Arch Pharm Res Vol 26, No 5, 416-420, 2003

Archives of Pharmacal Research
http://apr.psk.or.kr

Quantitative Analysis of Tiropramide in Human Blood by Gas Chromatography with Nitrogen-Phosphorus Detector

Oh-Seung Kwon, Young-Jin Park, Jae-Chun Ryu, and Youn Bok Chung[1]

Toxicology Lab., Bioanalysis and Biotransformation Research Center, Korea Institute of Science and Technology, Seoul 136-791, Korea and [1]Collge of Pharmacy, Chungbuk National University, Cheongju 361-763, Korea

(Received September 22, 2002)

The analytical method of antispasmodic agent tiropramide {(±)α-(benzoylamino)-4-[2-(diethylamino)ethoxy]-*N*,*N*-dipropylbenzenepropanamide hydrochloride} was developed by gas chromatography/nitrogen-phosphorus detector (GC/NPD) in human plasma. Two kinds of tiropramide tablets were orally administered to volunteers by Latin square crossover design, and blood was withdrawn as designed schedule. The plasma of 1 mL was loaded on Sep-pak C_{18} cartridge and eluted with methanol after washing with 30% methanol. The residue dissolved in 100 μL of methanol after evaporation was analyzed by GC/NPD. Precision (CV%) of intra-day was located within 2.6% and accuracy was less than 9.7%. Inter-day precision was below 8.7% and accuracy was relatively good as less than 14%. Plasma samples obtained from human volunteers were analyzed for the determination of tiropramide concentration by using this method. The method was sensitive, rapid and suitable enough to be applied for pharmacokinetic and bioequivalence studies of tiropramide in human volunteers.

Key words: Antispasmodic agent, Determination, Gas chromatography/nitrogen-phosphorus detector, Human plasma, Tiropramide

INTRODUCTION

Tiropramide {TRP; (±)α-(benzoylamino)-4-[2-(diethylamino) ethoxy]-*N*,*N*-dipropylbenzenepropanamide hydrochloride} has been known as a potent antispasmodic agent, and widely used for treatment of patients with disorders of the motility of the gastrointestinal, biliary and urinary tracts (Setnikar *et al.*, 1989a, 1989b; Suda *et al.*, 1992; Uruno *et al.*, 1992a). Pharmacological effects of the smooth muscle relaxant activity for TRP were associated with the increase of intracellular cyclic AMP levels and inhibition of Ca^{2+} influx in tissue (Uruno *et al.*, 1992b; Vidal *et al.*, 1981).

The concentration of TRP was determined mostly by ^{14}C-radiolabelled TRP in the various tissues, blood and urine of rats (Setnikar *et al.*, 1989c, 1989d, 1988), and rarely by gas-liquid chromatography/nitrogen-phosphorus detector (Arigoni *et al.*, 1986). Setnikar *et al.* (1989c) has analyzed metabolites of TRP after intravenous or oral administration of ^{14}C-radiolabelled TRP by thin layer chromatography, even if the chemical structure of its metabolites was unidentified. By using gas chromatography/mass spectrometry, Arigoni *et al.* (1988) identified three metabolites of TRP from eight metabolites.

At present, the method developed by gas chromatography with nitrogen-phosphorus detector, however, are not rapid enough to apply for preparation of large numbers of samples, not feasible for routine use in bioavailability studies, and requires expensive sample preparation technique because a relatively large volume of plasma sample (2 mL) and elution solvent (50 mL) were used to determine TRP in human plasma (Arigoni *et al.*, 1986).

Therefore, the purpose of the present work was focused on developing a simple, sensitive and rapid method suitable for the quantitation of TRP in large number of plasma samples for bioequivalence studies

MATERIALS AND METHODS

Chemicals

Tiropramide HCl {(±)α-(Benzoylamino)-4-[2-(diethylamino) ethoxy]-*N*,*N*-dipropylbenzenepropanamide hydrochloride}

