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High-performance liquid chromatographic method for determination of tramadol in human plasma

Geng-Chang Yeh[a], Ming-Thau Sheu[b], Chia-Lin Yen[a], Ya-Wen Wang[a], Cheng-Hsiung Liu[c], Hsiu-O Ho[b,*]

[a]*Department of Pediatrics, Taipei Medical College, Taipei, Taiwan, ROC*

[b]*Graduate Institute of Pharmaceutical Sciences, Taipei Medical College, 250 Wu-Hsing Street, Taipei, Taiwan, ROC*

[c]*School of Pharmacy, China Medical College, Taichung, Taiwan, ROC*

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Abstract

A modified high-performance chromatographic method using UV detection was developed for determination of tramadol concentration in human plasma. Plasma samples were extracted with ethyl acetate in a one-step liquid–liquid extraction (recovery 88.5±2.1%). Analysis of the extract was performed on a reversed-phase LiChrospher 60 RP-select B column with a particle size of 5 μm. The mobile phase consisted of 0.05 *M* KH_2PO_4 aqueous solution (pH 3.5) and acetonitrile in a ratio of 90:10 (v/v). Metoprolol was used as the internal standard and UV detection at 225 nm was employed. Accuracy of the assay in the concentration range examined was from 1.3 to 11.9% for the intra-day run and from 1.4 to 8.1% for the inter-day run. The precision of this method varied from 1.2 to 8.7%. The reproducibility of the method was determined to be from 0.8 to 7.2% over the six-day period. A limit of detection was 9 ng/ml at a signal-to-noise ratio of 3. This validated method was then applied to the determination of tramadol concentrations in healthy volunteers after oral administration of 100 mg of tramadol in capsules of Painlax® and Tramal®. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Tramadol HCl (*trans*-(±)-2-[dimethylamino] methyl)-1-(3-methoxyphenyl) cylcohexanol hydrochloride) is a centrally acting analgesic which possesses opioid properties and activates monoaminoergic spinal inhibition of pain. It may be administered orally, rectally, intravenously or intramuscularly. Many clinical studies have evaluated the therapeutic efficacy (analgesic effects) of tramadol in comparison with morphine and other analgesics and found tramadol to be effective for relief of postoperative pain (patient-controlled analgesic, PCA), moderate surgical pain, surgical pain in children, cancer pain control, obstetric pain, osteosynthesis, and chronic pain [1]. Tramadol was reported to be an effective analgesic in step 2 of the Word Health

*Corresponding author: Tel.: +886-2-2736-1661 (ext. 689); fax: +886-2-2377-1942; e-mail: hsiuoho@mail.tmc.edu.tw

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Organization's guidelines for the treatment of patients with cancer-related pain with a daily dose of 250 mg to 600 mg administered orally [1].

Several methods for determination of tramadol concentration in human plasma have been reported. These include gas chromatography with a nitrogen-selective detector [2], gas chromatography–mass spectrometry [3,4], and radiochromatography [3]. HPLC-based methods with UV detection have been employed for determination of higher concentrations of tramadol and its metabolites in urine [3,5]. Chiral high-performance liquid chromatography has been used for determination of enantiomeric ratios of the compounds mentioned [5].

There are several disadvantages to using HPLC with UV detection for determining tramadol concentrations in plasma. First, tramadol contains a weakly absorbing chromophore [6], which makes determination of low tramadol concentrations problematic. Furthermore, biological samples containing tramadol need to be extracted using multi-step pH-dependent procedures prior to HPLC, with recoveries of 50–87%. In addition, an internal standard used in GC and HPLC determinations [2,5], a higher homologue of tramadol, (*trans*-(±)-2[dimethylamino] methyl)-1-(3-ethoxyphenyl) cylcohexanol hydrochloride), was not readily available.

An improved high-performance liquid chromatographic method with fluorescence detection has recently been developed [7]. Human plasma samples containing tramadol and having been spiked with an internal standard of verapamil were extracted with *tert*.-butylmethyl ether. The recovery of extraction was 86% and the limit of quantification was 17 ng/ml. However, because of differences in emission wavelength of tramadol and verapamil, the detection of each of them has to be changed during the chromatographic run.

In the present study, we attempted to improve the sensitivity of the high-performance chromatographic method using UV detection at 225 nm with the modification of the extraction solvent and the composition of the mobile phase. In addition, this HPLC method was validated and used in a bioequivalency study (Painlax® vs Tramal®) for determination of tramadol concentrations in ten healthy Chinese volunteers.

