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Development and comparison of high-performance liquid chromatographic methods with tandem mass spectrometric and ultraviolet absorbance detection for the determination of cyclobenzaprine in human plasma and urine

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Abstract

Sensitive assays for the determination of cyclobenzaprine (I) in human plasma and urine were developed utilizing high-performance liquid chromatography (HPLC) with tandem mass spectrometric (MS–MS) and ultraviolet (UV) absorbance detections. These two analytical techniques were evaluated for reliability and sensitivity, and applied to support pharmacokinetic studies. Both methods employed a liquid–liquid extraction of the compound from basified biological sample. The organic extract was evaporated to dryness, the residue was reconstituted in the mobile phase and injected onto the HPLC system. The HPLC assay with MS–MS detection was performed on a PE Sciex API III tandem mass spectrometer using the heated nebulizer interface. Multiple reaction monitoring using the parent → daughter ion combinations of m/z 276 → 215 and 296 → 208 was used to quantitate I and internal standard (II), respectively. The HPLC–MS–MS and HPLC-UV assays were validated in human plasma in the concentration range 0.1–50 ng/ml and 0.5–50 ng/ml, respectively. In urine, both methods were validated in the concentration range 10–1000 ng/ml. The precision of the assays, as expressed as coefficients of variation (C.V.) was less than 10% over the entire concentration range, with adequate assay specificity and accuracy. In addition to better sensitivity, the HPLC–MS–MS assay was more efficient and allowed analysis of more biological fluid samples in a single working day than the HPLC-UV method.

1. Introduction

Cyclobenzaprine, 3-(5H-dibenzo[a,d]cyclohepten-5-ylidene)-N,N-dimethyl-1-propanamine (I, Fig. 1), is a skeletal muscle relaxant shown to reduce or eliminate skeletal muscle hyperactivity in laboratory animals [1–3] and clinically in man [4,5]. In order to provide bioanalytical support for pharmacokinetic evaluation of I at low (≤ 5 mg) therapeutic doses, an analytical method with a limit of quantitation (LOQ) of less than 1 ng/ml of plasma was required.

Compound I has been previously assayed in biological fluids by thin-layer chromatography [6], packed column or capillary gas chromatography (GC) with either flame-ionization or nitrogen-selective detection [7,8], or by HPLC with UV detection [9]. All these methods had a LOQ

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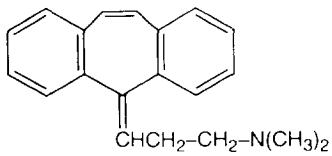
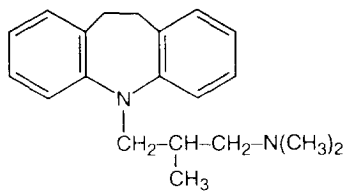


(I)



I.S. (II)

Fig. 1. Chemical structures of cyclobenzaprine (I) and internal standard (I.S., II).

of 1 ng/ml or greater, which was insufficient to quantitate the drug for a 24-h period following a 5-mg or lower dose of I. In addition, the GC method [8] was based on manual sample injection, required a relatively long analysis time (20 min), and allowed analysis of only a small number of clinical samples per day. The recently reported HPLC-UV assay [9] was based on liquid–liquid extraction of the drug from plasma and chromatography under normal-phase HPLC conditions. In order to achieve the LOQ of 1 ng/ml, it required 2 ml of plasma for extraction, injection of all of the aliquot (50 μl) onto the HPLC system, and a relatively long analysis time (20 min). The recovery of I from plasma was only 52%, and rigorous validation and assay specificity data were not presented or were not well established.

In order to develop an assay for I with an LOQ of less than 1 ng/ml of plasma two analytical techniques, HPLC with UV absorbance detection and HPLC–MS–MS, were employed and evaluated for sensitivity and reliability in supporting long term clinical trials. Initially, an HPLC-UV method, based on liquid–liquid extraction of I from basified plasma (1 ml), reversed-phase HPLC and detection at 229 nm, was developed. The LOQ of the HPLC-UV assay was 0.5 ng/ml, with high recovery (98%), and a relatively short run-time of 12 min.

