Sensitive HPLC–ESI-MS Method for the Determination of Tiotropium in Human Plasma

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Abstract

A sensitive high-performance liquid chromatography–electrospray ionization-mass spectrometry (HPLC–ESI-MS) assay is established for the determination of tiotropium in human plasma using benzyltriethylammonium chloride as the internal standard (IS). After being treated with C_{18} cartridges, plasma samples are separated by HPLC on a reversed-phase C_{18} column with a mobile phase of 40mM ammonium acetate buffer–methanol (56:44, v/v). Tiotropium is determined in a single-quadrupole MS. HPLC–ESI-MS is performed in the selected ion monitoring mode using target ions at *m/z* 392.0 for tiotropium and *m/z* 192.3 for the IS. The calibration curve is linear over the range 1.5–30 pg/mL. The intra- and inter-assay variability values are less than 10.1% and 13.6%, respectively. The mean plasma extraction recovery of tiotropium is 92.3 ± 5.0%. The method has been successfully applied to studying the pharmacokinetics of tiotropium in healthy Chinese volunteers.

Introduction

Tiotropium (Figure 1) bromide is a long-acting muscarinic antagonist for the treatment of patients with chronic obstructive pulmonary disease (COPD) (1–3). It blocks the M1, M2, and M3 subtypes of muscarinic receptors in the airway. Compared with ipratropium, tiotropium dissociates very slowly from lung muscarinic receptor M1 and M3. This property of tiotropium is thought to be the reason for its 24 h duration of action in vivo, which allows dosing 18 µg of the drug once daily in the management of COPD in clinical. Tiotropium offers the advantage of once daily administration over ipratropium, which needs 40 µg four times daily in the treatment of COPD (1–3). Both tiotropium and ipratropium showed a comparable terminal elimination half-life in rat urine (21–24 h) after single i.v. administration, which was much longer than the corresponding half-life in plasma (6–8 h) (4). Tiotropium bromide is a new inhaled anticholinergic agent approved for the treatment of bronchospasm associated with COPD. Inhaled drugs possess the major feature of having "local" effects on the target organ of the lung (5). Because the clinical dosage of tiotropium is very low, the concentrations of tiotropium in human plasma are at low pg/mL levels. To evaluate the pharmacokinetics of tiotropium in the volunteers, an extremely sensitive method is required. Turck et al. (6) developed a liquid chromatography–tandem mass spectrometry (LC–MS–MS) method for the determination of tiotropium in subjects with different degrees of renal impairment; the lower limit of quantitation (LLOQ) was 2.43 pg/mL in plasma, and the tiotropium plasma concentrations were determined up to 8 h after a single intravenous dose of 4.8

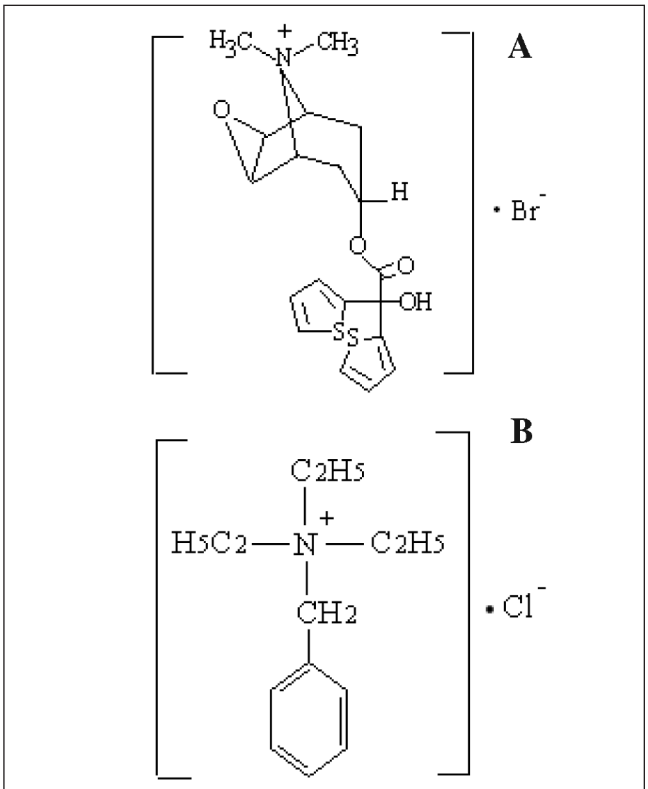

Figure 1. Chemical structures of tiotropium bromide (A) and benzyltriethylammonium chloride (B).

