Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 796 (2003) 395–400
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

# Determination of tiropramide in human plasma by liquid chromatography–tandem mass spectrometry

Hye Won Lee [a], Hye Young Ji [a], Hee Hyun Kim [a], Hea-Young Cho [b], Yong-Bok Lee [b], Hye Suk Lee [a,*]

[a] *Drug Metabolism and Bioanalysis Laboratory, College of Pharmacy and Medicinal Resources Research Center, Wonkwang University, Shinyongdong, Iksan 570-749, South Korea*
[b] *College of Pharmacy and Institute of Bioequivalence and Bridging Study, Chonnam National University, 300 Yongbong-Dong, Buk-Gu, Gwangju 500-757, South Korea*

Received 29 April 2003; received in revised form 15 July 2003; accepted 19 August 2003

**Abstract**

A rapid, sensitive and selective liquid chromatography–tandem mass spectrometric (LC/MS/MS) method for the determination of tiropramide in human plasma was developed. Tiropramide and internal standard, cisapride were extracted from human plasma by liquid–liquid extraction and analyzed on a Luna C8 column with the mobile phase of acetonitrile-ammonium formate (10 mM, pH 4.5) (50:50, v/v). The analytes was detected using an electrospray ionization tandem mass spectrometry in the multiple-reaction-monitoring mode. The standard curve was linear ($r = 0.998$) over the concentration range of 2.0–200 ng/ml. The intra- and inter-assay coefficients of variation ranged from 2.8 to 7.8 and 6.7 to 8.9%, respectively. The recoveries of tiropramide ranged from 50.2 to 53.1%, with that of cisapride (internal standard) being 60.9 ± 5.3%. The lower limit of quantification for tiropramide was 2.0 ng/ml using 100 μl plasma sample. This method was applied to the pharmacokinetic study of tiropramide in human.


*Keywords:* Tiropramide

## 1. Introduction

Tiropramide, α-(benzoylamino)-4-[2-(diethylamino)ethoxy]-*N*,*N*-dipropylbenzene-propanamide (Fig. 1), has been used as antispasmodic drug in the treatment of irritable colon and biliary dyskinesia [1,2]. Tiropramide was extensively metabolized to *N*-desethyltiropramide, *N*-desethyl-*N*-despropyltiropramide, *N*-despropyltiropramide and hydroxytiropramide via *N*-desethylation, *N*-despropylation, and hydroxylation at *N*-propyl after oral administration of tiropramide to rats [3] and human [4,5]. A few methods for the determination of tiropramide in biological fluids were reported using gas-liquid chromatography (GLC) [4], GLC/mass spectrometry (GLC/MS) [5] and semi-micro high-performance liquid chromatography (HPLC) method [6]. However, those methods require relatively long run time.

* Corresponding author. Tel.: +82-63-850-6817; fax: +82-63-851-2013.
*E-mail address:* hslee@wonkwang.ac.kr (H.S. Lee).

1570-0232/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2003.08.027

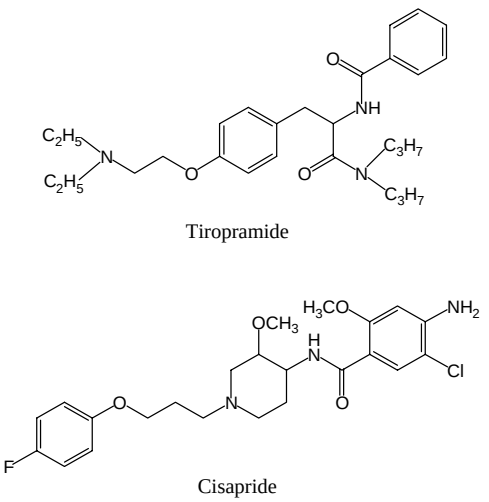


Fig. 1. Chemical structures of tiropramide and cisapride (internal standard).

Liquid chromatography–tandem mass spectrometry (LC/MS/MS) is recognized as a powerful tool for the quantitative determination of drugs and/or metabolites in biological matrices due to the selectivity, robustness and sample throughput. A sensitive, fast and reliable bioanalytical method is required for human pharmacokinetics and bioequivalence studies of tiropramide.

