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Determination of pirenzepine in human plasma using liquid chromatography with tandem mass spectrometric detection

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Abstract

A sensitive (LOQ = 1 ng ml^{-1}) and specific method based on liquid chromatography with tandem mass spectrometric (MS/MS) detection has been developed and validated for the analysis of pirenzepine (I) in plasma. Sample preparation involved liquid–liquid extraction of drug and internal standard (IS) from basified plasma. The organic extract was evaporated to dryness, and the residue was reconstituted in the mobile phase and then injected into the liquid chromatography/MS/MS system. Drug, IS, and endogenous impurities were separated using reverse-phase chromatography. A Sciex API III tandem mass spectrometer equipped with a heated nebulizer was operated in the positive ion mode. Multiple reaction monitoring using the parent → daughter ion combinations of *m/z* 352 → 113 and 629 → 422 was used to quantify **I** and IS, respectively. The method was validated in the concentration range of 1–100 ng ml^{-1} plasma with adequate assay precision and accuracy, and was utilized to support human safety and tolerability study with **I**.

1. Introduction

A number of studies have been performed to compare the effects of two muscarinic antagonists, atropine and pirenzepine (**I**, Fig. 1), on gastric and extragastric muscarinic receptors [1–4]. There is evidence that atropine prevents the development of myopia in humans, but this therapy has been limited owing to its ocular side effects [5,6]. It was hypothesized from the studies of Stone et al. [7] in a chick model that pirenzepine can prevent myopia by a similar mechanism to atropine, but without the disabling side effects, and can be considered as a topical agent to treat this disease.

Two liquid chromatography (LC) methods with ultraviolet (UV) detection for the determination of pirenzepine have been described in the literature. The first method was used for the analysis of **I** in dosage forms and biological fluids and had a limit of quantification (LOQ) of 1 μg ml^{-1} [8]. The second method [9] had a LOQ in plasma of 5 ng ml^{-1}, but the precision at the lowest concentration on the calibration line was unacceptably poor (RSD ≈ 25%). Another method based on radioimmunoassay [10] had a LOQ of 1.25 ng ml^{-1} in both plasma and urine. However, all of these methods lacked either the specificity or sensitivity required to quantify the drug at low ng ml^{-1} levels. In a clinical study in which **I** was orally dosed at 25 mg, it was found that the mean plasma level 24 h after dosing was 7.5 ng ml^{-1} [11]. Therefore, it was anticipated that systemic levels of pirenzepine after ocular administration will be ≤1 ng ml^{-1}, and an analytical method with an LOQ of at least 1 ng ml^{-1} was required.

In the last few years, atmospheric pressure ionization (API)/LC/MS and LC/MS/MS techniques have been widely utilized in biomedical