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µg tiotropium. Disse et al. (7) analyzed the tiotropium plasma samples by LC–ion spray-MS, the LLOQ was 4.8 pg/mL, and the tiotropium plasma concentrations were determined up to 8 h after a single inhalation dose of 10 µg tiotropium in the volunteers. This paper reports a sensitive high-performance liquid chromatography–electrospray ionization HPLC–ESI-MS method that can determine tiotropium plasma concentrations as low as 1.5 pg/mL and allows the determination of a tiotropium pharmacokinetic profile for 24 h.

Experiment

Materials

Tiotropium bromide monohydrate and the test formulation were obtained from Jiangsu Zhengdatianqing Pharmaceutical Company, Inc. (Lianyungang, China). The test formulation was tiotropium bromide inhalation powder packed in the capsule and intended for inhalation with the inhalation device. Each capsule contained 22.5 µg tiotropium bromide monohydrate (equivalent to 18 µg tiotropium). Benzyltriethylammonium chloride (internal standard [IS], Figure 1) was a gift from the Department of Pharmaceutical Chemistry, China Pharmaceutical University (Nanjing, China). The C_{18} cartridge solid-phase extraction (SPE) columns were 3-mL Supelclean LC-18 SPE Tubes supplied by Supelco (Bellefonte, PA). Methanol of HPLC grade was purchased from Merck KGaA. Triethylamine and acetic acid were analytical grade and purchased from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China).

Instrumental conditions

Solid-phase extraction was carried out using a 12-position Visiprep DL manifold (Supelco). HPLC–ESI-MS analysis was performed using an Agilent 1100 Series LC–MSD SL system (Agilent Technologies, Palo Alto, CA) with a Zorbax SB-C18 column, 4.6 × 25 mm, 5 µm (Agilent). The mobile phase was 40mM ammonium acetate buffer–methanol (56:44, v/v) at a flow rate of 0.6mL/min. The column temperature was maintained at 22°C. The HPLC–ESI-MS was controlled by a computer employing the HP ChemStation software (10.02 A) supplied by Agilent. HPLC–ESI-MS was carried out using nitrogen to assist nebulization. A quadrupole MS equipped with an ESI source was set with a drying gas (N_2) flow of 13 L/min, nebulizer pressure of 60 psi, drying gas temperature of 350°C, capillary voltage of 3.5 kV, and the positive ion mode. The fragmentor voltage was 200 V. HPLC–ESI-MS was performed in the selected-ion monitoring (SIM) mode using target ions at *m/z* 392.0 for tiotropium and *m/z* 192.3 for the IS.

Preparation of standard solutions, calibration standards, and quality control samples

Stock solutions of tiotropium (1.0 mg/mL) and internal standard (0.5 mg/mL) were prepared in methanol and stored at –20°C. A series of standard solutions of tiotropium was prepared at concentrations of 100 µg/mL, 10 µg/mL, 1 µg/mL, 100 ng/mL, 10 ng/mL, 2 ng/mL, and 0.5 ng/mL by diluting the stock solution of tiotropium with methanol. The internal standard working solution of 0.5 µg /mL was also prepared by diluting the stock solution of IS with methanol.

Calibration standards of tiotropium were prepared by spiking appropriate amount of the standard solutions in the blank plasma obtained from the healthy volunteers. Quality control (QC) samples were prepared in blank plasma at concentrations of 1.5, 6, 18, and 30 pg/mL and stored at –20°C.

