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Short communication

Improved HPLC method for the simultaneous determination of tramadol and *O*-desmethyltramadol in human plasma

Yongchuan Gu *, J. Paul Fawcett

School of Pharmacy, University of Otago, P.O. Box 913, Dunedin, New Zealand

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Abstract

This paper describes an HPLC method for the determination of tramadol and its major active metabolite, *O*-desmethyltramadol (ODT), in human plasma. Sample preparation involved liquid–liquid extraction with diethyl ether–dichloromethane–butanol (5:3:2, v/v/v) and back extraction with sulphuric acid. Tramadol, ODT and the internal standard, sotalol, were separated by reversed phase HPLC using 35% acetonitrile and an aqueous solution containing 20 mM sodium phosphate buffer, 30 mM sodium dodecyl sulphate and 15 mM tetraethylammonium bromide pH 3.9. Detection was by fluorescence with excitation and emission wavelengths of 275 and 300 nm, respectively. The method was linear for tramadol (3–768 ng/ml) and ODT (1.5–384 ng/ml) with mean recoveries of 87.2% and 89.8%, respectively. Intra- and inter-day precisions were 10.34% and 8.43% for tramadol and 9.43% and 8.75% for ODT at the respective limits of quantitation (3 and 1.5 ng/ml). Accuracy for tramadol ranged from 96.2% to 105.3%. The method was applied to a pharmacokinetic study of tramadol in human volunteers.

Keywords: Tramadol; *O*-Desmethyltramadol; Sotalol

1. Introduction

Tramadol hydrochloride, (±)-*trans*-2-[(dimethylamino) methyl]-1-(3-methoxyphenyl) cyclohexanol (Fig. 1A), is a centrally-acting analgesic used in the treatment of mild to moderate pain [1]. Its therapeutic plasma concentration is in the range 100–300 ng/ml [2]. Tramadol is rapidly and almost completely absorbed after oral administration but its absolute bioavailability is only 65–70% due to first-pass metabolism [3]. The metabolism of tramadol in human is mediated by cytochrome P4502D6 (CYP2D6) to *O*-desmethyltramadol (ODT, Fig. 1B) and *N*-desmethyltramadol (NDT). ODT is pharmacologically active and contributes to the analgesic efficacy of tramadol [4].

Although the tramadol molecule contains a benzene ring, UV detection is unsuitable for its analysis in plasma due to lack of sensitivity and selectivity [5]. Early analytical methods for tramadol and its metabolites in biological samples involved gas chromatography (GC) with nitrogen selective detection [6] and GC–mass spectrophotometry (GC–MS) [7]. More recent methods include the use of electrochemistry [8], mass spectrometry [9], capillary zone electrophoresis [10] and HPLC with fluorescence detection [5,11]. The latter provides good selectivity and sensitivity and was used to determine tramadol and ODT but it required an extensive washout period and different excitation and emission wavelengths for tramadol, ODT and the internal standard [11].

This paper describes an improved method for the simultaneous determination of tramadol and ODT in human plasma involving simple sample preparation followed by HPLC with fluorescence detection. The method is sensitive, rapid and suitable for routine application to pharmacokinetic, bioavailability and bioequivalence studies.

2. Experimental

2.1. Reagents and chemicals

Tramadol hydrochloride and sotalol hydrochloride were obtained from Sigma–Aldrich (St Louis, MO, USA). *O*-Des-

* Corresponding author. Fax: +64 3 4797034.
E-mail address: njguyc@hotmail.com (Y. Gu).

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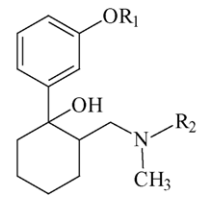

A. Tramadol (R_1=CH_3, R_2=CH_3)
B. ODT (R_1= H, R_2= CH_3)

Fig. 1. Chemical structures of tramadol and *O*-desmethyltramadol (ODT).