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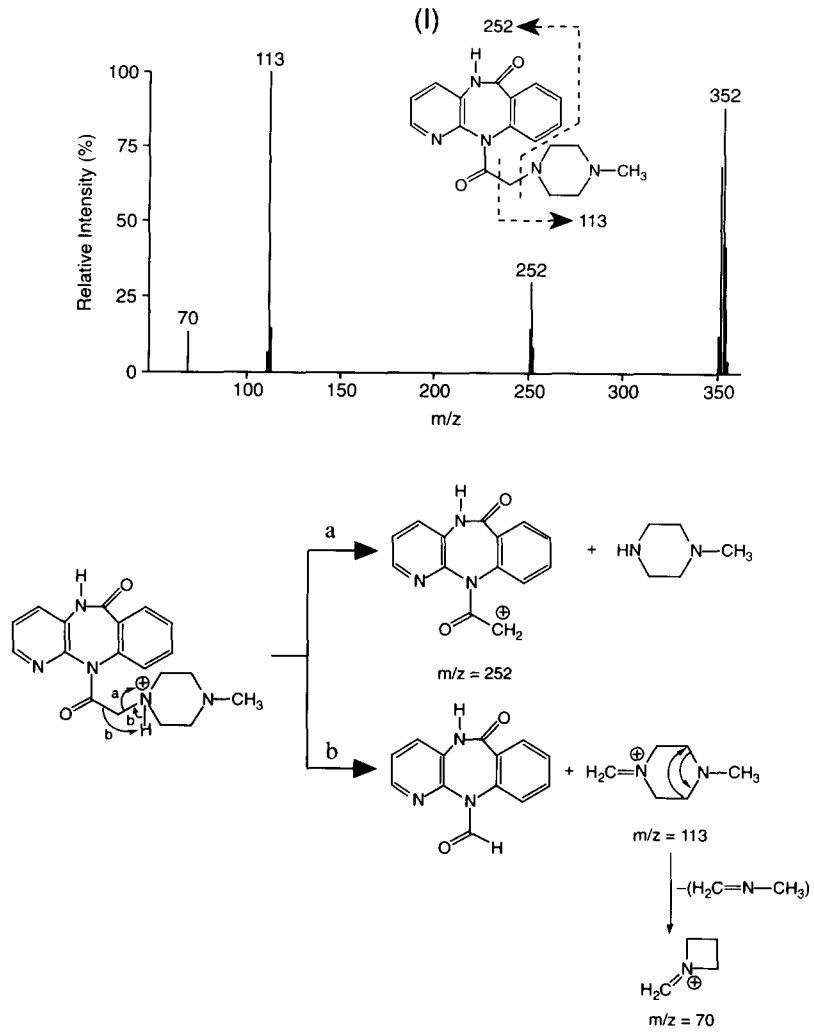

Fig. 1. Chemical structure and positive product ion mass spectrum of the protonated molecule of pirenzepine (I) (*m/z* 352) and the proposed mechanism of fragmentation.

fields for both the identification and quantitation of drugs and metabolites in biological fluids at very low concentrations [12–18]. This methodology provides both separation and highly specific and sensitive detection of non-volatile and polar compounds. Using the heated nebulizer interface, LC flow rates of 0.2–2.0 ml min^{-1} are easily accommodated and analytes are rapidly vaporized, usually without significant thermal degradation. Since chemical ionization of the sample molecules takes place at atmospheric pressure, ionization is highly efficient owing to high collision frequency [19]. In the positive ion mode, the primary reagent ions produced by gentle ionization of solvent vapor in the API source ionize analyte molecules by proton transfer. This creates a high abundance of pseudo-molecular ions $(M+H)^+$ which is favorable for sensitive MS analyses. By monitoring an intense product ion obtained from $(M+H)^+$ through collision with a neutral gas molecule (argon), a highly specific determination of an analyte in the tandem MS/MS mode could be achieved. In the multiple reaction monitoring mode (MRM), parent → daughter ions are monitored while the sample is eluting, thus making MS/MS a more selective and specific method than a single LC/MS.

The subject of this paper is the development of a highly efficient, sensitive, and specific analytical method for determination of **I** in human plasma with a LOQ of 1 ng ml^{-1} using LC/API/MS/MS. The LOQ is defined here as the lowest concentration on the calibration line for which intra-day assay precision (RSD) was less than 10% and assay accuracy was within ±10%.

2. Experimental

2.1. Materials and methods

Pirenzepine dihydrochloride, trimipramine maleate salt, and ammonium acetate were obtained from Sigma (St. Louis, MO, USA). The internal standard (IS, **II**) and cyclobenzaprine were synthesized in the Medicinal Chemistry Department of Merck Research Laboratories (Rahway, NJ, USA). Formic acid was purchased from Aldrich (Milwaukee, WI, USA). All other chemicals were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Instrumentation

The LC system consisted of a Perkin-Elmer Biocompatible Binary pump 250, and ISS 200 Autoinjector (Perkin-Elmer Corporation, Exton PA, USA), and an API III triple quadrupole tandem mass spectrometer (PE-Sciex, Thornhill, Canada) equipped with a heated nebulizer interface.