Sample preparation

Precondition of the SPE columns

The SPE columns were placed in numbered slots in the manifold. Each column was conditioned with 2 × 2 mL of methanol followed by 2 × 2 mL of distilled water. These were allowed to percolate through the sorbent under gravity. The level of distilled water was held just above the solid-phase sorbent bed to prevent it drying out.

SPE process of the samples

To a 2 mL aliquot of human plasma sample, 20 µL of the internal standard working solution were added. After being thoroughly vortex mixed, the plasma sample was loaded onto the previously conditioned solid-phase extraction column and allowed to pass through with the aid of gravity. After the sample passed through the solid phase column, the sorbent was washed with 2 × 2 mL of distilled water, then dried under full vacuum for 5 min. Tiotropium and internal standard were eluted from the column using an organic solvent mixture consisting of methanol–triethylamine–acetic acid (99:0.5:0.5, v/v/v). The volume of the elution solvent employed was 2 × 3 mL. The eluant was collected in a 10-mL test tube at a rate of approximately 1 mL/min, and was evaporated to dryness under a gentle stream of nitrogen at 50ºC. The residue was dissolved in 120 µL of the mobile phase. The reconstituted solution was transferred to an auto sampler vial and 90 µL aliquot of this solution were injected for analysis by HPLC–ESI-MS.

Assay validation (8)

Linearity and lower limit of quantitation

Calibration standards of tiotropium were prepared by spiking appropriate amounts of the standard solutions into 2 mL blank plasma obtained from the healthy volunteers. A standard curve was prepared in the range of 1.5–30 pg/mL for tiotropium at concentrations of 1.5, 3, 6, 12, 18, 24, and 30 pg/mL. The prepared calibration standards were extracted using the solid-phase extraction method described previously, and were injected for analysis by HPLC–ESI-MS. Peak-area ratios of tiotropium to the IS were obtained from selected-ion chromatograms. The tiotropium calibration curve was constructed by plotting the peak area ratios of tiotropium to the IS versus the concentrations (*C*) of tiotropium, using weighed least squares linear regression (weighing factor was 1/*C*). The calibration curve was prepared and assayed with QC and clinical plasma samples in each run batch when the analyses were performed. The lower limit of quantitation (LLOQ) was defined as the plasma sample concentration of tiotropium resulting in a peak height of ten times the noise (s/n = 10) and at which both precision and accuracy were less than or equal to 20%.

Precision, accuracy, specificity, and matrix effect

Validation samples were prepared and analyzed on three consecutive days (one run per day) to evaluate the accuracy, intra-run, and inter-run precision of the analytical method. The accuracy, intra-batch, and inter-batch precision of the method were determined by analyzing five replicates at 1.5, 6, 18, and 30 pg/mL of tiotropium along with one standard curve on each of 3 days. Assay precision was calculated using the relative standard deviation (RSD%). The accuracy is the degree of closeness of the determined value to the nominal or known true value under prescribed conditions. Accuracy is defined as the relative deviation in the calculated value (E) of a standard from that of its true value (T) expressed as a percentage (RE%). It was calculated using the formula: $RE\% = (E - T) / T \times 100$.

The accuracy of the assay was checked by preparing the QC samples at the start of the clinical study. These QC samples were assayed along with clinical samples in each run to monitor the performance of the assay and to assess the integrity and validity of the result of the unknown clinical samples analyzed.

The assay specificity was tested by analyzing six different batches of human blank plasma samples. Each blank sample was tested using the proposed extraction procedure and HPLC–ESI-MS conditions to ensure that no interference of tiotropium and IS occurred in the plasma.

The matrix effect (ME) on the ionization of tiotropium was evaluated by comparing the peak areas of tiotropium resolved in the blank plasma sample's reconstituted solution (the final solution of blank plasma after extraction and reconstitution) (A) with those resolved in the mobile phase (B). ME was calculated using the formula: $ME (\%) = A / B \times 100$. Three different concentration levels of tiotropium at 6, 18, 30 pg/mL were evaluated by analyzing five samples at each level. The blank plasma samples used in this study were from five different batches of human blank plasma samples. If the ME values exceed the range of 85%–115%, an exogenous matrix effect is implied.

