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Short communication

Determination of metolazone in human blood by liquid chromatography with electrospray ionization tandem mass spectrometry

Guangli Wei *, Shuhua Xiao, Changxiao Liu

Tianjin Key Laboratory of Pharmacokinetics and Pharmacodynamics, Tianjin Institute of Pharmaceutical Research, Tianjin 300193, PR China

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Abstract

A rapid, sensitive and accurate liquid chromatographic–tandem mass spectrometric method is described for the determination of metolazone in human blood. Metolazone was extracted from blood using ethyl acetate and separated on a C18 column interfaced with a triple quadrupole tandem mass spectrometer. The mobile phase consisting of a mixture of acetonitrile, 10 mmol/l ammonium acetate and formic acid (60:40:0.1, v/v/v) was delivered at a flow rate of 0.5 ml/min. Electrospray ionization (ESI) source was operated in positive ion mode. Selected reaction monitoring (SRM) mode using the transitions of *m/z* 366 → *m/z* 259 and *m/z* 321 → *m/z* 275 were used to quantify metolazone and the lorazepam (internal standard), respectively. The linearity was obtained over the concentration range of 0.5–500 ng/ml for metolazone and the lower limit of quantitation (LLOQ) was 0.5 ng/ml. For each level of QC samples, inter- and intra-run precision was less than 8.07 and 3.56% (relative standard deviation (RSD)), respectively, and the bias was within ±4.0%. This method was successfully applied to the pharmacokinetic study of metolazone formulation after oral administration to humans.

Keyword: Metolazone

1. Introduction

Metolazone (Fig. 1), (7-chloro-1,2,3,4-tetrahydro-2-methyl-4-oxo-3-*o*-tolyl-6-quinazoline-sulfonamide), is a diuretic agent. It has been clinically used for the treatment of hypertension and edema [1,2]. For the pharmacokinetic study of metolazone formulation in humans, an analytical method with simplicity and high sensitivity was required in our laboratory. Various methods have been reported for the determination of metolazone in biological samples, which involved high-performance liquid chromatographic method [3,4], liquid chromatography with fluorescence detection [5] and liquid chromatography–tandem mass spectrometry (LC–MS–MS) [6]. Taking into consideration the low levels of metolazone in human blood, LC–MS–MS method is the first choice for our purpose. But the published LC–MS–MS method [6] addressed the qualitative analysis of a metolazone metabolite, not the quantitative determination of metolazone. This paper describes a simple, specific and highly sensitive LC–MS–MS method with an electrospray ionization (ESI) source in selected reaction monitoring (SRM) mode for the determination of metolazone in human blood. The described method was validated in terms of matrix effect, selectivity, sensitivity, linearity, accuracy, precision and stability of analyte in human blood, and successfully applied to the pharmacokinetic studies of metolazone formulation in humans.

2. Experimental

2.1. Chemicals and reagents

Metolazone (purity, 99.0%) and tablets (2.5 mg, batch no. 20051124) were supplied by Tianjin Institute of Pharmaceutical Research (Tianjin, China). Lorazepam (purity, 99.5%, internal standard) was also supplied by Tianjin Institute of Pharmaceutical Research. HPLC-grade acetonitrile and formic acid were purchased from Tianjin Concord Tech Reagent Company (Tianjin, China). Ammonium acetate, analytical-grade, was purchased from Tianjin Chemical Reagent Co. Ltd. (Tianjin, China). Water was purified with a SYZ 550 quartz pure water distiller (Tianjin Xinzhou Tech Co. Ltd., China).

* Corresponding author. Tel.: +86 22 2300 6870; fax: +86 22 2300 6860.
E-mail address: weigl510@163.com (G. Wei).

