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Determination of pilocarpic acid in human plasma by capillary gas chromatography with mass-selective detection

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Abstract

A novel, highly sensitive method for the determination of pilocarpic acid (PA) in human plasma is described. In addition, the method provides for the conversion of the lactone, pilocarpine (P), to PA so that a total drug presence can be determined. Using novel high-performance liquid chromatographic conditions capable of separating P, isopilocarpine (I-P), PA and isopilocarpic acid (I-PA) from each other and from endogenous plasma impurities, it was confirmed that P exclusively and quantitatively converts to PA in heparinized human plasma during storage. For the determination of PA, the selective extraction of PA from protein-free plasma was accomplished using two different solid-phase extraction (SPE) cartridges in two consecutive SPE steps. After extraction, PA was lactonized with trifluoroacetic acid back to P, and both P and an internal standard were acylated using heptafluorobutyric anhydride (HFBA). The trifluoroacetylated derivatives were monitored using gas chromatography (GC) with mass spectrometric (MS) detection. This procedure allowed the sensitive and reliable determination of PA with a limit of quantification (LOQ) of 1 ng/ml, which could not be achieved using previously described methods. The assay was validated in the concentration range of 1 to 10 ng/ml with an intra-day precision (expressed as the coefficient of variation, C.V.) ranging from 9.9 to 0.5%. Inter-day precision for the quality control standard at 2.5 ng/ml showed a C.V. of 10.2%. Accuracy ranged from 94 to 102%. The assay was used to monitor the maximum systemic exposure to P, administered by the ocular route, in terms of total plasma PA (P and PA). © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Pilocarpic acid; Pilocarpine

1. Introduction

Pilocarpine (P) is widely used as a treatment for controlling elevated intraocular pressure associated with glaucoma [1]. The lactone ring of P is known to be highly unstable in biological fluids [2–6]. Presumably, enzymes present in the biological matrix cause the hydrolysis of the lactone, with the resulting formation of pilocarpic acid (PA) [7]. The sensitive determination of P in biological fluids is severely limited due to the chemical lability of the lactone.

Several methods for the determination of P in aqueous humor utilizing gas chromatography (GC) [8,9], high-performance liquid chromatography (HPLC) [3–5], and HPLC with mass spectrometric detection [2] have appeared in the literature. A

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sensitive method based on HPLC with atmospheric pressure chemical ionization (APCI) mass spectrometry (MS) had a limit of quantification (LOQ) of 10 ng/ml [2]. This method was capable of measuring both P and PA in aqueous humor and demonstrated a significant conversion to the acid. Other methods in aqueous humor had a LOQ of 25 [9] and 300 ng/ml [5]. The method based on the GC of an electron-capturing derivative [8] detected no P below 3 ng/g in aqueous humor. While the chromatographic conditions specified in some of these methods were capable of separating PA from P, the assays either used sample preparation procedures that were not suitable for the isolation of PA from human plasma or did not assess the potential for P to PA conversion during sample preparation, storage and/or derivatization.

Only two methods for the determination of P in dog [3] and in human [10] plasma have been reported in the literature. The method in dog plasma [3] had a LOQ of 10 ng/ml, but the detailed precision and accuracy data were not provided. In spite of attempts to stabilize P in plasma by the addition of sodium fluoride, an agent added to plasma to inactivate esterases and thought to catalyze P hydrolysis, the partial hydrolysis (<10%) of P during storage in plasma (−20°C for three months) was not avoided. However, it has been shown in vitro that the hydrolysis of P in plasma to PA can be completely inhibited by EDTA [7]. In a recently described method for the determination of P in human plasma [10], the direct determination of P was accomplished using chemical derivatization with 4-bromomethyl-7-methoxycoumarin. The LOQ of the method was 5 ng/ml per 3 ml plasma sample [10]. The method reportedly was not capable of separating the derivative of P from the derivative of isopilocarpine (I-P). Regardless, even though P converts stereospecifically to PA in plasma in the presence of esterases, metabolic epimerization should not a priori be discounted. The method used chloroform to extract P from the EDTA-stabilized plasma, in a manner similar to the procedure [8] that used methylene chloride to extract P from rabbit aqueous humor. PA was not extracted from the plasma. None of the reported plasma methods for determining P was suitable for determining PA and none had the requisite sensitivity for the systemic exposure study.

In order to avoid the shortcomings of assaying P in plasma directly, we describe in this paper that a relatively labile P was first converted to a more stable PA, followed by its extraction from plasma, derivatization and GC–MS detection. The approach permitted the assessment of the maximum systemic exposure to P in terms of total PA (P and PA in the biological fluid). The method for PA also provided an alternative strategy for determination of P (based on the difference between nonstabilized and stabilized samples) if the results for total PA warranted such an approach. The new methodology required development of a highly efficient extraction procedure for PA from plasma. This procedure was based on protein precipitation, followed by two separate solid-phase extraction (SPE) clean-up steps (silica and phenyl SPE cartridges) and an additional liquid–liquid extraction after derivatization.