2.3. Chromatographic conditions

The aqueous portion of the mobile phase was prepared by dissolving 0.77 g of ammonium acetate in 1000 ml of water with the addition of 820 µl formic acid. The mobile phase consisted of 60% of the aqueous portion and 40% acetonitrile, and was pumped at a flow rate of 1 ml min^{-1}. Chromatography was performed on a BDS-Hypersil C-18 20 × 4.6 mm, 5 µm guard column and BDS-Hypersil C-18 50 × 4.6 mm, 5 µm analytical column (Keystone Scientific Inc., Bellefonte, PA, USA). The total runtime was 5.5 min, with **I** eluting at 0.5 min and IS at 2.6 min after injection.

2.4. Mass spectrometric conditions

The mass spectrometer was interfaced to the LC system via a heated nebulizer probe that was maintained at 500 °C. Nebulizer (air) pressure was set at 80 psi, nebulizer flow at 0.6 l min^{-1}, and curtain gas (N_2) flow at 0.9 l min^{-1}. Positive chemical ionization was effected by a corona discharge needle (+4 µA) and the sampling orifice potential was set at +55 V. The first quadrupole, Q1, was set to monitor the protonated molecules $(M + H)^+$ at m/z 352 for drug and m/z 629 for IS with collision-induced fragmentation at Q2 (collision gas argon, 450×10^{12} atoms cm^{-2}), and monitoring of the product ions via Q3 at m/z 113 and m/z 422 for **I** and IS, respectively. The electron multiplier setting was −3.8 kV and detector electronics were set to counts of 10. The dwell time was 400 ms.

2.5. Data acquisition and analysis

Data acquisition and analyses were performed using RAD and MacQuan software (PE-Sciex). Unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by the weighted $(1/y^2)$ linear least-squares regression of the calibration line constructed from the peak area ratios of drug to IS vs. drug concentration.

2.6. Standard and sample preparation

A standard stock solution of **I** (1.0 mg ml^{-1}; 2.8×10^{-3} M) was prepared in water. Subsequent dilutions were made in water to give the following concentrations: 0.01, 0.02, 0.05, 0.10, 0.25, 0.5, and 1.0 µg ml^{-1}. A standard stock solution of IS was prepared as 1.0 mg ml^{-1} (1.6×10^{-3} M) in 15% acetonitrile and 85% water containing 0.1% formic acid and 10 mM ammonium acetate. Subsequent dilutions were made to prepare a 1.5 µg ml^{-1} working IS standard solution. Standards and quality control (QC) samples were prepared by the addition of known amounts of standard solutions (100 µl) to 1 ml of human control plasma.

Sample preparation involved liquid–liquid extraction of the drug from basified plasma. To 1 ml of plasma placed in a 45 ml glass centrifuge tube was added 100 µl of working IS standard. One ml of pH 9.8 carbonate buffer was added, followed by 10 ml of methylene chloride, and the mixture rotated (Glas-Col Laboratory Rotator, Beckman Instruments Inc., Palo Alto, CA, USA; speed setting of 8) for 20 min. After 15 min centrifugation at 4000 rpm, the aqueous layer was removed by vacuum suction. Seven ml of the organic layer was transferred to a clean tube, and after addition of 250 µl of 0.1 N HCl in methanol, the mixture was evaporated to dryness under a stream of nitrogen at 50 °C. The residue was redissolved in 250 µl of the mobile phase, vortexed for 1 min, sonicated for 15 min, vortexed and centrifuged for 5 min, and 100 µl of the

sample was injected into the LC/MS/MS system.

2.7. Precision, accuracy, linearity and recovery

The precision of the method was determined by replicate analyses ($n = 5$) of human plasma spiked with **I** at concentrations of 1, 2, 5, 10, 25, 50, and 100 ng ml^{-1}. Assay accuracy was assessed by comparing the mean calculated concentrations of standards, determined by replicate analyses, to the nominal concentration. Recovery was calculated by comparison of peak areas of **I** extracted from plasma with those of the directly injected standards.

