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Journal of Chromatography B, 830 (2006) 207–211
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Development and validation of a rapid HPLC method for simultaneous determination of tramadol, and its two main metabolites in human plasma

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Received 20 August 2005; accepted 26 October 2005
Available online 8 November 2005

Abstract

Tramadol, an analgesic agent, and its two main metabolites *O*-desmethyltramadol (M1) and *N*-desmethyltramadol (M2) were determined simultaneously in human plasma by a rapid and specific HPLC method. The sample preparation was a simple extraction with ethyl acetate. Chromatographic separation was achieved with a ChromolithTM Performance RP-18e 50 mm × 4.6 mm column, using a mixture of methanol:water (13:87, v/v) adjusted to pH 2.5 by phosphoric acid, in an isocratic mode at flow rate of 2 ml/min. Fluorescence detection (λ_{ex} = 200 nm/λ_{em} = 301 nm) was used. The calibration curves were linear ($r^2 > 0.997$) in the concentration range of 2.5–500 ng/ml, 1.25–500 ng/ml and 5–500 ng/ml for tramadol, M1 and M2, respectively. The lower limit of quantification was 2.5 ng/ml for tramadol, 1.25 ng/ml for M1 and 5 ng/ml for M2. The within- and between-day precisions in the measurement of QC samples at four tested concentrations were in the range of 2.5–9.7%, 2.5–9.9% and 5.9–11.3% for tramadol, M1 and M2, respectively. The developed procedure was applied to assess the pharmacokinetics of tramadol and its two main metabolites following administration of 100 mg single oral dose of tramadol to healthy volunteers.

Keywords: Tramadol; *O*-Desmethyltramadol; *N*-Desmethyltramadol; Plasma; Monolithic; Pharmacokinetics

1. Introduction

Tramadol hydrochloride *trans*-(±)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)-cyclohexanol hydrochloride (Fig. 1), is a centrally acting analgesic with efficacy and potency ranging between weak opioids and morphine. The drug acts as an opiate agonist by selective activity at the μ-opioid receptors. In addition to opiate agonist activity, tramadol inhibits reuptake of norepinephrine and serotonin, which appears to contribute to the drug's analgesic effect [1,2]. Its therapeutic plasma concentration is in the range of 100–300 ng/ml [3]. Tramadol is rapidly and almost completely absorbed after oral administration but its absolute bioavailability is only 65–70% due to first-pass metabolism [4]. This analgesic is rapidly and extensively metabolized in the liver. The principal metabolic pathways, *O*- and *N*-desmethylation, involve cytochrome P-450 isoenzymes 2D6, 2B6 and 3A4, respectively [5]. *O*-Desmethyltramadol (M1) and *N*-desmethyltramadol (M2) are main metabolites in most species. These primary metabolites may be further metabolized to three additional secondary metabolites namely, *N*, *N*-didesmethyltramadol (M3), *N*, *N*, *O*-tridesmethyltramadol (M4), and *N*, *O*-desmethyltramadol (M5). All metabolites are further conjugated with glucuronic acid and sulfate before excretion in urine [6]. Only one of these metabolites, *O*-desmethyltramadol (M1 metabolite), is pharmacologically active [6]. Approximately 10–30% of the parent drug is excreted unmetabolised in the urine [6,7].

A number of high-performance liquid chromatographic (HPLC) methods with ultraviolet [6,8,9], fluorescence [10,11], electrochemical [12] and MS detection [13] have been

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doi:10.1016/j.jchromb.2005.10.039

described. Achiral HPLC analytical methods [6,8,9,14–20] are routinely used in pharmacokinetic and bioequivalence studies, while chiral HPLC methods [10–13] are very important for the determination of the enantiomeric ratios of tramadol and its metabolites. Although the tramadol molecule contains a benzene ring, UV detection is unsuitable for its analysis in plasma due to lack of sensitivity [14,9]. Nobilis et al. [17] reported a HPLC method that provides good selectivity and sensitivity, although it requires an extensive washout period and different excitation and emission wavelengths for tramadol, M1 and the internal standard. Among all published methods, two methods reported for the simultaneous determination of tramadol and its two main phase I metabolites (M1 and M2). The first method described by Elsing and Blaschke [8] determines these compounds only in urine and in the second method stereoselective separation has been reported [11]. Nonstereoselective analytical methods are still useful in bioequivalence studies as there is no guideline by FDA to necessitate the setereoselective study of drugs with stereoselective pharmacokinetics. More over pre-separation of tramadol and its two metabolites in a very short time may increase their resolution in chiral analysis. Surprisingly, to our knowledge, there is no validated achiral HPLC method available for the quantification of these three compounds in plasma simultaneously.