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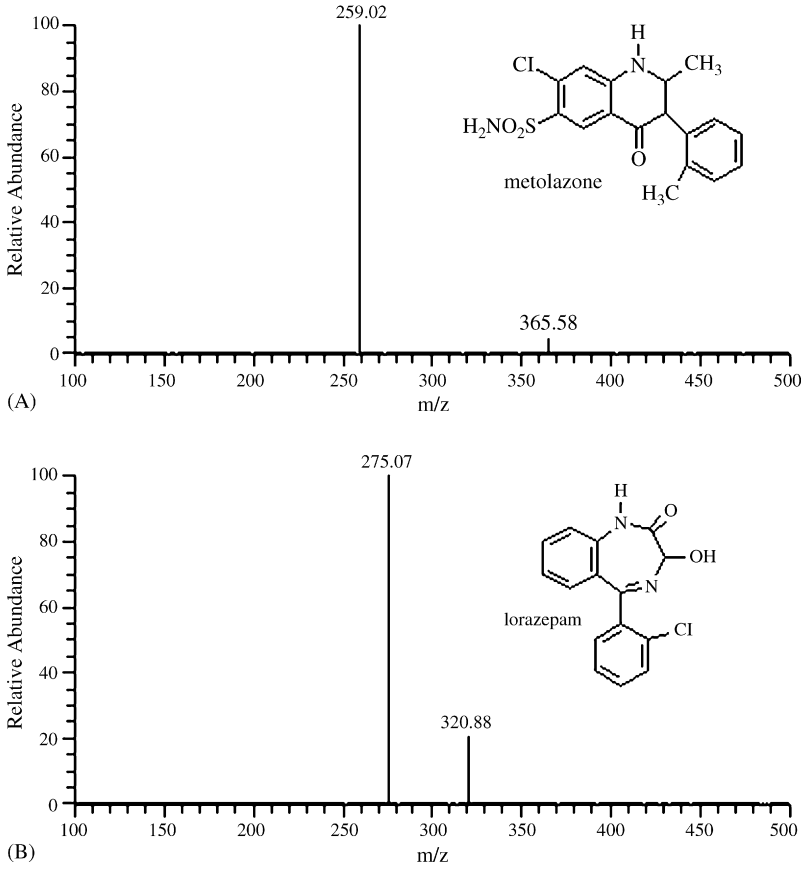

Fig. 1. Full-scan product ion spectra of $[M+H]^+$ of: (A) metolazone; (B) lorazepam.

2.2. Instrumentation

A Surveyor LC pump and a Surveyor Autosampler were used for the solvent delivery and samples injection. A Finnigan TSQ Quantum triple quadrupole tandem mass spectrometer with an electrospray ionization source (Thermo Finnigan, San Jose, CA, USA) was used for quantitative determination of metolazone in human blood. Data collection was performed with Xcalibur 1.1 software (Thermo Finnigan). Peak integration and calibration were made with LCQuan software (Thermo Finnigan).

2.3. Chromatographic conditions

Chromatographic separation was performed on a Diamonsil C18 column (200 mm × 4.6 mm i.d., 5 μm, Dikma Technologies, China). The mobile phase consisting of a mixture of acetonitrile, 10 mmol/l ammonium acetate and formic acid (60:40:0.1, v/v/v) was delivered at a flow rate of 0.5 ml/min. The column temperature was maintained at 30 °C. The injection volume was 10 μl.

2.4. Mass spectrometric conditions

ESI in positive ion detection mode was used as the ionization source. The capillary temperature was maintained at 325 °C. The spray voltage was 3.5 kV. High purity nitrogen served both as sheath gas with an operating pressure of 40 psi and as auxiliary gas with a flow rate of 10 l/min. Argon (Ar) was used in the studies of collision induced dissociation (CID) with a collision gas pressure of 0.7 mTorr and collision energy of 25 eV. Selected reaction monitoring mode was used for the quantitation. The transitions selected were *m/z* 366 → *m/z* 259 for metolazone and *m/z* 321 → *m/z* 275 for the internal standard, respectively.

