ELSEVIER

Journal of Chromatography B, 724 (1999) 83–89

JOURNAL OF CHROMATOGRAPHY B

Simultaneous determination of tramadol and its major active metabolite *O*-demethyltramadol by high-performance liquid chromatography with electrochemical detection

M. Valle[a], J.M. Pavón[a], R. Calvo[a], M.A. Campanero[b], I.F. Trocóniz[c,*]

[a]*Department of Pharmacology, School of Medicine, University of the Basque Country, Leioa 48940, Bizkaia, Spain*
[b]*Department of Clinical Pharmacology, University Hospital, University of Navarra, Pamplona 31080, Spain*
[c]*Department of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, University of Navarra, Irunlarrea s/n, Pamplona 31080, Spain*

Received 3 August 1998; received in revised form 25 November 1998; accepted 26 November 1998

Abstract

A novel, highly sensitive method was developed for simultaneous determination of tramadol and its main active metabolite *O*-demethyltramadol (ODMT) in rat plasma. The method involves a single-step extraction procedure and a specific determination by high-performance liquid chromatography with electrochemical detection, using an ethoxy analogue of tramadol (L-233) as internal standard. The dual-electrode detector was operated in the oxidation-screening mode. Absolute recoveries of tramadol and ODMT were about 80%. Calibration curves were linear over a concentration range of 10–1000 ng/ml for ODMT and 10–10 000 ng/ml for tramadol with intra- and inter-day coefficients of variation not exceeding 10% and 15%, respectively. The limit of quantification for tramadol and ODMT was lower than 15 ng/ml and 10 ng/ml using 100 μl of plasma, respectively. The described method allows an adequate characterization of the plasma vs. time profiles for both compounds.

Keywords: Tramadol; *O*-Demethyltramadol

1. Introduction

Tramadol hydrochloride [*rac*-1-(e)-(*m*-methoxyphenyl)-2-(e)-(dimethylaminomethyl) cyclohexan-1-(a)-ol hydrochloride] is a centrally acting analgesic agent widely used in the treatment of chronic pain [1]. It shows a selective interaction with μ receptors [2] and acts synergistically on neuramine transmission by inhibiting norepinephrine re-uptake [3] and inducing serotonine release [4]. Tramadol shows an efficacy which is not very different from other drugs with a similar mechanism of action like buprenorphine [5], but with a lower incidence of adverse respiratory effects.

After oral administration, tramadol is almost completely absorbed, and approximately 85% of an oral dose is metabolized by the liver in healthy volunteers [1]. Tarradel et al. [6] reported a mean value of tramadol concentration, at the time when maximum effect was observed (120 min after a bolus of 100 mg), of 249 ng/ml and a significantly lower mean

*Corresponding author. Tel: +34-948-425-600; fax: +34-948-425-649; e-mail: itroconiz@unav.es

0378-4347/99/$ – see front matter
PII: S0378-4347(98)00547-7

value of *O*-demethyltramadol (ODMT) (35 ng/ml) at the same time. ODMT has been shown to be the main metabolite of tramadol. In addition, several studies have demonstrated that ODMT possesses in vitro affinity for μ receptors [7] and also in vivo analgesic activity [8].

On the basis of these findings, the simultaneous determination of tramadol and ODMT in plasma could be considered of importance in order to describe and predict the time course of the analgesic effect. Several methods have been described in the literature for the determination of tramadol in different biological fluids: Lintz and Uragg [9] reported a gas chromatographic–mass spectrometric method for the determination of tramadol in human serum, plasma and whole blood samples. Becker and Lintz [10] developed a method for the determination of tramadol by capillary gas chromatography with nitrogen-selective detection in human serum, which included a three-step liquid–liquid extraction with a lower detection limit of 3 ng/ml using 1-ml samples. Nobilis et al. [11] described a simpler method consisting of a one-step liquid–liquid extraction of tramadol from human plasma samples, in which determinations were performed on reverse-phase silica gel using ion-pair chromatography and fluorescence detection. Recently, two analytical methods including solid-phase extraction with capillary gas chromatography and mass selective detection in the electron-impact ionization mode [12] and chiral liquid chromatography with both UV and fluorescence detection [13] have been described. However, only in two techniques has the possibility of extracting and determining both compounds been reported, although not simultaneously. The first was described by Paar et al. in 1996 [14]. In this method both compounds were determined by high-performance liquid chromatography (HPLC) with fluorescence detection after extraction of the compound from liver tissue. The second, described by Elsing and Blaschke [15], make it possible to determine not only tramadol and ODMT but also *N*-demethyltramadol in urine. Surprisingly, to our knowledge there is no validated HPLC method available for the quantification of both compounds in plasma samples after one-step extraction.