The objective of this study was to develop a new HPLC method for simultaneous determination of tramadol and its two main metabolites in human plasma.

2. Experimental

2.1. Materials

The pure substances of tramadol, M1, M2 and *cis*-tramadol (Fig. 1) as internal standard were kindly supplied by Grünenthal (Stolberg, Germany). HPLC-grade acetonitrile and methanol, and analytical grade ethyl acetate and phosphoric acid (85%) were supplied by Merck (Darmstadt, Germany).

2.2. Preparation of standard solutions

Tramadol, M1, M2 and *cis*-tramadol in concentrations of 1 mg/ml were prepared separately by dissolving 10 mg of each analyte in methanol. Intermediate stock standards of 100 and 10 μg/ml were prepared using water as solvent. Four standard solutions of tramadol and its main phase I metabolites (10, 50, 100 and 300 ng/ml) were made by further dilution of the intermediate stock solution with appropriate volumes of water. The standard solution of *cis*-tramadol (1 μg/ml) was similarly prepared. Standard and stock solutions of all compounds were stored at 4 °C.

2.3. Apparatus and chromatographic condition

The chromatographic apparatus consisted of a low-pressure gradient HPLC pump, a fluorescence detector [excitation

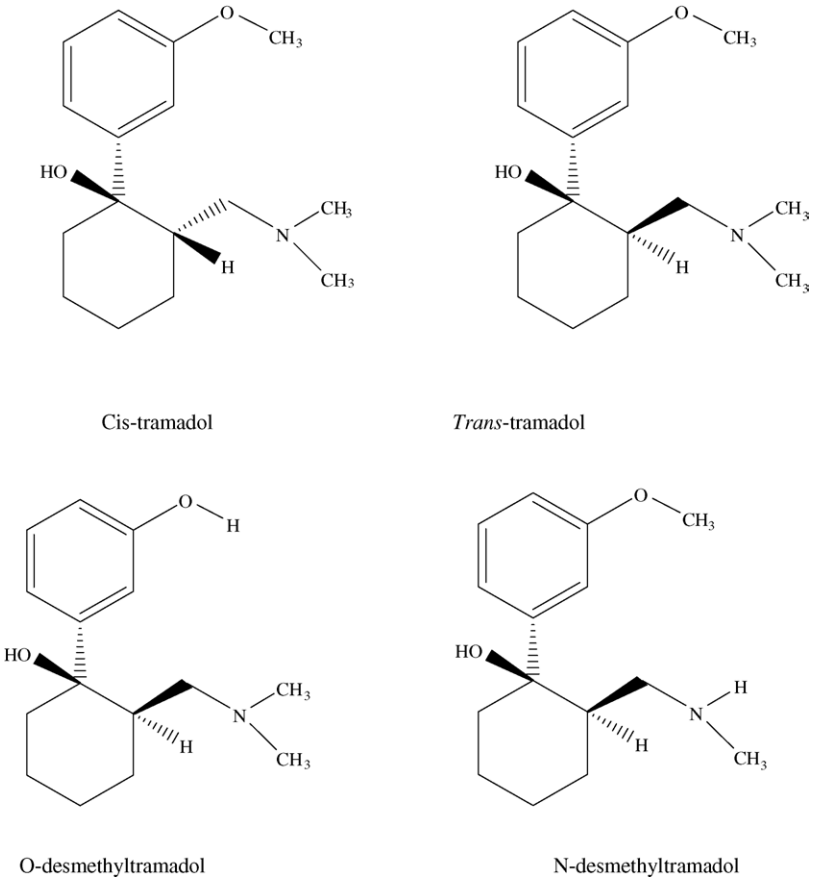

Fig. 1. Chemical structure of *cis*-tramadol (IS), *trans*-tramadol, and its two metabolites.

wavelength (λ_{ex}) 200 nm/emission wavelength (λ_{em}) 301 nm] and an online degasser, all from Knauer (Berlin, Germany). A Rheodyne model 7725i injector with a 100 μl loop was used. The data was acquired and processed by means of ChromGate chromatography software (Knauer, Berlin, Germany).