Combination of HPLC with atmospheric-pressure chemical ionization (APCI) tandem mass spectrometry was recently shown to provide a convenient and highly efficient method for the quantitation of drugs in biological fluids [10–14]. A number of examples illustrating the applicability of this technique for the subnanogram quantitation of various drugs in support of human pharmacokinetic studies were reported [15–19]. Use of APCI-MS–MS with heated nebulizer probe and corona discharge results in high ionization efficiencies, and is compatible with flow-rates of HPLC mobile phases of up to 2 ml/min. With the availability of HPLC–MS–MS instrumentation, it was of interest to evaluate tandem mass spectrometric detection for the quantitation of I. Using HPLC–MS–MS methodology, an assay for I in human plasma with an LOQ of 0.1 ng/ml and a short run-time of 3 min was developed. The details of the HPLC–MS–MS method, its comparison with the HPLC-UV assay, and its application to pharmacokinetic studies in human subjects with low doses ($\leq$5 mg) of I are described in this paper.

2. Experimental

2.1. Materials

Cyclobenzaprine (I) was synthesized at the Merck Research Laboratories (Rahway, NJ, USA). All solvents and reagents were of HPLC or analytical grade and were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The drug free human heparinized plasma originated from Biological Specialties Corporation (Lansdale, PA, USA). The β-glucuronidase and the maleate salt of the internal standard (I.S.) (II, 10,11-dihydro-N,N-β-trimethyl-5H-dibenz[b,f]-azepine-5-propanamine, trimipramine) were purchased from Sigma (St. Louis, MO, USA). Air (hydrocarbon-free), nitrogen (99.999%) and argon (99.999%) were purchased from Matheson (Morris Plains, NJ, USA).

2.2. Instrumentation

A Waters Associates (Waters-Millipore, Milford, MA, USA) 6000E HPLC system equipped with a WISP 715 autoinjector and Applied Biosystems (Foster City, CA, USA) 785 variable-wavelength UV detector was employed for all HPLC-UV analyses. The detector output was interfaced with a Perkin-Elmer (PE) Nelson (Cupertino, CA, USA) Access-Chrom data system via a PE-Nelson 900 series interface, for data collection, peak integration, and analyses. A Keystone Scientific (Bellefonte, PA, USA) C_{18} base-deactivated silica (BDS) 250 × 4.6 mm I.D., 5 μm analytical column, coupled with a 20-mm C_{18} BDS guard column, were used in the HPLC-UV assay.

A PE Sciex (Thornhill, Ont., Canada) API III tandem mass spectrometer equipped with heated nebulizer interface and a Perkin-Elmer (Norwalk, CT, USA) advanced LC sample processor (ISS 200) and Perkin-Elmer biocompatible binary pump (Model 250) were used for all HPLC–MS–MS analyses. The data was processed using MacQuan software (PE Sciex) on a MacIntosh Quadra 900 microcomputer. HPLC was performed using a Keystone Scientific C_{18} BDS 50 × 4.6 mm I.D., 5 μm analytical column coupled with a C_{18} BDS 20 × 4.6 mm I.D., guard column.

2.3. Chromatographic conditions

The mobile phase in the HPLC-UV assay consisted of 50:50 or 43:57 (v/v) acetonitrile–water containing 0.085% phosphoric acid (adjusted to pH 6.5 with triethylamine) for plasma and urine, respectively. The mobile phase was delivered at a flow-rate of 1 ml/min. Prior to use the mobile phase was filtered through a 0.2-μm Nylon filter. The retention times for I and II were 7.8 and 10.5 min, respectively, for the assay in plasma and 9.6 and 12.8 min for the assay in urine.

The mobile phase for the HPLC–MS–MS assay consisted of acetonitrile–water (90:10, v/v) containing 0.1% formic acid and 10 m*M* ammonium acetate, and was delivered at a flow-rate of 1 ml/min. The retention times for I and II were 1.9 and 2.2 min, respectively, for both plasma and urine.