Correspondence to: Oh-Seung Kwon, Ph.D., Toxicology Lab., Bioanalysis and Biotransformation Research Center, Korea Institute of Science and Technology, Hawokgok-dong 39-1, Sungbuk-ku, Seoul 136-791, Korea
Tel: 82-2-958-5184, Fax: 82-2-958-5059
E-mail: oskwon@kist.re.kr

was obtained from Dongkoo Pharm. Co. (Seoul, Korea). (±)α-(Benzoylamino)-4-[2-(dimethylamino)ethoxy]-*N*,*N*-dipropylbenzenepropanamide hydrochloride used as internal standard was obtained from Doping Control Center of Korea Institute of Science and Technology (Seoul, KIST). Methanol was purchased from J.T. Baker (Phillipsburg, NJ, USA). Sep-pak C_{18} was purchased from Waters (Milford, MA, USA). Sodium bicarbonate and potassium carbonate were obtained from Sigma (St. Louis, MO, USA). The other agents used for tiropramide analysis were of analytical grade.

Equipment

Tiropramide analysis was performed with a gas chromatography with nitrogen phosphorus detector (HP5890A, Hewlett Packard, DE, USA). A capillary column (Ultra-1, 12.5 m, L. × 0.2 mm, I. D. × 0.3 μm, film thickness, Hewlett Packard) was used. The flow rate of carrier gas (He) was 1 mL/min. The splitless mode was used and septum purge was in the rate of 5 mL/min. Gas flow rates for the detector of air, hydrogen, and helium (make-up) were 100, 3.5, and 30 mL/min, respectively. Both the injector and detector temperature were set to 300°C. The gradient oven temperature was used: initial temperature was set at 200°C without holding time and increased by 20°C per min to 300°C of final temperature at which the temperature was maintained for 5 min.

Oral administration of tiropramide tablets to human volunteers

One tablet of tiropramide (total 100 mg tiropramide) was orally taken to volunteers fasted for 12 h with about 200 mL of drinking water. The volunteers agreed to attend this work were medically examined by a doctor and stayed in the hospital during blood sampling. Blank blood was withdrawn prior to the administration of a tiropramide tablet. After oral administration of tiropramide about 7 mL of blood was collected at 0, 0.33, 0.66, 1, 1.5, 2, 2.5, 3, 5, 8 and 12 h and was centrifuged to obtain plasma. The plasma was stored at -70°C until analyzed.

Preparation of calibration curve of tiropramide in human plasma

To 1 mL of the tiropramide-free blank plasma, 0, 5, 10, 25, 50, 100, 150 and 200 ng of tiropramide prepared in methanol and internal standard (5 μg/mL, 20 μL) were added. The clean-up procedure was the same as described below.

Determination of tiropramide in human plasma

The thawed plasma of 1 mL was added to a Eppendorf tube. Internal standard (5 μg/mL, 20 μL) and 250 μL of 1 N perchloric acid were added to the tube and mixed on a vortex-mixer (Maxi Mix II, Thermolyne Co., Dubuque, IA, USA). The tube was centrifuged (HM 150-IV, Hanil Industrial Co., Seoul, Korea) at 4,000 g for 10 min. The supernatant was transferred to the tube with 300 μL of 0.5 M sodium bicarbonate/0.5 M potassium carbonate (v/v, 5:1) to adjust the pH value to 9.5-10. This solution was loaded to the activated Sep-pak C_{18} cartridge. The cartridge was activated with the order of each 1 mL of methanol and distilled water. After loading the sample, the cartridge was washed with 30% methanol (1 mL × 3). Elution was made by methanol (1 mL × 3) at the flow rate of 1 mL/min under a vacuum extraction manifold (Waters, Milford, MA, USA). The methanol eluate was dried on heating block (Dri-Block, Techne Inc., Princeton, NJ, USA) at 80°C under nitrogen stream. The residue was dissolved in 100 μL of methanol. Two μL of the solution was injected by an auto liquid sampler. The plasma concentration of tiropramide was calculated from area ratios of tiropramide to internal standard by a calibration curve.

