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Simultaneous quantification of tiloronoxim and tilorone in human urine by liquid chromatography–tandem mass spectrometry

Xianhua Zhang[a,b], Li Yang[a], Suodi Zhai[a], Jingli Duan[a,b,*]

[a] Department of Pharmacy, The Third Hospital, Peking University, Beijing 100083, PR China
[b] Center for Therapeutic Drugs Monitoring and Clinical Toxicology, Beijing 100083, PR China

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ABSTRACT

A simple, sensitive and specific HPLC method with tandem mass spectrometry (HPLC/MS/MS) detection has been developed and validated for the simultaneous quantification of tiloronoxim and its major active metabolite, tilorone, in human urine. The analytes, together with metoprolol, which was employed as an internal standard (IS), were extracted with a mixture solvent of chloroform/ethyl ether (1/2, v/v). The chromatographic separation was performed on a narrow-bore reversed phase HPLC column with a gradient mobile phase of methanol/water containing 15 mM ammonium bicarbonate (pH 10.5). The API 3000 mass spectrometer was equipped with a TurboIonSpray interface and was operated on positive-ion, multiple reaction-monitoring (MRM) mode. The mass transitions monitored were m/z 426.3 → 100.0, m/z 411.3 → 100.0 and m/z 268.3 → 116.1 for tiloronoxim, tilorone and the IS, respectively. The assay exhibited a linear dynamic range of 1–100 ng/ml for both tiloronoxim and tilorone based on the analysis of 0.2 ml aliquots of urine. The lower limit of quantification was 1 ng/ml for both compounds. Acceptable precision and accuracies were obtained for concentrations over the standard curve ranges. Run time of 8 min for each injection made it possible to analyze a high throughput of urine samples. The assay has been successfully used to analyze human urine samples from healthy volunteers.

1. Introduction

Silicosis is a fibrosing pulmonary disease caused by inhalation of crystalline silica dust [1–4]. It is a disabling, progressive, non-reversible and sometimes fatal lung disease. The great harm of silicosis has been gradually recognized and many drugs have been synthesized for the treatment of silicosis [5,6]. Tiloronoxim is a newly synthesized tilorone derivative to treat silicosis and its structure is shown in Fig. 1A. Pre-clinic study has demonstrated the efficacy of tiloronoxim with low chronic toxicity [7]. It is rapidly and widely distributed in the body and is excreted in urine. It is metabolized to several metabolites in human, and among these, tilorone (Fig. 1B) is a pharmacologically active one [8]. The concentration level of tiloronoxim and its metabolite is very low after oral administration, and therefore, a sensitive analytical method was required for the measurement of tiloronoxim and its metabolites in order to understand the metabolism or to perform pharmacokinetics studies.

To date, only a few publications have reported the detection of tiloronoxim alone in blood or plasma using the HPLC/UV methods [9–11]. The reported lowest quantification limit is 40 ng/ml and this is not likely to provide sufficient sensitivity for the measurement of tiloronoxim and tilorone at low levels. Furthermore, lack of specificity in biological fluids and long analysis time do not meet the high throughout needs of modern drug development, which requires a rapid feedback of analytical information from pharmacokinetics studies.

This paper describes a sensitive, specific and rapid LC–MS/MS method for the simultaneous determination of tiloronoxim and tilorone in an aliquot of 0.2 ml human urine using metoprolol (Fig. 1C) as the internal standard (IS). Neither of the two compounds has been determined by HPLC–MS/MS methods before. To increase the sensitivity, an enrichment procedure with liquid–liquid extraction was performed and a strong polarity extraction solvent was used. This method has been successfully applied to real samples from three healthy volunteers.

* Corresponding author at: Department of Pharmacy, Peking University Third Hospital, Beijing, 100191, P.R. China. Tel: +86 10 8226 6673; fax: +86 10 8226 6673.
E-mail address: Jingliduan@yahoo.com.cn (J. Duan).

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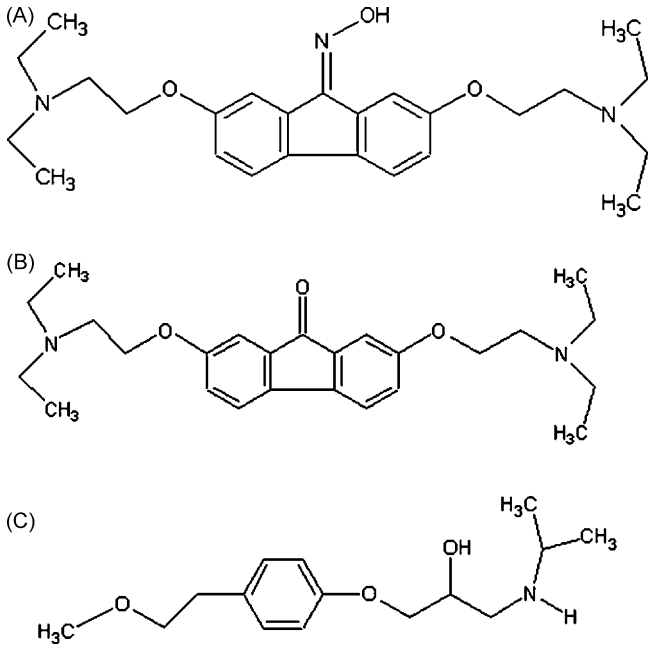

Fig. 1. Chemical structures of (A) tiloronoxim, (B) tilorone and (C) the IS.