2.4. HPLC–MS–MS conditions

A PE Sciex triple quadrupole mass spectrometer was interfaced via a Sciex heated nebulizer probe to the HPLC system, and gas-phase chemical ionization was effected by a corona discharge needle (+4 μA) using positive ion APCI. The heated nebulizer probe was maintained at 500°C. The nebulizing gas (air) pressure and auxiliary flow were set at 80 psi and 2.0 l/min, respectively. Curtain gas-flow (nitrogen) was 0.9 l/min, and the sampling orifice potential was set at +45 V. The dwell time was 400 ms, and the temperature of the interface heater was set at 60°C. The mass spectrometer was programmed to admit the protonated molecular ions $[M + H]^+$ at m/z 276 (I) and m/z 295 (II), via the first quadrupole filter (Q1), with collision-induced fragmentation at Q2 (collision gas argon, $355 \cdot 10^{12}$ atoms cm^{-2}), and monitoring the product ions via Q3 at m/z 215 and 208 for I and II, respectively. The electron multiplier setting was −3.7 kV. Peak-area ratios obtained from multiple-reaction monitoring of analyte (m/z 276 → 215)/(m/z 295 → 208) were utilized for the construction of calibration curves, using weighted ($1/y$) linear least square regression of the plasma or urine concentrations and the measured area ratios. Data collection, peak integration and calculations were performed using MacQuan PE-Sciex software.

2.5. Standard solutions

A stock solution of I (1 mg/ml) was prepared in methanol. This solution was further diluted with methanol to give a series of working standards with concentrations of 1, 2, 5, 7.5, 10, 25, 50, 100, 200, and 500 ng/ml for plasma assay, and 100, 250, 500, 1000, 2500, 5000, and 10 0000 ng/ml for the urine assay. The internal standard II was also prepared as a stock solution (1 mg/ml) in methanol by dissolving 10 mg of solid II in 10 ml of methanol. A working standard of 500

ng/ml was prepared by serial dilutions of stock standard with methanol and was used for all analyses. All standards were prepared once a month and stored at 5°C.

A series of quality control (QC) samples at 0.2 and 40.0 ng/ml for the plasma assay and 20 and 750 ng/ml for the urine assay were prepared. Aliquots (1.25 ml) of these solutions were placed in 2-ml plastic tubes, stored at −15°C, and analyzed daily with clinical samples. The calculated concentrations of the QC samples were compared on a day-to-day basis.

2.6. Sample preparation

Plasma

A 1-ml aliquot of plasma was pipetted into a 15-ml centrifuge tube and 100 μl of the working standard of II (equivalent to 50 ng/ml of I.S.) followed by 1 ml of 0.2 *M* carbonate buffer (pH 9.8) were added. After addition of 5 ml of hexane and capping tubes with PTFE-lined caps, the mixture was mixed and rotated for 15 min. The tubes were then centrifuged for 5 min at 3000 *g* and the organic layer was transferred to a clean centrifuge tube. The organic extract was evaporated to dryness under a stream of nitrogen at 50°C, the residue was reconstituted in 150 μl (HPLC–MS–MS) or 300 μl (HPLC-UV) of the mobile phase, and a 75- or 150-μl aliquot was injected onto the HPLC–MS–MS or HPLC-UV system, respectively.

Urine

A 1-ml aliquot of urine was pipetted into a 15-ml centrifuge tube and 1 ml solution containing β-glucuronidase (7200 Fishman Units) in 0.02 *M* phosphate buffer (pH 6.5) was added. After incubation at 37°C for 24 h, the sample was basified with 100 μl of 10 *M* sodium hydroxide and extracted with 5 ml of hexane. Following a similar procedure as in plasma the residue, after evaporation of the extract to dryness, was reconstituted in 300 μl or 1 ml of the mobile phase for injection onto the HPLC-UV (150 μl) or HPLC–MS–MS (50 μl) system, respectively.

2.7. Precision, accuracy, recovery, and specificity

The precision of the method was determined by replicate analyses ($n = 5$) of human plasma and urine containing I at all concentrations utilized for constructing the calibration curves. The linearity of each standard was confirmed by plotting the peak-area ratio of the drug to I.S. versus drug concentration. The unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by weighted ($1/y^2$ for plasma or $1/y$ for urine) linear regression of the standard line. The standard curve was prepared and assayed daily with quality control and unknown samples. The accuracy of the method was expressed by: (mean observed concentration)/(spiked concentration) · 100%. Assay specificity was assessed by running blank control and patients pre-dose biological fluid samples. No endogenous interferences were observed. The recovery was determined by comparing the peak area of I extracted from biological fluids to that of standards injected directly.

