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Validation of a high-performance liquid chromatography method for tramadol and *o*-desmethyltramadol in human plasma using solid-phase extraction

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Abstract

An HPLC system using solid-phase extraction and HPLC with UV detection has been validated in order to determine tramadol and *o*-desmethyltramadol (M1) concentrations in human plasma. The method developed was selective and linear for concentrations ranging from 50 to 3500 ng/ml (tramadol) and 50 to 500 ng/ml (M1) with mean recoveries of 94.36±12.53% and 93.52±7.88%, respectively. Limit of quantitation (LOQ) was 50 ng/ml. For tramadol, the intra-day accuracy ranged from 95.48 to 114.64% and the inter-day accuracy, 97.21 to 103.24%. Good precision (0.51 and 18.32% for intra- and inter-day, respectively) was obtained at LOQ. The system has been applied to determine tramadol concentrations in human plasma samples for a pharmacokinetic study.

Keywords: Tramadol; *o*-desmethyltramadol

1. Introduction

Tramadol hydrochloride, (±)-*trans*-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol, is a centrally-acting analgesic agent used in the treatment of mild to moderate pain [1]. Its therapeutic concentration is within 100 to 300 ng/ml [2]. After a single bolus infusion of 100 mg tramadol, concentrations in plasma can be detected instantaneously. Elimination is slow, being characterised by an elimination half-life of 6 h [2].

It has been shown that the hepatic *o*-demethylation of tramadol is carried out by the isoenzyme cytochrome P4502D6 (CYP2D6). The gene encoding for CYP2D6 is known to show polymorphism [3] and the existence of different alleles result in functionally different enzymes. This is the basis of large inter-individual differences of the metabolism of those drugs requiring CYP2D6 to be eliminated from the body. CYP2D6 polymorphism is the most extensively studied oxidation polymorphism which was discovered by the identification of so-called ''poor metabolisers'' of the antihypertensive drug debrisoquine. More than 30 drugs are substrates of CYP2D6, including drugs with narrow therapeutic range. In the case of tramadol, poor metabolisers of sparteine/debrisoquine (affecting about 5–10% of Caucasians) are virtually unable to demethylate it to its pharmacologically active metabolite, *o*-desmethyl-

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tramadol (M1). Tramadol is not truly a prodrug; it acts on both the serotonergic and noradrenergic system with M1 showing 2–4-fold higher analgesic potency in animals as it acts mainly via the μ_1-opioid receptors [4]. Therefore, poor metabolisers may exhibit either an absent or at least a weaker analgesic efficacy after administration of i.v. tramadol.

Tramadol contains a weakly absorbing chromophore in its molecule, rendering UV detection unsuitable for the determination of its concentration in plasma samples [5]. Recent methods for its determination employed gas chromatography (GC) with a nitrogen-selective detector [6] and GC–mass spectrophotometry (GC–MS) [7]. These require tedious recrystallisation, synthetic and purification process. Furthermore, not all institutions have GC or GC–MS due to the high cost. More recent progress includes the use of a fluorescence detector [5] and capillary zone electrophoresis [8].

We describe a newly developed method and its validation for the determination of tramadol and M1 in the human plasma using low wavelength UV detection on an HPLC. The developed method was applied to a pharmacokinetic study of i.v. tramadol injections used for relieving postoperative pains in patients undergoing orthopaedic operations in our hospital. The randomised controlled trial on this group of 53 patients confirmed its usage.

2. Experimental

2.1. Chemicals and reagents

Tramadol hydrochloride standard (Fig. 1A) and the metabolite M1 (Fig. 1B) were gifts from Grunenthal, (Aachen, Germany). The internal standard used was phenacetin (Fig. 1C) (N-[4-ethoxyphenyl]acetamide) purchased from Sigma (St Louis, MI, USA). Potassium dihydrogen phosphate (KH_2PO_4) was of analytical-reagent grade from Merck® (Germany). Methanol, acetonitrile and triethylamine were of HPLC grade (Merck®). Water was doubly distilled and purified using the Water Prodigy System (Labconco®, MI, USA).