2.5. Preparation of calibration standards and quality control samples

Stock solutions of metolazone and lorazepam (internal standard, I.S.) were individually prepared at 1 mg/ml in methanol. The stock solution of metolazone was further diluted with methanol to give a series of standard solutions with concentration of 0.01, 0.1, 1, 10.0 and 100.0 μg/ml. In order to prevent photodegradation, all solutions of metolazone were stored with brown volumetric flask in the dark. A solution containing 200 ng/ml I.S. was also prepared using methanol. Calibration standards of metolazone (0.5, 2.0, 5.0, 20.0, 50.0, 200.0 and 500.0 ng/ml) were prepared by spiking appropriate amount of the standard solutions in blank human blood. Quality control (QC) samples were prepared using the pooled blood at concentrations of 2.0, 20.0 and 200.0 ng/ml. The spiked samples were then treated following the sample preparation procedure as indicated in Section 2.6.

2.6. Sample preparation

A 200 μl aliquot of each blood sample was transferred to a 10 ml glass tube. A 200 μl of internal standard solution (200 ng/ml) and a 2 ml of ethyl acetate was added, the mixure was vortexed for 1 min and centrifuged at 2000 × *g* for 5 min, then the 1.5 ml organic layer was transferred to another tube and dried with nitrogen in a 40 °C water bath. The residue of each sample was reconstituted in 150 μl of the mobile phase, and a 10 μl aliquot was injected onto the LC–MS–MS system.

2.7. Method validation

Validation runs were conducted on three separate days. Each validation run consisted of a set of the spiked standard samples at seven concentrations over the concentration range (each in triplicate), QC samples at three concentrations ($n = 5$, at each concentration), blank samples. Standard samples were analyzed at the beginning and at the end of each validation run and other samples were distributed randomly throughout the run. The results from QC samples in three runs were used to evaluate the accuracy and precision of the method developed. Concentrations of the analyte in blood samples were determined by back-calculation of the observed peak-area ratios of the analyte and internal standard from the best-fit calibration curve using a weighted ($1/x^2$) linear regression. During routine analysis, each analytical run included a set of standard samples, a set of QC samples in duplicate and blood samples to be determined. The matrix effect was investigated by extracting blank blood from six different sources, reconstituting the final extract in the mobile phase containing a known amount of the analyte, analyzing the reconstituted extracts and then comparing the peak areas of the blood standards versus non-blood standards. The extraction recovery of metolazone was determined at low, medium and high concentrations by comparing the responses from blood samples spiked before extraction with those from blood samples spiked after extraction. In both cases, the internal standard was added after extraction to eliminate bias introduced by sample processing. Sample stability was tested by analyzing QC samples (including the samples after three freeze (−20 °C)–thaw (room temperature) cycles, short-term room temperature exposure and extracted) at concentrations of 2.0, 20.0 and 200.0 ng/ml.

2.8. Application of the LC–MS–MS method

The LC–MS–MS method developed was successfully applied to the pharmacokinetic studies of metolazone formulation in humans. After an overnight fast (12 h), six healthy volunteers were given a single oral dose of metolazone tablets (7.5 mg). No food was allowed until 4 h after oral administration, while water intake was controlled. About 2 ml of blood samples were collected into heparinized tubes before (0 h) and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h after dosing. Blood was immediately shielded from light and kept frozen at −20 °C until analysis.

3. Results and discussion

3.1. Method development

Liquid–liquid extraction (LLE) was used for the sample preparation in this work. LLE can be helpful in producing a clean sample and avoiding the introduction of non-volatile materials onto the column and MS system. Clean samples are essential for minimizing ion suppression and matrix effect in LC–MS–MS analyses. Two organic solvents, ethyl acetate and chloroform were evaluated. Finally, ethyl acetate was found to be optimal, which can produce a clean chromatogram for a blank blood sample and yield the highest recovery for the analyte from the blood. The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes of analytes as well as short run time. Finally, a mixture of acetonitrile, 10 mmol/l ammonium acetate and formic acid (60:40:0.1, v/v/v) was adopted as the mobile phase. Internal standard is necessary for determination of analyte with LLE process in biological samples. In initial stage of the study, several compounds were tried to find a suitable internal standard and finally lorazepam was found to be optimal. Clean chromatograms were obtained and no significant matrix effect was found. For the quantitation of metolazone in human blood, some parameters related with tandem mass spectrometry were investigated. Parameters involving spray voltage, capillary temperature, flow rate sheath and auxiliary gas were optimized to obtain the protonated molecules of metolazone and lorazepam. The collision energy was optimized to achieve maximum response of the fragment ion peak. Selected reaction monitoring was used for the detection of metolazone and lorazepam.

