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Short communication

Determination of tramadol in human plasma by capillary gas chromatography–mass spectrometry using solid-phase extraction¹

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Abstract

An analytical method using solid-phase extraction, capillary gas chromatography and mass selective detection in the electron-impact ionization (EI) mode was developed for the determination of tramadol (2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol) in human plasma. The advantages of this method are the high sensitivity and selectivity and the linearity over the concentration range 2–500 ng/ml. Quantification was made using nefopam as an internal standard, and the detection limit was found to be 1 ng/ml. The standard deviations of the intra-day precision test ranged from 4.5 to 6.0% with respect to the concentration. Accuracy ranged from 1.0 to 4.0% (inter-day). The method was used for the determination of tramadol in a bioequivalence study.

Keywords: Tramadol

1. Introduction

Tramadol hydrochloride (2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol hydrochloride) is a centrally acting analgesic from the group of opiantagonists. Its activity is comparable to that of pentazocine, dextropropoxyphene and codeine. It is well tolerated and does not produce respiratory depression or cardiovascular effects of clinical relevance when given in therapeutic doses [1].

After oral administration, almost all the tramadol is completely absorbed from the gastrointestinal

tract, reaching peak plasma concentrations in 15 to 45 min. The mean peak plasma concentration of racemic tramadol is about 280 ng/ml, which was obtained 1.6 to 2 h after a 100-mg dose was administered orally to a group of healthy volunteers. Tramadol is metabolized extensively in the liver and eliminated through the kidneys, with an elimination half-life of approximately 6 h [1,2].

For the determination of tramadol, gas chromatographic (GC) methods with nitrogen–phosphorus detector (NPD) or mass spectrometric (MS) detection have been previously reported [3,4]. These methods include a three-step liquid–liquid extraction procedure with *n*-hexane and 0.1 M acetic acid. The limit of quantification was 12.5 ng/ml and linearity was observed within a concentration range of 12.5 ng/ml to 200 ng/ml. The GC–MS method [4] has

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been described using $[2\text{H}_2, 15\text{N}]$ tramadol hydrochloride as an internal standard and single ion-monitoring mode after chemical ionization (CI) with isobutane. The method gave good precision and accuracy because the isotopic internal standard was used. The sample preparation (liquid–liquid extraction) was relatively long and time-consuming.

The aim of our work was to develop a fast and simple method for routine analysis of tramadol in biological samples, especially important in bioequivalence and pharmacokinetics studies.

The present paper describes a very sensitive, specific and quantitative extraction procedure for the separation of tramadol from plasma using a reversed-phase C_{18} solid-phase extraction column. Tramadol and the internal standard nefopam were analysed by capillary gas chromatography and mass selective detection in the electron-impact ionization (EI) mode.

In our work the emphasis was placed on developing a sample preparation method that uses a very low quantity of organic solvent, because of new regulatory restrictions on the use of solvents in the workplace.

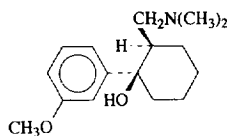
2. Experimental

2.1. Materials

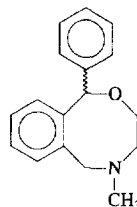
Lichrolut RP-18 (200 mg) extraction columns were purchased from Merck (Darmstadt, Germany), a Vac Elut vacuum manifold (SPE-12G) by J.T. Baker (Phillipsburg, PA, USA). Automatic pipettes were supplied by Eppendorf (Hamburg, Germany). A vortex mixer was purchased from Tehnica (Železniki, Slovenia). A centrifuge Megafuga 1.0R and centrifugal evaporator Gyrovap GT were supplied by Heraeus Instruments (Hanau, Germany) and Howe (Banbury, UK), respectively.

2.2. Chemicals and reagents

Tramadol hydrochloride batch No. 56473124 was obtained from Krka Pharmaceuticals (Novo mesto, Slovenia) and the internal standard nefopam hydrochloride batch No. N-0391 was purchased from Sigma (St. Louis, MO, USA) (Fig. 1).



(a) tramadol; 2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol



(b) internal standard; 3,4,5,6-tetrahydro-5-methyl-1-phenyl-1H-2,5-benzoxazine

Fig. 1. Structural formulae of tramadol (a) and internal standard (b).

Ammonia (25%, extra pure), methanol (gradient grade), sodium hydroxide (analytical grade), 1-butanol (for chromatography) and water (HPLC-grade) were purchased from Merck. 2-Propanol (for residue analysis) was purchased from J.T. Baker. Helium, quality 6.0, was supplied by Messer-Griessheim (Magdeburg, Germany).