2. Experimental

2.1. Drugs and reagents

Tramal® 50 mg capsules (Lot. No. 619D) were obtained from Grunenthal (Stolberg, Germany). Painlax® 50 mg capsules (Lot. No. TR001) were from B&F Pharmaceutical and Chemical Co. (Taipei, Taiwan). Potassium monobase phosphate, sodium hydroxide, and phosphoric acid were from Merck (Germany). Acetonitrile, and ethyl acetate were from BDH Laboratory Supplies (Poole, UK), and metoprolol was from Sigma Chemical Co. (St. Louis, MO, USA). All solvents were HPLC grade and all chemicals were AR grade.

2.2. Chromatography

A high-performance liquid chromatographic system equipped with a pump (Model 510, Waters, Milford, MA, USA) and a Waters 717 plus Autosampler was used. A 15 cm×4.6 mm I.D. reversed-phase LiChrospher 60 RP-select B column (LiChroCart, Darmstadt, Germany) with a particle size of 5 μm was employed. The mobile phase consisted of 0.05 *M* KH_2PO_4 aqueous solution (adjusted to pH 3.5 with H_3PO_4) and acetonitrile in a ratio of 90:10 (v/v). The flow-rate was set at 1.0 ml/min. The eluent was detected with a Water 486 UV detector at a wavelength of 225 nm. The HPLC system was controlled by a PC workstation installed with Millennium 2010 Chromatographic Management computer software (Waters). The peak area ratio (PAR) of tramadol to the internal standard (metoprolol) was used to construct the calibration curve and calculate the tramadol concentration in plasma samples.

2.3. Internal standard solution and sample preparation

Metoprolol HCl, the internal standard, was freshly prepared at a concentration of 2.0 μg/ml in deionized water. Seventy-five microliters of metoprolol were added to 1 ml of plasma sample or plasma standard and the solution was vortexed for several seconds. The pH of the mixture was then adjusted to 12 with 0.1 *M* NaOH, followed by thorough vortex-

ing. Six milliliters of organic solvents, either ethyl acetate, chloroform, *n*-hexane, or methylene chloride, was added for extraction. The mixture was then vortexed and centrifuged (Sorvall RT 6000D centrifuge, DuPont, Boston, MA, USA) at 3000 rpm (1000 *g*) for 15 min. The supernatant (the organic phase) was transferred to a clean glass tube and the organic solvent was evaporated with nitrogen gas at room temperature until the tube was dry. The extract was reconstituted with 200 μl of the mobile phase, and vortexed again for several seconds until completely dissolved. One hundred microliters of this solution was injected into the HPLC for analysis.

2.4. Calibration

The standard stock solution (1mg/ml of tramadol) was diluted with deionized water to a calibration series of tramadol/metoprolol mixtures with tramadol concentrations of 12.5, 25.0, 50.0, 100.0, 200.0, 400.0, 600.0, and 800.0 ng/ml and with a constant metoprolol concentration (150 ng/ml) at each calibration level. The same concentrations were used to make a calibration curve with drug-free human plasma spiked with tramadol and metoprolol. The extraction followed the same procedure described above.

2.5. Volunteers

Ten healthy Chinese adult males (age: 21–26 years old, average 23.6±1.2 years old; mean body weight 66.9±7.4 kg) participated in this study. All volunteers first underwent medical screening consisting of a series of examinations including a physical examination, biochemistry (blood and urine) tests and chest X-ray (as necessary). A physician explained the study, the reported possible side effects of tramadol, and volunteers' privileges to the volunteers prior to the study. Consent forms were obtained from all volunteers when they decided to participate in this study. During the study, volunteers were advised not to take any medication (at least 1 week before the study) or caffeine-containing beverages or food (24 h before the study). On the study day, volunteers had fasted at least 10 h before and 4 h after oral administration of the study drugs. Water was freely supplied during the study. The washout period was at least 1 week.

2.6. Study design

A randomized, single-dose, two-treatment, two-period, two-sequence crossover study design was employed. Each volunteer received a single oral dose of tramadol 100 mg (two 50 mg capsules of either the reference product or the test product) in each period, with 200 ml water. Blood samples were collected pre-dose and at 30, 60, 90, 120, 150, 180, 240, 360, 480, 600, 720, 840, and 1440 min after oral administration. An 8–10 ml blood sample was withdrawn at each time point from a venous vein of the forearm, through a three-way stopcock. Plasma was obtained by centrifugation at 3000 rpm (1000 *g*) for 10 min. Plasma samples were stored at −20°C until analysis.