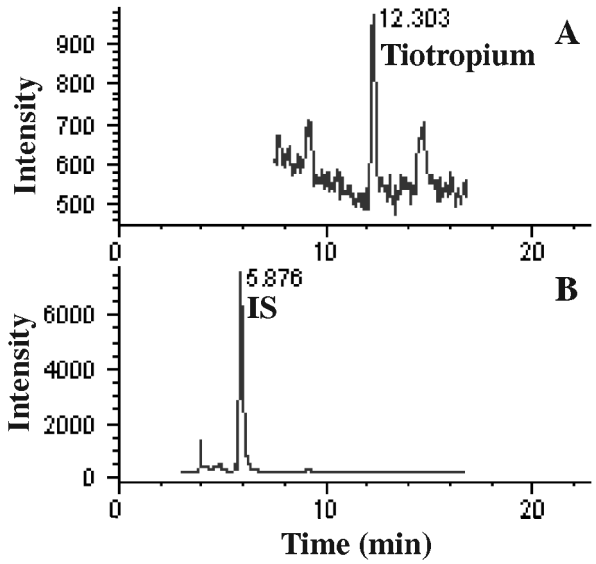

Figure 2. Typical SIM mass chromatogram of blank plasma spiked with tiotropium (1.51 pg/mL) (A) and IS (5.0 ng/mL) (B).

Extraction recovery

The extraction recovery of tiotropium was determined at low, medium, and high concentrations, respectively. Recovery was calculated by a comparison of the peak areas of tiotropium extracted from plasma samples with those of injected standards.

Stability

The stability of tiotropium in plasma was studied under a variety of storage and handling conditions at the concentrations of 1.5, 6, 18, and 30 ng/mL. The long-term stability was performed at –20°C in plasma. Freeze-thaw stability (–20°C in plasma) was checked through three cycles. The stability at ambient temperature was tested for 10 h. The stability of tiotropium and the IS in extraction solution at ambient temperature were observed for 24 h. The stock solutions of tiotropium and the IS at –20°C were also evaluated.

System suitability test

Prior to running each batch of clinical plasma samples, the instrument performance (e.g., sensitivity, reproducibility of chromatographic retention and separation, plate number, and tailing factor) was determined by the analysis of the reference standard of tiotropium, IS, blank plasma, and the plasma samples spiked with tiotropium and IS.

Clinical study design and pharmacokinetic analysis

Twelve healthy Chinese volunteers participated in this study, six female and six male. The mean age of the volunteer was 23.7 ± 2.2 (total range: 20~27), and the mean body weight of the volunteer was 58.3 ± 5.2 kg (total range: 50~66 kg). After an overnight fast, each volunteer inhaled 18 µg tiotropium by the inhalation device. Blood was sampled predose and at 5, 15, 30, and 45 min and 1, 1.5, 2, 4, 6, 9, 12, 15, and 24 h postdose for the determination of plasma concentration of tiotropium. Model-independent pharmacokinetic parameters were calculated for tiotropium.

Results and Discussion

Conditions of HPLC

Usually, we prefer choosing a compound that has similar chemical structure to the analyte or is chemically similar to the analyte as the internal standard. In fact, the necessary requirements for a proper internal standard include that it should be well resolved from the analyte and other peaks, and mimic the analyte in any sample preparation steps, but does not have to be chemically similar to the analyte (9). Moreover, in the LC–MS analysis, a critical requirement for a proper internal standard is that it should have a similar ionization efficiency to the analyte. Benzyl-triethylammonium chloride was chosen as the IS in our method, for it has a quaternary ammonium structure like tiotropium bromide, a similar ionization efficiency to tiotropium, and mimics tiotropium in any sample preparation steps.