2. Experimental

2.1. Chemicals and reagents

Tiloronoxim and tilorone were provided by Chinese Center for Disease Control and Prevention (Beijing, China). Metoprolol was purchased from National Institute for the Control of Biological Products (Beijing, China). Methanol (Fisher Chemical, USA) and ammonium bicarbonate (Sigma) of HPLC grade were used. NaOH (Beijing Chemical Reagents Company, China), chloroform (Beijing Chemical Reagents Company, China) and ethyl ether (Tianjin Chemical Reagents Factory, China) were of analytical grade. The mobile phase was filtrated with a 0.45-μm film before use. Purified water was prepared using a Milli-Q purification system (Bedford, USA).

2.2. Instrumentation

Separation of the compounds was performed using an Agilent 1100 HPLC system (Agilent Technology, USA) consisting of binary pumps, an autosampler and a vacuum degasser. The HPLC system is coupled to an API 3000 triple quadrupole mass spectrometer equipped with a TurboIonSpray (TIS, Applied Biosystems, Foster City, CA) source, under the control of the Analyst software (version 1.4).

2.3. Chromatographic conditions

Chromatographic separation was carried out on a narrow-bore reversed phase Xterra MS C18 column (50 mm × 2.1 mm, 5 μm, Waters, USA). The gradient mobile phase consisted of 15 mM ammonium bicarbonate (pH 10.5) as mobile phase A and methanol as mobile phase B. The pump was run at a flow rate of 0.3 ml/min from 30% B to 100% B over 0.2 min and remained at 100% B for 3.8 min. Then it returned to the original 30% B at 5.5 min and remained at 30% B for another 2.5 min. The column effluent was directly introduced into the mass spectrometer. The injection volume was 20 μl.

2.4. Mass spectrometry conditions

The mass spectrometer was operated on positive-ion, multiple reaction-monitoring (MRM) mode. The source temperature and flow rate of the heated gas was adjusted to 500 °C and 7500 units (set by the Analyst), respectively. The ionspray voltage was 5000 V. Nitrogen was used as nebulizer gas, curtain gas and collision gas with flow rates at 4, 10 and 8 units, respectively. Collision energy was 37 V for tiloronoxim and 38 V for tilorone. The collision exit potential was 17 V for tiloronoxim and 18 V for tilorone. Singly charged precursor-product ion transitions were monitored at *m/z* 426.3 → 100.0 (tiloronoxim), *m/z* 411.3 → 100.0 (tilorone) and *m/z* 268.3 → 116.1 (IS). The dwell time was 200 ms for each analytes and the IS. The Q1 and Q3 quadrupoles were maintained at unit resolution.

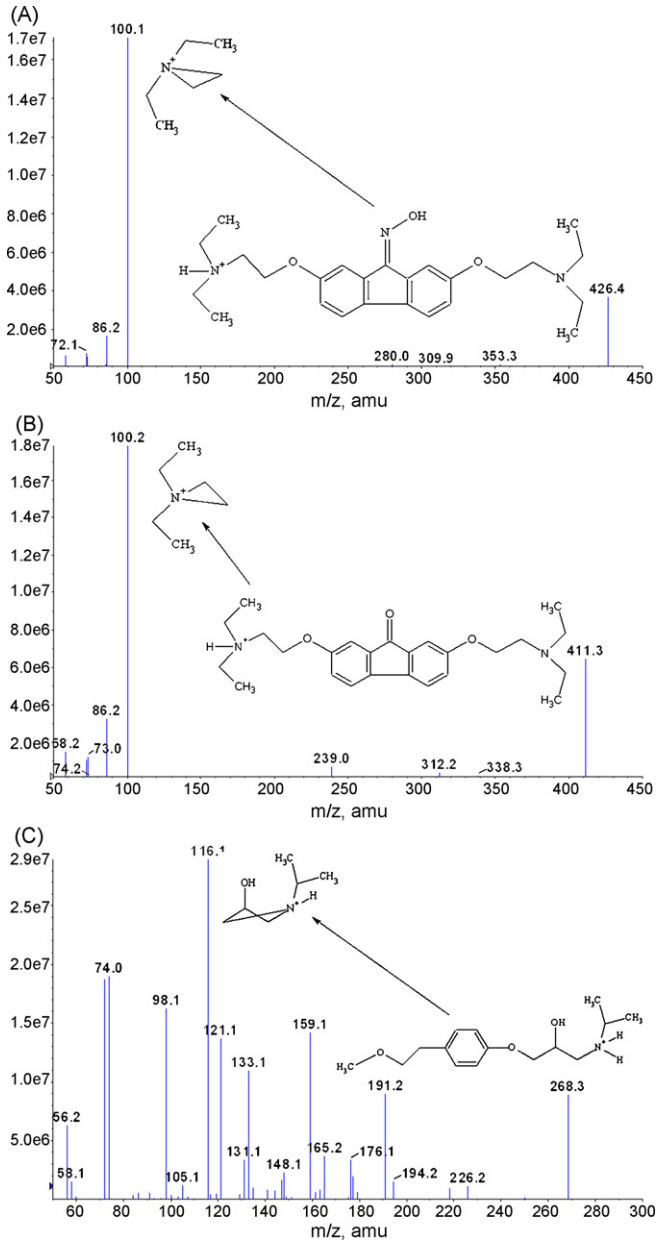

Fig. 2. Product spectra of (A) tiloronoxim, (B) tilorone and (C) the IS.

2.5. Preparation of solutions, calibration standards and validation QC standards

Stock solutions of tiloronoxim (5 mg/ml), tilorone (5 mg/ml) and the IS (0.4 mg/ml) were separately prepared in volumetric flasks with methanol and stored at 4 °C. Combined working solutions were prepared from the stock solutions at concentrations of 10, 20, 50, 100, 200, 500 and 1000 ng/ml for both analytes with methanol as solvent. The stock- and working solutions were stable for at least 3 months when stored at 4 °C. The IS working solution was prepared by diluting the stock solution with methanol on each day of analysis with a concentration of 2500 ng/ml.