The purpose of this paper was to develop and validate a LC/MS/MS method using liquid–liquid extraction for the quantitative analysis of tiropramide in human plasma. The present method has been successfully applied to the evaluation of tiropramide pharmacokinetics in human.

## 2. Experimental

### 2.1. Materials

Tiropramide hydrochloride and cisapride (internal standard) were obtained from Daewoong Pharm. Co. (Seoul, Korea) with >99.9% purity. Acetonitrile and methyl *t*-butyl ether (HPLC grade) were obtained from Burdick & Jackson Inc. (Muskegon, MI, USA) and other chemicals were of HPLC grade or the highest quality available. Drug-free human plasma containing sodium heparin as the anticoagulant was obtained from healthy volunteers.

### 2.2. Preparation of calibration standards and quality control samples

Primary stock solutions of tiropramide and cisapride (1 mg/ml) were prepared in water and methanol, respectively. Working standard solutions of tiropramide were prepared by diluting each primary solution with water. The working solution for internal standard (50 ng/ml) was prepared by diluting an aliquot of stock solution with methanol. All tiropramide and cisapride solutions were stored at ca. 4 °C in polypropylene bottles in the dark when not in use.

Human plasma calibration standards of tiropramide (2.00, 5.00, 10.0, 20.0, 50.0, 100 and 200 ng/ml) were prepared by spiking appropriate amount of the working standard solutions into a pool of 10 lots of drug-free human plasma. Quality control (QC) samples at 6.00, 30.0 and 150 ng/ml were prepared in bulk by adding 50 μl of the appropriate working standard solutions (0.6, 3.0 and 15 μg/ml) to drug-free human plasma (4950 μl). The QC samples were aliquoted (100 μl) into polypropylene tubes and stored at −20 °C until analysis.

### 2.3. Sample preparation

One hundred microlitres of blank plasma, calibration standards and QC samples were mixed with 10 μl of internal standard working solution and 100 μl of 50 μM NaOH. The samples were extracted with 800 μl of methyl *t*-butyl ether in 2 ml polypropylene tubes by vortex-mixing for 5 min at high speed and centrifuged at 5000 × *g* for 5 min at room temperature. The organic layer was pipette transferred and evaporated to the dryness under nitrogen at 35 °C. The residues were dissolved in 40 μl of 50% acetonitrile in water by vortex-mixing for 2 min, transferred to injection vials, and 10 μl were injected onto LC/MS/MS system.

### 2.4. LC/MS/MS analysis

For LC/MS/MS analysis, the chromatographic system consisted of a Nanospace SI-2 pump, a SI-2 autosampler and an S-MC system controller (Shiseido, Tokyo, Japan). The separation was performed on a Luna C8 column (3 μm, 2 mm i.d. × 100 mm, Phenomenex, Torrance, CA, USA) using a mixture

of acetonitrile-ammonium formate (10 mM, pH 4.5) (50:50, v/v) at a flow rate of 0.2 ml/min. The column and autosampler tray temperature were 30 and 4 °C, respectively. The analytical run time was 3 min. The eluent was introduced directly to the tandem quadrupole mass spectrometer (Quattro LC, Micromass UK Ltd., UK) through the positive ionization electrospray interface. The ion source and desolvation temperature were held at 120 and 250 °C, respectively. The optimum cone voltages for ionization of tiropramide and cisapride were 42 and 45 V, respectively. Multiple-reaction-monitoring (MRM) mode using specific precursor/product ion transitions was employed for the quantification. The molecular ions of tiropramide and cisapride were fragmented at collision energy of 24 eV using argon as collision gas. Detection of the ions was performed by monitoring the transitions of *m*/*z* 469 to 367 for tiropramide and *m*/*z* 467 to 184 for cisapride. Peak areas for all components were automatically integrated using MassLynx Version 3.1 (Micromass UK Ltd., UK). Linear regression analysis with a weighting of 1/peak area ratio was used to generate calibration curves from nine standard lines and calculate the concentrations of quality control samples.

### 2.5. Method validation

Batches, consisting of triplicate calibration standards at each concentration, were analyzed on three different days to complete the method validation. In each batch, QC samples at 6.00, 30.0 and 150 ng/ml were assayed in sets of six replicates to evaluate the intra- and inter-day precision and accuracy. The percentage deviation of the mean from true values, expressed as relative error (RE), and the coefficient of variation (CV) serve as the measure of accuracy and precision.