3. Results and discussion

3.1. HPLC-UV assay

The UV spectra of I in methanol indicated the presence of two absorption bands with maxima at 226 and 292 nm and molar absorption coefficients (ε) of 38 500 and 11 800 $M^{-1}\,cm^{-1}$, respectively. This significant absorption of I in the accessible UV region permitted highly sensitive detection of the drug. About 30 pg of I injected on the HPLC column was detected at 226 nm, at a signal-to-noise ratio of 3:1, allowing development of assays in biological fluids at levels below 1 ng/ml.

The isolation of the drug from biological fluids was based on a simple one-step liquid–liquid extraction of I and II from basified plasma and/or urine, evaporation of the extract to dryness, reconstitution of the residue in the mobile phase and injection onto the HPLC system. By minimizing the number of extraction steps the adsorption problems commonly associated with the

isolation of tricyclic amine compounds [20,21] were eliminated as indicated by the high recovery of the drug. The mean recoveries of I were 98% and 99% for plasma and urine, respectively, and were practically the same at all concentrations within the standard curve range.

The retention times of I and II were highly dependent on the pH and the organic content of the mobile phase, and even small changes in these parameters would dramatically alter the retention times of I and II in relationship to the endogenous interferences extracted from plasma or urine. Under the conditions described in the Experimental section, assay specificity was maintained for all plasma and urine samples originating from various subjects and different sources.

The validation of the assay in urine was performed after pre-treatment of the urine samples with β-glucuronidase to convert the cyclobenzaprine glucuronide present in post-dose urine samples to I. Only 1.8% of the radioactivity in the 0–24 h urine was accounted for as unchanged drug after dosing human subjects with ^{14}C-labelled I, whereas the glucuronide of I represented 48.5% of the urinary ^{14}C for a given time period [22]. In order to determine the total concentration of I in urine in the form of unchanged drug and its glucuronide, the method utilizing pretreatment of urine samples with β-glucuronidase was employed.

Following the procedure described in the Experimental section, the HPLC-UV assay was validated in the concentration range 0.5–50 ng/ml in plasma and 10–1000 ng/ml in urine. The intra-day precision, expressed as the coefficient of variation (C.V., %) was less than 10% at all concentrations within the standard curve range (Tables 1 and 2).

Typical equations for the calibration curves were $y = 0.127x - 0.0021$ in plasma (with the correlation coefficient $r^2 = 0.9998$), and $y = 0.0148x - 0.007$ in urine ($r^2 = 0.9994$). Representative chromatograms are shown in Figs. 2 and 3.

The limit of quantitation (LOQ) of the assay in plasma was 0.5 ng/ml. The LOQ was defined here as the lowest concentration on the standard curve for which precision of the determination, expressed as coefficient of variation (C.V., %), was less than 10%, with an adequate assay accuracy ($\leq 15\%$). The LOQ of I in urine was 10 ng/ml, and was sufficient to monitor relatively high concentration of I excreted predominantly in the form of glucuronide.

3.2. HPLC–MS–MS assay

The positive-daughter mass spectra of the protonated molecular ions $[(M+H)^+]$ of cyclobenzaprine (*m/z* 276) and the internal stan-

Table 1
Intra-day precision[a] and accuracy of the HPLC-UV and HPLC–MS–MS assays of I in human plasma

Spiked concentration (ng/ml)	HPLC-UV		HPLC–MS–MS	
	C.V.[a] (%)	Accuracy[b] (%)	C.V.[a] (%)	Accuracy[b] (%)
0.10	[c]	[c]	4.6	98
0.20	[c]	[c]	5.9	95
0.50	7.3	104	2.7	92
0.75	5.9	103	4.9	96
1.00	3.6	99	3.2	97
2.50	7.4	100	5.7	99
5.00	2.0	104	5.7	98
10.00	2.0	103	1.8	98
20.00	3.7	101	2.5	102
50.00	2.4	98	2.6	104

[a] Coefficient of variation ($n = 5$).
[b] Expressed as: (mean observed concentrations/spiked concentration) · 100 ($n = 5$).
[c] Below the limit of quantitation (LOQ) of the assay.

Table 2
Intra-day precision[a] and accuracy of the HPLC-UV and HPLC–MS–MS assays of I in human urine

Spiked concentration (ng/ml)	HPLC-UV		HPLC–MS–MS	
	C.V. (%)	Accuracy[b] (%)	C.V. (%)	Accuracy[b] (%)
10	3.0	101	2.2	92
25	4.2	98	5.3	101
50	1.9	97	3.4	97
100	3.8	98	7.1	103
250	3.6	103	5.0	101
500	4.1	96	4.5	99
1000	2.0	100	2.9	99

[a] Coefficient of variation ($n = 5$).
[b] Expressed as: (mean observed concentrations)/(spiked concentration) · 100 ($n = 5$).

dard (m/z 295) indicated the presence of intense product ions at m/z 215 for cyclobenzaprine and m/z 208 for internal standard (Fig. 4).