Calibration standard were freshly prepared on each day of analysis by adding 20 μl of the appropriate combined standard working solution to a 180-μl aliquot of blank urine. Standards were prepared at concentrations of 1, 2, 5, 10, 20, 50 and 100 ng/ml for both compounds. Validation quality control (QC) samples were prepared in the same way at levels of 2, 10 and 50 ng/ml.

2.6. Extraction procedures for urine samples

Frozen urine samples were thawed in a water bath and the thawed samples were vortexed for 1 min. Aliquots of 200 μl of urine were placed in a 1.5-ml polypropylene tube and 10 μl of the IS was added. The mixture was basified with 200 μl of NaOH (2 M) and then was extracted with 600 μl of extraction solvent (chloroform/ethyl ether = 1/2, v/v). After being vortexed for 2 min and centrifuged for 5 min, the organic layer was transferred to another polypropylene tube and the aqueous layer was extracted with another 600 μl of extraction solvent. Then the organic fractions were combined and dried under a gentle stream of nitrogen. The residue was dissolved in 100 μl of the mobile phase and 20 μl of the reconstituted sample was injected into the HPLC–MS/MS system for analysis.

2.7. Validation procedures

The method was validated for selectivity, linearity, intra- and inter-batch precision and accuracy. The lower limit of quantification (LLOQ), matrix effect and stability of the analytes in urine were also tested.

Selectivity of the method was investigated by analyzing blank urine from six different individuals for endogenous interferences with the analytes. The matrix effect was investigated by infusing analytes solution through a T tube and the blank urine extract was injected through HPLC system. Negative or positive deflects indicated suppression or enhancement of ionization and the retention time of the analytes was adjusted away from the affected areas to minimize the matrix effect. Further experiments were also performed to examine the absolute matrix effect by comparing the MS/MS responses of the analytes spiked *postextraction* into the blank urine extracts (A_1) at three levels to that presented in the mobile phase (A_2). A value of >100% indicates an ionization enhancement and a value of <100% indicates an ionization suppression. The assessment of relative matrix effect was made based on the direct comparison of the MS/MS responses of the analytes spiked into the extracts (A_1) originating from different sources of blank urine.

Seven calibration standards were prepared and analyzed by using linear weighted analysis ($y = 1/X$). The urine standards were prepared at concentrations of 1, 2, 5, 10, 20, 50 and 100 ng/ml.

Accuracy and precision were determined for each analyte by analysis of five replicates of combined validation QCs at defined concentration in three validation batches. The investigated concentration levels in urine were 2, 10 and 50 ng/ml for both analytes.

The recovery experiment was performed by comparing the response of QC samples with spiking solution of the same concentration in extracted blank matrix.

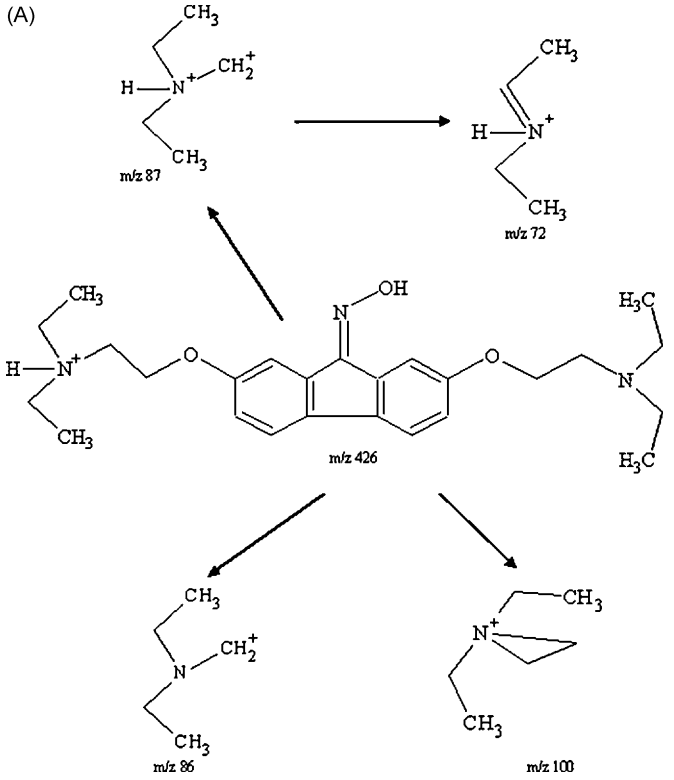

Fig. 3. Proposed fragmentation pathways of (A) tiloronoxim, (B) tilorone and (C) the IS.