In order to monitor the conversion of P to PA, new HPLC conditions were developed which permitted the baseline separation of P, I-P, PA and I-PA from each other, and from endogenous plasma impurities. It was demonstrated that complete conversion of P to PA in heparinized human plasma occurs during storage, in concurrence with previously reported observations [10]. The details of the isolation of PA from plasma and assay validation are the subject of this paper.

2. Experimental

2.1. Materials

Pilocarpine hydrochloride (P HCl) and trifluoroacetic acid (anhydrous, protein sequencing grade) were obtained from Sigma (St. Louis, MO, USA). The internal standard (Fig. 1), 3-methyl-4-[(4-ethyltetrahydro-3-furanyl)methyl]-1*H*-imidazole nitrate was provided by Merck Sharp and Dohme (Chibret,

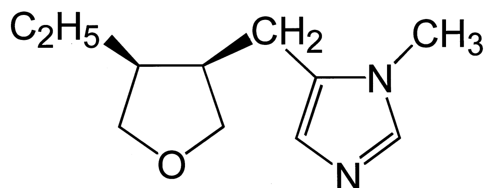

Fig. 1. Chemical structure of internal standard.

France). Heptafluorobutyric anhydride (HFBA; Pierce, Rockford, IL, USA) was used as received. Isopilocarpine nitrate and triethylamine (gold label grade) were from Aldrich (Milwaukee, WI, USA). Triethylamine was distilled over KOH pellets and stored in aliquots under nitrogen in crimped-top vials in a desiccator at −20°C. Q12 ion pair cocktail (dodecyltriethylammonium phosphate) was purchased from Regis (Morton Grove, IL, USA). Sylon CT was obtained from Supelco (Bellefonte, PA, USA).

Methylene chloride (Fisher Scientific, Pittsburgh, PA, USA; Optima GC–MS/HPLC grade) was dried over a molecular sieve (3 Å). All other solvents were of HPLC or GC grade from either Fisher Scientific or Burdick and Jackson (Muskegon, MI, USA) and were used as received. Ammonium carbonate was also from Fisher Scientific. Drug-free heparinized human control plasma was purchased from Sera Tec Biologicals (New Brunswick, NJ, USA). All other reagents were of analytical reagent grade or better.

Solid phase phenyl (500 mg, 3 ml) and silica gel (500 mg, 3 ml) extraction cartridges were obtained from J.T. Baker (Phillipsburg, NJ, USA). Disposable polypropylene tubes were purchased from Sarstedt (Newton, NC, USA). A Tecam dri-block DB-3 heater was purchased from Techne (Princeton, NJ, USA).

2.2. Instrumentation

The HPLC system consisted of a Waters (Milford, MA, USA) 6000 pump, a Waters 712 automatic injector and an Applied Biosystems (Foster City, CA, USA) Model 783A UV detector. The analog detector output was connected to a PE Nelson Access*Chrom data acquisition system (Perkin Elmer, PE Nelson Division, San José, CA, USA) by a PE Nelson Model 941 interface.

The GC chromatographic system consisted of a Hewlett Packard (Atlanta, GA, USA) 5890 Series II gas chromatograph equipped with a Hewlett Packard 5971A mass selective detector (MSD) and a Hewlett Packard 7673 autosampler. A Hewlett Packard Chem Station was used for data acquisition.

2.3. HPLC conditions

The separation of P, PA, I-P and I-PA was achieved by reversed-phase ion pair chromatography on a 5-μm BDS Hypersil C_{18} bonded silica column (250×4.6 mm I.D., Keystone Scientific, State College, PA, USA) using an acetonitrile–water (15:85, v/v) mobile phase containing 0.01 *M* $(NH_4)_2CO_3$ and 0.002 *M* Q12 ion pair cocktail at a flow-rate of 1.2 ml/min. Detection was via UV absorbance at 214 nm.

2.4. GC conditions

Helium was used as a carrier gas with a back pressure of 16 p.s.i. (1 p.s.i.=6894.76 Pa). Splitless injections (60 s periods) were made at 250°C. The HP Ultra 5 (crosslinked 5% phenyl silicone) fused-silica capillary column (Hewlett Packard; 25 m×0.2 mm I.D.×0.5 μm film thickness) was maintained at 100°C for 1 min, then the oven temperature was increased by 35°C/min up to 300°C, where it was held for 20 min. After cooling to 100°C, the column was equilibrated for 30 s between each injection. The MSD interface temperature was 300°C, while the ion source temperature was 190°C. The electron multiplier voltage was 200 to 600 V above the calibration voltage. The ion source pressure was 1.5×10^{-5} Torr and the dwell time was 100 ms during selected ion monitoring. Data were acquired from 6 to 10 min following each injection with selected ion monitoring at m/z 291.