3.2. Specificity

The specificity of the method was investigated by comparing chromatograms of six different sources of human blood. The positive product ion mass spectra of the molecular ions of metolazone and the internal standard are shown in Fig. 1. The most intensive product ion was observed at *m/z* 259 for metolazone and *m/z* 275 for the internal standard. Representative chromatograms are shown in Fig. 2, indicating no interferences from endogenous substances in human blood with the analyte and internal standard. Metolazone and internal standard exhibited retention time of 6.3 and 7.4 min, respectively. By monitoring the precursor-to-product ion transitions *m/z* 366 → *m/z* 259 for metolazone and *m/z* 321 → *m/z* 275 for the internal standard in the SRM mode, a highly sensitive assay for metolazone was developed.

3.3. Matrix effect

The ion suppression caused by the blood matrix was evaluated. By comparing the peak areas of blood standards versus non-blood standards, no matrix effect and interferences from endogenous compounds were detected for six different sources of human blood. The relative standard deviation (RSD) value

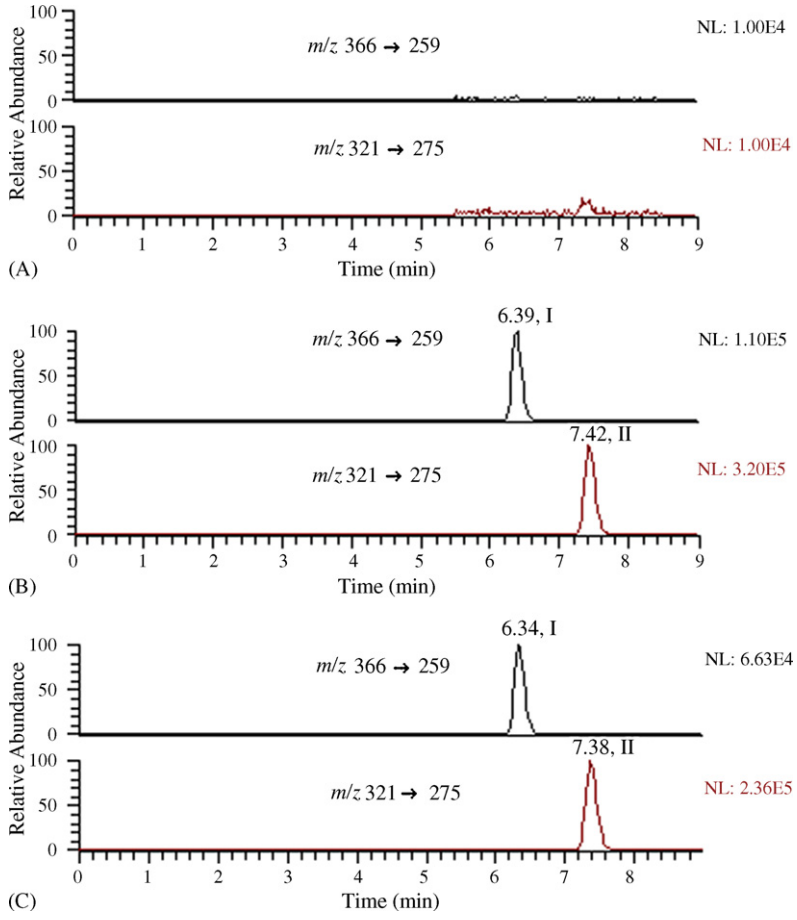

Fig. 2. Representative SRM chromatograms of: (A) blank human blood; (B) blank human blood spiked with metolazone (20.0 ng/ml) and lorazepam (200 ng/ml); (C) human blood at 3 h after an oral dose of 7.5 mg metolazone tablets to a volunteer. Peak I: metolazone and peak II: lorazepam (internal standard).

for the peak areas of the six reconstituted samples was 4.6%, indicating that the extracts did not interfere with the ionization of the analyte.