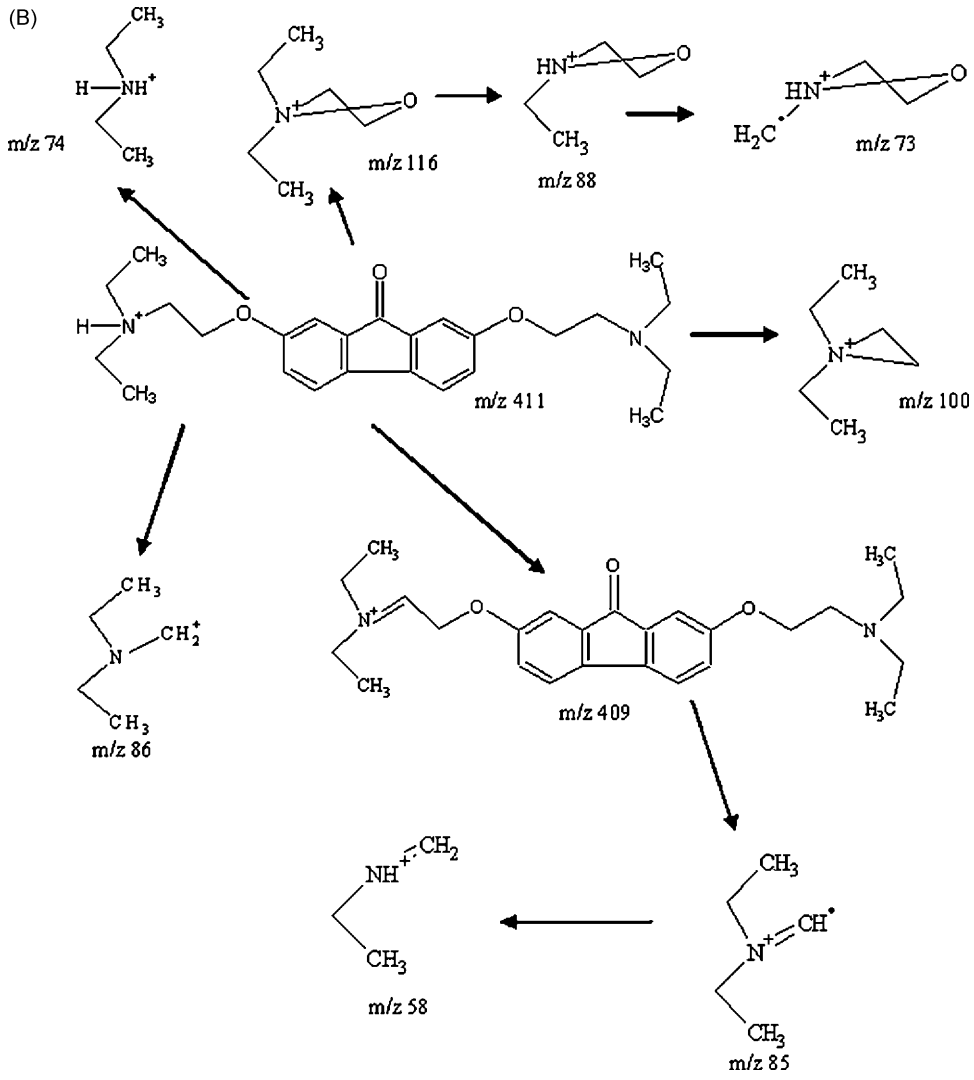

Fig. 3. (*Continued*)

Pre-treatment stability experiments were carried out by placing the QC urine samples at room temperature for 4 and 8 h. The freeze–thaw stability was also examined in the experiment. QC samples were subjected to freeze–thaw cycles consisting of a thaw to room temperature (more than 4 h), vortexing, and then refreezing (−40 °C) at least overnight. After each freeze–thaw cycle, the samples were analyzed. The auto sampler stability was investigated as well. After being placed at ambient temperature for 8 h, the QC samples were analyzed. The long-term stability was evaluated to establish the storage conditions for the clinical samples. QC samples were stored at −40 °C for 60 days and analyzed against the fresh prepared calibration curve.

To extend the upper limit of the method, a pool of 200 ng/ml of urine sample was prepared as the dilution QC. It was diluted to be within the calibration range with urine blank before being analyzed.

2.8. Application

A test study was done to measure the tiloronoxim and tilorone levels in urine from three healthy male volunteers with an oral administration of 100 mg tiloronoxim. Urine samples were collected before drug administration and at the end of the following intervals after drug intake: 0–6, 6–12, 12–24, 24–30, 30–36, 36–48, 48–60 and 60–72 h. Urine samples were stored at −40 °C until analysis. After addition of 10 μl IS to 0.2 ml aliquot of urine, the samples were extracted and analyzed with the described method.

3. Results and discussion

3.1. MS/MS detection

Positive detection mode was employed in the experiments. In this mode, the very soft ionization process in the TIS source produces the precursor ions $[M+H]^+$. The precursor ions observed were $m/z = 426.3$ and $m/z = 411.3$ for tiloronoxim and tilorone, respectively. Each of the precursor ions was subjected to collision-induced dissociation in order to obtain product ions. The collision conditions were optimized and the MS/MS peaks of the product ions for the analytes and the IS were acquired (Fig. 2A–C). The probable fragmentation mechanism for tiloronoxim and tilorone is proposed in Fig. 3A and B. Based on the product spectra and the mechanism, transitions of m/z 426.3 → 100.0 was selected for tiloronoxim and 411.3 → 100.0 for tilorone, respectively. For the IS, the process of dissociation was very complex (Fig. 3C) and transition of m/z 268.3 → 116.1 was selected for quantification.