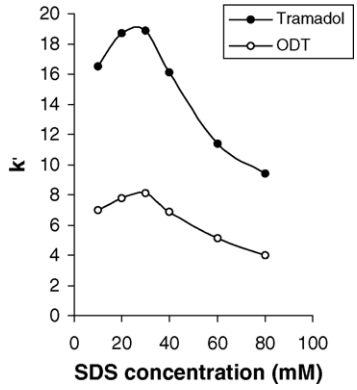

Fig. 2. Plots showing the variation in capacity factor, k', with sodium dodecyl sulfate concentration (SDS) for tramadol and ODT using a mobile phase containing 35% acetonitrile.

methyltramadol hydrochloride, *N*-desmethyltramadol and *O*,*N*-didesmethyltramadol (ONDT) were purchased from Toronto Research Chemicals Inc. (Ontario, Canada). Acetonitrile, methanol, diethyl ether, dichloromethane and 1-butanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS), sodium dihydrogenphosphate and tetraethylammonium bromide (TEA) were analytical grade from BDH (Poole, UK). Deionized water was produced by a Milli-Q Millipore Water System (Millipore, MA, USA).

2.2. Chromatography

The HPLC system was an LC Workstation Class LC10 (Shimadzu, Kyoto, Japan) consisting of a SIL-10ADvp autosampler maintained at 4 °C, LC-10ADvp pump, CTO-10Avp column oven, SCL-10Avp system controller and RF-10A spectrofluorometric detector. Data was processed by an LC solution Workstation (Shimadzu Corporation). Chromatographic separation was performed on a Hypersil C18 analytical column (100 mm × 2.1 mm i.d., 5 μm particle size) maintained at 30 °C. The mobile phase consisted of acetonitrile:buffer (20 mM sodium dihydrogenphosphate, 30 mM sodium dodecyl sulphate and 15 mM tetraethylammonium bromide adjusted to pH 3.9 with phosphoric acid) (35:65, v/v) at a flow rate of 0.5 ml/min. Fluorescence detection employed excitation and emission wavelengths of 275 and 300 nm, respectively.

To achieve stable ion pair chromatography, the mobile phase was optimised for the content of SDS (10–80 mM) at various acetonitrile concentrations (20–60%). Capacity factors were obtained using sodium benzenesulfonate as unretained substance. A typical plot of capacity factor (k') versus SDS concentration is shown in Fig. 2. At the optimum SDS concentration, the addition of TEA (5–30 mM) and variation in buffer pH (2.5–7.0) were evaluated to optimise peak shape and retention on the column. Sotalol was chosen as the internal standard (IS) after comparing with verapamil, metoprolol and citalopram.

2.3. Stock solutions

Stock solutions of tramadol and ODT (both 1.0 mg/ml as free bases) were prepared by dissolving 11.38 and 11.46 mg of the respective hydrochlorides in 10 ml methanol. Stock solutions containing 16 μg/ml tramadol and 8 μg/ml ODT were prepared by dilution. An IS stock solution (1.0 mg/ml) was prepared by dissolving 10.0 mg sotalol hydrochloride in 10 ml methanol. The working IS solution (8 μg/ml) was prepared by successive 1:10 and 0.8:10 dilutions with water. All stock solutions (tramadol, ODT and IS) were stored at −16 °C and other solutions were prepared daily.

2.4. Sample preparation

Into a 10 ml glass tube was added 1.0 ml plasma followed by 100 μl IS solution and 0.5 ml 0.2 M borate buffer (pH 9.3). After vortex-mixing, 7.0 ml of extraction solvent (diethyl ether:dichloromethane:1-butanol 5:3:2) was added and the tube shaken for 20 min and then centrifuged for 10 min at 2600 × *g*. The organic layer was transferred to a clean 10 ml glass conical tube, shaken with 200 μl back-extraction solvent (0.05 M H_2SO_4:acetonitrile 9:1) for 20 min and centrifuged for 10 min at 2600 × *g*. The aqueous phase (100 μl) was injected into the HPLC system.

2.5. Validation procedures

Outdated Blood Bank plasma (Dunedin Hospital) was screened to ensure the absence of interfering peaks at the retention times of tramadol, ODT and the IS The assay was also tested for potential interference from the following basic drugs; propranolol, metoprolol, gallopamil, carvedilol, naftopidil and sertraline.

Calibration standards were prepared by spiking plasma with tramadol (3, 6, 12, 24, 48, 96, 192, 384, 691.2 and 768 ng/ml) and ODT (1.5, 3, 6, 12, 24, 48, 96, 192, 345.6 and 384 ng/ml). QC samples containing LOQ, low, medium and high concentrations of tramadol (3, 9, 74 and 614 ng/ml) and ODT (1.5, 4.5, 37 and 307 ng/ml) were similarly prepared.