2.5. Base hydrolysis of pilocarpine

P HCl (5 ml of a 10 mg/ml, free base, aqueous solution) was mixed with 200 μl of 15% (w/w) sodium hydroxide and the solution was allowed to stand at room temperature for a minimum of 40 min, to allow completion of hydrolysis and partial epimerization. The sodium hydroxide was neutralized by adding 150 μl of an aqueous phosphoric acid solution (1:4, v/v). The contents of the volumetric flask were diluted to the mark with water to yield a 5-mg/ml stock solution containing a 4:1 ratio of PA and I-PA, as determined by the HPLC conditions described above.

A solution of I-PA was prepared in a similar manner from the hydrolysis of I-P. In contrast to P, hydrolysis of I-P yielded only I-PA, i.e. no PA was detected.

2.6. Sample preparation

2.6.1. HPLC analysis of human plasma

A 100-μl aliquot of plasma sample was pipetted into a 75×12 mm polypropylene conical test tube. Acetonitrile (200 μl) was added to the tube in order to precipitate proteins. Following vortex-mixing for 30 s, the tube was centrifuged for 10 min at 2500 *g*. A 100-μl aliquot of the supernatant was evaporated to dryness under nitrogen in a 50°C water bath. The sample was reconstituted in 500 μl of mobile phase and 100 μl were injected onto the HPLC system.

2.6.2. Extraction of PA from human plasma for GC–MS analysis

Samples were thawed at room temperature for approximately 2 h. A 1-ml aliquot of each plasma sample was pipetted into a 75×12 mm polypropylene conical test tube. Acetonitrile (1.5 ml) was added to the tube in order to precipitate plasma proteins. Following vigorous vortex-mixing, the tube was centrifuged for 10 min at 2500 *g*. The supernatant (aliquot) was decanted into a 75×12 mm polypropylene round bottom test tube.

A silica gel SPE cartridge, positioned on a ten-port vacuum manifold, was conditioned by aspirating one column volume each of methanol, acetonitrile, water and 50:50 (v/v) acetonitrile–water through the cartridge. The sample was then slowly drawn through the cartridge using a vacuum of less than 200 mbar (<20 kPa). The SPE column was washed with 1 ml of acetonitrile. The analyte was eluted into a 100×15.7 mm polypropylene tube using two 1.0 ml aliquots of methanol. The eluate was evaporated to dryness.

The residue was reconstituted in 1 ml of 10 m*M* phosphate buffer solution, pH 3. A phenyl SPE cartridge was conditioned by aspirating one column volume each of methanol, water and 10 m*M* phosphate buffer solution, pH 3, through the column. The reconstituted sample was then drawn through the conditioned phenyl column, followed by two column volumes of water. The SPE column was centrifuged for 10 min at 3000 rpm (2500 *g*) in order to remove all water from the column. Acetonitrile (1.5 ml) and 2.5 ml of methanol were then each drawn through the column using centrifugal force (5 min at 2500 *g*). The SPE cartridge was returned to the vacuum manifold. The analyte was eluted into a 100×13 mm silanized glass screw cap tube by drawing 2.5 ml of a 1% NH_4OH–methanol (v/v) solution through the SPE column under a vacuum of less than 200 mbar (<20 kPa). The eluate was evaporated to dryness.

2.7. Derivatization of pilocarpic acid

All glassware used for the derivatization was silanized by treatment with Sylon CT followed by rinsing with methanol. The autosampler glass vial inserts were additionally rinsed three times with methanol before use.

A methanolic solution of the internal standard (30 μl, 300 ng/ml) and 1 ml of 1% trifluoroacetic acid (TFA)–methanol (v/v) were added to the residue from the SPE and then evaporated to dryness. To the residue, 200 μl of dried methylene chloride and 10 μl of HFBA derivatizing reagent were added and the sample was vortex-mixed. A 50-μl volume of a triethylamine–methylene chloride solution (80 μl:1000 μl) was added. The sample tubes were capped, vortex-mixed and heated on a dri-block for 30 min at 50°C.