The linearity of each standard curve was confirmed by plotting the peak area ratio of drug to IS vs. drug concentration. A calibration line was prepared and assayed daily with QC and clinical samples.

3. Results and discussion

Liquid–liquid extraction was utilized for the isolation of **I** from plasma. A number of solvents, namely toluene, ethyl acetate, hexane, methylene chloride, and methyl *t*-butyl ether (MTBE), were evaluated, but only methylene chloride and MTBE showed adequate recoveries of the drug. Methylene chloride was chosen as the extraction solvent over MTBE, owing to better precision and consistent recovery (approximately 78%) of the drug from plasma at various concentrations. The extraction of **I** and IS from the aqueous phase into the organic solvent was greatly improved when neither molecule was ionized; therefore, the pH of plasma was adjusted to pH 9.8 by the addition of carbonate buffer. In order to prevent the adsorption of the drug onto glassware during evaporation of the organic extract to dryness, a 0.1 N methanolic solution of HCl was added to the organic extract. In the presence of the acid, the amino groups of **I** are protonated, decreasing possible interactions with the silanol groups present on the glass surface and allowing more efficient reconstitution of the analytes. Under these conditions a considerable improvement in assay precision was observed.

Several compounds structurally related to **I** were initially evaluated as potential internal standards. Both trimipramine (**III**) and cyclobenzaprine (**IV**) (Fig. 2) behaved similarly to pirenzepine in terms of extractability from plasma. Compound **III** separated from **I** on a short (5 cm × 4.6 mm) BDS C-8 or C-18 column in 2 min, but under the LC conditions utilized an endogenous plasma peak interfering with the quantification of **I** at m/z 352 → 113 was observed. This endogenous peak from plasma was not separated from **I**. In addition, the standard of **III** utilized in this study (10 ng in 1 ml of plasma) gave an undesired response at the parent → daughter ion combination (m/z 352 → 113) characteristic to **I** and at the same retention