The present study describes a rapid, sensitive and highly selective HPLC method with electrochemical detection, for the determination of both tramadol and ODMT in a small volume of plasma sample, with one-step extraction. This procedure could be very useful for carrying out simultaneous studies of the pharmacokinetics and pharmacodynamics of tramadol and ODMT.

2. Experimental

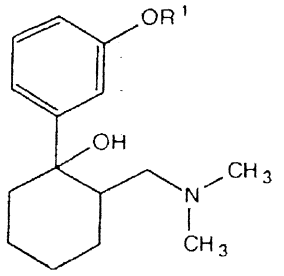
2.1. Reagents and standards

Racemic tramadol hydrochloride, racemic ODMT and racemic L-233 (ethoxy analogue of tramadol) used as internal standard (Fig. 1) were kindly provided by Grünenthal (Stolberg, Germany).

HPLC grade methanol and *n*-hexane were purchased from Scharlau (Barcelona, Spain) and ethyl acetate from Merck (Barcelona, Spain). All other chemicals were obtained from commercial sources and were of analytical grade. Buffer solution (disodium tetraborate anhydrous, Merck) was prepared in deionized water.

2.2. Calibration standards

Stock aqueous solutions of tramadol (100 μg/ml) and ODMT (100 μg/ml) were prepared and stored



R^1	Compound
CH_3	Tramadol **(1)**
H	O-Demethyltramadol **(2)**
C_2H_5	Ethoxy analogue **(3)**

Fig. 1. Chemical structures of tramadol (1), *O*-demethyltramadol (2) and the ethoxy analogue of tramadol (internal standard) (3).

frozen at −20°C until use. These stock solutions were diluted with deionized water to obtain the working standard solutions. Calibration standards were prepared by spiking 25 μl of tramadol working standard solutions and 25 μl of ODMT working standard solutions to aliquots of pooled rat drug-free plasma (450 μl) to give final concentrations ranging between 10 and 10 000 ng/ml of tramadol and between 10 and 1000 ng/ml of ODMT.

Internal standard: a 24 μg/ml solution of L-233 free base was prepared and further diluted with deionized water to obtain a working solution of 6 μg/ml.

2.3. Sample preparation

Ten μl of internal standard working solution was added to 25, 50 or 100 μl of plasma spiked with tramadol and ODMT, followed by 200 μl of ammonium hydroxide to adjust the pH. After the addition of 3 ml of ethyl acetate–*n*-hexane (40:60, v/v) the samples were shaken on a vortex mixer for 2 min and then centrifuged for 10 min at 1000 *g*. The organic phase was transferred to a 5-ml tube and evaporated down at vacuum in an Automatic Environmental Speed Vac (AES 100 Savant, Pacisa, Barcelona, Spain) evaporator. The dried residues were reconstituted in 100 μl of water, and an aliquot (50 μl) was injected into the chromatographic system.

2.4. Chromatographic conditions

The chromatographic system consisted of a HPLC 420 pump (Kontron Instruments) fitted with a Rheodyne manual sample injector (Model 7125, Rheodyne) equipped with a 50-μl loop. Detection was performed with an ESA Coulochem Model 5200A electrochemical detector consisting of a Model 5010 dual-electrode analytical cell operating in the oxidation-screening mode. The potential of the first electrode was set at +610 mV (for ODMT detection) and the second electrode at +850 mV (for tramadol and internal standard detection). The signal of both electrodes was recorded simultaneously by two different channels using the Data System 450-MT (Kontron Instruments). Due to the extreme flow sensitivity of the electrochemical detector, a pulse dampener was placed after the pump.