Linearity of calibration curves based on peak height ratios (tramadol/IS) was assessed by weighted least squares regression analysis ($1/y^2$). Limit of detection (LOD) and quantita-

tion (LOQ) were determined as analyte concentrations giving signal-to-noise ratios of 3 and 10, respectively. Intra- and inter-day precision (expressed as relative standard deviation (R.S.D.)) and accuracy (expressed as percentage of the nominal value) were determined by analysis of replicates ($n = 6$) of LOQ, low, medium and high QC samples on three different days.

Recovery was assessed by comparing extracted solutions with solutions of tramadol and ODT in back-extraction solvent at concentrations of 45, 370 and 3070 ng/ml for tramadol and 22.5, 185 and 1535 ng/ml for ODT. The same procedure was followed for the IS solution (4 μg/ml).

Stability of stock solutions containing tramadol, ODT and IS was tested over 6 h at room temperature ($22 \pm 2\,^{\circ}C$) and over 32 days at $-16\,^{\circ}C$. Freeze–thaw stability in plasma was evaluated using spiked samples stored at $-70\,^{\circ}C$ and subjected to three freeze–thaw cycles. Long-term stability in plasma was assessed using spiked samples stored in the freezer at $-70\,^{\circ}C$ over 22 weeks. Stability in back extraction solvent was evaluated in the autosampler at $4\,^{\circ}C$ (post-preparative stability) over 48 h.

2.6. Application of the method

Tramadol and ODT levels in plasma samples from 24 healthy volunteers were measured as part of a bioequivalence study. The volunteers were administered a single oral dose of tramadol as a 100 mg sustained release tablet. Plasma concentration–time profiles for tramadol and ODT were determined over 32 h.

3. Results and discussion

Several HPLC methods using one-step liquid–liquid or solid phase extraction have been reported for analysis of tramadol in biological samples. However, the assay reported here is the first to report a sample preparation method that does not require evaporation and reconstitution of the samples. Although ion-pair chromatography and fluorescence was used by Nobilis et al. [5], their assay determined only tramadol. The method reported here has the high sensitivity and selectivity associated with ion pair chromatography and fluorescence detection as shown by the fact that interference from a number of potentially concomitant basic drugs was absent.

Typical HPLC chromatograms of blank plasma, spiked plasma and plasma samples from a volunteer after a single oral administration are shown in Fig. 3. Tramadol, ODT and IS were free of interference from endogenous compounds in plasma. Baseline resolution was obtained with retention times of 13.1, 10.5, 6.6, 5.5 and 3.4 min for NDT, tramadol, ONDT, ODT and IS, respectively. Peaks were identified by comparison with the chromatograms of reference substances and, in the case of NDT, by the mass spectrum of collected fractions.