To the resulting solution, 2 ml of methylene chloride and 1 ml of water were added and the mixture was shaken for 1 min. An additional 1 ml of 5% NH_4OH–H_2O (v/v) was added and the mixture was shaken again for 5 min and then centrifuged (2500 *g* for 5 min). The upper aqueous layer was aspirated to waste and the remaining organic layer was partially evaporated under nitrogen until approximately 0.5 ml remained. The methylene chloride layer was then transferred to a clean silanized glass vial and evaporated to dryness under nitrogen. To the residue was then added 0.5 ml of TFA–methanol solution, which was subsequently removed under nitrogen. The resulting residue was reconstituted in 100 μl of 20:80 ethyl acetate–hexane (v/v) and dissolution was ensured by thorough vortex-mixing followed by placing of the vial in an ultrasonic bath for 5 min. A 1-μl volume of the final solution was injected into the GC–MSD for analysis.

Recovery of the extraction procedure was determined by derivatizing both neat P standards and extracted PA standards (as P equivalent) from plasma and comparing their responses.

2.8. Preparation of standards

Since P hydrolyzes completely to PA in the presence of heparinized human plasma [10] and a pure standard of PA was unavailable, standards for analysis were prepared by adding P to plasma and allowing the compound to completely hydrolyze.

A primary stock solution of 10 mg/ml P (free base) was prepared by weighing P HCl, dissolving it in water and storing at 4°C. The primary stock solution was stable for at least three months.

Secondary (0.1 mg/ml) and tertiary stock solutions (5 and 1 μg/ml) were prepared with water.

Aliquots of the 1 and 5 μg/ml tertiary stock solutions were diluted in human control plasma (heparin anticoagulant) to yield plasma standards with concentrations of 1, 2.5, 5 and 10 ng/ml of PA as P equivalent (free base). Working standards were prepared at room temperature and stored in polypropylene tubes at −20°C, and thawed at room temperature for analysis. Exposure to room temperature was for a minimum of 3.5 h.

PA quality control samples at 2.5 ng/ml were prepared in a similar manner.

2.9. Quantitation of pilocarpic acid

The PA standards and quality controls were extracted, derivatized and analyzed daily along with study samples. The linear regression equation calculated from a plot of the peak area ratio of analyte to internal standard versus concentration in standard PA solution was used to calculate the concentration of PA in the quality control and study samples. The standard curve range was from 1 to 10 ng/ml, and the LOQ was defined as the lowest concentration on