The analytical separation was performed by isocratic separation at room temperature using an Asahipack ODP-50 column (5 μm particle size, 125 mm×4.0 mm I.D.), Hewlett-Packard. The mobile phase, consisting of 0.01 *M* borate buffer (pH=9)–methanol (40:60, v/v), was filtered through a 0.22-μm membrane filter (Millipore, Spain) and degassed by sonication. The flow-rate was 0.7 ml/min, and the mobile phase was recycled to conserve the solvents.

2.5. Linearity and calibration

Three calibration curves, using different volumes of calibration standards and 10 μl of internal standard, were prepared. The first, corresponding to the lowest concentrations (10–50 ng/ml) was prepared using 100 μl of plasma. The second, corresponding to concentrations ranging between 25–500 ng/ml, with 50 μl of plasma. In the third, corresponding to concentrations of tramadol ranging between 500–10 000 ng/ml and 500–1000 ng/ml of ODMT, 25 μl of plasma was used. Each point on the calibration curves was based on three determinations.

Linearity was evaluated by least-squares linear regression of the drug-to-internal standard ratios of peak areas versus the respective concentrations of tramadol and ODMT. Peak areas were determined with the Data System 450-MT (Kontron Instruments). The linearity was confirmed by correlation coefficients of calibration curves higher than 0.99. A Fisher test was used to establish the statistical significance of the curves and a significance level of 5% was selected.

2.6. Recovery

Calibration standards containing a combination of 10, 25, 250, 500 and 1000 ng/ml of tramadol and ODMT and 600 ng/ml of internal standard were prepared in blank plasma. Absolute recovery, the overall extraction efficiency of the system, was measured by comparison of peak areas obtained from an extracted sample with those measured with equivalent amounts of each analyte in water.

2.7. Precision and accuracy

Intra-day accuracy and precision were determined by assaying spiked plasma samples at four different concentrations (10, 25, 500 and 1000 ng/ml). Inter-day precision was determined on four different days at 1000 ng/ml and 10 ng/ml.

Precision was calculated as the percentage of coefficients of variation (C.V.) of drug to internal standard ratio of peak areas, while accuracy was determined as relative error (%) from the following expression:

$$\text{Accuracy (\%)} = (C_{\text{real}} - C_{\text{determined}})/C_{\text{real}} \cdot 100.$$

2.8. Stability

The stability of tramadol and its metabolite was determined before and after extraction: (i) before extraction calibration standard samples of 1000 ng/ml were analyzed for seven days after freezing (−20°C) or refrigerating (+4°C). These frozen and refrigerated samples were compared to freshly prepared tramadol and ODMT plasma standard samples; (ii) stability of samples after extraction was performed over a 30-day period. Pooled plasma samples were extracted and stored in a freezer at −20°C until the time of analysis, with and without being reconstituted in water.

Samples were considered stable until there was a loss of more than 5% of the product when stored samples were compared with freshly prepared samples.

2.9. Interference studies

As tramadol is often used concomitantly with a large number of drugs, we studied the possible interference from the following agents: propranolol, buprenorphine, morphine, methadone, verapamil, nimodipine and felodipine.

2.10. Application to a pharmacokinetic study

A study was conducted in male Sprague–Dawley rats ($n=5$) weighing 220–250 g to determine whether the technique was sufficiently sensitive to characterize the pharmacokinetic behavior of tramadol and ODMT after a 5-min i.v. infusion of 26 mg/kg of tramadol. This dose was previously established in our laboratory to produce significant analgesia in the rat (unpublished results). Blood samples (225 μl) were obtained immediately before, during and after the infusion at the following times: 0, 5, 7.5, 10, 15, 20, 35, 50, 65, 95, 125, 275 and 375 min for simultaneous determination of both tramadol and ODMT.

Blood was immediately centrifuged for 15 min at 1000 *g* and plasma was separated. Internal standard was added to 100 μl of plasma and tramadol and ODMT were extracted as has been explained above. The dried extracts were stored in a freezer at −20° until their analysis. The concentrations of tramadol were established using calibration curves ranging between 10 and 10 000 ng/ml and ODMT concentrations using calibration curves ranging between 10 ng/ml and 1000 ng/ml.