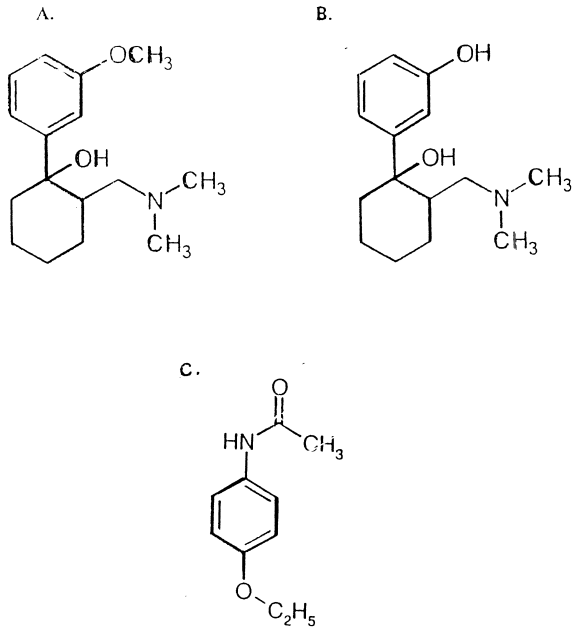

Fig. 1. Chemical structure of (A) tramadol (B) *o*-desmethyltramadol and (C) phenacetin (internal standard).

2.2. Instrumentation

The HPLC system consisted of a 307 Gilson pump coupled to a 115 Gilson Variable UV detector which was set at 218 nm. The sensitivity was 0.005 and the time constant set at 0.5. The analytical column was LiChrosorb reverse phase (RP-18) (CA, USA) with particle size of 5 μm (250×4.6 mm, I.D.). It was coupled to an RP-18 Supelcosil guard column (Bellefonte, USA) (5 μm: 4×4 mm I.D.).

The control of the HPLC system and data collection was performed by use of an IBM-compatible computer equipped with Unipoint® software (version 2.00).

2.3. Chromatographic conditions

The mobile phase was a mixture of 70% phosphate buffer (0.01 *M*), 30% acetonitrile with the addition of 0.1% triethylamine (v/v). Phosphate buffer was prepared fresh in a 1000 ml volumetric flask by dissolving 1.36 g of KH_2PO_4 in doubly distilled water. The pH of the final mixture was

adjusted to pH 5.90. The solution was degassed prior to use under vacuum by filtration through a cellulose filter (0.45 μm) (Sartorius, Germany) and was prepared fresh daily.

Guard columns were replaced after every 100 injections due to the dirty plasma samples used. A flow-rate of 0.75 ml/min was employed throughout with 20 μl injection each time.

2.4. Preparation of stock solutions and working standard solutions

Stock solutions of tramadol and M1 (100 μg/ml each) were prepared monthly by dissolving 10 mg of each drug, respectively, in 100 ml methanol and kept stored at 4°C. Tramadol concentrations in the working standard solutions chosen for the calibration curve were 0.05, 0.1, 0.5, 1 and 3.5 μg/ml while that of M1 were 0.05, 0.1, 0.16, 0.3 and 0.5 μg/ml. These working solutions were made by further dilution of the stock solutions in methanol. They were prepared fresh daily.

A stock solution of the internal standard (100 μg/ml each) was prepared by dissolving 10 mg of phenacetin in 100 ml methanol and kept stored at 4°C. From here, working standard solution of 20 μg/ml in methanol was prepared daily.

2.5. Preparation of plasma standards and samples

Frozen human plasma samples were left on the bench to thaw naturally and were vortexed prior to their use. Quality control samples were prepared by spiking drug-free human plasma with the different working standard solutions of tramadol and M1 while phenacetin was added at 1 μg/ml throughout.

2.6. Solid-phase extraction (SPE)

Solid-phase extraction of samples was carried out on disposable non-end-capped C_{18} 100 mg/1 ml cartridges produced by International Sorbent Technology (Isolute, UK). The procedure was described below.