3. Results and discussion

3.1. Chromatography

To increase the sensitivity of tramadol detection, an UV wavelength of 225 nm was chosen. However, the interference at this wavelength was expected to be larger than that at the wavelength of 275 nm, which was chosen by a previous study. This could be minimized with an appropriate selection of solvents for extraction. The response of the drug and interference in the chromatographic region of the analytes of interest were compared to drug-free plasma extracted with various organic solvents. A typical chromatogram of blank plasma, plasma spiked with metoprolol, and plasma spiked with tramadol and metoprolol is presented in Fig. 1, using ethyl acetate as the extraction solvent and the same mobile phase (acetonitrile–0.05 *M* phosphate buffer pH 3.5, 10:90) for elution. The results demonstrate that extraction with ethyl acetate yielded the highest response. Table 1 lists the relative recovery of tramadol and metoprolol from plasma with different solvents when compared with water ($n=3$). The results again demonstrate that ethyl acetate was the optimal choice for tramadol and metoprolol extraction.

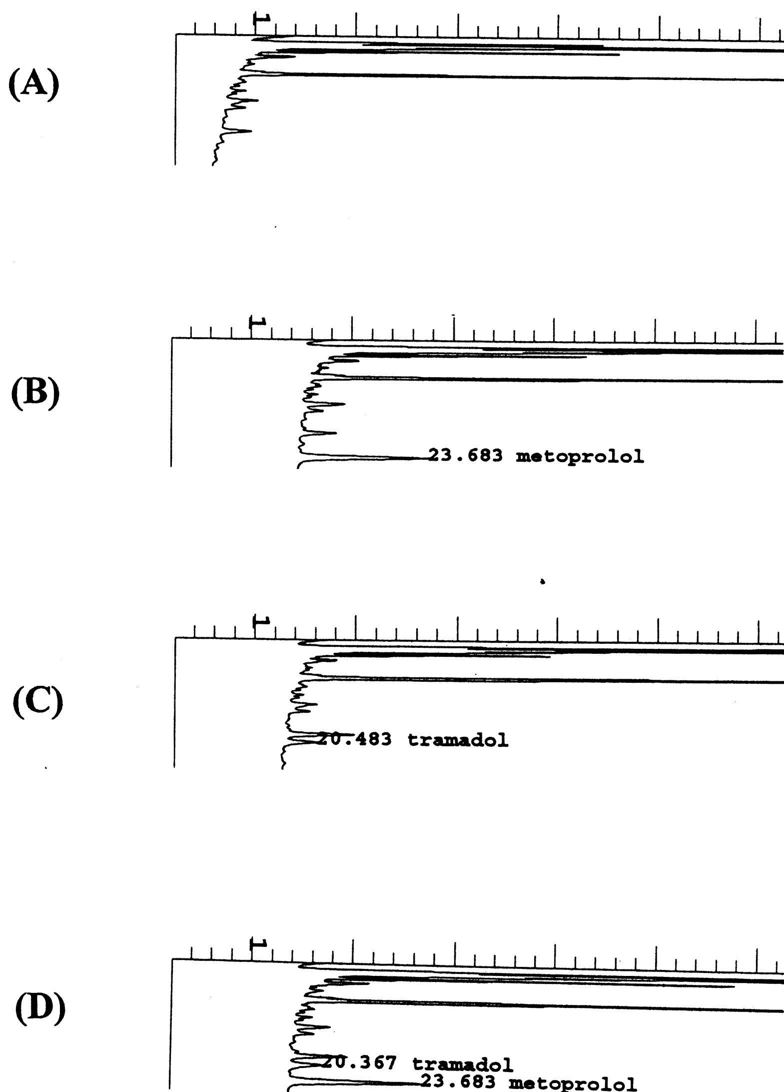

Fig. 1. Chromatograms of extracted plasma standards eluted with phosphate buffer (pH 3.5, 0.05 *M*)– acetonitrile (90:10). (A) blank plasma; (B) blank plasma with metoprolol (100 ng/ml); (C) blank plasma with tramadol (150 ng/ml); (D) blank plasma with tramadol and metoprolol.