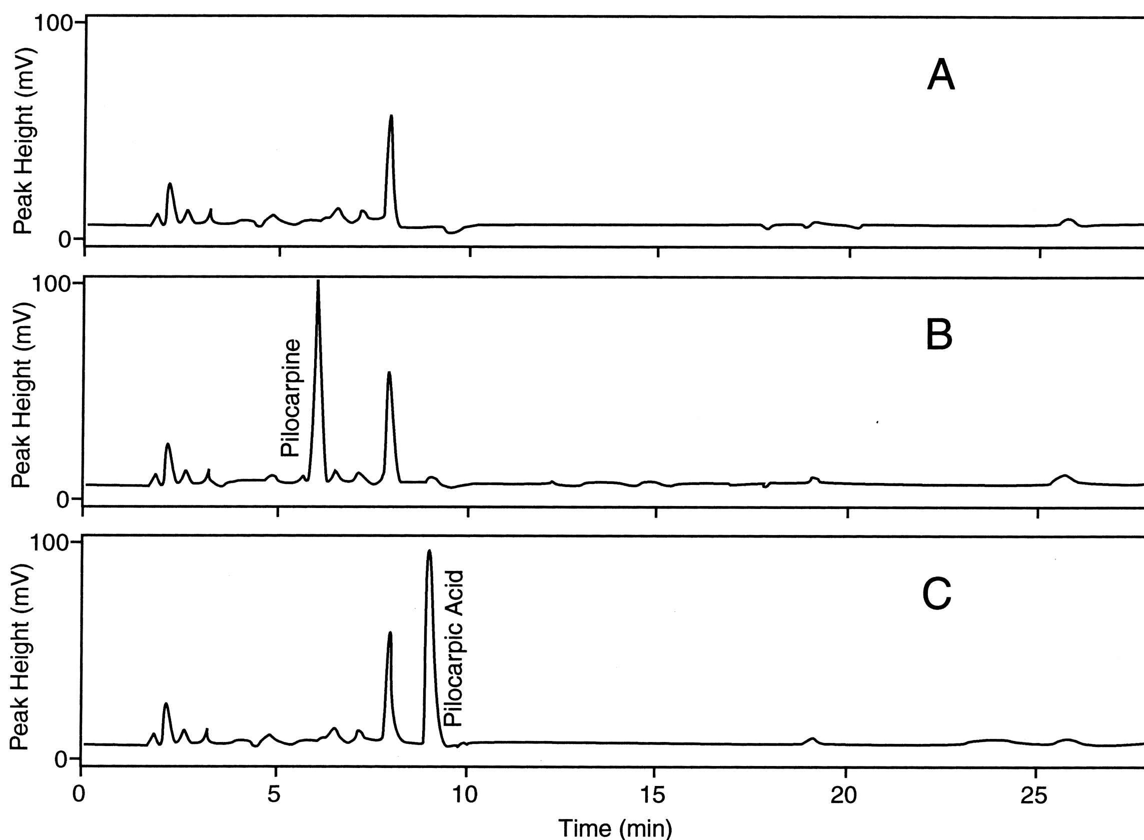

Fig. 2. HPLC chromatograms obtained from extracts of (A) control plasma; (B) plasma freshly spiked with pilocarpine, 150 μg/ml; and (C) plasma stored with pilocarpine, 150 μg/ml, for 24 h at room temperature.

the standard curve for which replicate determinations had a coefficient of variation (C.V.) of less than 10% and for which accuracy of back calculated assay values was within 15% of the nominal concentration.

2.10. Clinical samples

In a randomized, two-period crossover study, a pharmacokinetic comparison of P plasma exposure was made between TIMPILO4 (0.5% Timolol–4% pilocarpine, ophthalmic solution, Merck, twice a day (b.i.d.); total daily dose, 8 mg of P) and ISOPTOCARPINE (4% pilocarpine ophthalmic solution, Alcon, four times a day (q.i.d.); total daily dose, 16 mg of P) after $7\frac{1}{2}$ days of dosing in normal volunteers. TIMPILO4 was administered at 0 and 12 h daily and ISOPTOCARPINE was administered at 0, 4, 8 and 12 h daily. Blood samples were collected over 12 h on the last day of dosing in vacutainer tubes containing heparin. The samples were centrifuged immediately after collection. Following centrifugation, the plasma layer was transferred to a polypropylene cryotube and frozen at −20°C until analysis.

3. Results and discussion

3.1. Hydrolysis of pilocarpine

P is known to degrade rapidly in aqueous alkaline solutions [11–14]. The primary degradation product, PA, is the product of the hydrolysis of the P lactone ring. The secondary product, I-PA, results from the hydrolysis of I-P, which is initially formed under basic conditions through epimerization at the α-carbon of the lactone ring of P. Thus, chromatographic conditions capable of separating P, I-P, PA and I-PA were required to study the degradation of P in heparinized plasma.

Numerous HPLC separations of the degradants of P appear in the literature [4,13,15–17]. However, in the majority of these methods, PA and I-PA elute near the solvent front, a region where numerous endogenous components elute when extracts of plasma are chromatographed. In order to overcome this problem, reversed-phase ion pair chromatography was used to separate P, I-P, PA and I-PA under the chromatographic conditions described earlier [18], with some modifications. In this system, PA and I- PA had retention times that were longer than that of P.

Standards of PA and I-PA were not commercially available. As was published previously [1,4,13,18], the reference standard solution for PA, a 4:1 PA–I-PA mixture, was produced from the base hydrolysis of P. Similarly, in good agreement with the literature [4,13], a solution containing greater than 97% I-PA and less than 3% PA was produced from the hydrolysis of I-P.

Representative HPLC chromatograms (Fig. 2) illustrate that P was totally hydrolyzed to PA in plasma that was allowed to remain at room temperature for 24 h. The hydrolysis of P to PA in heparinized plasma was quantitative (>99.6% peak area of initial) and no epimerization of P was observed. This confirmed previous reports [7].

The hydrolysis of P in heparinized plasma to PA was found to be both time- and temperature-dependent. Exposure to room temperature for a minimum of 3.5 h resulted in the quantitative hydrolysis of P to PA in different lots of plasma studied.

Due to the unavailability of analytical standards of PA and based on the results of the hydrolysis study, the standards of PA in plasma were prepared by spiking P into plasma and letting the hydrolysis occur prior to analysis.

3.2. GC–MS analysis

The concentration of PA in plasma samples collected following ocular administration of P was expected to be below 10 ng/ml. Hence, a procedure with a LOQ of about 1 ng/ml was required for the analysis of clinical samples. GC–electron capture detection (ECD) methods with low ng limits of detection have been reported for the determination of P in aqueous humor [8,9]. They were based on acylation of the imidazole ring of P with HFBA and determination of the derivative using GC–ECD. The GC–MS monitoring of the derivative in contrast to GC–ECD [8] provided a more robust method that was less sensitive to electron-capturing impurities and residual derivatizing reagents. Since the sensitivity of GC–MS detection was sufficient for low ng/ml detection of PA, this technique was employed for the development of an assay in plasma.