The columns were conditioned with 1 ml of methanol followed by 1 ml of water. Following that, 1 ml of plasma spiked with tramadol and M1 was loaded. The columns were washed thrice with 1 ml of water. This was followed by washing with 250 μl of acetonitrile (ACN):ethylacetate (EtAc) (60:40) combination for three times. Elution was carried out four times with 250 μl of the mixture ACN:EtAc (60:40) with added triethylamine 1%. They were collected into one tube. A volume of 50 μl of phenacetin was then spiked from a 20 μg/ml working stock solution as internal standard. The mixture was vortexed before drying under a stream of nitrogen. The sample was reconstituted in 50 μl of mobile phase and injected into the HPLC system.

2.7. Validation

The criteria established for the development of our analytical procedure include: (1) using the smallest amount of mobile phase possible; (2) restricting k' values to between 1 and 10; (3) using solvents that allow detection at low wavelengths for a weak chromophoric drug as tramadol; (4) ionization suppression between drug molecule and residual silanol groups on the surface of silica.

The following parameters were determined for the validation of analytical method developed for tramadol and M1 in human plasma: selectivity, linearity, range, precision, accuracy, LOQ, recovery and stability [9].

2.8. Application of the method

The developed method has been applied to pharmacokinetic studies in which the concentrations of tramadol and M1 were measured in more than 500 plasma samples. As an example, we show some of the results obtained in a randomized controlled trial of tramadol hydrochloride given to post-operative orthopaedic patients (refer to Section 3.9.). In this study, 100 mg of i.v. formulation developed by Boehringer-Mannheim (Grunenthal, Germany) was administered slowly (over 2 min) to 53 patients immediately after their operations. Blood samples were collected at 0, 0.1, 0.25, 0.5, 0.75, 1, 2, 4, 8, 16, 20 and 24 h after drug administration. After each blood sampling, plasma was separated by centrifugation at 2200 g for 15 min and stored at −20°C until assay.

3. Results and discussion

3.1. Optimisation of the chromatographic conditions

To ensure the stability of basic tramadol with pK_a value of 8.3 [8], we buffered the mobile phase to pH 5.9. Although increasing the pH above this increases the capacity factor and area for both the drug and its metabolite, peak tailing was more significant with total run time increased. Asymmetric peaks were obtained for compounds having pK_a values above 6 because these compounds were partly protonated and hence more strongly-bound to the silanol groups [10]. On the other hand, the internal standard (a neutral drug) was not much affected by pH variation. However, at higher pH its retention dropped, bringing its peak nearer to tramadol thus affecting the resolution. Furthermore, silica-based particles are unstable at high pH [11]. Therefore, we have used pH 5.9.

Triethylamine, a very basic compound (p$K_a \approx$ 11.0) interacts with silanol groups and competes with basic drug. Its addition results in an improvement of the peak shape [11] as illustrated in our result.

We observed excellent base line separation between tramadol, M1 and the internal standard peaks in our chromatogram (Table 1). There were also no interfering peaks from the serum matrix in the analysis (Fig. 2.). The average retention time for tramadol, M1 and internal standard were 11.40 min, 6.20 min and 14.10 min, respectively, with total run time set at 17.00 min.

3.2. Solid-phase extraction

Solid-phase extraction technique has many benefits which include quick sample processing, elimination of some of the glassware necessary with liquid–liquid extraction methods, reduction of analysts exposure to organic solvents besides consuming much less solvent and producing more reproducible results.

We obtained excellent recovery using the solid-phase extraction method described. Different solid-phase extraction cartridges both end-capped and non-end-capped (C_2, C_8, C_{18} and PH) has been tested. C_{18} cartridges (non-end-capped) have enhanced secondary silanol interactions which can be very useful for a basic compound such as tramadol. They showed the highest recovery for both drug and metabolite under the assay conditions and thus were selected. Table 2 shows their percentage recoveries at various elution steps and also their cumulative recovery.