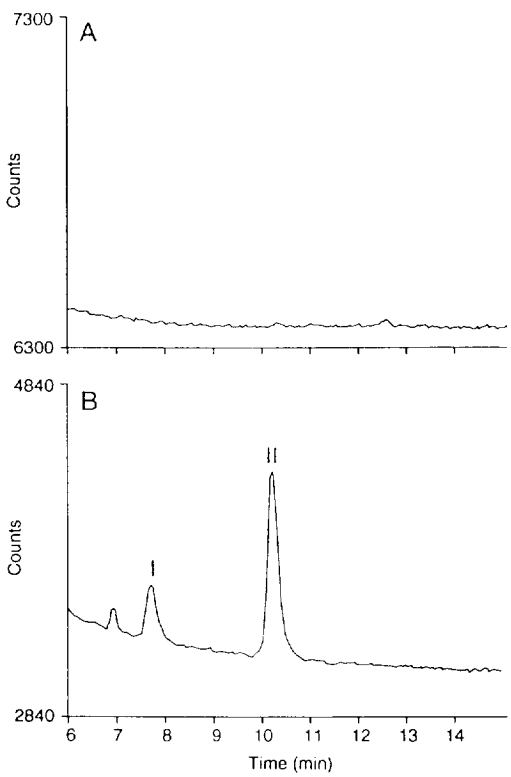


Fig. 2. Representative chromatograms of cyclobenzaprine (I) extracted from human plasma using HPLC-UV method. (A) Blank control plasma; (B) plasma (1 ml) spiked with 2.5 ng of I and 50 ng of I.S. (II).

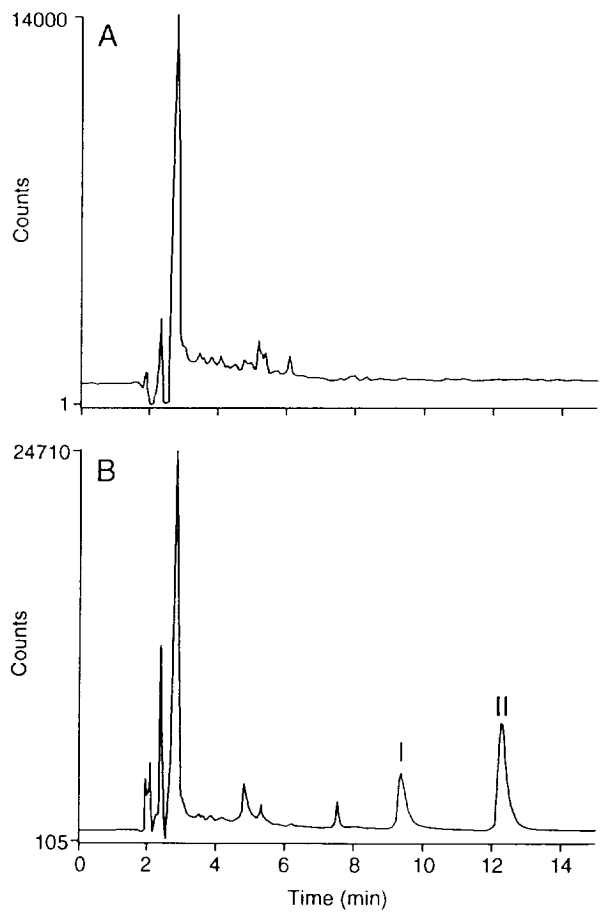


Fig. 3. Representative chromatograms of cyclobenzaprine (I) extracted from human urine using HPLC-UV method. (A) Blank control urine; (B) urine (1 ml) spiked with 40 ng of I and 500 ng of I.S. (II).