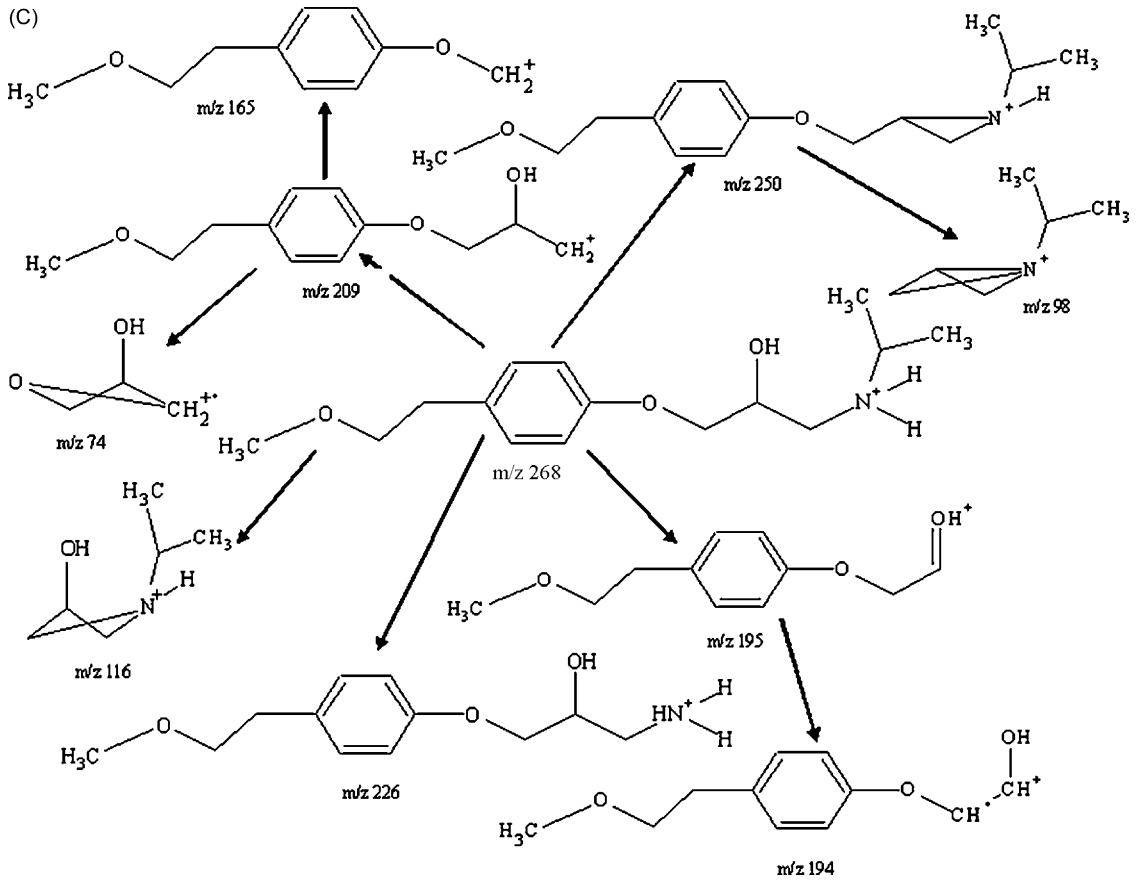

Fig. 3. (*Continued*).

Table 1
Results of matrix effect

Compound	Nominal concentration (ng/ml)	Absolute matrix effect $A_1/A_2 \times 100\%$	Relative matrix effect CV (%)
TLW	2	112.80	3.66
	10	106.01	8.71
	50	104.00	2.95
TLL	2	94.48	9.27
	10	90.82	7.46
	50	85.58	1.54
IS	125	96.06	9.29

Table 2
Back-calculated concentrations and correlation coefficient of tiloronoxim (TLW) and tilorone (TLL)

Compound	Spiked concentration (ng/ml)	Mean (ng/ml, $n=5$)	R.S.D. (%)	Accuracy (%)	Correlation coefficient ($n=5$)
TLW	1	1.09	5.9	109.2	
	2	1.99	4.5	99.6	
	5	4.84	9.8	96.7	
	10	9.77	8.0	97.7	0.9985 ± 0.0011
	20	18.98	7.0	94.6	
	50	48.47	4.0	96.9	
	100	101.72	5.7	101.7	
TLL	2	2.13	6.7	106.3	
	5	4.31	6.7	86.2	
	10	10.31	4.8	103.1	
	20	18.94	4.9	94.7	0.9985 ± 0.0007
	50	46.68	5.6	93.4	
	100	101.28	4.3	101.3	

Table 3
Intra- and inter-batch precisions and accuracy of tiloronoxim and tilorone in human urine

Compound	Nominal concentration (ng/ml)	Intra-batch (n = 5)			Inter-batch (n = 3)		
		Measured concentration mean ± S.D. (ng/ml)	R.S.D. (%)	Accuracy (%)	Measured concentration mean ± S.D. (ng/ml)	R.S.D. (%)	Accuracy (%)
TLW	2	2.22 ± 0.08	3.39	111.00	2.12 ± 0.11	4.96	106.33
	10	9.60 ± 0.78	8.07	96.03	10.36 ± 0.70	6.76	101.12
	50	46.22 ± 3.23	6.98	92.43	51.17 ± 4.43	8.66	98.40
TLL	2	1.94 ± 0.11	5.85	97.00	1.99 ± 0.06	2.96	99.43
	10	8.93 ± 0.35	3.87	89.26	9.59 ± 0.80	8.30	95.95
	50	50.36 ± 1.68	3.34	100.72	47.49 ± 2.83	5.96	94.99

Table 4
Summary of stability

Stability	Compound	Nominal concentration (ng/ml)	Calculated concentration (ng/ml)	Accuracy (%)
Freeze–thaw stability	TLW (1 cycle)	2	2.13 ± 0.18	106.38
		10	9.67 ± 0.11	96.73
		50	42.53 ± 1.76	85.05
	TLL (1 cycle)	2	1.84 ± 0.08	91.98
		10	9.61 ± 0.43	96.08
		50	45.93 ± 3.07	91.83
	TLW (3 cycles)	2	1.78 ± 0.08	89.00
		10	9.05 ± 0.48	90.50
		50	45.28 ± 1.84	90.55
	TLL (3 cycles)	2	1.79 ± 0.03	89.53
		10	9.14 ± 0.38	91.38
		50	46.98 ± 2.85	93.93
Pre-treatment stability at room temperature	TLW (4 h)	2	1.85 ± 0.18	92.67
		10	10.05 ± 0.63	100.50
		50	52.20 ± 3.98	104.40
	TLL (4 h)	2	2.03 ± 0.21	101.30
		10	8.77 ± 0.25	87.73
		50	55.00 ± 0.71	110.00
	TLW (8 h)	2	1.86 ± 0.03	93.17
		10	10.00 ± 0.10	100.00
		50	48.73 ± 0.40	97.47
	TLL (8 h)	2	2.00 ± 0.18	100.20
		10	9.48 ± 0.62	94.80
		50	49.43 ± 1.86	98.87
Auto sampler stability	TLW (8 h)	2	2.14 ± 0.220	106.83
		10	9.55 ± 0.47	95.47
		50	50.30 ± 2.61	100.60
	TLL (8 h)	2	2.03 ± 0.16	101.25
		10	9.38 ± 0.67	93.83
		50	44.84 ± .98	89.68
Long-term stability	TLW (60 days)	2	1.94 ± 0.16	96.60
		10	10.71 ± 1.12	107.05
		50	44.87 ± 0.74	89.80
	TLL (60 days)	2	2.13 ± 0.07	106.50
		10	9.94 ± 1.51	99.35
		50	51.15 ± 2.90	102.30