RESULTS AND DISCUSSION

Tiropramide separation

Retention times of TRP (shown as peak 2 in Fig. 1) and its internal standard (peak 1 in Fig. 1) were 6.69 and 5.93 min, respectively. No interfering peaks were found and showed good separation between these peaks. These GC/NPD chromatograms were shown in Fig. 1. Total run time was about 10 min, corresponding to the analysis of one plasma sample. Peaks 3 and 4 were metabolites of TRP that their structures were not identified. The peak of the internal standard with 2-(dimethylamino)ethoxy moiety on its structure instead of 2-(diethylamino)ethoxy moiety of TRP was eluted early, compared to the peak of TRP.

Intra- and inter-day precision and accuracy

Intra- and inter-days precision and accuracy data were presented in Table I and Table II, respectively. Precision (CV%) of intra-day was located within 2.6% and accuracy was less than 9.7% except for 22.5% of the lowest concentration (5 ng/mL). Inter-day precision was below 8.7% and accuracy was relatively good as less than 14%. Calibration curve prepared from 1 mL of plasma spiked 5~200 ng of authentic TRP gave good linearity ($y = 0.00795 x + 0.0117$, $r^2 = 0.9996$, $n = 6$/point). Quantitation limit of TRP by this method was 5 ng/mL based on the ratio of signal to noise (S/N = 10).

Establishment of optimal elution volume of tiropramide

From a Sep-pak C18 cartridge, arbitrary peak areas for TRP and its internal standards were plotted by the number of fraction that was consisted of 1 mL of methanol.