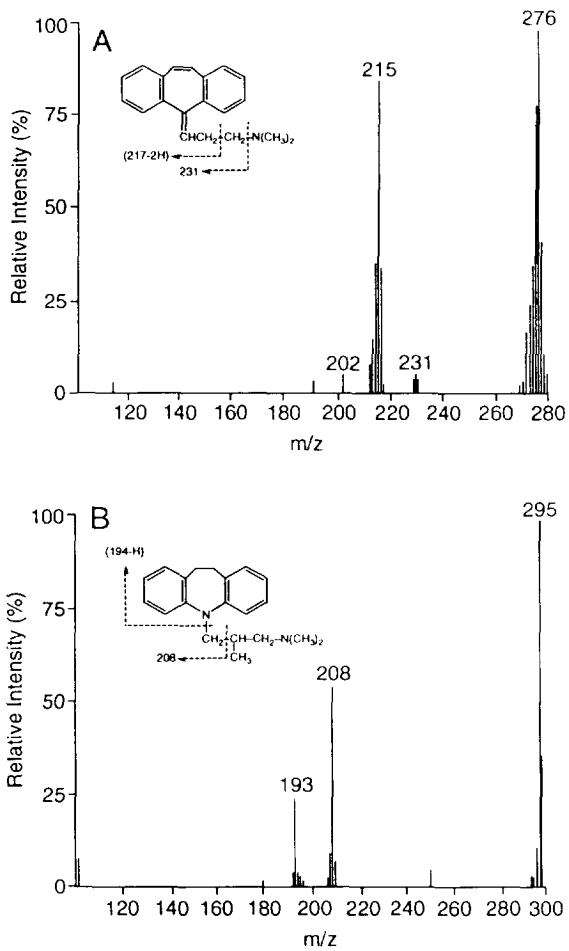


Fig. 4. Positive product-ion mass spectra of the protonated molecular ions of cyclobenzaprine (m/z 276, A) and internal standard (m/z 295, B).

By monitoring these parent → daughter pairs at m/z 276 → 215 for I and m/z 295 → 208 for II in the multiple-reaction monitoring mode, a highly sensitive assay for I in plasma with an LOQ of 0.1 ng/ml was developed. The assay was validated in plasma in the concentration range of 0.1–50 ng/ml, and 10–1000 ng/ml in urine. The intra-day precision and accuracy data, presented in Tables 1 and 2, indicated that the C.V.s at all concentrations within the standard curve range in both plasma and urine were below 8%, and accuracy was within the range 92–104%. Inter-day variability, as measured by the concentration of QC standards, was also ≤ 10% (Table 3).

In addition, the analyses of QC standards indicated that I was stable in urine and plasma for at least one and three months, respectively, when stored at −20°C (data not shown). The representative chromatograms of plasma and urine extracts analyzed using HPLC–MS–MS method are presented in Figs. 5 and 6.

Typical equations for the calibration curves for the HPLC–MS–MS assays were $y = 0.095x - 0.004$ in plasma (with the correlation coefficient of $r^2 = 0.9992$), and $y = 0.020x - 0.01$ in urine ($r^2 = 0.9999$).

3.3. Analyses of samples from clinical studies

The HPLC–MS–MS method was chosen to support clinical pharmacokinetic studies because of its increased sensitivity (LOQ = 0.1 ng/ml) for assaying I in plasma in comparison with the

Table 3
Inter-day variability for the HPLC–MS–MS assay of quality control samples spiked with I

Biological specimen	Spiked concentration (ng/ml)	Number of determinations	Mean calculated concentration (ng/ml)	C.V. (%)
Plasma	0.5	16[a]	0.48	9.2
Plasma	40.0	16[a]	38.1	8.4
Urine	18.4	10[b]	18.5	3.6
Urine	739.0	10[b]	709.0	5.8

[a] Over a period of 8 days.
[b] Over a period of 5 days.

HPLC-UV method (LOQ = 0.5 ng/ml) and the ability to analyze a large number of samples (150) per day. The performance of the HPLC–MS–MS assay was tested by analyzing more than 1000 plasma and 300 urine samples from a clinical study with I. As an example, representative concentrations of I in plasma after oral administration of I in selected human subjects participating in a single-dose pharmacokinetic study are presented in Table 4.