The absolute recoveries of tiropramide were determined by comparing the peak area of six extracted samples at the concentrations of 6.00, 30.0 and 150 ng/ml with the mean peak area of recovery standards. Three replicates of each of the recovery standards were prepared by adding tiropramide and internal standard to blank human plasma extracts.

To evaluate the three freeze–thaw cycle stability and room temperature matrix stability, six replicates of QC samples at each of the low and high concentrations (6.00 and 150 ng/ml, respectively) were subjected to three freeze–thaw cycles or were stored at room temperature for 4 h before processing, respectively. Six replicates of QC samples at each of the low and high concentrations were processed and stored under autosampler conditions for 24 h were assayed to assess post-preparative stability.

### 2.6. Application

Four healthy male volunteers, fasted for 12 h, received a single oral dose of tiropramide hydrochloride (100 mg tablet) with 240 ml of water. Blood samples (1 ml) were withdrawn from the forearm vein at 0, 0.33, 0.67, 1, 1.5, 2, 2.5, 3, 4, 6, 8 and 12 h post dosing, transferred to Vacutainer$^{TM}$ plasma glass tubes (sodium heparin, BD, NJ, USA) and centrifuged. Following centrifugation (3000 × *g*, 15 min, 4 °C), plasma samples were transferred to polypropylene tubes and stored at –20 °C prior to analysis. Drug concentrations were determined as the mean of duplicate samples. The peak concentration ($C_{max}$) and the time to peak concentration ($T_{max}$) were determined by visual inspection from each volunteer's plasma concentration–time plots for tiropramide. Area under the plasma concentration–time curve (AUC) was calculated by the linear trapezoidal method from 0 to 12 h.

## 3. Results and discussion

### 3.1. LC/MS/MS

The electrospray ionization of tiropramide and cisapride produced the abundant protonated molecular ions ($MH^+$) at *m*/*z* 469 and 467, respectively under positive ionization conditions, without any evidence of fragmentation. $MH^+$ ions from tiropramide and cisapride were selected as the precursor ion and subsequently fragmented in MS/MS mode to obtain the product-ion spectra yielding useful structural information (Fig. 2). The fragment ions at *m*/*z* 367 (the loss of dipropylamino group from [$MH^+$] ion) and 184 (5-chloro-4-amino-2-methoxy-phenyl-ketone) were produced as the prominent product ions for tiropramide and cisapride, respectively. The quantification of the analytes was performed using the

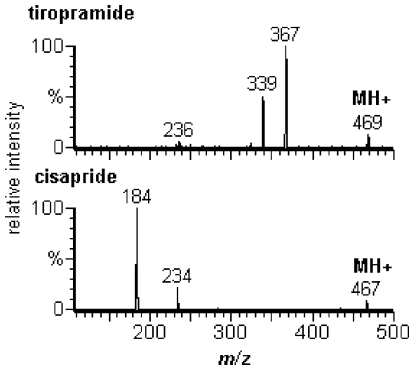


Fig. 2. Product-ion mass spectra of tiropramide and cisapride (internal standard).

MRM mode due to the high selectivity and sensitivity of MRM data acquisitions, where the precursor and product ions are monitored. Two pairs of MRM transitions were selected: *m/z* 468 → 367 for tiropramide and *m/z* 467 → 184 for cisapride (internal standard).

Fig. 3 shows the representative LC/MS/MS MRM chromatogram obtained from the analysis of blank human plasma, human plasma spiked with tiropramide at 2.0 ng/ml and a human plasma sample obtained 2 h after oral administration of tiropramide hydrochloride (100 mg). The analysis of blank plasma samples from six different sources did not show any interference at the retention times of tiropramide (2.0 min) and cisapride (1.9 min) (Fig. 3a), confirming the specificity of the present method.

### 3.2. Method validation

This method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation [7]. Calibration curves were obtained over the concentration range of 2.0–200 ng/ml of tiropramide in plasma. Linear regression analysis with a weighting of 1/peak area ratio gave the optimum accuracy of the corresponding calculated concentrations at each level (Table 1). The low coefficients of variation (CV) value for the slope indicated the repeatability of the method (Table 1).