The mobile phase composition was optimized by adjusting the ammonium acetate buffer concentration to limit the tailing phenomena of chromatographic peaks of the analytes

and by adjusting the buffer/organic proportion to produce adequate separation. Finally, an acceptable retention and resolution of the analytes was obtained by using an elution system of 40mM ammonium acetate buffer–methanol (56:44, v/v) as the mobile phase. Representative selected-ion chromatograms are shown in Figures 2 and 3, in which the retention time is approximately 12.2 min for tiotropium and 5.8 min for the IS.

Conditions for ESI-MS

Because tiotropium possesses a quaternary ammonium in its structure, the positive-ion monitoring mode was adopted in the LC–MS. Usually, the ESI is used for medium- to high-polarity analytes (10–11). Furthermore, tiotropium is ready to form tiotropium cation in the mobile phase and offers abundant positive ions in the ESI procedure. Thus, the ESI mode was selected for the assay of tiotropium. ESI produced abundant molecular ions of $[M]^+$ *m/z* 392.0 for tiotropium and $[M]^+$ *m/z* 192.3 for the IS in the SIM mode.

In order to determine the optimal fragmentation voltage, the intensity of tiotropium molecular ion $[M]^+$ *m/z* 392 was compared at fragmentation voltages of 30, 50, 70, 90, 110, 130, 150, 170, 200, and 250 V. The highest sensitivity was obtained using a 170 V fragmentation voltage. But at this voltage, tiotropium was interfered by the endogenous substances from the plasma, and the baseline noise in the chromatogram was serious. By raising the fragmentation voltage from 170 V to 200 V, the interference of endogenous substances from the plasma was avoided. The highest sensitivity for tiotropium was achieved by using 200 V fragmentor voltage.

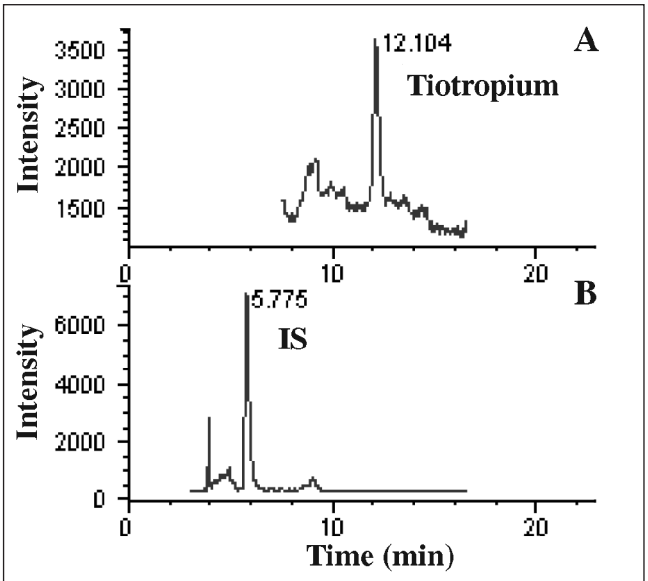

Figure 3. SIM mass chromatograms of plasma obtained from a volunteer at 30 min after inhalation of a single dose of 18 µg tiotropium. The plasma concentrations were estimated to be 5.89 pg/mL for tiotropium (A) and 5.0 ng/mL for IS (B).

Table I. Precision and Accuracy of the Assay for the Determination of Tiotropium in Plasma (*n* = 3 Days, Five Replicates Per Day)

Added to plasma (ng/mL)	Mean measured concentration (ng/mL)	RE (%)	Intra-assay RSD%	Inter-assay RSD%
1.5	1.590	5.5	10.1	13.6
6	6.238	3.5	9.8	11.3
18	17.61	–2.7	6.2	6.7
30	30.37	0.7	3.3	3.8

Method validation

The calibration curve of tiotropium is linear over the range 1.5–30 pg/mL. The typical calibration curve was $y = 0.03621 - 0.00154x$ with the correlation coefficients > 0.99, where y represents the ratio of tiotropium peak area to that of the IS and x represents the plasma concentrations of tiotropium. The LLOQ for tiotropium in plasma, defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%, is 1.5 pg/mL.