2.3. Instrumentation and chromatographic conditions

A HP 5890 Series II plus gas chromatograph (Hewlett-Packard, Waldbronn, Germany) with an electronic pressure programmer, split–splitless injector and HP 7673 autosampler was used. A J.T. Baker solid-phase extraction manifold using RP-18 columns (3-ml capacity) was used for plasma sample preparation. Separation was achieved using a HP-5-MS capillary column (30 m×0.25 mm I.D., 0.25 μm film thickness) with a HP 5972 MSD Hewlett-Packard mass-selective detector. Data analysis was performed using the Hewlett-Packard Chemstation software.

2.4. Conditions

The oven temperature was set at 140°C, increased at a rate of 24°C/min to 260°C and then held for 4 min. The injector temperature was 250°C. Pressure

program of the carrier gas for inlet: 207 kPa initial pressure, 689 kPa/min to 90 kPa, 1 min constant, then 11 kPa/min to 137 kPa, 4 min constant. Injection was performed in the splitless mode, purge off time 1 min. The carrier gas flow was 1 ml/min.

The MS detector parameters were: GC–MS transfer line temperature, 280°C; solvent delay time, 5 min; electron energy, 70 eV; emission current, 35 μ A; mode: selected ion monitoring from 5 min to a time between the tramadol and the internal standard peak group 1 (m/z 263), from this time to the end group 2 (m/z 179); electron multiplier voltage, 1600–2000 V. The detector was turned off at 7.5 min.

2.5. Plasma sample collection

Tramadol suppositories were rectally administered as a single 100-mg dose to healthy volunteers, from whom written informed consent was obtained, and the study protocol was approved by the National Medical Ethic committee of the Republic of Slovenia. Plasma samples were collected at set intervals for a period of 36 h after dosing, centrifuged within 10 min after venipuncture and then immediately frozen at -20°C .

2.6. Stock solution

A stock solution of tramadol and the internal standard was prepared monthly at a concentration of 1 mg/ml in water and stored at 4°C . No change in the stability of the stock solution over 1 month was observed.

2.7. Plasma calibration curve and quality control samples

Drug-free human plasma was prepared from heparinized whole blood samples collected from healthy volunteers and stored at -20°C . After thawing, the plasma was spiked daily with the diluting stock solution to give a final concentrations from 2 to 500 ng/ml of tramadol and 100 ng/ml of the internal standard. To ensure the stability of the assay quality control samples with tramadol concentrations of 7.5, 150 and 300 ng/ml were prepared daily. All cali-

bration samples, the quality control samples and the unknown samples were prepared according to the described method.

2.8. Sample preparation

The RP-18 columns were connected to a Vac Elut and conditioned with 2 ml of methanol, followed by 3 ml of 0.001 M NaOH. A 1-ml volume of the plasma sample was applied to the RP-18 column. The sample was forced through the bed at a low flow-rate by applying a vacuum and then washed with 3 ml of 0.001 M NaOH. The sorbent was dried for 20 min under 400 mm Hg vacuum. Analytes were eluted with 2 ml of 2-propanol. The extract was evaporated to dryness under a stream of nitrogen at 50°C and a pressure of 1.01 kPa in a centrifugal evaporator. The residue was redissolved in 50 μ l of 1-butanol and a 2- μ l volume was injected into the GC–MS system.

3. Results and discussion

3.1. Extraction procedure

The solid-phase extraction procedure described herein provides a fast, reproducible and quantitative method for isolating tramadol and the internal standard from spiked plasma samples. The reversed-phase C_{18} retains the substances by a mixed-mode interaction mechanism. The cleanliness of the extract is due to the fact that most of the interfering substances are removed during column rinsing.

The recovery of the extraction procedure was determined by comparing the peak areas for tramadol extracted from plasma and a standard solution of tramadol in 1-butanol at the same concentration.

The average recovery of tramadol over a concentration range from 5 to 300 ng/ml was 68% with a R.S.D. of 7.9%. The average recovery for internal standard at a concentration of 100 ng/ml was found to be 50.1% with a R.S.D. of 6.5% ($n=8$).

Solid-phase extraction procedure was used to shorten the sample preparation time and to reduce the amount of organic solvent needed.

3.2. Chromatography

The tramadol and the internal standard peaks were well resolved and the endogenous plasma components did not cause any interferences.

Fig. 2 shows chromatograms of (a) blank plasma, (b) blank human plasma spiked with 5 ng/ml of tramadol and I.S., and (c) human plasma sample containing 300 ng/ml of tramadol and I.S.