For six samples of blank plasma from six independent sources with tiropramide at 30 ng/ml, CV and RE

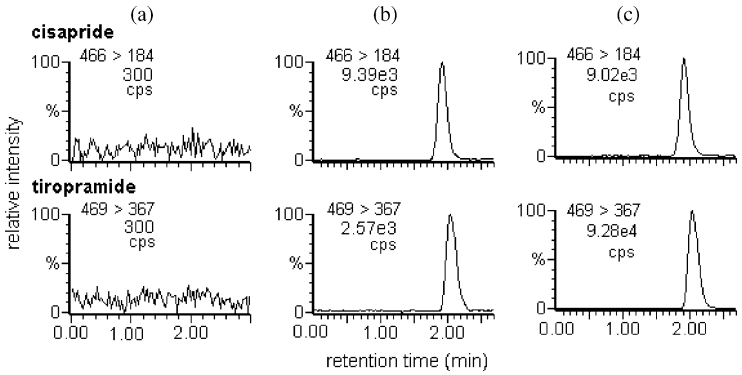


Fig. 3. MRM LC/MS/MS chromatograms of (a) a blank human plasma, (b) human plasma sample spiked with 2.00 ng/ml of tiropramide and (c) a human plasma sample obtained 2 h after oral administration of tiropramide hydrochloride (100 mg).

Table 1
Calculated concentrations of tiropramide in calibration standards prepared in human plasma ($n = 9$)

| | Theoretical concentration (ng/ml) | | | | | | | Slope | $r$ |
|---|---|---|---|---|---|---|---|---|---|
| | 2.00 | 5.00 | 10.0 | 20.0 | 50.0 | 100 | 200 | | |
| Mean (ng/ml) | 2.05 | 4.91 | 10.1 | 20.3 | 50.6 | 104 | 199 | 0.1110 | 0.998 |
| CV (%) | 6.5 | 5.8 | 8.6 | 7.1 | 7.9 | 5.8 | 4.9 | 8.0 | |
| RE (%) | 2.5 | −1.8 | 1.0 | 1.5 | 1.2 | 4.0 | −0.5 | | |

Table 2
Precision and accuracy of tiropramide in quality control samples

| | Intra-batch ($n = 6$) | | | | Inter-batch ($n = 18$) | | |
|---|---|---|---|---|---|---|---|
| QC (ng/ml) | 2.00 | 6.00 | 30.0 | 150 | 6.00 | 30.0 | 150 |
| Mean (ng/ml) | 1.95 | 5.64 | 29.4 | 153 | 5.62 | 28.6 | 144 |
| CV (%) | 5.6 | 2.8 | 7.8 | 6.3 | 8.1 | 8.9 | 6.7 |
| RE (%) | −2.5 | −6.0 | −2.0 | 2.0 | −6.3 | −4.7 | −4.0 |

were 3.3 and 1.6%, respectively. These tight CV and RE values indicate no significant lot-to-lot variation in matrix effects.

Table 2 shows a summary of intra- and inter-batch precision and accuracy data for QC samples containing tiropramide. Both intra- and inter-assay CV values ranged from 2.8 to 8.9% at three QC levels. The inter- and inter-assay RE values for tiropramide were −6.3 to 2.0% at three QC levels. These results indicated that the present method has an acceptable accuracy and precision.

The lower limit of quantitation (LLOQ) was set at 2.0 ng/ml for tiropramide using 100 μl of human plasma. Representative chromatogram of an LLOQ is shown in Fig. 3b and the signal-to-noise ratio for tiropramide is about 20 at 2.0 ng/ml. CV and RE at the LLOQ level were 5.6 and −2.5%, respectively (Table 2).

The extraction recoveries of tiropramide from spiked human plasma were determined at the concentrations of 6.0, 30.0 and 150 ng/ml in six replicates. The recoveries of tiropramide ranged from 50.2 to 53.1%, with that of cisapride (internal standard) being 60.9 ± 5.3% (Table 3). The one-step liquid–liquid extraction with methyl *t*-butyl ether at basic pH has been successfully applied to the extraction of tiropramide from human plasma.

Table 3
Absolute recoveries of tiropramide and cisapride (internal standard) from spiked human plasma

| Concentration (ng/ml) | Recovery (%, mean ± S.D., $n = 6$) | |
|---|---|---|
| | Tiropramide | Cisapride |
| 6.00 | 53.1 ± 3.9 | – |
| 30.0 | 50.9 ± 2.9 | – |
| 150 | 50.2 ± 3.9 | – |
| 5.00 | – | 60.9 ± 5.3 |

(–) Not assayed.