Although the ideal elution number was shown to be eight for M1 and six for tramadol respectively, our method has employed only up to four elutions which give sufficiently high percentage recoveries (82.6 and 94.30% for the metabolite and tramadol, respectively). This was due to the fact that there was increased interference at the same time beyond elution number 4 leading to noisy baselines.

3.3. Selectivity

The pre-dose samples of the patients in the pharmacokinetic study did not show any relevant interference. By comparing the retention times, our HPLC assay was found to be free from possible interferences from other analgesics used in the wards such as pethidine, morphine, diclofenac, acetominophen and mefenamic acid. It was also free from other agents used during anaesthesia (fentanyl, tracium, propofol, lignocaine, atropine, neostigmine, succinylcholine) and the standard pre-medication (midazolam). Other drugs used post-operatively which include anti-emetic such as metoclorpramide, antibiotic such as cefuroxime and also from the possible contamination from the anticoagulant

Table 1
System suitability parameters

Compound	Retention time (min)	Capacity factor (k')	Resolution (Rs)	N (plate count)	T (tailing factor)
M1	6.20	0.68	2.30	42815.47	1.50
Tramadol	11.40	2.09	3.10	14705.60	1.62
Phenacetin	14.10	2.82	1.60	56885.50	1.25

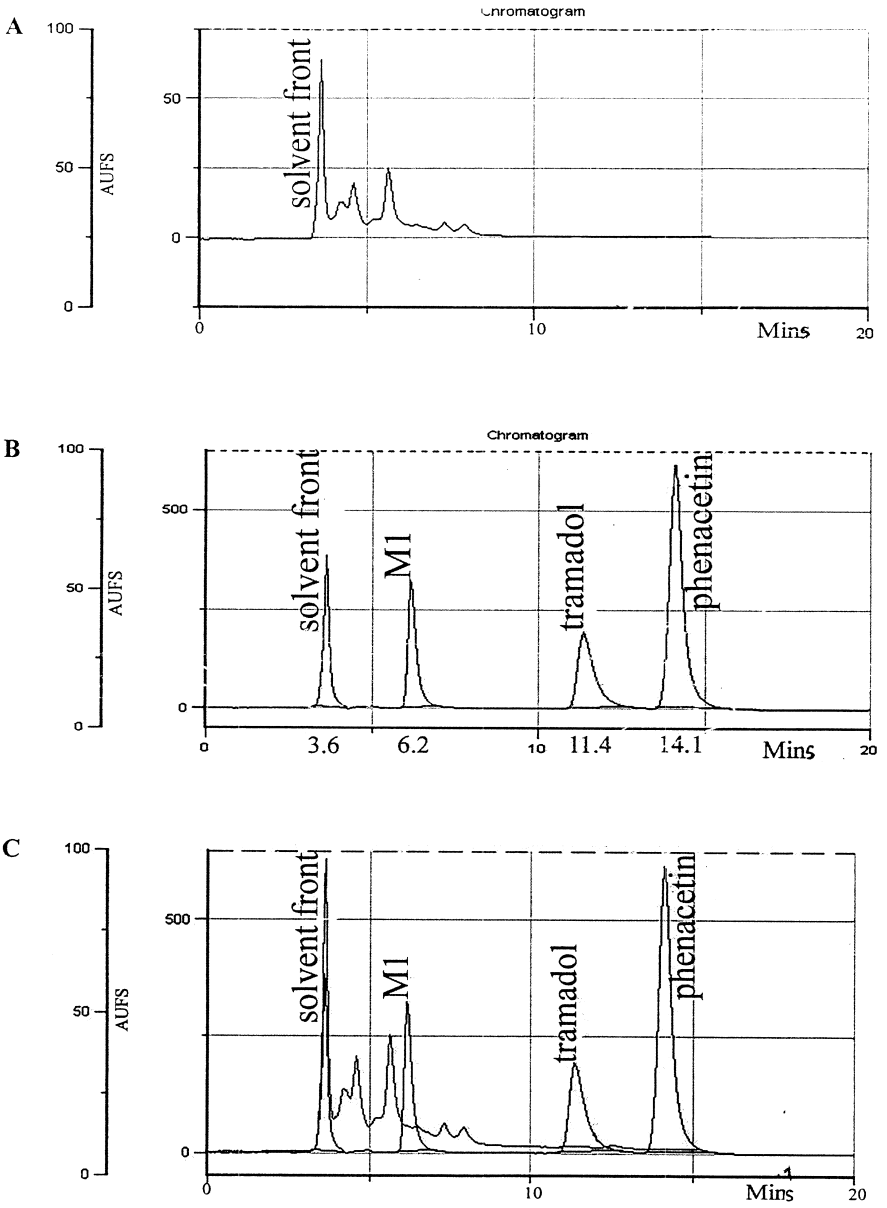

Fig. 2. Representative chromatograms obtained from (A) drug-free human plasma and (B) human plasma spiked with tramadol (0.5 μg/ml), M1 (0.5 μg/ml) and internal standard (1 μg/ml) and (C) the two graphs superimposed on one another.

heparin have also been tested and were not found to interfere with the analysis.

The peaks for both drug and metabolite were found to be pure (peak purity more than 990) determined by means of a Gilson photo-diode array detector.

3.4. Linearity and range

A calibration graph was constructed in duplicates in the range of 50 to 3500 ng/ml ($n=5$ each) for tramadol (Table 3) and 50 to 500 ng/ml ($n=5$ each) for the metabolite (Table 4). The linearity of the