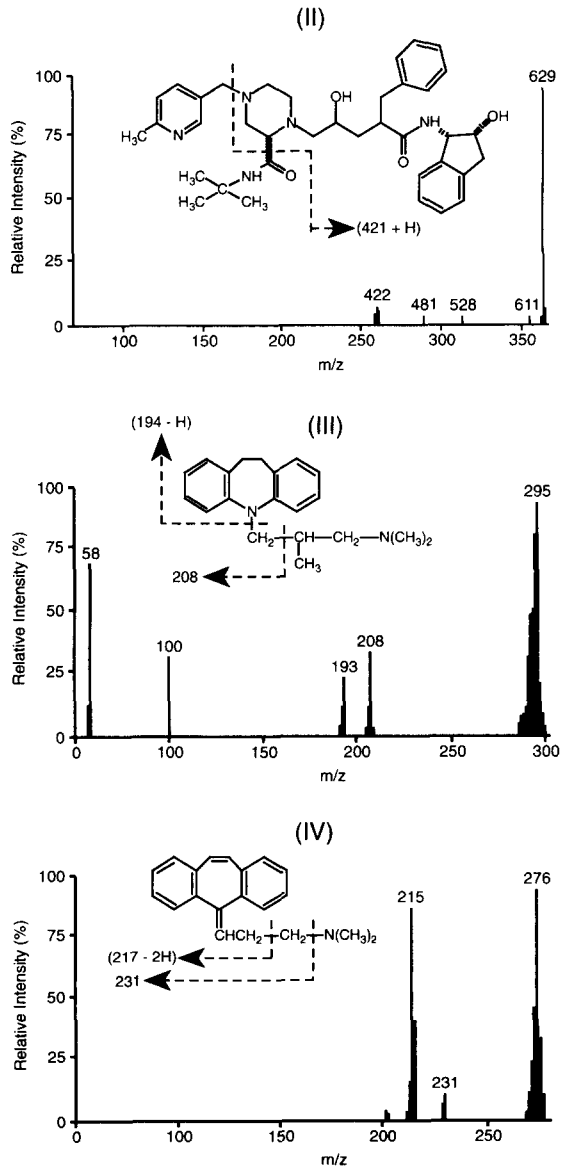

Fig. 2. Chemical structures and positive product ion mass spectra of the protonated molecules of the internal standard (**II**) (m/z 629), trimipramine (**III**) (m/z 295), and cyclobenzaprine (**IV**) (m/z 276).

time as **I**. This "cross talk" effect contributed to about 0.14% of the peak obtained when 100 ng of **I** was injected. Based on these data, compound **III** could not be utilized as an IS for the quantitation of **I** at the 1 ng ml^{-1} level.

Cyclobenzaprine (**IV**) was also initially extensively evaluated as an IS for the assay of **I**. Owing to the presence of pyridyl and piperazine groups in **I**, pirenzepine differs significantly from the majority of other tricyclic compounds and exhibits highly hydrophilic properties [20] at the low pH of the mobile phase utilized in the LC system. Therefore, a gradient elution method was necessary to separate **I**, **IV**, and other endogeneous compounds from the plasma. Initially, an assay based on gradient chromatography and using **IV** as an IS was developed, but the LC analysis time required was 16 min. In addition, the efficient of extraction of **IV** from different batches of plasma was highly variable (up to ±30%). Therefore, another internal standard was evaluated that gave reproducible recovery from different batches of plasma and was similar to **I** in terms of chromatographic behavior, allowing isocratic LC analyses with a short analysis time. Compound **II** containing pyridyl and piperazine groups, was chosen and was found to meet all the criteria necessary for the development of a sensitive, specific, and highly efficient assay. Using **II** as an IS, the run time was 5.5 min per sample, allowing analysis of approximately 90 biological fluid samples over an 8 h period.

The positive ion mass spectra of both **I** and **II** predominantly yielded the protonated molecules at m/z 352 and 629, respectively. The MS/MS product ion mass spectra of these ions showed mostly intense fragment ions at m/z 113 and 422, respectively (Figs. 1 and 2). The proposed fragmentation mechanism of the protonated molecule of **I** is also shown in Fig. 1. Multiple reaction monitoring using the parent → daughter combinations of m/z 352 → 113 and 629 → 422 were used to quantify **I** and IS, respectively.

The method was validated in human plasma with intra-day precision of less than 10% at all concentrations within the standard curve range and with adequate assay accuracy (Table 1). The method has been applied to the determination of **I** in the plasma of subjects dosed for 4 weeks with 2% pirenzepine ocular solution. Plasma concentrations of pirenzepine from three subjects are listed in Table 2. These data confirmed that the concentrations of **I** in systemic circulation after ocular administration were much lower than after oral administration of **I** at clinically useful doses of 50–150 mg [20]. Representative ion chromatograms of blank plasma, plasma spiked with drug and IS, and a plasma sample from a subject participating in a clinical study are shown in Fig. 3.

Table 1
Precision[a] and accuracy of the assay of pirenzepine in human plasma

Nominal conc. (ng ml^{-1})	Calculated conc. (ng ml^{-1})	RSD (%)	Accuracy (%)[b]
1.0	1.0	4.3	100.0
2.0	2.0	6.8	100.0
5.0	5.0	5.5	100.0
10.0	10.0	6.0	100.0
25.0	24.6	7.4	98.4
50.0	51.6	7.4	103.2
100.0	101.4	5.3	101.4

[a] Expressed as RSD; $n = 5$.
[b] Expressed as [(mean calculated concentration)/(spiked concentration)] × 100.