Derivatization of P with HFBA yields a product having a mass spectrum consistent with that shown in reference [8] (previously presented) in which the structure was shown to be consistent with acylation of the imidazole ring. Mass spectral analysis of the heptafluorobutyric (HFBA) derivative of PA, following lactonization to P (Fig. 3), confirmed this assignment [8]. A molecular ion with a mass to charge ratio (m/z) of 404 was observed with a major fragment having a m/z of 291, assigned to the loss of the acylated imidazole ring [8]. The m/z 291 fragment was used for quantitation, in a selected ion monitoring (SIM) mode. The mass spectra of the derivative of the internal standard also contained a major fragment at m/z 291, hence the same ion was monitored for internal standard.

3.3. Analysis of PA from plasma

In order to determine P in human plasma in the form of PA, an effective isolation procedure for PA from plasma was required. PA in plasma supernatant following protein precipitation was effectively retained on a silica gel SPE column. No retention was observed on reverse phase packings such as C_8, C_{18} or cyano. The silica SPE column could be washed with acetonitrile prior to elution of the analyte with methanol.

Further purification of the plasma extracts using phenyl-bonded SPE cartridges was necessary because samples still contained peaks that interfered with quantitation of PA. Centrifugal force was used to remove residual water from the column. Following the drying step, it was possible to wash the column with acetonitrile and methanol before elution of PA. Chromatograms of extracts prepared using the combined silica–phenyl SPE column procedure were free of peaks that interfered with quantitation. The internal standard was added to the extracted sample prior to derivatization.

Attempts to derivatize PA directly after extraction from plasma yielded irreproducible results. Therefore, the PA was converted to P by acidification of the extract with TFA. The conversion of PA to P was quantitative, as confirmed by HPLC analysis. This finding confirmed similar observations made earlier by Nunes and Brochman-Hansen [11] based on the NMR analysis of products.

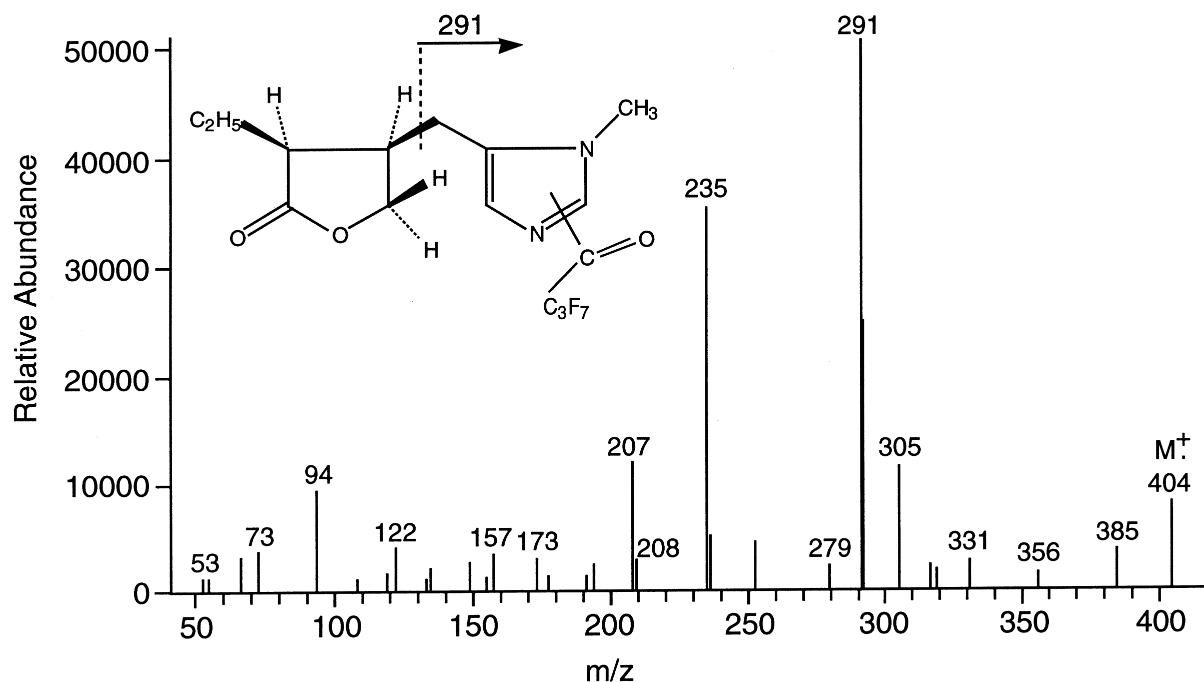

Fig. 3. Representative electron ionization mass spectrum of the HFB derivative of pilocarpine.