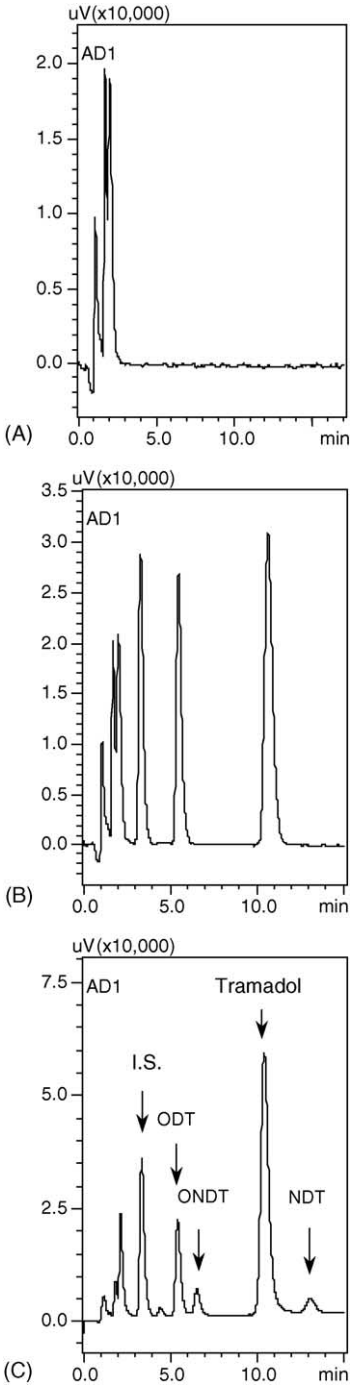

Fig. 3. Chromatograms of (A) blank plasma, (B) plasma spiked with tramadol (96 ng/ml) and *O*-desmethyltramadol (48 ng/ml) and (C) plasma from a healthy volunteer 1.0 h after oral administration of a 100 mg sustained release tablet of tramadol (114.6 ng/ml tramadol, 27.9 ng/ml *O*-desmethyltramadol).

Calibration curves gave linear regression equations of $y = 0.0076 + 0.0121x$ for tramadol ($r^2 > 0.999$) and $y = 0.0055 + 0.0213x$ for ODT ($r^2 > 0.999$), where y is relative peak height and x is concentration (ng/ml). LOD and LOQ were found to be, respectively, 1 and 3 ng/ml for tramadol and 0.5 and 1.5 ng/ml for ODT. Intra- and inter-day

Table 1
Intra- and inter-day precision and accuracy for tramadol and *O*-desmethyltramadol (ODT) in human plasma

Tramadol concentration (ng/ml)				ODT concentration (ng/ml)			
Added	Measured	Accuracy (%)	R.S.D. (%)	Added	Measured	Accuracy (%)	R.S.D. (%)
Intra-day							
3.0	3.1	103.3	10.34	1.5	1.4	93.3	8.43
9.0	9.1	101.1	7.52	4.5	4.4	97.8	7.08
74.0	77.9	105.3	6.39	37.0	37.6	101.6	2.77
614.0	603.8	98.3	3.48	307.0	296.1	96.4	2.04
Inter-day							
3.0	2.9	96.7	9.43	1.5	1.5	100.0	8.75
9.0	9.2	102.2	8.86	4.5	4.4	97.8	7.22
74.0	75.4	101.9	5.78	37.0	37.2	100.5	4.41
614.0	603.6	98.3	3.66	307.0	295.3	96.2	2.38

Data are mean values based on six replicates analysed on three different days.

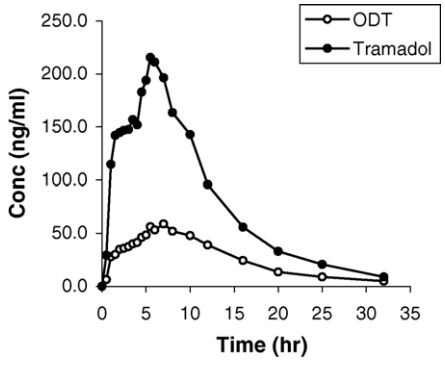

Fig. 4. Plasma concentration–time profiles of tramadol and *O*-desmethyltramadol in a healthy male subject after oral administration of a 100 mg sustained released tablet of tramadol.

precision and accuracy for tramadol and ODT were good (Table 1). The recoveries of tramadol at 9, 74 and 614 ng/ml were 87.5%, 87.7% and 86.5%, respectively. The recoveries of ODT at 3, 37 and 384 ng/ml were 90.6%, 88.0% and 90.7%, respectively. The mean recovery of the IS was 82.2%. In terms of stability, no significant degradation of tramadol and ODT was observed under any of the storage conditions evaluated.

The concentration–time profile for one of the subjects is illustrated in Fig. 4. Maximal concentrations (C_{max}) of tramadol and ODT were 215.5 and 58.9 ng/ml at 5.5 and 7.0 h, respectively. The corresponding elimination half-lives were 6.1 and 7.1 h. Area under the concentration–time curve ($AUC_{0\rightarrow\infty}$) for tramadol and ODT were 2590 and 869 μg h/l, respectively. The results for tramadol are consistent with values previously obtained in a dose linearity study which included a 100 mg sustained release capsule [12].

In conclusion a rapid, simple, robust and reproducible method has been developed for the determination of tramadol and ODT in human plasma with limits of quantitation of 3 and 1.5 ng/ml for tramadol and ODT, respectively. The method was validated according to FDA guidelines for human studies [13] and was successfully used in a clinical study in healthy volunteers.

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