Table 1
The relative recovery (%) of tramadol and metoprolol from plasma

Solvent	Tramadol	Metoprolol
Ethyl acetate	88.5(2.1)[a]	84.8(5.1)
n-Hexane	79.7(4.8)	37.6(3.4)
Chloroform	58.1(10.9)	79.6(13.9)
Dichloromethane	61.6(4.8)	76.7(3.4)

[a] Mean (SD), $n=3$.

The resolution between tramadol and metoprolol was further examined by adjusting the ratio of buffer to organic solvent. The relative retention times for tramadol with respect to metoprolol were 0.86, 0.91, 0.99, and 1.0 with phosphate buffer (0.05 *M*, pH= 3.5) to acetonitrile ratios of 90:10, 87.5:12.5, 85:15, and 80:20, respectively. Evaluation of the pH effect of the buffer solution on the elution of tramadol and metoprolol revealed that the optimal pH was 3.5.

Table 2
Accuracy and precision of tramadol assay: intra-day

Concentration spiked (ng/ml)	Concentration found (mean±SD, n=6) (ng/ml)	Accuracy (%)	Precision (%)
12.5	14.0±0.7	11.9	5.0
25	27.2±1.4	9.0	5.3
50	47.8±4.2	4.5	8.7
100	93.3±5.5	6.7	5.9
200	196.0±6.6	2.0	3.3
400	412.1±6.4	3.0	1.6
600	605.1±13.8	0.9	2.3
800	792.0±9.1	1.0	1.2

Further, there were no interfering peaks in the chromatographic region of the analytes of interest. Therefore, it was concluded that the optimal solvent for extraction is ethyl acetate and the optimal mobile phase consists of 0.05 *M* KH_2PO_4 buffer (pH 3.5) and acetonitrile in the proportion of 90:10 (v/v).

3.2. Linearity of response

A good linearity (r^2=0.999) within the concentration range of 12.5 to 800 ng/ml was found. This linear relationship was demonstrated by the statistical analysis of linear regression model of $y=a+b*x$. The homogeneity of slopes for different days was validated to demonstrate the reproducibility of responses (PAR). Mean values (standard deviation) for the slope and the intercept were 0.004104 (0.000125) and −0.01641 (0.01119), respectively. Both slopes and intercepts were examined to be statistically no different for calibration curves of different days. But slopes were significantly different from the zero and intercepts were not. The limit of detection for tramadol was 9 ng/ml at a signal-to-noise ratio of 3.

3.3. Accuracy

The found concentrations obtained from the standard concentrations of tramadol are shown in Tables 2 and 3 for intra-day and inter-day runs, respectively. The percent absolute deviations of the concentrations calculated from the true values are presented in Tables 4 and 5. The percent absolute difference from the true value for the analysis of any standard value from 12.5 to 800 ng/ml of tramadol ranged from 1.3 to 11.9% for the intra-day run and from 1.4 to 8.1% for inter-day run.

Using the deviation of the absolute differences between the concentration determined with HPLC and the true concentration, as well as the *t*-value

Table 3
Accuracy and precision of tramadol assay: inter-day

Concentration spiked (ng/ml)	Concentration found (mean±SD, n=6) (ng/ml)	Accuracy (%)	Precision (%)
12.5	12.7±0.9	1.8	7.0
25	24.2±1.8	3.0	7.2
50	46.3±3.0	2.3	6.5
100	93.9±4.7	6.5	5.0
200	200.7±8.1	0.3	4.0
400	414.0±12.8	3.5	3.1
600	607.0±12.3	1.2	2.0
800	788.6±6.2	1.4	0.8

Table 4
Tramadol percent deviation at different concentrations: intra-day

Concentration spiked (ng/ml)	Deviation (%)			95% C.I.[b]	
	Mean	SD	MAD[a]	ng/ml	Deviation (%)
12.5	11.9	5.5	1.5±0.7	±0.7	5.8
25	9.0	5.7	2.2±1.4	±1.5	6.0
50	8.3	3.4	−2.2±4.2	±4.4	8.7
100	6.8	5.2	−6.7±5.5	±5.7	5.7
200	2.9	2.3	−4.1±6.6	±6.9	3.4
400	3.0	1.6	12.1±6.4	±6.7	1.7
600	2.2	0.5	5.1±13.8	±14.5	2.4
800	1.3	0.8	−8.0±9.1	±9.5	1.2

[a] MAD: Mean absolute difference from actual value±SD.
[b] C.I.: Confidence interval.

from the two-tailed student's t distribution table, 95% confidence limits were estimated for each concentration (Tables 4 and 5). These calculations indicated that the results of any intra-day single determination of plasma tramadol levels would differ from the true value by 1.2 to 8.7% for all concentrations within the range studied. The results of any inter-day single determination for plasma tramadol concentration would differ from the true value by 0.1 to 7.5%.