Following lactonization, the resulting P, along with internal standard, was derivatized with HFBA [8]. The concentrations of reagents required for efficient derivatization and the kinetics of the reaction were optimized. Excess derivatization reagent was removed by the addition of aqueous ammonia [9]. The

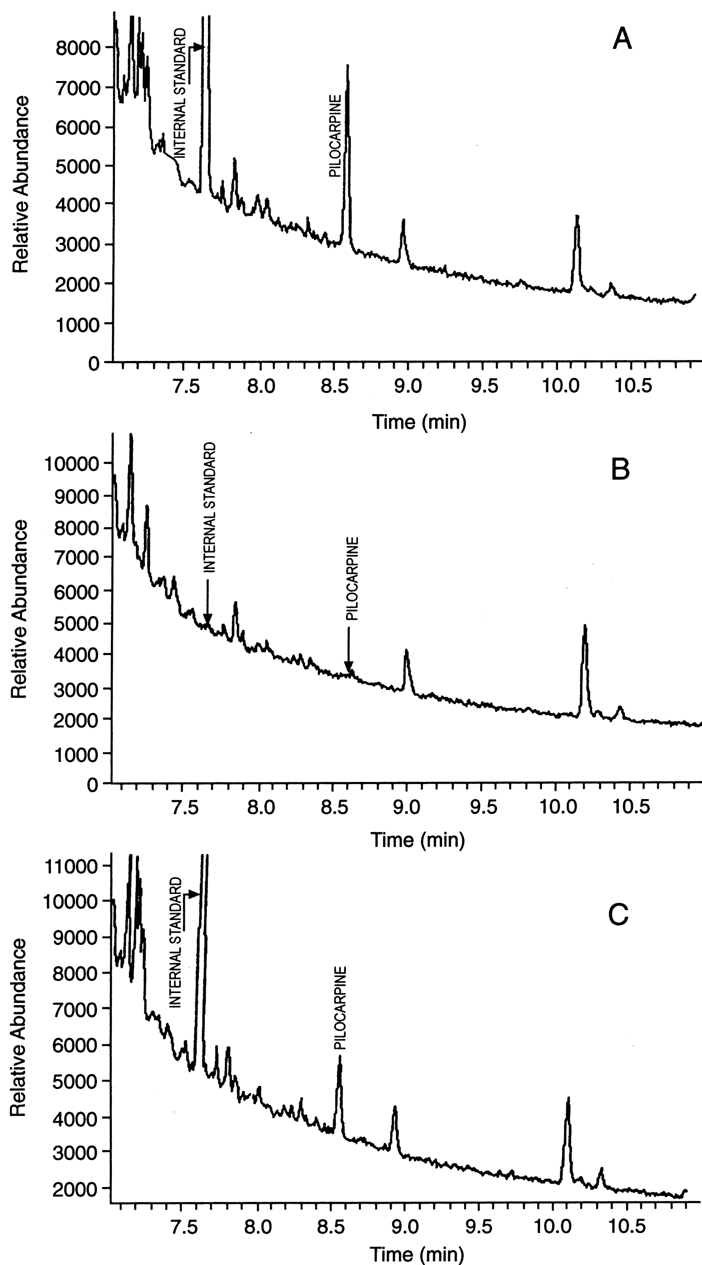

Fig. 4. GC–MS/SIM chromatograms (ion at m/z 291 was monitored) of HFB derivatives of pilocarpine and internal standard of (A) 5.0 ng/ml standard in control plasma containing 9 ng/ml of internal standard; (B) predose plasma sample taken on day 1 before dosing with pilocarpine and (C) plasma sample taken from a subject on day 8, 1 h after dosing for $7\frac{1}{2}$ days, one drop each eye, b.i.d., with a solution of Timolol (0.5%) and pilocarpine (4%) (TIMPILO4); the concentration of pilocarpic acid was equivalent to 1.97 ng/ml.

organic layer was evaporated, dried, acidified with a TFA–methanol solution, redried and reconstituted with 20:80 ethyl acetate–hexane (v/v). A portion of the extract was subjected to GC–MS analysis.

3.4. Specificity and selectivity

Fig. 4 shows representative GC–MSD chromatograms of a pre-dose plasma sample and a plasma sample obtained following the ocular administration of P to a human subject. A comparison of the chromatograms 4B and 4C indicates that no endogenous peaks co-eluted with the analytes.

The HFB derivative of I-PA (retention time, $RT=$ 8.40 min) eluted before the HFB derivative of PA ($RT=8.55$ min). I-PA was not detected in any plasma samples, confirming that the hydrolysis of P in plasma yielded only PA.

3.5. Assay validation

Standards of PA in plasma were analyzed using the sample preparation/derivatization scheme. Regression analysis of a plot of the peak area ratios of PA to internal standard yielded correlation coefficients greater than 0.995 over the concentration range of 1 to 10 ng/ml. Attempts to extend the standard curve range to 20 ng/ml resulted in nonlinearity. This relatively narrow standard concentration range was suitable for the analysis of clinical samples.

