethylene diamine. Cloni-dine HCl (Sigma, Missouri, USA) and 3,5-bis(trifluoromethyl)benzoyl chloride (Aldrich, Wisconsin, USA) were purchased from commerical sources. All the solvents used were the highest grade available and reagents, and the other chemicals were of analytical grade. All the glassware used to prepare the drug solutions was silylated.

2.2. Calibration standards and quality controls

Stock solutions of brimonidine tartrate (1 mg ml^{-1}), brimonidine-d_4 (0.5 mg ml^{-1}) and clonidine HCl (0.1 mg ml^{-1}), and working standards were prepared in methanol. Eight calibration standards of brimonidine (2, 4, 10, 25, 50, 100, 500 and 1000 pg base ml^{-1}) were prepared by spiking 10 ml of drug-free plasma (EDTA-treated) with the appropriate brimonidine working standards. Quality controls of brimonidine (8, 50 and 500 pg base ml^{-1}) were obtained by spiking 100 ml of blank human plasma with appropriate aliquots of brimonidine working standards. The working internal standard (WIS) solution used for drug quantitation consisted of brimonidine-d_4 (1.52 ng ml^{-1}) and clonidine (2.02 ng ml^{-1}) in methanol.

2.3. Probe human study

Four healthy male volunteers (mean age of 28 years) participated in a probe study to investigate the plasma concentration–time profile after topical dosing. Following an overnight fast before dosing, a single drop of 0.50% brimonidine tartrate solution was instilled into each eye of individual subjects. Prior to dosing, a blood sample was taken as baseline and additional 5 ml blood samples were withdrawn at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, 8.0, 10, 12 and 24 h post-dosing. The blood samples were collected in EDTA-treated tubes and centrifuged to separate plasma. The human test samples were stored briefly at −20 °C at the clinical site and at −80 °C at the bioanalyical site before analysis.

2.4. Plasma extraction

Twenty five microliters of WIS solution was added to 1 ml aliquots of human plasma calibration standards, quality controls, and probe study samples. After vortexing for 10 s and equilibrating for 10 min, 0.5 ml of 1 M sodium carbonate was added followed by the addition of 7 ml of ethyl acetate. The samples were extracted by rotating at 32 rpm for 15 min and centrifuging at 2000*g* for 10 min at 20 °C. The

organic phase was transferred to silylated culture tubes containing 0.5 ml of 0.1 M HCl. The acidified extracts were vortexed for 2 min and centrifuged at 1000*g* for 10 min. The sample tubes were frozen at −80 °C for 10 min and, subsequently, the top organic layer was poured off and discarded when thawed. Upon complete thawing of the bottom aqueous layer, 0.5 ml of 1 M sodium carbonate solution was added and mixed well, followed by the addition of 8 ml of methylene chloride. The tubes were then vortexed for 2 min, followed by centrifugation at 2000*g* for 10 min at 20 °C. The top aqueous layer was aspirated off, and the remaining organic layer was poured into a silylated conical-bottom screw-top tube and evaporated to dryness with N_2.

2.5. Derivatization

After complete drying of the organic layer, 50 µl of freshly prepared derivatizing reagent (prepared by adding 100 µl of bis(3,5-trifluoromeththyl)benzoyl chloride to 5 ml of toluene) was added to the concentrated extract and the mixture heated at 60 °C for 30 min. Subsequently, the samples were centrifuged for 5 min and evaporated to dryness under N_2 before reconstitution in 25 µl of ethyl acetate. One µl of the derivatized extract was injected into the GC/MS apparatus by an autosampler.