Table 5
Drug levels in three healthy volunteers' urine after oral administration of 100 mg tiloronoxim

Time (h)	Person 1 (ng/ml)		Person 2 (ng/ml)		Person 3 (ng/ml)		mean (ng/ml)	
	TLW	TLL	TLW	TLL	TLW	TLL	TLW	TLL
0–6	23.6	19.7	23.2	27.3	20.3	46.0	22.4	31.0
6–12	36.3	22.7	32.9	42.0	44.9	94.6	38.0	53.1
12–24	45.3	41.8	37.9	38.4	38.8	52.1	40.7	44.1
24–30	35.3	15.4	58.1	99.4	41.0	66.4	44.8	60.4
30–36	60.2	21.5	28.9	51.9	23.0	81.3	37.4	51.6
36–48	62.5	38.4	84.6	163.0[a]	31.4	45.6	59.5	82.3
48–60	10.5	6.71	23.4	42.8	10.0	17.4	14.6	22.3
60–72	26.6	20.8	26.6	36.7	13.3	18.5	22.2	25.3

[a] Re-assayed after dilution.

3.2. Optimization of the chromatographic conditions

Several analytical columns of different sizes and brands and different mobile phases were evaluated to find which one produced the sharpest, most symmetrical peaks with appropriate retention time. The Xterra MS C18 column (50 mm × 2.1 mm, 5 μm, Waters) and a gradient mobile phase (as described in Section 2.3) consisting of ammonium bicarbonate buffer (15 mM, pH 10.5, A) and methanol (B) with a flow rate of 0.3 ml/min were found to produce adequate retention and good peak shapes for the analytes and the IS. Representative chromatograms of the analytes and the IS from a spiked urine sample are shown in Fig. 4. The retention times of tiloronoxim, tilorone and the IS were typically 6.29, 6.48 and 4.42 min, respectively. The total analysis time was 8 min for each injection.

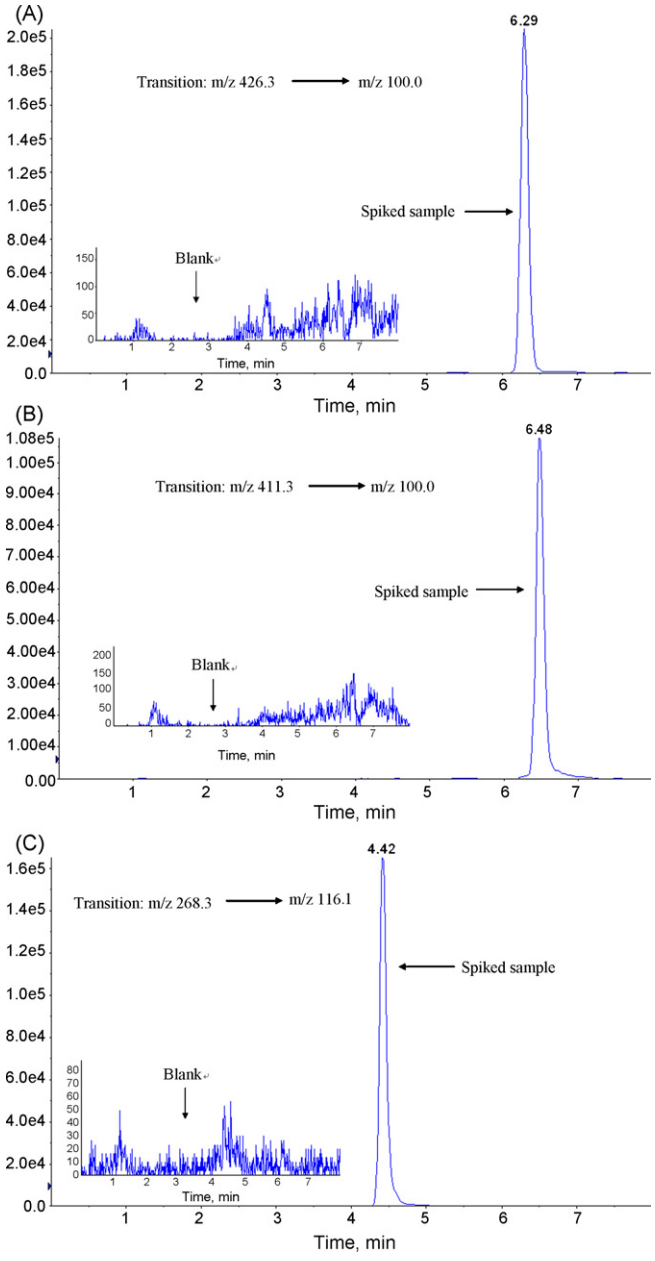

Fig. 4. Representative chromatograms of (A) tiloronoxim, (B) tilorone and (C) the IS in a spiked urine sample and a blank urine sample.