Maximal concentration achieved (C_{max}) was obtained directly from the measured concentrations. The area under the curve (AUC) of the observed tramadol and ODMT concentrations from $t=0$ min to $t=375$ min was determined using the linear trapezoidal rule.

3. Results and discussion

A sensitive and specific method for the determination of tramadol and its active metabolite ODMT in plasma with rapid sample preparation is described. This assay offers significant advantages over previously reported HPLC methods for the determination of tramadol and ODMT. The main advantage of the present method is its capability for simultaneous extraction, determination and quantification of the parent drug and its active metabolite. Several other methods have been described in the literature for the determination of tramadol in different biological fluids [9–13], but to our knowledge only two techniques extract and determine both compounds [14–15]. However, a validated technique permitting the simultaneous extraction and determination of both compounds in plasma has not yet been published.

Figs. 2A and 3A show the chromatograms obtained from blank plasma samples at +610 and +850 mV, respectively. No interference with endog-

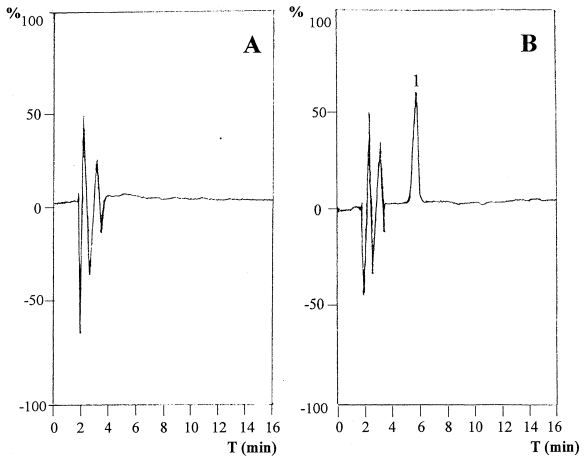


Fig. 2. Representative chromatograms of blank plasma (A) and a plasma sample taken from to a rat at min 35 after the beginning of a 5-min i.v. infusion of tramadol (B) obtained from the analysis after extraction at +610 mV. 1=*O*-Demethyltramadol peak.

enous plasma substances was found at the retention times of the studied compounds in their corresponding oxidation potentials. The reasons for using two different oxidation potentials were: (i) the selected potentials +610 and +850 for ODMT and tramadol, respectively were those showing the higher sensitivity to the products and (ii) because there appears to be an interference in the chromatogram of

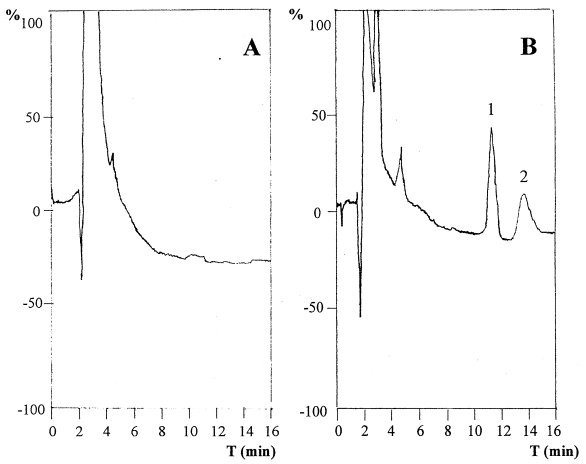


Fig. 3. Representative chromatograms of blank plasma (A) and a plasma sample taken from to a rat at min 35 after the beginning of a 5-min i.v. infusion of tramadol (B) obtained from the analysis after extraction at +850 mV. Peaks: 1=Tramadol, 2=internal standard.

tramadol and internal standard at the time where ODMT would elute.

As can be observed in the chromatograms represented in Figs. 2B and 3B, an adequate separation of tramadol and ODMT from the internal standard was achieved. The approximate retention times of ODMT, tramadol and internal standard were 5, 10.5 and 14 min, respectively. The total duration of the chromatogram was 16 min. These retention times allow a fast and repeated determination of both compounds, as is necessary in pharmacokinetic studies.