2.6. GC/MS conditions

The GC/MS system used for the assay was a Hewlett-Packard 5890A GC coupled to a Finnigan TSQ 4500 mass spectrometer. Gas chromatography was carried out on an RT_x-35 capillary column (15 m × 0.32 mm i.d., 0.25 µm film) (Restek Corporation, Bellefonte, USA). Helium was the carrier gas with an inlet pressure of 10 psi. The injector temperature was held constant at 290 °C. The column oven temperature was programmed at 25 °C min^{-1} from 250 (1.0 min hold) to 290 °C (1.5 min hold). The transfer line and source temperatures were 290 °C and 190 °C, respectively. The mass spectrometer was operated in the negative ion chemical ionization mode using methane (0.65 Torr) as the reagent gas. The di-TFMB derivative of brimonidine was monitored at *m/z* 691 $[M - HBr]^-$. The di-TFMB derivative of brimonidine-d_4 was monitored at *m/z* 694 $[M - DBr]^-$. The total GC/MS run time for each sample vial injected was approximately 4 min.

2.7. Extraction recovery

The extraction recovery of brimonidine from plasma was determined by comparing the peak area ratios for extracted plasma standards over the concentration range studied with those of unextracted standards of brimonidine in ethyl acetate. The external standard, brimonidine-d_4, was added to the ethyl acetate solution after extraction or directly to ethyl acetate without extraction, and samples derivatized for GC/MS analysis.

2.8. Data analysis

The calibration curves were obtained by plotting the ion peak area ratios (*m/z* 691 to *m/z* 694) of brimonidine to brimonidine-d_4 di-TFMB derivatives against the plasma drug concentrations of brimonidine, using linear least-square regression and a weighting factor of 1/concentra- $tion^2$. The isotopic purity of brimonidine-d_4 and contribution of brimonidine to *m/z* 694 of the internal standard was assessed during the validation. The mean, standard deviation (SD) and relative standard deviation (RSD) were calculated. The RSD was used to assess intra-day and inter-day assay precision for replicate analysis of quality control samples. Assay accuracy (% nominal) for analysis of quality control samples was calculated as observed concentration/nominal concentration × 100. The observed concentrations of calibration standards were calculated from the daily calibration curves to assess the inter-day reproducibility and accuracy of analysis of calibration standards and the limit of quantitation. The observed maximum plasma concentrations, the corresponding time and the elimination half-life obtained from the probe study were calculated using standard methods [5].

3. Results

3.1. Assay specificity

As shown in Fig. 1, the individual negative ion mass spectrum of the derivatized brimonidine and brimonidine-d_4 is characterized by the highly abundant *m/z* 691 $[M - HBr]^-$ and *m/z* 694 $[M - DBr]^-$. The single ion chromatograms of *m/z* 691 and *m/z* 694 resulting from the injection of an extracted blank are