Table 2
Percentage recovery of tramadol and *o*-desmethyltramadol with the number of elutions

	Percentage of M1 recovered in respective elutes (cumulative %)	Percentage of tramadol recovered in respective elutes (cumulative %)
Elution no. 1	3.72 (3.72)	7.46 (7.46)
Elution no. 2	20.71 (24.43)	38.48 (45.94)
Elution no. 3	39.48 (63.91)	44.30 (90.24)
Elution no. 4	18.69 (82.60)	4.06 (94.30)
Elution no. 5	7.80 (90.40)	2.80 (97.10)
Elution no. 6	4.36 (94.76)	0.82 (97.92)
Elution no. 7	2.42 (97.18)	0.00 (97.92)
Elution no. 8	2.38 (99.56)	0.00 (97.92)
Elution no. 9	0.00 (99.56)	0.00 (97.92)

Table 3
Linearity data for tramadol-calibration standard response values

Calibration	Set 1 (n=5)	Set 2 (n=5)	SD	SE	Mean
(Day 1)					
Intercept	0.0110	0.0078	0.0023	0.0016	0.0094
Slope	0.0005	0.0005	0.0000	0.0000	0.0005
Correlation coefficient (r^2)	0.9999	0.9998	–	–	0.9998
(Day 2)					
Intercept	−0.0081	0.0291	0.0263	0.0186	0.0137
Slope	0.0005	0.0004	0.0000	0.0005	0.0005
Correlation coefficient (r^2)	0.9999	0.9984	–	–	0.9890
(Day 3)					
Intercept	−0.0174	−0.0108	0.0047	0.0033	−0.0141
Slope	0.0005	0.0005	0.0000	0.0000	0.0005
Correlation coefficient (r^2)	0.9999	1.0000	–	–	0.9998

Table 4
Linearity data for *o*-desmethyltramadol calibration standard response values

Calibration	Set 1 (n=5)	Set 2 (n=5)	SD	SE	Mean
(Day 1)					
Intercept	−0.0122	−0.0071	0.0036	0.0026	−0.0097
Slope	0.0004	0.0004	0.0000	0.0000	0.0004
Correlation coefficient (r^2)	0.9895	0.9973	–	–	0.9914
(Day 2)					
Intercept	0.0022	0.0183	0.0114	0.0080	0.0185
Slope	0.0003	0.0003	0.0000	0.0000	0.0003
Correlation coefficient (r^2)	0.9874	0.9812	–	–	0.9836
(Day 3)					
Intercept	0.0294	0.0314	0.0014	0.0010	0.0305
Slope	0.0003	0.0003	0.0000	0.0000	0.0003
Correlation coefficient (r^2)	0.9986	0.9988	–	–	0.9986

calibration graph was demonstrated by the good determination coefficient (r^2) obtained for the regression line.