3.4. Comparison of HPLC-UV and HPLC–MS–MS methods

The LOQ for the assay of I in plasma using HPLC–MS–MS was 0.1 ng/ml, and was lowered ten-fold compared with the previously reported GC method [8] (LOQ = 1 ng/ml), and five-fold compared with the HPLC-UV method (LOQ = 0.5 ng/ml) described in this paper. Additionally, the HPLC–MS–MS assay offered several advantages over conventional HPLC-UV and GC methods [8]. First, the analysis time in the HPLC–MS–MS method (3 min) was far less than that of the HPLC-UV (12 min) and GC (20 min) assays, enabling analyses of about 150 clinical samples per day as compared to less than 50 samples analyzed by the HPLC-UV or GC methods. In addition, due to the high specificity of the tandem mass spectrometric detection, the presence of interferences from post-dose biological fluid samples originating from a large number of subjects was greatly reduced in comparison with the HPLC method based on UV absorbance detection at 229 nm.

In conclusion, two highly sensitive methods based on HPLC–MS–MS and HPLC-UV detection for the determination of cyclobenzaprine in

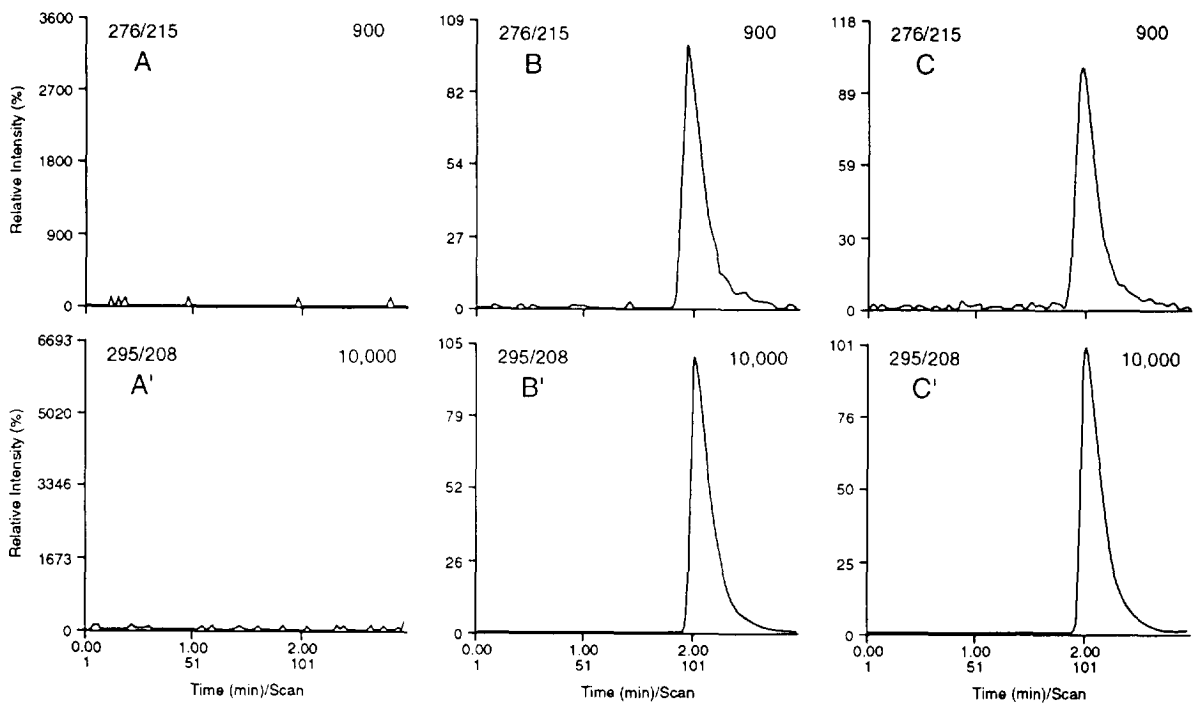


Fig. 5. Representative HPLC–MS–MS chromatograms of plasma (1 ml) extracts obtained by multiple-reaction monitoring at m/z 276 → 215 (channel "a") for cyclobenzaprine and m/z 295 → 208 (channel "b") for internal standard (II). (A,A') Blank control plasma monitored at channels "a" and "b", respectively; (B,B') control plasma spiked with 1 ng of I and 50 ng of II monitored at channels "a" and "b", respectively; (C,C') plasma sample of a subject 48 h after a 5-mg dose of I spiked with 50 ng II and monitored at channels "a" and "b", respectively; concentration of I was 0.9 ng/ml. The numbers in the upper right-hand corner of the chromatograms correspond to the peak heights expressed in arbitrary units.