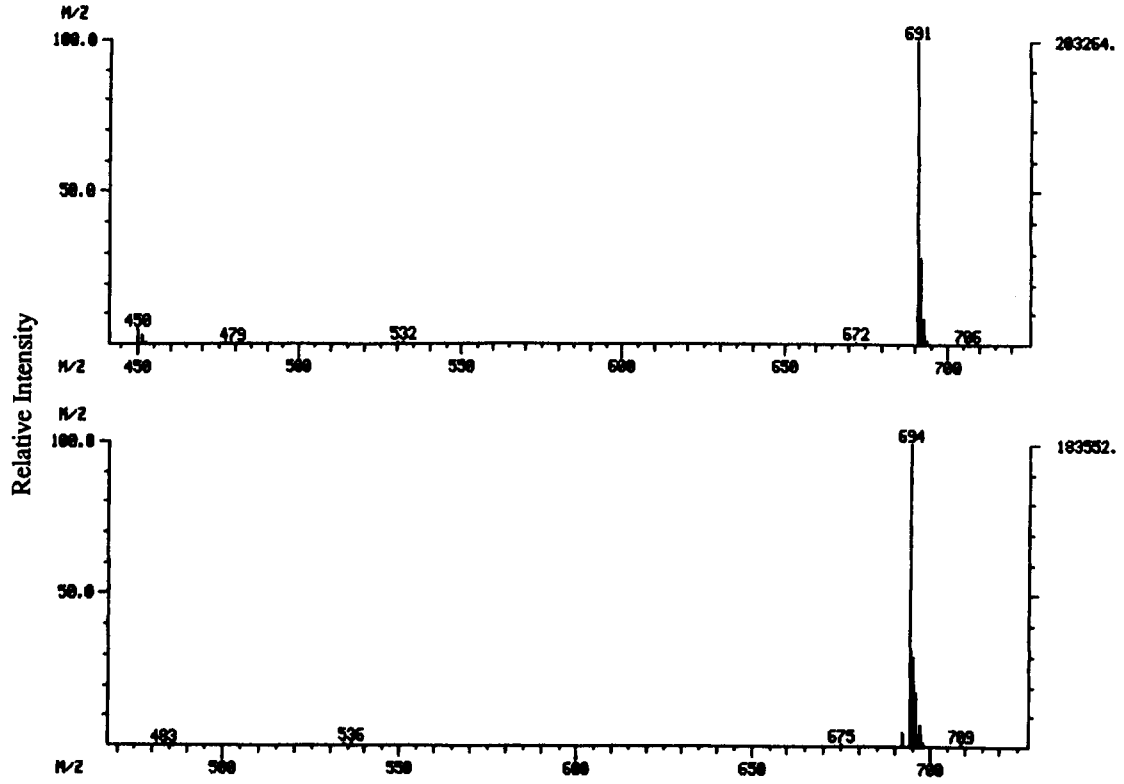

Fig. 1. Negative ion mass spectra of the 3,5-bis(trifluoromethyl)benzoyl derivatives of brimonidine (upper plot) and brimonidine-d_4 (lower plot).

shown in Fig. 2, indicating that there was little or no interfering endogenous compound present at approximately 2.5 min retention time for derivatized brimonidine and brimonidine-d_4. The clonidine peak eluted after more than 3 min. Typical ion chromatograms for a human plasma sample containing 2 pg ml^{-1} of brimonidine (m/z 691) and 38 pg ml^{-1} of brimonidine-d_4 (m/z 694) are shown in Fig. 1, indicating the sensitivity of the assay. The ion chromatograms in Fig. 3 correspond to much higher concentrations of brimonidine (500 pg ml^{-1}) and 38 pg ml^{-1} of brimonidine-d_4 in plasma.

3.2. Linearity of calibration curve

The calibration curves obtained on the three validation days were relatively similar. They showed excellent linearity in the range of concentration values from 2 pg ml^{-1} to 1 ng ml^{-1}. The coefficient of determination (r^2) ranged from 0.982 to 0.996 across the three validation runs. The slope of the calibration curves varied across the runs, ranging from 0.0281 to 0.0319 ml pg^{-1}. The intercept values of 0.0138–0.0231 were close to zero. The mean correlation coefficient (r), slope, and intercept of the three validation runs were 0.995, 0.0305, and 0.0176, respectively.

3.3. Precision and accuracy

The intra-day assay precision (RSD) for assay of replicates of the 8, 50 and 500 pg ml^{-1} quality control samples ranged from 7.5 to 15% (Table 1), indicating acceptable precision. The inter-day assay precision (RSD) for analysis of plasma controls was no more than 12.0% (Table 1). The assay accuracy based on inter-day analysis of plasma controls ranged from 97.4 to 104% of nominal values. The extraction efficiency of brimonidine from plasma was greater than 80% over the calibration range. Analysis of the 8, 50 and 500 pg ml^{-1} human plasma controls stored in aliquots at −80 °C showed no significant change in nominal concentrations over 1 year period of storage.

3.4. Limit of quantitation

Table 2 lists the precision and nominal data for mean observed concentrations of calibration standards over the three days. The lower limit of quantitation of the assay was 2 pg ml^{-1}, the lowest concentration in the cali-