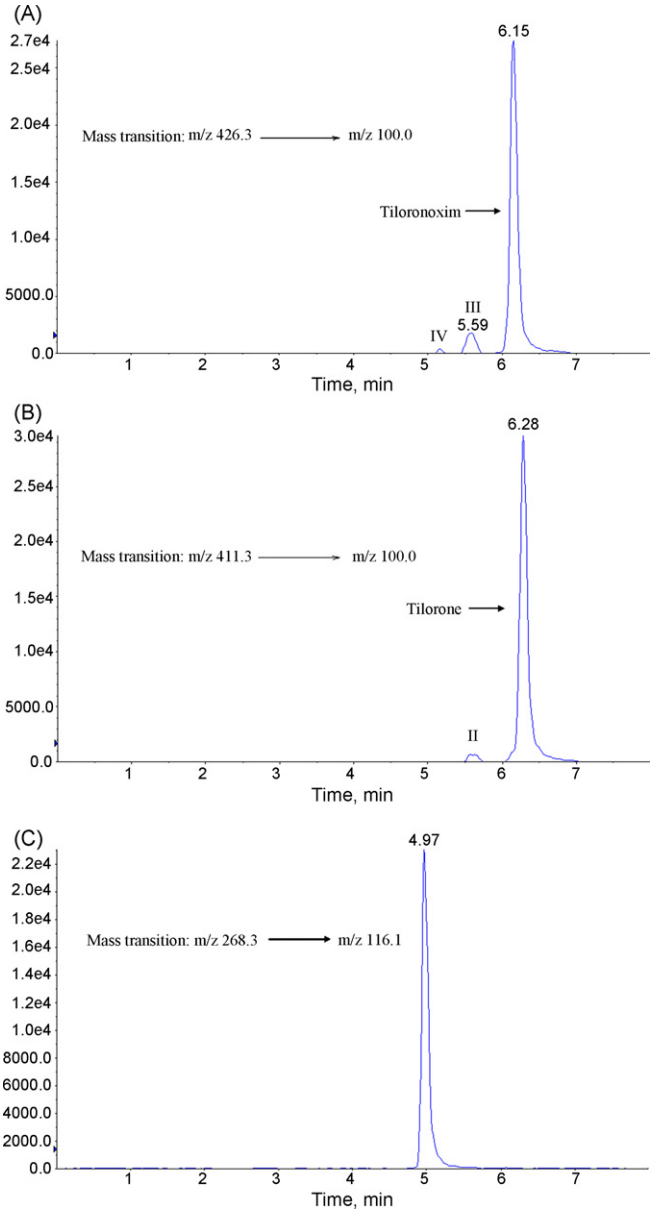

Fig. 5. Chromatograms of a urine sample from a volunteer (A) tiloronoxim, (B) tilorone and (C) the IS.

3.3. Method validation

3.3.1. Specificity

No endogenous inferences were observed in any extracts from the blank human urine. The miniature in Fig. 4 shows the representative chromatograms of a blank urine sample, demonstrating that the developed LC–MS/MS method is highly selective.

Post-column infusing method was used to check the matrix effect and we adjusted the retention of the analytes and the IS away from the suppression regions to minimize the matrix effect. Further experimental results showed that the absolute matrix effect ranged from 85.58 to 112.80% under current conditions and the relative matrix effect was less than 9.29% for the analytes and the IS (Table 1). It indicated that significant matrix effect was absent.

3.3.2. Linearity, precision and accuracy

The method was linear for both compounds within the range of seven calibrators used. The weighted regression calibration ($1/X$)