3.5. Precision and accuracy

The precision of the test was evaluated by determining the inter-day and intra-day RSD of the measured peak area ratios for different concentrations. Accuracy was expressed as mean percentage of analyte recovered in the assay. The results are presented in Table 5.

The explicit assay error pattern, obtained by plotting the standard deviations of the concentrations against their respective concentrations with a polynomial fitting (Fig. 3), suggests that error is higher at both the lower and upper ends. This may be explained by the fact that at the lower end extraction efficiency may be much lower. There may also be a higher tendency for measurement or pipetting errors at lower concentrations. On the other hand, at the upper end UV detector saturation may have occurred.

The concentration of interests as defined by the reported therapeutic range has fallen in the middle portion of the graph rendering more strength to the validation.

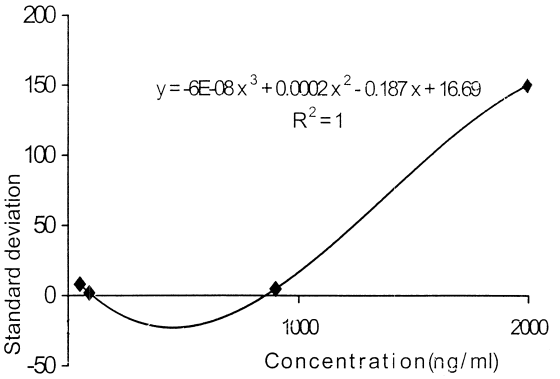

Fig. 3. Explicit assay error pattern for tramadol.

3.6. Limit of quantitation (LOQ)

The LOQ, defined in the presented experiment as the lowest plasma concentration in the calibration

Table 5
Precision and accuracy of the method for the determination of tramadol and *o*-desmethyltramadol in human plasma

Concentration (ng/ml)	Mean	SD	Precision RSD (%)	Accuracy (%)	Percentage difference
Tramadol inter-day (between batch) (n=5)					
50	51.62	9.46	18.32	103.24	3.24
90	87.48	9.98	11.41	97.21	−2.79
900	904.73	8.92	0.99	100.53	0.53
3000	3049.47	95.88	3.14	101.65	1.65
M1 inter-day (between batch) (n=5)					
50	52.22	4.71	9.02	104.43	4.43
90	80.26	6.41	7.99	89.18	−10.82
190	189.60	20.67	10.90	99.79	−0.21
480	484.44	41.74	8.62	100.93	0.93
Tramadol intra-day (within batch) (n=2)					
50	57.32	0.53	0.93	114.64	14.64
90	85.93	1.42	1.65	95.484	−4.52
900	913.83	4.69	0.51	101.54	1.54
3000	3107.00	50.12	1.61	103.57	3.57
M1 intra-day (within batch) (n=2)					
50	50.53	6.43	12.72	101.06	1.06
90	78.03	6.46	8.28	86.70	−13.30
190	194.48	7.06	3.63	102.36	2.36
480	436.92	3.11	0.71	91.02	−8.98

curve that can be measured routinely with acceptable precision (RSD<20%) and accuracy (80–120%) was 50 ng/ml for both the drug and the metabolite (Table 5).

3.7. Recovery

Absolute recovery was calculated by comparing peak areas obtained from freshly prepared sample extracts with those found by direct injection of aqueous standard solutions of the same concentration [12]. Recovery data was determined in triplicates at three concentrations as recommended by the Centre for Drug Evaluation and Research CDER [13]. Table 6 summarises the percentage recovery and its standard deviations at different concentrations.

Using the solid-phase extraction method described, phenacetin suffered from a low recovery, explained by the fact that it is chemically unrelated to the drugs. Therefore, we have spiked in the internal standard just prior to injection as suggested by Karnes et al. [14]. Due to this fact, we have not determined its recovery.