Chromatographic separation was achieved by a ChromolithTM Performance RP-18e 50 mm × 4.6 mm column (Merck, Darmastadt, Germany) protected by a ChromolithTM Guard Cartridge RP-18e 5 mm × 4.6 mm.

For the mobile phase, a mixture of methanol:water (13:87, v/v) adjusted to pH 2.5 by phosphoric acid (final acid concentration of about 1.5 mM), was delivered in isocratic mode at 2 ml/min flow rate.

2.4. Sample preparation

The preparation of plasma samples was by liquid–liquid extraction (LLE). The conditions consisted of mixing 250 μl of plasma with 50 μl *cis*-tramadol as internal standard (1 μg/ml) and 100 μl NaOH (1N) in a 2 ml Eppendorf polypropylene tube and then extracting with 1.25 ml of ethyl acetate. After vertical agitation (10 min) and centrifugation (10,000 × *g*, 2 min), the upper organic layer was transferred into a conical glass tube. The extraction was repeated by another 1.25 ml of ethyl acetate. The organic phase was then evaporated under a gentle stream of air and reconstituted in 250 μ1of a mobile phase. A 100 μ1 aliquot was injected on to the HPLC system.

2.5. Preparation of calibration standards

Starting from pooled stock solution of tramadol, M1 and M2 (10 μg/ml) in water, standards were prepared using pooled human drug free plasma obtained from healthy volunteers as diluent. The calibration curve was performed with standards of the final concentrations of 1.25, 2.5, 5, 10, 20, 50, 100, 150, 200, 300, 400 and 500 ng/ml in human plasma.

2.6. Accuracy, precision, limit of quantification (LOQ) and recovery

Accuracy, between- and within-day precisions of the method were determined for each compound according to FDA guidance for bioanalytical method validation [21]. Five replicate spiked plasma samples were assayed between- and within-day at four different concentrations (10, 50, 100 and 300 ng/ml) for each analyte. Accuracy was calculated as deviation of the mean from the nominal concentration. Between- and within-day precision were expressed as the relative standard deviation of each calculated concentration. For the concentration to be accepted as LOQ the percent deviation from the nominal concentration (accuracy) and the relative standard deviation has to be ±20% and less than 20%, respectively. Average recovery of each compound was determined by comparing AUC obtained after injection of the processed QC samples with those achieved by direct injection of the same amount of drug in distilled water at different concentrations (five samples for each concentration level).

2.7. Application of the method

Three volunteers were included in this study. The study protocol was approved by the Ethics Committee of Tehran University of Medical Sciences and written informed consent was obtained from the volunteers. Volunteers were not allowed to take any other medication for 2 weeks before and throughout the study. The volunteers received 100 mg Tradolan tablets (Lannach, Austria) after an overnight fasting. Intake of food was delayed for 3 h after medication. Peripheral venous blood samples were taken from each volunteer at predetermined intervals and plasma samples were stored at −20 °C until analysis.

2.8. Calculation of pharmacokinetic parameters

Plasma concentration–time curves of tramadol and its metabolites were evaluated by non-compartmental analysis. Maximum plasma concentration C_{max} and the time to C_{max} (T_{max}) were obtained directly from the individual plasma concentration versus time curves. The terminal half-life, $t_{1/2}$ was obtained from log–linear regression analysis of the plasma concentration–time curves in the terminal phase. The area under plasma concentration–time curve up to last quantifiable plasma concentration ($AUC_{lqc)}$ was determined according to the linear trapezoidal method.

3. Results

3.1. Selectivity

The separation achieved using the experimental conditions of the present assay for tramadol and its main metabolites are presented in Fig. 2. Selectivity was indicated by absence of any endogenous interference at retention times of peak of interest as evaluated by chromatograms of control human plasma and plasma spiked with compounds. Retention times for tramadol, M1, M2 and IS were 3.1, 1.3, 4.0 and 2.1 min, respectively.

3.2. Linearity

Twelve point calibration curves for tramadol and its metabolites on separate days were linear over the concentration range of 2.5–500 ng/ml for tramadol, 1.25–500 ng/ml for M1 and 5–500 ng/ml for M2. The equations for means ($n = 3$) of three standard curves for each analyte are: tramadol; $y = 0.0051x - 0.0099$ ($r^2 = 0.999$), M1; $y = 0.0035x + 0.0066$ ($r^2 = 0.999$) and M2; $y = 0.0035x + 0.0047$ ($r^2 = 0.997$). R.S.D. (%) values (slope, intercept) were (4.28, 9.5), (4.15, 10.5) and (9.08, 12.8) for tramadol, M1 and M2, respectively.