3.4. Linearity and sensitivity

The linearity of each calibration curve was determined by plotting the peak-area ratio (y) of metolazone to internal standard versus the nominal concentration (x) of metolazone. The calibration curves were obtained by weighted ($1/x2$) linear regression analysis. To evaluate the linearity of the LC–MS–MS method, blood calibration curves were determined in triplicate on three separate days. Representative regression equation for the calibration curve was $y = -0.00091 + 0.01056x$ with a correlation coefficient of 0.999. Good linearity was observed over the concentration range of 0.5–500.0 ng/ml for metolazone. The lower limit of quantitation (LLOQ) for determination of metolazone in human blood, defined as the lowest concentration analyzed with an accuracy less than ±10% and a precision less than 5%, was found to be 0.5 ng/ml, which is sufficient for pharmacokinetic study of metolazone in humans.

3.5. Accuracy and precision

The accuracy and precision of the method were evaluated by a one-way analysis of variance (ANOVA) based on the data from QC samples in three validation runs. The accuracy was determined by calculating the percentage deviation observed in the analysis of QC samples and expressed in the bias. The intra- and inter-run precision was expressed as the relative standard deviation. As shown in Table 1, for each QC level of metolazone, the intra- and inter-run precision was less than 3.6 and 8.1%,

Table 1
Accuracy and precision for the determination of metolazone in human blood ($n = 3$ days, five replicates per day)

Nominal amount (ng/ml)	Calculated amount (ng/ml)	Intra-run RSD[a] (%)	Inter-run RSD (%)	Bias (%)
2.0	1.92	3.56	8.07	−4.0
20.0	20.0	3.30	6.02	0
200.0	202.6	3.18	7.91	1.3

[a] Relative standard deviation.

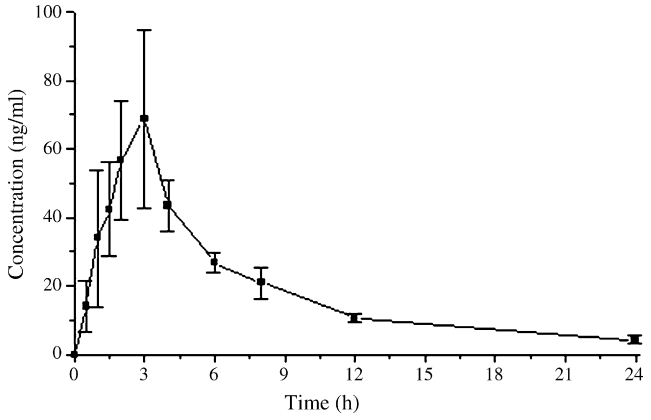

Fig. 3. Mean blood concentration–time profiles of metolazone after oral administration of metolazone tablets (7.5 mg) to six humans.

respectively, and the accuracy was within ±4.0%, indicating the acceptable accuracy and precision of the method developed.

3.6. Extraction recovery

The extraction recovery of metolazone from human blood was determined by comparing peak areas from blood samples spiked before extraction with those from blood samples spiked after extraction. The results showed that the extraction recoveries of metolazone from human blood were (74.6 ± 10.3)%, (69.8 ± 6.2)% and (74.7 ± 5.0)% at concentrations of 2.0, 20.0 and 200.0 ng/ml, respectively.

3.7. Stability

The stability of metolazone in human blood and mobile phase was investigated. Metolazone in human blood was found to be stable after three freeze–thaw cycles and at room temperature for at least 8 h. Metolazone in the reconstitution mobile phase was also found to be stable for at least 24 h at room temperature. The change of the analyte was less than 5%.

3.8. Application

The LC–MS–MS method developed for the determination of metolazone in human blood has been successfully applied to the pharmacokinetic study of metolazone tablets following oral administration to six humans. The mean blood concentration–time profiles for metolazone tablets is shown in Fig. 3.

4. Conclusions

A rapid, sensitive and accurate liquid chromatography with electrospray ionization tandem mass spectrometry was developed for the determination of metolazone in human blood. The method was successfully applied to the pharmacokinetic studies of metolazone formulation in humans.

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