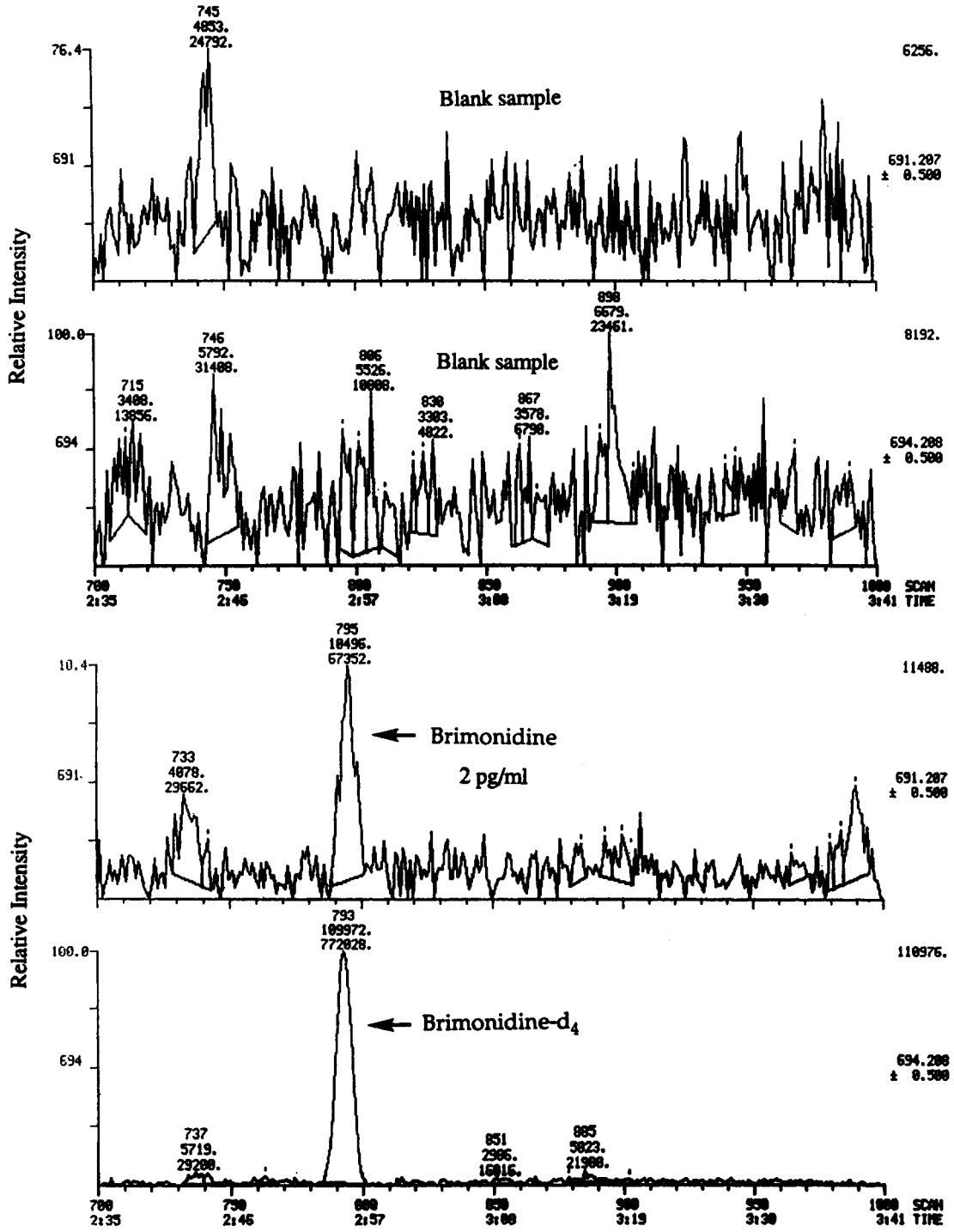

Fig. 2. Representative chromatograms obtained from blank human plasma (upper plot), and human plasma sample containing 2 pg ml^{-1} of brimonidine and 38 pg ml^{-1} of internal standard (lower plot).

bration curve. Duplicate analysis of the 2 pg ml^{-1} standards at three different days showed an inter-day assay precision (RSD) of 11.9% and a nominal accuracy of 96.5%. The analysis of six replicates of a 2 pg ml^{-1} control sample yielded similar assay precision and accuracy. The 2 pg ml^{-1} concentration produced a peak area with a signal-to-noise ratio of 8:1 (Fig. 2), indicating a limit of detection significantly lower than 2 pg ml^{-1}.