Table 2
Plasma concentrations (ng ml^{-1})[a] of subjects receiving 2% ocular solution of pirenzepine b.i.d. (one drop in each eye) for 29 days

Subject No.	Day 29					
	0 h	1 h	2 h	4 h	6 h	8 h
S-101	0.0	0.0	1.1	1.0	0.0	0.0
S-117	0.0	0.0	1.0	1.1	1.1	0.0
S-125	0.0	1.0	1.0	1.2	1.3	0.0

[a] A zero value represents a sample with less than 1.0 ng pirenzepine per ml of plasma.

In conclusion, a sensitive and specific LC/MS/MS method has been developed for the determination of pirenzepine in human plasma in the concentration range of 1–100 ng ml^{-1}. This method was applied to the analysis of **I** in the plasma of human subjects participating in a safety and tolerability study of pirenzepine ophthalmic solution.

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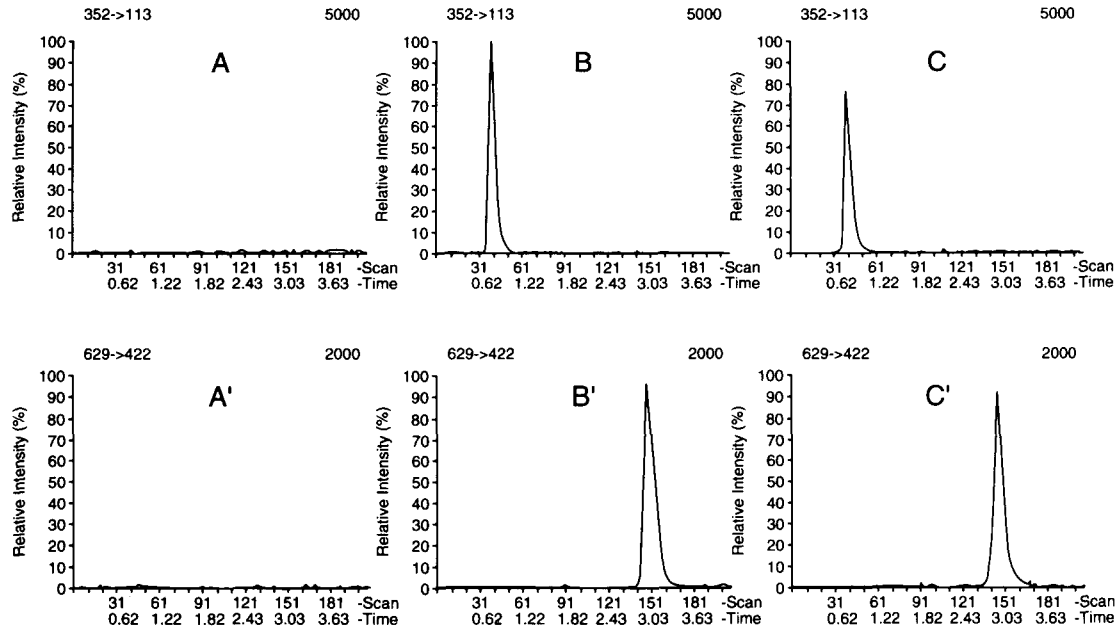

Fig. 3. Representative LC/MS/MS chromatograms of the plasma extracts obtained by multiple reaction monitoring at m/z 352→113 for pirenzepine and m/z 629→422 for internal standard. Chromograms A and A′ — extracts of control plasma; chromatograms B and B′ — extracts of control plasma spiked with 2 ng ml^{-1} of **I** and 150 ng ml^{-1} of **II**; chromatograms C and C′ — plasma extract of a subject after receiving a 2% ocular dose of **I** for 29 days 3 h after the previous dose, spiked with 150 ng ml^{-1} of II; concentration of **I** equivalent to 1.1 ng ml^{-1}. The numbers in the upper right-hand corner of the chromatograms correspond to the peak heights expressed in arbitrary units.

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