3.3. Limit of quantification

LOQs as defined previously were 2.5 ng/ml for tramadol, 1.25 ng/ml for M1 and 5 ng/ml for M2. The LOQ values for three analytes are reported in Table 1.

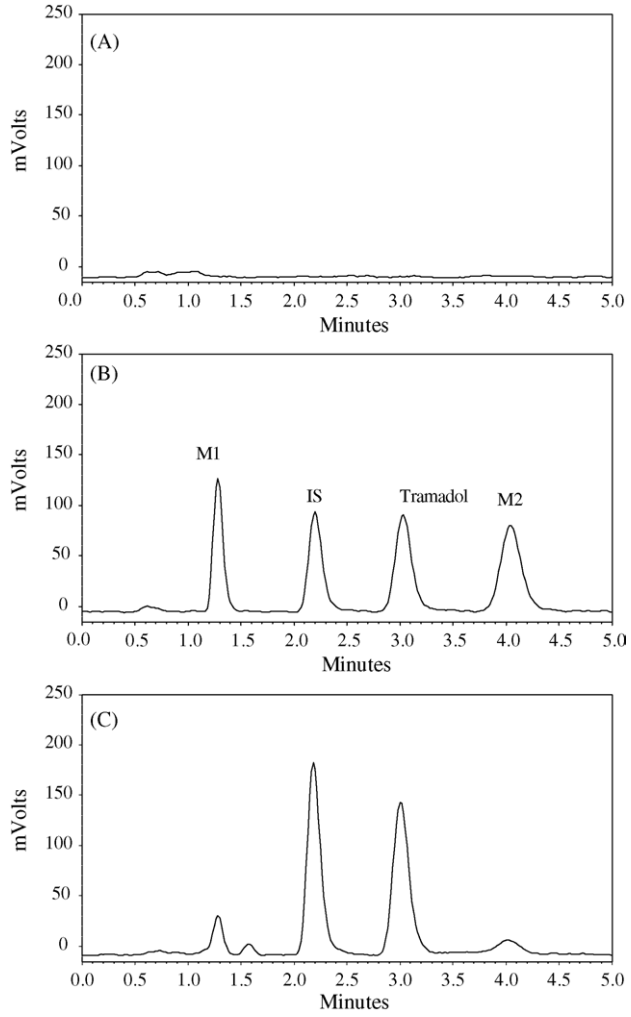

Fig. 2. Chromatograms of (A) blank human plasma, (B) plasma spiked with *cis*-tramadol (IS), 100 ng/ml of tramadol, M1 and M2 and (C) plasma of the same person 2.5 h after single oral administration of 100 mg tramadol (tramadol: 155 ng/ml, M1: 45 ng/ml and M2: 20 ng/ml).

3.4. Recovery, accuracy and precision

The results from the validation of the method in human plasma are listed in Table 2. The method proved to be accurate and precise. Accuracy at four concentration levels ranged from 80.3 to 115.3% for all compounds . The within- and between-day precision ranged from 2.5 to 9.7%, 2.5 to 9.9% and 5.9 to 11.3% for tramadol, M1 and M2, respectively.

Table 1
Limit of quantitation (LOQ) for tramadol, *O*-desmethyltramadol and *N*-desmethyltramadol ($n = 5$)

	Concentration (ng ml^{-1})	Between-day R.S.D. (%)	Accuracy (%)
Tramadol	2.5	14.9	88.1
O-Desmethyltramadol	1.25	12.1	81.3
N-Desmethyltramadol	5	6.7	85.9

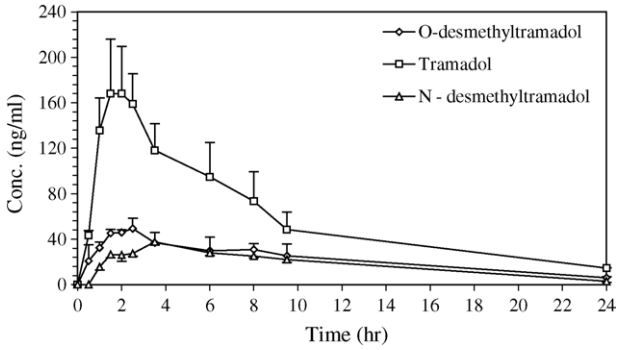

Fig. 3. Mean (±S.D.) concentration–time profile of tramadol, M1 and M2 after administration of 100 mg single oral dose of tramadol to 3.