The detector was turned off after elution of tramadol and the internal standard because of later eluting polar compounds. Selected ion monitoring was employed using the molecular ion m/z 263 for tramadol and the basic fragment ion m/z 179 for the internal standard chosen from mass spectra of both compounds (Fig. 3). While monitoring these ions no interfering peaks were observed in the representative window. Peak-area ratios of tramadol/internal standard were generated using Chemstation software.

3.3. Validation

3.3.1. Specificity

The specificity was studied by checking the chromatograms obtained from eight different blank plasma samples. As shown by the chromatograms in Fig. 2, good separation of tramadol from the internal standard and from endogenous plasma substances was achieved and at the retention times of tramadol and internal standard no interfering peaks were observed.

3.3.2. Calibration curves

The least square linear regression analysis of the peak-area ratios versus the concentration of tramadol showed that this method has a broad linear range from 2 to 500 ng/ml with a correlation coefficient of $r^2=0.9989$ (S.D.=0.0013). A calibration sequence of eight curves gave a slope of 0.00361 (S.D.=0.00025) and an intercept of -0.00033 (S.D.=0.0032).

3.3.3. Precision and accuracy

Assay of accuracy and intra-day precision were determined by analysis of replicates ($n=8$) of spiked plasma samples containing known concentrations of

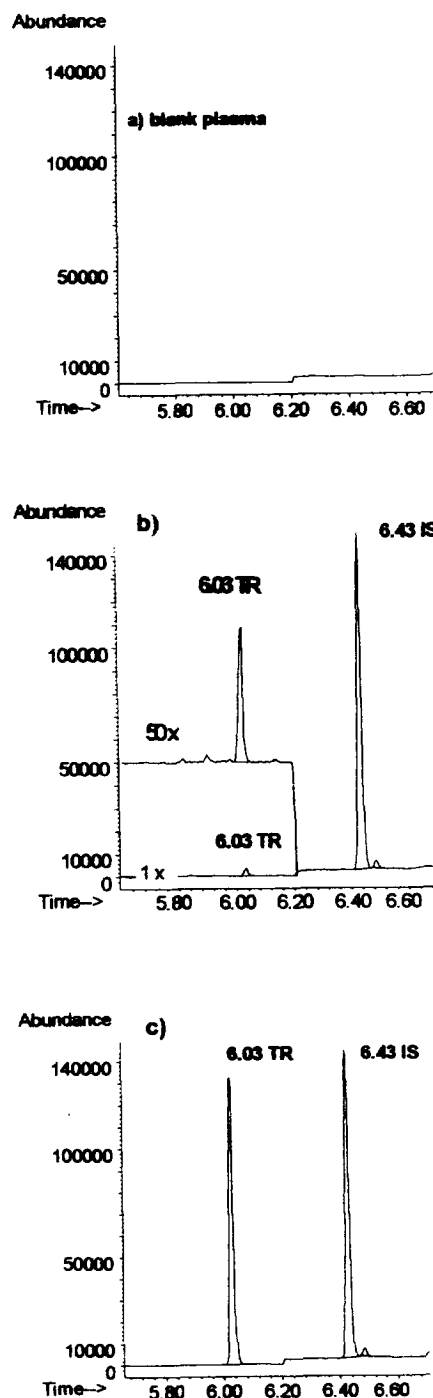


Fig. 2. Chromatograms of (a) blank plasma; (b) blank plasma spiked with 5 ng/ml of tramadol and 100 ng/ml of I.S., and (c) human plasma sample containing 300 ng/ml of tramadol and 100 ng/ml of I.S.

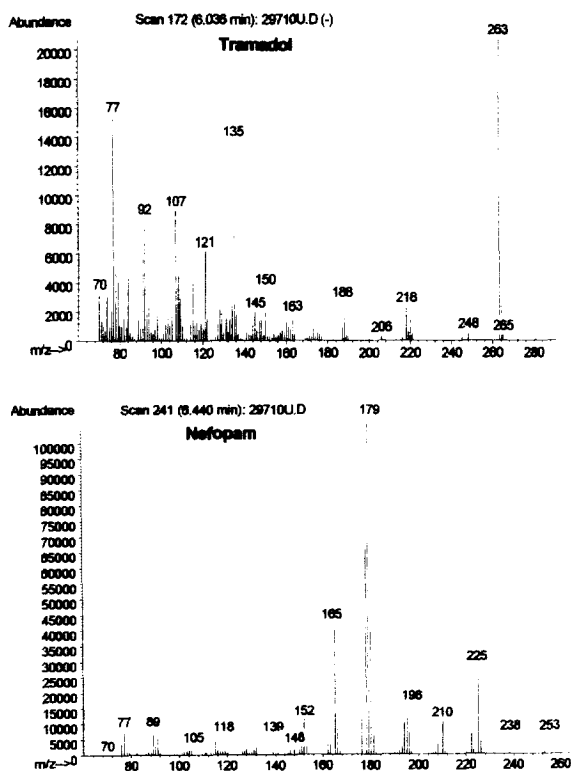


Fig. 3. EI mass spectra of tramadol and internal standard.