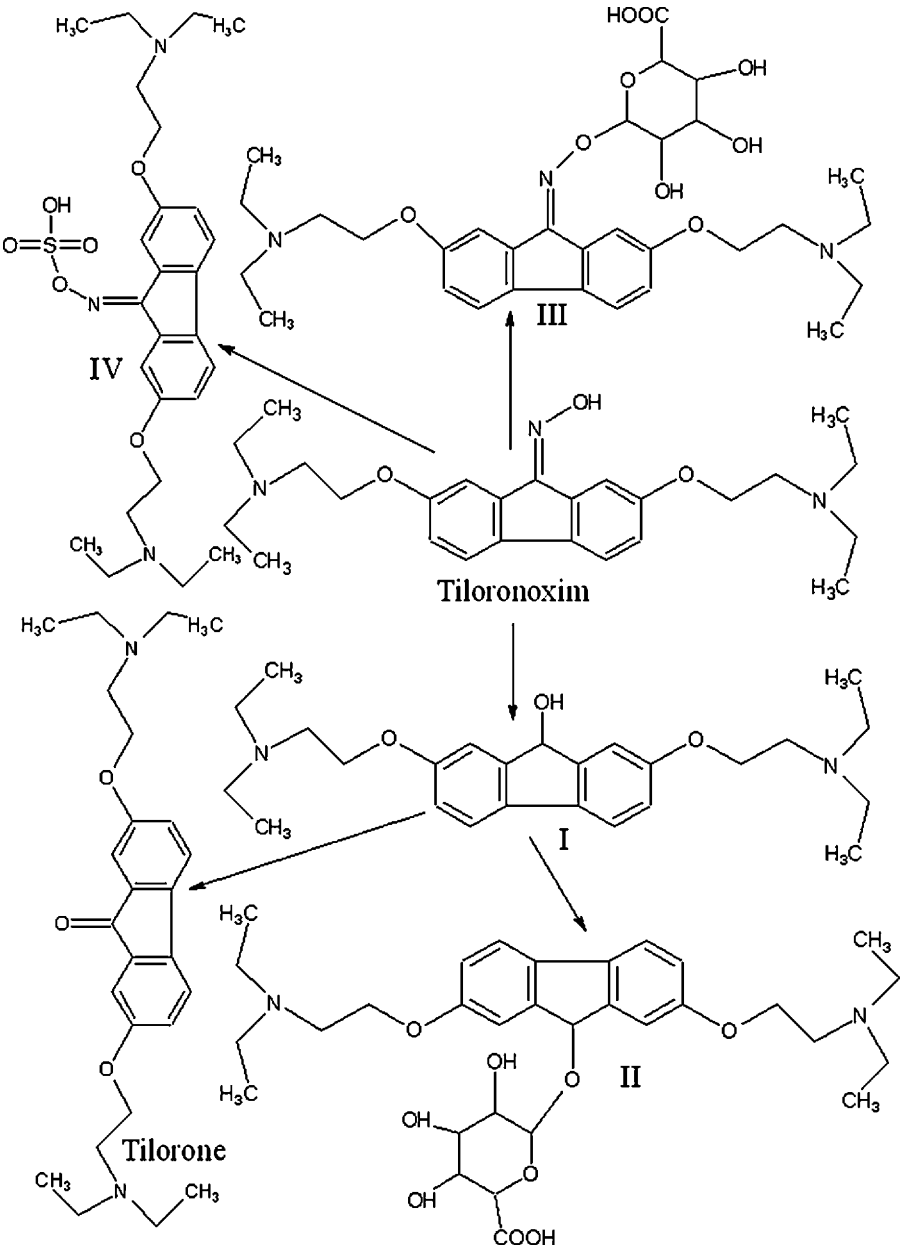

Fig. 6. Proposed metabolic pathway of tiloronoxim.

equations are listed below. Back calculated concentration and data of accuracy are given in Table 2.

$$Y = (0.0732 \pm 0.0007)\,X - (0.0024 \pm 0.0018) \quad (n = 5)\, r = 0.9985$$

$$Y = (0.0061 \pm 0.0004)X - (0.0027 \pm 0.0019) \quad (n = 5)\, r = 0.9985$$

Data of precisions are presented in Table 3. The intra- and inter-batch precisions of tiloronoxim ranged from 3.39 to 8.07% and 4.96–8.66%, respectively. For tilorone, the intra- and inter-batch precisions were 3.34–5.85% and 2.96–8.30%, respectively. Accuracies for tiloronoxim and tilorone were 92.43–111.00% and 89.26–100.72%, respectively. Lower limits of quantification (LLOQ) were 1 ng/ml for both tiloronoxim and tilorone.

3.3.3. Extraction recovery

The recovery was calculated from the response ratio of QC samples to pure solutions in matrix extracts at the same concentration. The mean recoveries for tiloronoxim and tilorone were 61.46 and 61.53%, respectively.

3.4. Stability

The results of the stability evaluation in human urine are summarized in Table 4. The accuracy of tiloronoxim ranged between 89.00 and 90.55% after three freeze–thaw cycles. For tilorone, accuracy ranged between 89.53 and 93.93% after three freeze–thaw cycles. The results demonstrate that tiloronoxim and tilorone were stable in urine after enduring three freeze–thaw cycles. And there is no degradation for both compounds after storage in human urine at room temperature for up to 8 h and at −40 °C for up to 2 months.

Extracts were stable at room temperature in auto sampler for at least 8 h (Table 4).

3.5. Dilution

The upper limit of calibration can be extended with acceptable precision and accuracy to 200 ng/ml by fivefold dilution with human urine blank. For the dilution experiment, the precision at 200 ng/ml was 1.75 and 2.55% and accuracy was 91.67 and 111.67% for tiloronoxim and tilorone, respectively. These data suggested that samples whose concentrations are greater than the upper limit of the standard curves could be reanalyzed by dilution to obtain acceptable data.

3.6. Application

Urine samples were obtained from three male healthy volunteers who were treated with tiloronoxim orally at a dose of 100 mg. The tiloronoxim and tilorone levels in urine were measured with the developed method. The quantification results are summarized in Table 5. Samples with concentration higher than the upper limit of quantification were diluted into the range of the calibration curve with the pre-dosed urine. These results indicate that the assay was appropriate for the measurement of tiloronoxim and tilorone in human urine. Fig. 5 shows the representative chromatograms of a real urine sample. It is interesting that in the extracted chromatogram of tiloronoxim (Fig. 5A), two more small peaks were observed before the retention of tiloronoxim, while in the spiked urine samples, these two peaks do not exist. Similar phenomenon was observed for tilorone and one more peak appears before the retention of tilorone (Fig. 5B). False positive results were excluded for both compounds. In the MRM mode, that several peaks in one chromatogram implies the presence of different compounds with the same precursor/product pairs and they may be other metabolites of tiloronoxim besides tilorone. However, the mechanism of tiloronoxim in human is unclear yet to date and possible mechanism is presumed. Glucuronidation and sulfation are common phase II metabolic pathways for oximes [12], and these two kinds of metabolites can often obtain the same precursor/product ion pairs with drug prototype. Therefore, the metabolic pathway was proposed (see Fig. 6) based on the experimental results and also the reasoning. But further studies need to be performed to verify the metabolism of tiloronoxim.

4. Conclusion

An LC–MS/MS method for the quantification of tiloronoxim and its main metabolite, tilorone, in human urine was developed and validated. This method was rapid, selective and highly sensitive with a LLOQ of 1 ng/ml for both compounds. Only 0.2 ml of urine is needed with this method, which greatly facilitated the collection of samples. The method allowed the quantification of both tiloronoxim and tilorone over the range of 1–100 ng/ml. It has been successfully applied to evaluate tiloronoxim and tilorone levels in urine from healthy volunteers. It provides a practical tool for *in vivo* detection of tiloronoxim and tilorone.

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