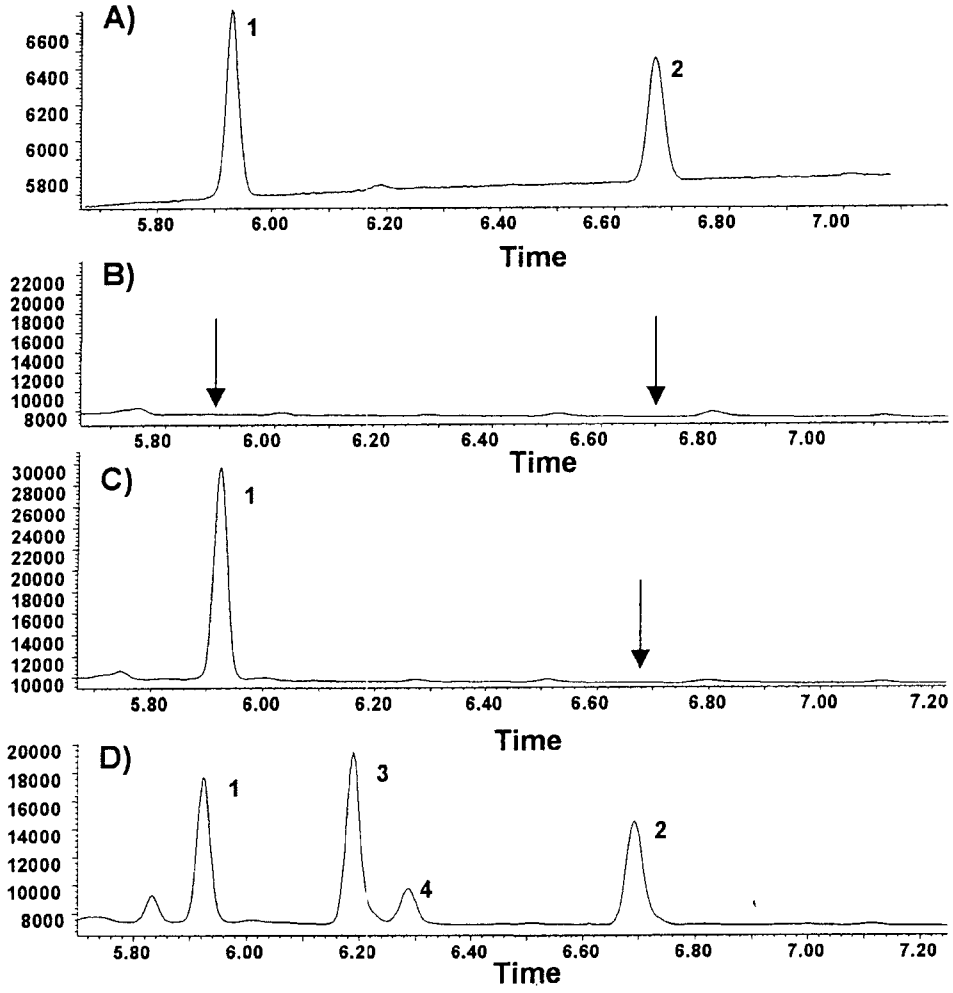

Fig. 1. Gas chromatography/nitrogen-phosphorus detector chromatograms obtained from authentic standard of tiropramide (A), tiropramide-free blank of human plasma without (B) or with (C) spiked internal standard, and a plasma sample of human volunteer taken tiropramide tablet (D). Retention times of tiropramide (peak 2) and internal standard (peak 1) were 6.69 and 5.93 min, respectively.

Table I. Intra-day precision and accuracy for analysis of tiropramide spiked in human plasma

Tiropramide (ng/mL)	Ratio[a] (Mean ± SD)	CV%	Accuracy (Bias %)
5	0.048 ± 0.001	2.1	22.5
10	0.086 ± 0.001	1.2	9.7
25	0.196 ± 0.002	1.0	0.02
50	0.416 ± 0.011	2.6	6.2
100	0.807 ± 0.014	1.7	3.0
150	1.178 ± 0.025	2.1	0.2
200	1.548 ± 0.040	2.6	-1.3

[a]Mean ± standard deviation of peak area ratios of tiropramide to internal standard (n = 3).

Table II. Inter-day precision and accuracy for analysis of tiropramide spiked in human plasma

Tiropramide (ng/mL)	Ratio[a] (Mean ± SD)	CV%	Accuracy (Bias %)
5	0.046 ± 0.004	8.7	14.0
10	0.092 ± 0.004	4.3	14.0
25	0.209 ± 0.008	3.8	3.6
50	0.453 ± 0.028	6.2	12.3
100	0.798 ± 0.049	6.1	-1.1
150	1.200 ± 0.111	9.3	-0.9
200	1.612 ± 0.113	7.0	0.1

[a]Mean ± standard deviation of peak area ratios of tiropramide to internal standard (n = 3).

TRP showed the highest peak at the second fraction and the peak area was gradually decreased until the fourth fraction as shown in Fig. 2. Peak area of internal standard was the highest at the first fraction and almost complete elution was achieved at the third and fourth fractions (Fig. 2). Based on this data the optimal fraction volume of

methanol was decided to be 3 mL (1 mL × 3). This results in recovery of >90%.

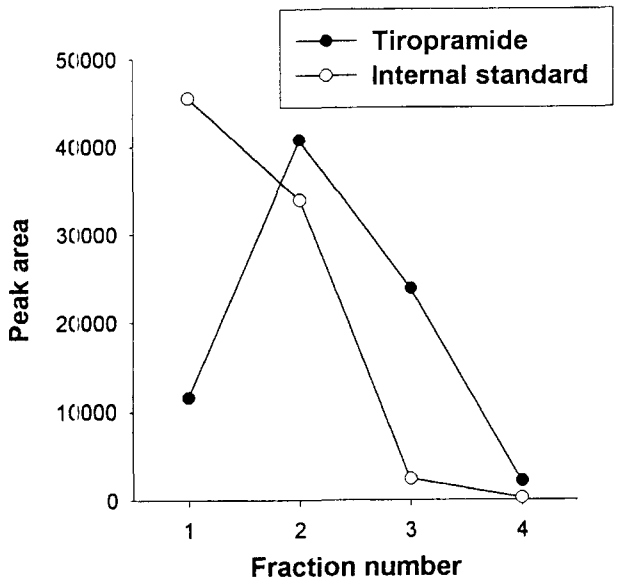

Fig. 2 Effects of elution volume on extraction of tiropramide using Sep-pak C_{18} cartridge. The plasma (1 mL) spiked tiropramide and internal standard was loaded on pre-activated Sep-pak C_{18} cartridge. The cartridge was washed with 30% methanol (1 mL × 3) and eluted by 1 mL of methanol three times. Two compounds were determined in each fraction. This procedure was described in detail in Materials and Methods.