The absolute recoveries ranged from 74.7 to 80.8%, 76.9 to 87.3% and 85.5 to 94.5% for tramadol, M1 and M2, respectively.

3.5. Application of the method

To apply the developed and validated method, the pharmacokinetics of tramadol and its metabolites was assessed in three volunteers. Plot of tramadol, M1 and M2 mean plasma concentrations as a function of time following oral dosing is shown in Fig. 3.

The pharmacokinetic parameters of tramadol and its metabolites derived by non-compartmental analysis are summarized in Table 3.

4. Discussion

The limitation of flow rate applied to routine HPLC columns may result in long run time in most of HPLC reported methods. Considering this limitation, most HPLC methods for achiral analysis of tramadol have reported run times of not shorter than 15 min [14–20]. Flow limitation may be resolved by using newly developed monolithic HPLC columns. The ChromolithTM column, as a monolithic HPLC column, has a biporous structure which offers a high porosity compared to usual columns (http://www.chromolith.com) which allows high flow-rates without loss of performance or limitations due to increased pressure. Monolithic columns therefore may provide faster separation than those of conventional HPLC columns. The described method was established as a rapid analytical tool in a pharmacokinetic study requiring short retention time, high precision, sensitivity and small volumes of plasma for analysis. This method also provides simultaneous determination of tramadol, M1 and M2. The achiral separation of the later compound in human plasma has not been reported yet. The parameters of the assay obtained in the course of validation processes presented above in the results section were considered satisfactory for its clinical application. A simple analytical procedure based on two-step extraction and a total run time of 4.5 min allows the possibility of determination of 50 samples a day.

Table 2
Between- and within-day variability, accuracy, and recovery for determination of tramadol, *O*-desmethyltramadol and *N*-desmethyltramadol

Concentration (ng/ml)	Between-day precision ($n = 5$)		Within-day precision ($n = 5$)		Recovery ($n = 5$)	
	R.S.D. (%)	Accuracy (%)	R.S.D. (%)	Accuracy (%)	%	R.S.D. (%)
Tramadol						
10	5.4	91.4	4.6	87.6	74.7	6.1
50	9.7	98.1	2.5	97.4	80.8	11.2
100	2.5	102.5	3.5	99.2	79.3	12.9
300	3.3	103.5	3.7	102.5	77.1	4.7
O-Desmethyltramdol						
10	2.5	80.3	5.0	80.3	87.3	6.8
50	3.8	101.1	9.4	106.3	81.2	4.5
100	4.1	95.9	7.5	100.27	79.8	8.3
300	3.8	91.7	9.9	102.8	76.9	16.3
N-Desmethyltramadol						
10	11.3	115.3	11.3	115.3	85.5	3.8
50	7.4	106.4	7.4	106.4	90.5	8.2
100	6.3	98.3	6.3	98.3	90.9	6.7
300	5.9	95.4	5.9	95.4	94.5	4.9

Table 3
Pharmacokinetic data (mean ± S.D.) obtained from healthy volunteers following oral administration of 100 mg tramadol ($n = 3$)

	T_{max} (h)	C_{max} (ng/ml)	AUC_{lqc} (ng h/ml)	$t_{1/2}$ (h)
Tramadol	1.8 ± 0.4	170.4 ± 44.5	1398.4 ± 480.3	5.2 ± 0.9
O-Desmethyltramdol	2 ± 0.7	51.6 ± 5.7	538.2 ± 151.9	7.6 ± 1.1
N-Desmethyltramadol	3.8 ± 0.6	35.5 ± 4.0	400.6 ± 38.3	6.1 ± 1.2

5. Conclusion

This HPLC method for tramadol, *O*-desmethyltramadol (M1) and *N*-desmethyltramadol (M2) in human plasma may be fully recommended for pharmacokinetic studies as well as for therapeutic drug monitoring.

Acknowledgements

This work was fully supported by a grant from Tehran University of Medical Sciences. The authors wish to thank Grünenthal Co. for their kind donation of *cis*- and *trans*-tramadol and the metabolites. Technical assistance of Mrs. Lida Hakemi is highly appreciated.

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