Table 4
Stability of samples ($n = 6$)

| Statistical variable | Theoretical concentration (ng/ml) | |
|---|---|---|
| | 6.00 | 150 |
| Freeze–thaw stability | | |
| Mean | 5.70 | 145 |
| CV (%) | 5.8 | 7.2 |
| RE (%) | −5.0 | −3.3 |
| Short-term temperature stability (4 h at room temperature) | | |
| Mean | 5.61 | 143 |
| CV (%) | 3.6 | 6.9 |
| RE (%) | −6.5 | −4.7 |
| Post-preparative stability (24 h at room temperature) | | |
| Mean | 5.89 | 146 |
| CV (%) | 5.1 | 5.6 |
| RE (%) | −1.8 | −2.7 |

Stability of tiropramide during sample handling (freeze–thaw and short-term temperature) and the stability of processed samples were evaluated (Table 4). Three freeze–thaw cycles and room temperature storage of the QC samples for 4 h before analysis, had little effect on the quantification. Extracted QCs and calibration standards were allowed to stand at ambient temperature for 24 h prior to injection without affecting the quantification.

### 3.3. Clinical application

This method has been successfully used to the pharmacokinetic study of tiropramide after a single oral dosing of tiropramide hydrochloride (100 mg) to four healthy male volunteers. Fig. 4 shows mean plasma concentration profiles of tiropramide in four healthy male volunteers. $C_{max}$, $T_{max}$, AUC and $t_{1/2}$ of tiropramide were 77.4 ± 33.0 ng/ml, 1.6 ± 0.6 h, 319 ± 147 ng h/ml and 2.7 ± 0.5 h, respectively.

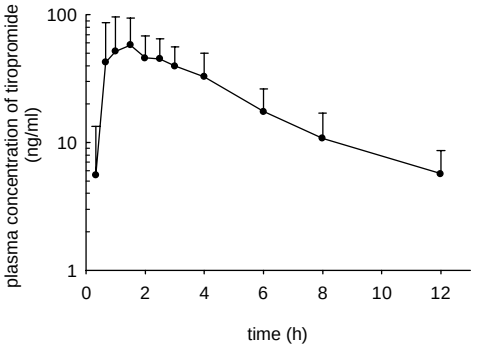


Fig. 4. Mean plasma concentration–time plots of tiropramide after a single oral dose of tiropramide hydrochloride (100 mg tablet) to four male volunteers. Each point represents mean ± S.D.

## 4. Conclusion

A sensitive and reliable LC/MS/MS method for the analysis of tiropramide in human plasma has been successfully developed and validated. To extract tiropramide from the plasma, a liquid–liquid extraction with methyl *t*-butyl ether at basic pH was used. The LLOQ for tiropramide was 2.00 ng/ml. The method was proved to be suitable for the clinical pharmacokinetic study of tiropramide.

## Acknowledgements

This work was partially supported by the Project from the Center for Biological Modulators (CBM-02-B-8), Ministry of Science and Technology, Republic of Korea.

## References

[1] S. Tinozzi, M.G. Valesi, Minerva Med. 75 (1984) 23.
[2] E. Trabucchi, C. Baratti, A. Centemero, M. Zuin, E. Rizzitelli, R. Colombo, Pharmatherapeutica 4 (1986) 541.
[3] I. Setnikar, F. Makovec, R. Chiste, C. Giachetti, G. Zanolo, Arznemittel-Forsch. 38 (1988) 1815.
[4] R. Arigoni, R. Chisté, A. Drovanti, F. Makovec, P. Senin, I. Setnikar, Arznemittel-Forsch. 36 (1986) 738.
[5] R. Arigoni, R. Chisté, F. Makovec, I. Setnikar, E. Benfenati, R. Fanelli, Biomed. Environ. Mass Spectr. 15 (1988) 205.
[6] S.K. Baek, S.S. Lee, E.J. Park, D.H. Sohn, H.S. Lee, J. Pharm. Biomed. Anal. 31 (2003) 185.
[7] Guidance for Industry-Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Verternary Medicine, May 2001, http://www.fda.gov/cder/guidance/index.htm.