Plasma concentration-time profile of tiropramide

Two different formulation of TRP were orally administrated to volunteers by Lain-square crossover design. Fig. 3A and 3B present the time-plasma concentration curves of TRP in Formulations I and II, respectively. The area under the curve (AUC) in Formulation I was similar or little higher than the Formulation II in two volunteers, as known by comparison of the area under the curves of TRP (Table III). In practice, when volunteers were increased (n = 16), differences between AUC of two formulations were located within 20% (data not shown). If compared two volunteers, absorption in volunteer A3 was more rapid than that in volunteer A1 in all two Formulations.

Except for reports by Arogoni *et al.* (1986), there are no reports relating to the determination of TRP in human plasma by GC/NPD. However, Arigoni *et al.* (1986) had used large scale of extraction for TRP analysis in human plasma and urine. Increased sensitivity of tiropramide

Table III. Principal pharmacokinetic parameters of tiropramide in human volunteers

Formulations (Tablets)	Human volunteers	AUC (ng/mL · h)	C_{max} (ng/mL)	T_{max} (h)
I	A1	466.4	119.7	1.5
	A3	812. 7	186.6	0.66
II	A1	267.7	87.3	2
	A3	737.7	191.3	0.66

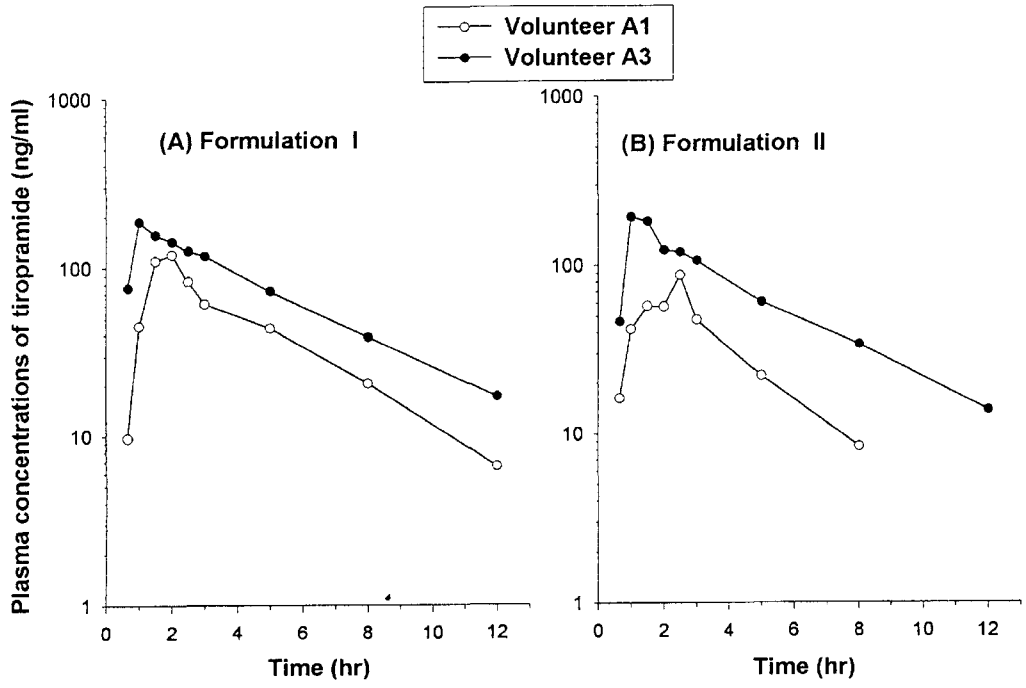

Fig. 3 The typical time-plasma concentration curves of tiropramide in volunteers orally taken two kinds of tiropramide formulations by Latin square cross-over design. Formulations I and II were shown in panels A and B, respectively. Principal pharmacokintic parameters obtained from these curves were shown in Table III.

detection by GC/NPD may be due to the use of relatively short length (12.5 m) of GC column, selection of splitless mode on GC, and addition of washing step to Sep-pak C_{18} cartridge with 30% methanol. By using this method, about 48 samples can be prepared and analyzed for a day. Our method is much more simple, rapid and reproducible in the analysis of TRP in biological fluids in human compared to the method by Arigoni *et al.* (1986). The developed method is suitable for and can be applied for pharmacokinetic or bioeqivalence studies of TRP.

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