Five replicate plasma standards at all concentrations used for constructing the calibration curve were analyzed for PA to assess the accuracy and within-day variability of the assay. The accuracy of the mean assayed standard concentrations ranged from 94.0 to 101.6% (Table 1). The coefficients of variation of the replicate analyses were found to be under 10%, indicating good assay precision.

A 2.5-ng/ml quality control sample was prepared by adding P HCl to human heparinized plasma and allowing hydrolysis to occur at room temperature. Aliquots were pipetted into polypropylene centrifuge tubes and stored at −20°C. Two quality control samples were extracted and derivatized daily with study samples to assess accuracy and inter-day variability. The quality controls were analyzed six times over a period of three weeks. The results (Table 2) indicate that the inter-day variability of the assay, as measured by the coefficient of variation, was under 11%. The mean assay accuracy over the three week period was 93.6%.

The limit of quantitation of the assay, defined as the lowest concentration that yielded a within-day precision of less that 10% C.V., and a within-day accuracy of between 90 and 110% of nominal concentration, was 1 ng/ml of PA.

Recovery of the extraction procedure was 67.0±1.0% ($n=3$) and 67.7±2.0% ($n=3$) for 2.5 and 10 ng/ml PA plasma standards, respectively.

Greater than 95% of the initial concentration of PA was found in plasma samples assayed after 12 months of storage at −20°C. Freeze/thaw cycles ($n=3$) were not found to alter PA concentrations.

3.6. Analysis of clinical samples

Fig. 5 shows a representative concentration versus time profile of PA in plasma for one healthy volunteer over a dosing interval following $7\frac{1}{2}$ days of ocular dosing with 8 mg of P (TIMPILO4) two times a day. The assay for PA was sensitive enough to determine PA concentrations above the LOQ, as total PA, in plasma for at least 4 h following the adminis-

Table 1
Accuracy and precision data for pilocarpic acid (pilocarpine equivalent) in human heparinized control plasma

Intra-day variability ($n=5$)			
Nominal concentration (ng/ml)	Calculated concentration (ng/ml)	Coefficient of variation (%)	Accuracy[a] (%)
1.0	0.94	9.9	94.0
2.5	2.52	2.0	100.8
5.0	5.08	2.0	101.6
10.0	9.96	0.5	99.6

[a]Determined as [(mean assayed concentration)/(nominal spiked concentration)×100].

Table 2
Accuracy and precision data for the analysis of pilocarpic acid (pilocarpine equivalent) quality control samples in human heparinized control plasma

Intra-day variability (n = 5)

Quality control standard (ng/ml)	Calculated concentration (ng/ml)	Coefficient of variation (%)	Accuracy[a] (%)
2.5	2.42	2.9	96.8

Inter-day variability (n = 12)

Quality control standard Mean calculated concentration (ng/ml)	Number of days of analysis	Coefficient of variation (%)	Accuracy[a] (%)
2.34	6	10.2	93.6

[a]Determined as [(mean assayed concentration)/(nominal spiked concentration) × 100].

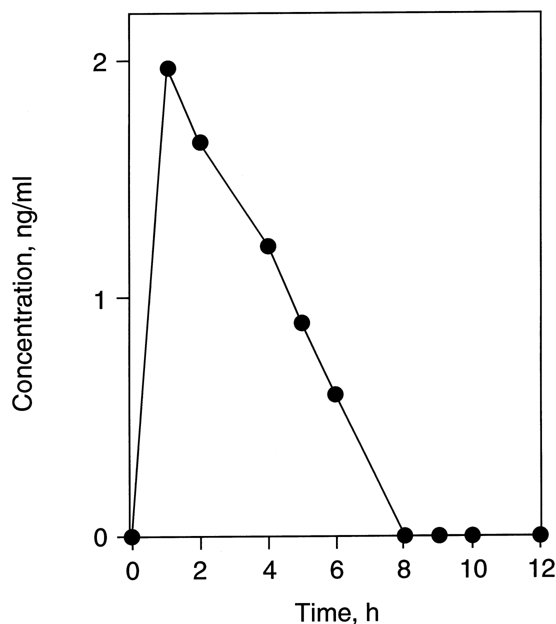

Fig. 5. Representative concentration vs. time profile of pilocarpine in plasma following dosing of one healthy volunteer with a solution of Timolol (0.5%) and pilocarpine (4%) (TIMPILO4) for $7\frac{1}{2}$ days, one drop to each eye, b.i.d.

tration of P. Additionally, the concentrations were far less (maxima almost 20-fold less) than maximum plasma concentrations for P (54 ng/ml) observed after a 10 mg oral dose [9]. There was no evidence of I-PA in these samples.

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