the analytes at three different concentrations: 5, 100 and 300 ng/ml. Inter-day precision and accuracy were calculated from the quality control samples during 30 days over a 3-month period. Both the precision and accuracy were satisfactory (Tables 1 and 2). Precision parameters were 4.5–6.0% (intra-day) and 7.1–9.7% (inter-day). Accuracy (difference between the found and added concentration) was found to be from 1.0% to 4.0% (inter-day).

3.3.4. Limit of detection and quantification (LOD, LOQ)

The limit of detection was 1 ng/ml at a signal-to-noise ratio $S/N=5$. The limit of quantification was 2 ng/ml at a $S/N=15$ and a R.S.D. of 8.9%.

3.3.5. Stability samples

Stability testing was determined for two concentrations under the following storage conditions: freshly prepared, after one freeze–thaw cycle (spiked plasma frozen at -18°C , thawed at ambient temperature for 1 h), after two freeze–thaw cycles (spiked plasma frozen at -18°C , thawed at ambient temperature for 1 h, frozen again for 24 h and thawed at ambient temperature for 1 h), while storing the

Table 1

Intra-day precision and accuracy data for tramadol at three different concentrations

Added concentration (ng/ml)	Found concentration (ng/ml) (mean \pm S.D.)	R.S.D. (%)	Accuracy (%)
Intra-day ($n=8$)			
5	4.44 \pm 0.20	4.5	-11.2
100	90.6 \pm 5.2	5.7	-9.4
300	300 \pm 18	6.0	0

Table 2

Results of the inter-day precision and accuracy test of the quality control samples during 30 days over a three-month period

	Added concentration (ng/ml)	Found concentration (ng/ml) (mean \pm S.D.) ($n=30$)	R.S.D. (%)	Accuracy (%)
QC1	7.5	7.33 \pm 0.52	7.1	-2.3
QC2	150	144 \pm 14	9.7	-4.0
QC3	300	303 \pm 26	8.6	1.0

Table 3
Stability of plasma samples under different conditions ($n=8$)

Storing conditions	Added concentration (ng/ml)	
	5	300
	Found concentration (ng/ml) (mean \pm S.D.)	Found concentration (ng/ml) (mean \pm S.D.)
Fresh	4.99 \pm 0.27	291 \pm 14
One freeze–thaw cycle	5.75 \pm 0.21	271 \pm 14
Two freeze–thaw cycles	4.92 \pm 0.78	312 \pm 18
After storage in autosampler for 12 h	4.54 \pm 0.22	318 \pm 26

sample extract in an autosampler at ambient temperature for 12 h. The results of stability are presented in Table 3. None of the experiments leads to a result with a deviation exceeding 15% of the added concentration.

4. Conclusion

A method for determining tramadol in human plasma has been developed that is rapid and has a low limit of quantification of 2 ng/ml for 1 ml of plasma.

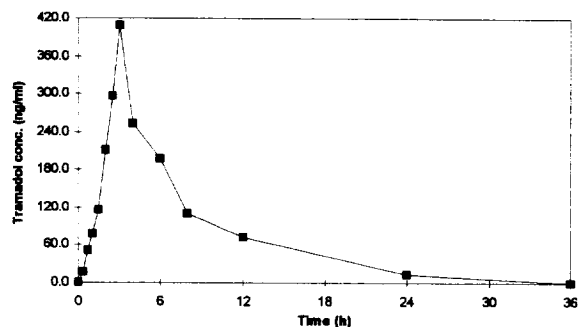


Fig. 4. Concentration–time profile of a healthy volunteer over a 36-h interval after administration of a 100-mg tramadol suppository.

Solid-phase extraction using a RP-18 cartridge allowed a significant reduction in both the sample preparation time and the amount of organic solvents required. Extraction recovery for tramadol was 68% over the concentration range from 5 to 300 ng/ml with a R.S.D. of 7.9%.

Nefopam was found to be a suitable internal standard for the quantification of tramadol in human plasma. The method was linear between 2 and 500 ng/ml.

This method has been used in bioequivalence study of two suppositories of tramadol in which over 700 samples were analysed. Fig. 4 shows a concentration–time profile for a healthy volunteer over a 36-h interval after administration of a 100-mg tramadol suppository.

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