3.4. Precision and reproducibility

The precision (intra-day variation) and the reproducibility (inter-day variation) are optimally demonstrated using the data obtained from the analysis of standard values. For each concentration, a mean value and percentage coefficient of variation were calculated on each analysis day ($n=6$) and over the course of the six-day study. The precision of this method varied from 1.2 to 8.7% for plasma tramadol concentrations.

The reproducibility of the method was determined using the variability of the mean of each concentration analyzed over the course of the validation day. Over the six-day period, the relative standard deviation for plasma tramadol varied from 0.8 to 7.2%.

3.5. Pharmacokinetics

The area under the curve from $t=0$ to 24 h (AUC_{0-24}) was determined by the linear trapezoidal rule. The extrapolation to infinity ($AUC_{0-\infty}$) was calculated by summing AUC_{0-24} and the ratio of the concentration of the last sampling point to the

Table 5
Tramadol percent deviation at different concentrations: inter-day

Concentration spiked (ng/ml)	Deviation (%)			95% C.I.[b]	
	Mean	SD	MAD[a]	ng/ml	Deviation (%)
12.5	5.2	4.8	0.2±0.9	±0.9	7.5
25	5.8	4.4	−0.8±1.8	±1.8	7.3
50	8.1	4.6	−3.7±3.0	±3.2	6.3
100	6.1	4.7	−6.1±4.7	±4.9	4.9
200	2.9	2.6	0.7±8.1	±8.5	4.3
400	4.0	2.4	14.0±12.8	±13.5	3.4
600	1.7	1.5	7.0±12.3	±12.9	2.1
800	1.4	0.8	−11.4±6.2	±6.5	0.1

[a] MAD: mean absolute difference from actual value±SD.
[b] C.I.: confidence interval.

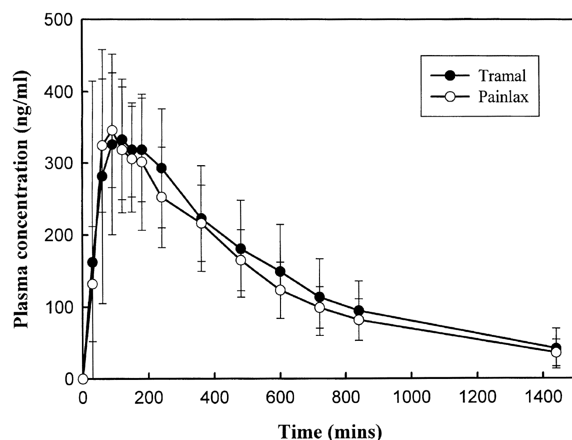

Fig. 2. The mean plasma concentration versus time of Tramal and Painlax.

elimination rate constant. The latter was calculated by employing a linear regression model from at least the last three points that had been logarithmically transformed. The maximal concentration achieved (C_{max}) was determined directly from the measured concentrations without interpolation. The mean plasma concentration profiles of tramadol for both products are compared in Fig. 2.

These parameters (AUC_{0-24}, $AUC_{0-\infty}$, and C_{max}) of test (Painlax®) and reference (Tramal®) formulations and their ratio were computed without transformation and presented in Table 6. These confidence intervals estimated by two one-sided t tests were calculated for bioequivalence testing. They were well within the bioequivalence range of 80–120% of the reference product, indicating that Painlax® and Tramal® are bioequivalent.

4. Conclusion

The data presented in this study indicate that the assay procedure described herein is reliable, sensitive, and selective for assessment of the bioavailability of tramadol, and for clinical use. The limit of quantitation was close to that of the method using fluorescence detection.

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Table 6
Pharmacokinetic data of tramadol products

	Painlax®	Tramal®	Ratios	90% Confidence interval (%)
AUC_{0-24} (ng h/ml)	3253.0	3534.4	0.9470	84.8–102.4
	(796.7)	(1087.0)	(0.1339)	
$AUC_{0-\infty}$ (ng h/ml)	3716.2	3987.2	0.9810	87.7–101.4
	(1156.8)	(1437.0)	(0.1588)	
C_{max} (ng/ml)	377.4	395.9	0.9243	89.8–101.4
	(68.4)	(92.0)	(0.1485)	