We observed no carry-over in our assay. When a water sample (reconstitution liquid) was injected into the system after a sample containing 1000 ng/ml of tramadol and ODMT, no peaks were detected.

The calibration curves of tramadol and ODMT extracted from plasma showed a good linearity over the studied concentration range. Correlation coefficients were higher than 0.99 ($p<0.01$). The obtained correlation curves were:

(a) Tramadol:
10–50 ng/ml: $y=0.059x-0.230$; $r=0.9981$
25–500 ng/ml: $y=0.011x+0.013$; $r=0.9987$
500–10 000 ng/ml: $y=0.0061x-0.02$; $r=0.9992$

(b) ODMT:
10–50 ng/ml: $y=0.0213x+0.050$; $r=0.9986$
25–500 ng/ml: $y=0.0118x+0.216$; $r=0.9991$
500–1000 ng/ml: $y=0.0012x-1.016$; $r=0.9998$

The recoveries from plasma for tramadol, ODMT and internal standard were found to be pH-dependent (data not shown). The best recoveries were found at pH values higher than 13. Table 1 lists the absolute recoveries: 70 to 83% and 76 to 87% for tramadol

Table 1
Absolute recovery (%) of tramadol and ODMT in plasma

Concentration (ng/ml)	Absolute recovery (%) (mean±SD)	
$n=3$	Tramadol	ODMT
1000	80.9±9.6	79.6±5.8
500	90.5±4.6	86.8±6.1
250	79.3±4.7	80.4±1.1
25	82.8±4.2	73.0±5.2
10	71.4±5.1	77.5±5.2

and ODMT, respectively, which where found to be concentration independent. The recovery of internal standard at 600 ng/ml was 65%. The obtained recovery data were similar to those reported in other studies [14,15]. However, the present method shows an advantage over other techniques that require more than one step for the plasma sample to be cleaned up. In the present method it is possible to extract both tramadol and ODMT in one step without any interference of plasma compounds.

Table 2 lists the results obtained from the intra-day precision and accuracy studies. For all the concentrations studied, intra-day C.V.s were lower than 10% and for all concentrations of both compounds the accuracy was higher than 90%. The overall inter-day mean C.V.s of the high 1000 ng/ml and low 10 ng/ml concentrations for tramadol were 5.46 and 10.1%, respectively and for ODMT these values were 8.5 and 13.9%, respectively.

On the basis of a signal-to-noise ratio of 3 the detection limits of our assay were 4 ng/ml and 3 ng/ml for tramadol and ODMT, respectively when 100-μl plasma aliquots were used. Quantification limits considered as 10-times the signal-to-noise ratio were 12 ng/ml and 10 ng/ml for tramadol and ODMT, respectively when 100-μl plasma aliquots were used.

Some authors have reported that tramadol is not stable after extraction [10]. Nevertheless, the present method shows that both tramadol and ODMT are stable in plasma before and after extraction: (i) when plasma samples containing tramadol and ODMT were stored at −20°C, both compounds were found to be stable for at least one week; if plasma samples were stored at +4°C the stability of the compounds was reduced to four days; (ii) after extraction, the stability was found to be increased to at least 10 or 15 days if the samples were stored dried or reconstituted with 100 μl of deionized water, respectively. On the basis of these results, it could be concluded that if it is necessary to store samples after extraction, reconstitution in deionized water is preferred.

Our method showed adequate selectivity since none of the compounds tested during the interference study (propranolol, buprenorphine, morphine, methadone, verapamil, nimodipine and felodipine) showed a detectable signal at the oxidation potentials used in the current study.

One of the major problems of pharmacokinetic studies in animals (rat) is the need to determine low concentrations of the drug in the lowest possible volume of plasma. The described method has a limit of quantification of 12 and 10 ng/ml for both tramadol and ODMT using only 100 μl of plasma.