The intra- and inter-batch precision and accuracy are summarized in Table I. The precision was calculated using one-way-analysis of variance (11). The results in Table I demonstrate that the precision and accuracy of this assay are acceptable (8).

The clean-up of the target component from biological matrix before HPLC–MS is a prerequisite for successful analysis. Because of a quaternary ammonium in its structure, the liquid–liquid extraction efficiency of tiotropium is very low. In order to improve the extraction efficiency of tiotropium, the C_{18} cartridge was adopted to extract tiotropium in plasma. The

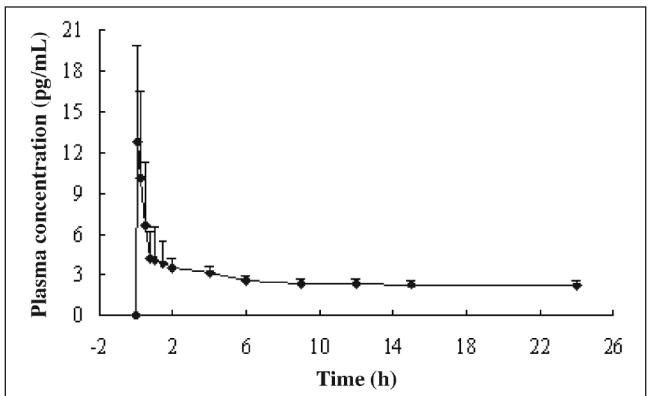

Figure 4. Mean tiotropium plasma concentration-time profile in 12 healthy volunteers after inhalation of a single dose of 18 µg tiotropium (Mean ± SD).

Table II. Mean Pharmacokinetic Parameters for 12 Volunteers After Inhalation of a Single Dose of 18 µg Tiotropium (Mean ± SD)

Parameters	Female volunteers (*n* = 6)	Male volunteers (*n* = 6)
C_{max} (pg/mL)	16.6 ± 8.2	16.3 ± 3.1
T_{max} (min)	8.3 ± 5.2	8.3 ± 5.2
$t_{1/2}$ (h)	104 ± 15	111 ± 15
$AUC_{0–24}$ (pg h/mL)	60.9 ± 15.1	62.4 ± 7.8

extraction recovery of tiotropium was evaluated at low, medium, and high concentration levels. Recovery was calculated by a comparison of the peak areas of tiotropium extracted from plasma samples with those of injected standards. In this assay, the mean plasma extraction recovery of tiotropium is 92.3 ± 5.0%.

The stability of tiotropium was studied under a variety of storage and handling conditions. The results show that no significant degradation occurred at ambient temperatures for 10 h and during the three freeze-thaw cycles for tiotropium plasma samples. Tiotropium in plasma at –20ºC is stable for at least 21 days. Tiotropium and IS in extraction solution at ambient temperature are stable for at least 24 h. The stock solutions of tiotropium and IS at –20ºC are stable for at least one month.

Pharmacokinetic study

The method described previously was successfully applied in the pharmacokinetic study in which plasma concentrations of tiotropium in 12 healthy Chinese volunteers were determined up to 24 h after inhalation of a single dose of 18 µg tiotropium. The mean plasma concentration-time curve of tiotropium is shown in Figure 4. The mean pharmacokinetic parameters of 12 volunteers are summarized in Table II. The maximum plasma concentrations (C_{max}) of tiotropium and the time to those (T_{max}) were noted directly. The elimination rate constant (k_e) was calculated by linear regression of the terminal points of the semi-log plot of plasma concentration against time. The elimination half-life ($t_{1/2}$) of tiotropium in human body was calculated using the formula $t_{1/2} = 0.693/ k_e$. The area under the plasma concentration-time curve ($AUC_{0–24}$) to the last measurable plasma concentration was calculated by the linear trapezoidal rule.

Conlusion

In this study, a specific and sensitive assay is presented for the determination of tiotropium in human plasma. The method is suitable for the pharmacokinetic study of tiotropium in human subjects.

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