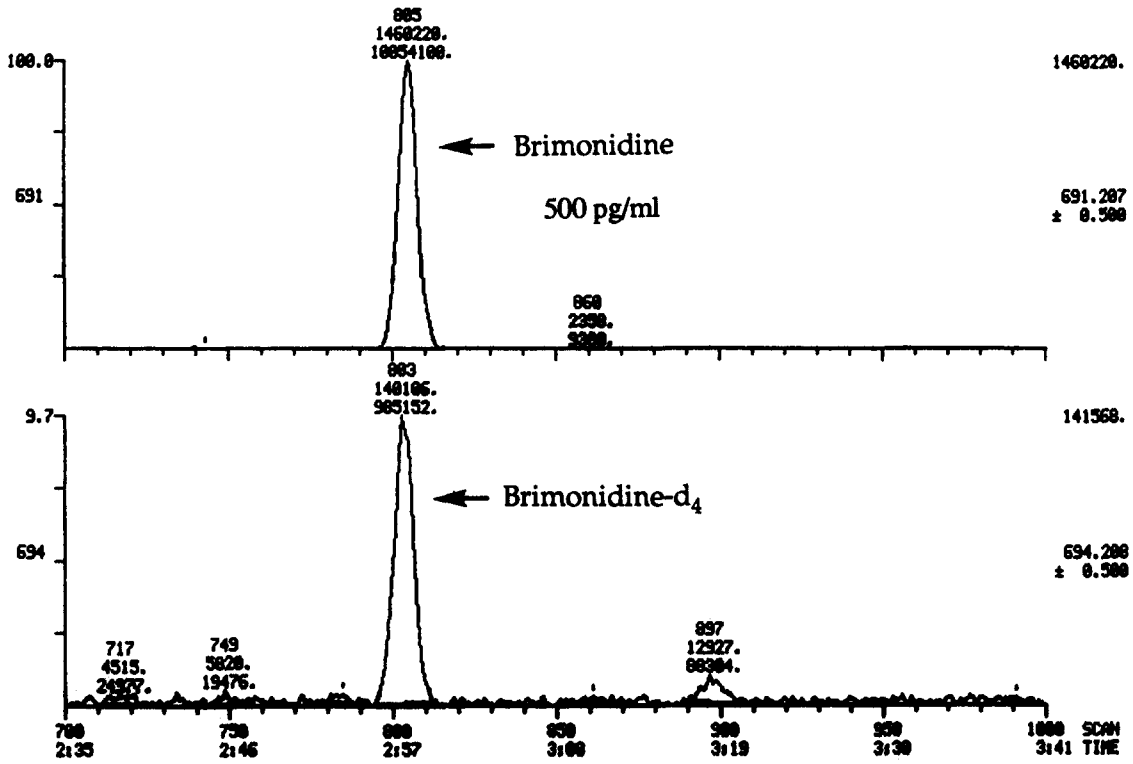

Fig. 3. Representative chromatograms obtained for human plasma sample containing 500 pg ml^{-1} of brimonidine and 38 pg ml^{-1} of internal standard.

3.5. Application of assay to probe human study

Fig. 4 shows the plasma concentration–time profile for the four individual subjects used in the probe study. The results revealed that brimonidine was absorbed systemically and rapidly eliminated after ocular dosing. The maximal plasma concentrations occurred between 1 and 4 h after dosing, and were less than 0.3 ng ml^{-1}. After reaching a peak, the plasma concentrations declined rapidly with an apparent elimination half-life of approximately 2 h. The plasma concentrations of brimonidine at 24 h were below the limit of quantitation of 2 pg ml^{-1}.