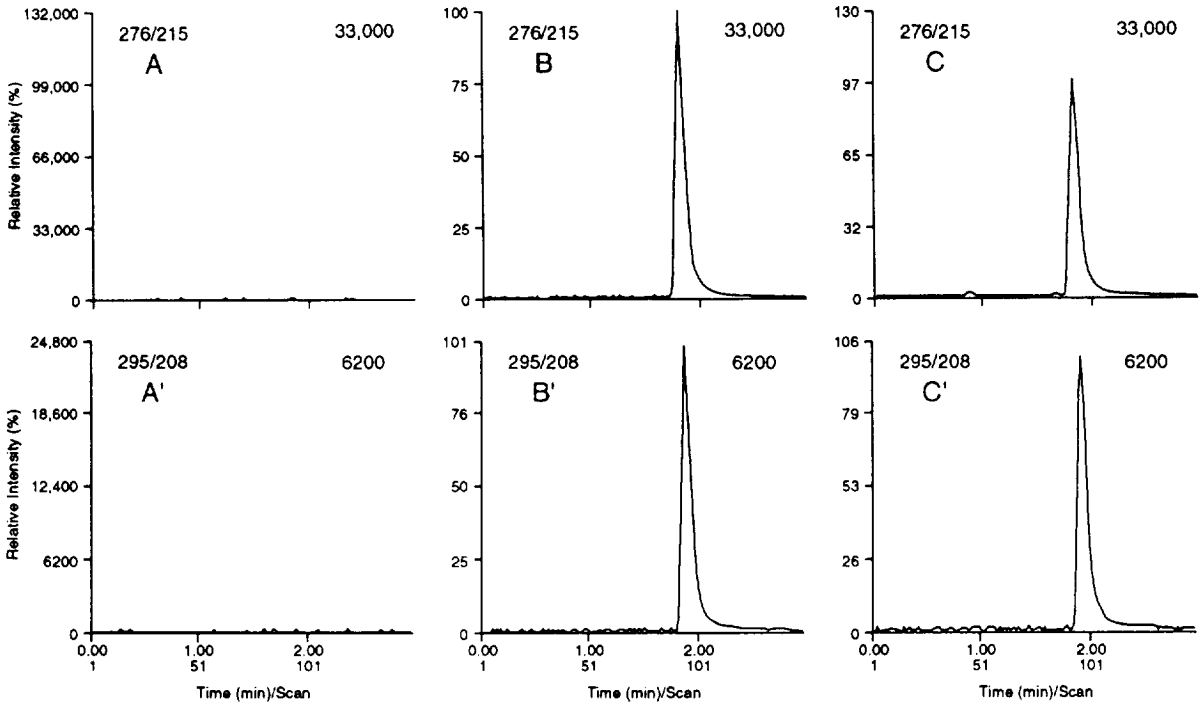


Fig. 6. Representative HPLC–MS–MS chromatograms of urine (1 ml) extracts obtained by multiple reaction monitoring at m/z 276 → 215 (channel "a") for cyclobenzaprine and m/z 295 → 208 (channel "b") for internal standard (II). (A,A') Blank control urine monitored at channels "a" and "b", respectively; (B,B') control urine spiked with 250 ng of I and 50 ng of II monitored at channels "a" and "b", respectively; (C,C') urine sample (0–8 h collection) of a subject on day 15 of dosing with 5 mg of I, spiked with 50 ng of II and monitored at channels "a" and "b", respectively; concentration of I was 193 ng/ml. The numbers in the upper right-hand corner of the chromatograms correspond to the peak heights expressed in arbitrary units.

human plasma and urine were developed. The limits of quantitation in plasma were 0.1 and 0.5 ng/ml for the HPLC–MS–MS and HPLC-UV methods, respectively. The HPLC–MS–MS assay was utilized for the analyses of clinical samples in support of pharmacokinetic studies with 2.5-mg doses of cyclobenzaprine.

Table 4
Concentration of I in plasma of selected human subjects after a 2.5-mg oral dose of I

Time post-dose (h)	Concentration (ng/ml)	
	Subject 7	Subject 12
0	0.0	0.0
1	0.0	0.0
2	0.8	1.1
3	2.0	1.8
4	2.3	1.5
6	1.8	1.4
8	1.5	1.0
12	1.1	0.7
24	0.6	0.4

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