The utility of the described method was demonstrated by applying it to determine tramadol and ODMT for at least 6 h after an i.v. infusion of tramadol in rats. Fig. 4 shows an example of the time course of plasma concentrations of tramadol and ODMT in one rat after a 5-min i.v. infusion of tramadol. Pharmacokinetic analysis of the data indicated a mean C_{max} of 10 μg/ml for tramadol and of 850 ng/ml for the metabolite. The mean AUCs of

Table 2
Intra-day precision (expressed as C.V.) and accuracy (expressed as relative error, %) of tramadol and ODMT

	Concentration (ng/ml)	Precision (C.V., %)	Accuracy (%)
Tramadol	1000	4.30	7.07
	500	4.00	5.88
	25	2.55	10.55
	10	8.76	10.30
O-Demethyltramadol	1000	7.56	3.87
	500	2.77	3.10
	25	2.44	5.40
	10	1.51	9.83

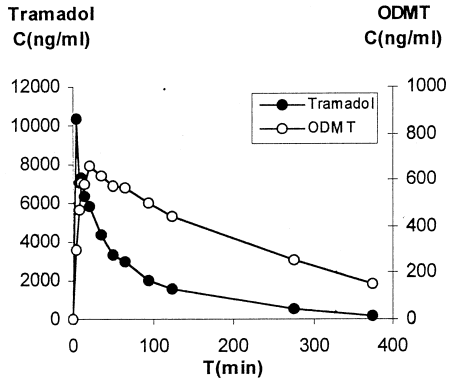


Fig. 4. Time course of the arterial concentrations of tramadol (●) and *O*-demethyltramadol (○) after a 5-min i.v. infusion administration of tramadol (dose: 26 mg/kg) to one rat.

tramadol and ODMT were 493 μg/ml min and 119 μg/ml min, respectively.

4. Conclusion

In conclusion, LC–electrochemical detection is a rapid, specific, sensitive and accurate method for simultaneous quantification of tramadol and ODMT in a small volume of plasma, which, on the basis of the pharmacodynamic properties of ODMT [7] could be very useful in performing pharmacokinetic/pharmacodynamic studies of tramadol.

Acknowledgements

The authors thank Carlos Pérez for his assistance in the preparation of the manuscript. This work was supported by a grant from the University of the Basque Country (026.327 EB 231/96). M.V. was supported by a fellowship from the University of the Basque Country.

References

[1] C.R. Lee, D. McTavis, E. Sorkin, Drugs 46 (1993) 313.
[2] A. Mattia, T. Vanderah, R.B. Raffa, J.L. Vaught, R.J. Tallarida, F. Porreca, Drug Dev. Res. 28 (1993) 176.
[3] W. Reimann, H.H. Hennies, Biochem. Pharmacol. 47 (1994) 2289.
[4] H.H. Hennies, E. Friderichs, K. Wilsmann, L. Flohe, Biochem. Pharmacol. 31 (1982) 1654.
[5] A.V. Bono, S. Cuffari, Drugs 53 (1997) 40.
[6] R. Tarradell, O. Pol, M. Farré, E. Barrera, M. Puig, Methods Find. Exp. Clin. Pharmacol. 18 (1996) 211.
[7] H.H. Hennies, E. Friderich, J. Schneider, Arzneimittel-Forschung/Drug Res. 38 (1988) 877.
[8] M. Valle, R. Calvo, J.M. Pavón, I.F. Trocóniz, Methods Find. Exp. Clin. Pharmacol. 19 (Suppl. A) (1997) PB-74.
[9] W. Lintz, H. Uragg, J. Chromatogr. 341 (1985) 65.
[10] R. Becker, W. Lintz, J. Chromatogr. 377 (1986) 213.
[11] M. Nobilis, J. Pastera, P. Anzenbacher, D. Svodoba, J. Kopeky, F. Perlik, J. Chromatogr. B 681 (1996) 177.
[12] M. Merslavic, A. Zupancic-Kralj, J. Chromatogr. B 693 (1997) 222.
[13] A. Ceccato, P. Chiap, P.H. Hubert, J. Crommen, J. Chromatogr. B 698 (1997) 161.
[14] W.D. Paar, P. Frankus, H.J. Dengler, J. Chromatogr. B 686 (1996) 221.
[15] B. Elsing, G. Blaschke, J. Chromatogr. 612 (1993) 223.