4. Discussion

The analyte was extracted from plasma with organic solvent and the initial extract cleaned up by back extraction. Formation of the di-TFMB derivatives of brimonidine in extracts was quantitative and rapid, requiring heating of derivatization vials at 65 °C for 30 min. Brimonidine may be susceptible to degradation to the aryl guanidine derivative at a higher temperature of derivatization [6]. The 2-aminoimidazoline compounds, such as brimonidine and clonidine, are capable of forming mono and di-derivatives using silyl, benzoyl and acyl reagents [1,2,4,6], depending on the reaction conditions and concentration of analyte. Evidence for the formation of the *N,N*-di-TFMB derivative of brimonidine was determined from the mass spectra obtained in the scan mode (Fig. 1). The most intense peak in the mass spectrum was the $[M - MBr]^-$ for brimonidine or $[M - DBr]^-$ ion for brimonidine-d_4.

The limit of quantitation for the assay was 2 pg ml^{-1} of brimonidine in plasma. To achieve a very sensitive and reproducible assay at the very low pg ml^{-1} concentrations, special attention was paid to resolve the problem of adsorption of low amounts of compound during extraction and GC/MS analysis by using clonidine as a main carrier to mask the adsorption sites, in addition to the presence of low concentrations of the tetradeuterated brimonidine. For pg ml^{-1} plasma concentrations, the choice of a stable isotope-labeled compound as the internal standard for quantitative GC/MS assay of brimonidine was an effective way to reduce assay variability.

The GC/MS method using a 1 ml sample aliquot was validated according to the guide-

Table 1
Intra-day and inter-day assay precision and accuracy for analysis of plasma quality controls

Nominal conc. (pg ml^{-1})	Mean observed conc. (pg ml^{-1})	Precision		Accuracy (%)	n
		SD (pg ml^{-1})	RSD (%)		
Intra-day					
8.00	7.78	0.56	7.53	97.2	4
50.0	44.4	6.7	15.2	88.8	4
500	490	49	9.92	98.0	4
Inter-day					
8.00	8.33	0.83	9.92	104	11
50.0	48.7	5.8	12.0	97.4	12
500	502	44	8.63	100	12

Table 2
Inter-day assay precision and accuracy of the standard curves and back-calculated concentrations of brimonidine

Nominal conc. (pg ml^{-1})	Mean observed conc. (pg ml^{-1})	Precision		Accuracy (%)	n
		SD (pg ml^{-1})	RSD (%)		
2.00	1.93	0.23	11.9	96.5	6
4.00	3.86	0.37	9.61	96.5	5
10.0	11.9	0.3	2.89	119	6
25.0	25.3	1.1	4.19	101	6
50.0	48.5	3.0	6.16	97.0	6
100	93.7	9.0	9.59	93.7	6
500	448	44	9.80	89.6	6
1000	1000	106	10.6	100	6

lines of the pharmaceutical industry [7]. The assay development involved determining the operating conditions for the GC/MS assay as well as optimizing for a low limit of quantitation of brimonidine in plasma. The short run time also allowed about 80 plasma samples to be run overnight. The specificity of the GC/MS method was demonstrated by the lack of significant chromatographic peak from blank human plasma at the retention times of brimonidine and tetradeuterated brimonidine. The RSD and percentage error in the 2 pg ml^{-1} to 1000 ng ml^{-1} range demonstrate an assay with high precision and good accuracy, suitable for routine analysis of brimonidine concentrations.

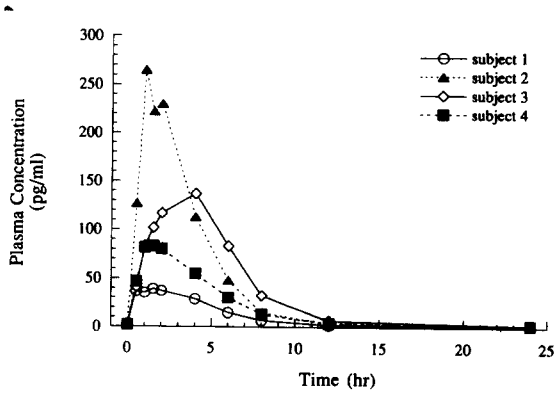

Fig. 4. Plasma brimonidine concentration–time curves for four human subjects who had been administered an eye-drop of 0.5% brimonidine tartrate solution topically to both eyes.

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