3.8. Stability

We have followed the guidelines laid by the FDA [9] in carrying out the stability testing.

A set of standard samples was prepared from a freshly prepared stock solution of the analyte in drug-free plasma. Three aliquots at both low and high concentrations were prepared for each study and kept frozen at −20°C.

For short-term stability study, the samples were left on the bench to thaw unassisted for 5 h and then analysed.

During freeze–thaw stability testing, the samples were left on the bench to thaw unassisted. When completely thawed, they were transferred back to the original freezer and kept refrozen for 12 to 24 h. The experiment was repeated for a total of three times. Measurement was done after the third freeze–thaw cycle.

For long-term stability testing, enough samples were prepared and stored in the freezer for thawing and analysis at 1 week and then at 1 month.

Table 7 summarises the measured concentration of each replicate and their standard deviations.

The results showed that tramadol and M1 were stable in plasma under the storage condition. This result agrees with that obtained by Lintz [7] stating that tramadol concentration was detectable when stored at −20°C even up to one year.

3.9. Application of the method

This procedure has been applied successfully to the analysis of samples from a pharmacokinetic study. In a study of 53 patients, human plasma samples together with calibration standards and quality control samples were analysed for tramadol and *o*-desmethyltramadol content.

Table 6
Recovery studies for tramadol and M1

Concentration (ng/ml) (n=3)	Mean area ratio (standard)	SD	Mean area ratio ratio (extract)	SD	% Recovery
(A) Tramadol					
50	0.0280	0.0039	0.0302	0.0109	107.96
100	0.0568	0.0027	0.0522	0.0147	91.83
3500	2.1058	0.0537	1.7537	0.1093	83.28
Mean recovery	–	–	–	–	94.36±12.53
(B) o-*desmethyltramadol*					
50	0.0212	0.0024	0.0204	0.0194	96.39
100	0.0442	0.0026	0.0440	0.0189	99.57
500	0.2230	0.0149	0.1887	0.0093	84.61
Mean recovery	–	–	–	–	93.52±7.88

Table 7
Stability study

Quality control sample	Low concentration		High concentration	
	M1 100 ng/ml	Tramadol 100 ng/ml	M1 600 ng/ml	Tramadol 2000 ng/ml
A) Short-term stability				
Replicate 1	106.42	147.78	624.45	1854.27
Replicate 2	108.66	126.20	683.48	1809.44
Replicate 3	119.04	121.99	704.68	1834.15
Mean	111.37	131.99	670.87	1832.62
SD	6.73	13.84	41.57	22.45
CV	6.05	10.48	6.20	1.23
B) Freeze-thaw cycle				
Replicate 1	102.92	113.46	730.69	1914.37
Replicate 2	121.42	100.54	687.39	1844.26
Replicate 3	111.72	115.43	687.69	1886.60
Mean	112.02	109.81	701.92	1881.74
SD	9.25	8.09	24.92	35.30
CV	8.26	7.37	3.55	1.88
C) One week long-term stability				
Replicate 1	112.74	92.78	607.61	1931.92
Replicate 2	108.58	80.14	618.24	2001.28
Replicate 3	104.38	79.15	609.96	2046.59
Mean	108.57	84.02	611.94	1993.26
SD	4.18	7.60	5.58	57.75
CV	3.85	9.05	0.92	2.90
D) One month long-term stability				
Replicate 1	101.68	80.39	527.90	2259.60
Replicate 2	99.66	72.29	539.44	2210.54
Replicate 3	100.01	75.63	554.67	2373.69
Mean	100.45	76.10	540.67	2281.28
SD	1.08	4.07	13.43	83.71
CV	1.07	5.34	2.48	3.67

Fig. 4 represents the concentration–time profile (fitted by a log-linear relationship) for tramadol in three of the patients while Fig. 5 represents the concentration–time profile for its metabolite in the same patients.

4. Conclusions

The assay for tramadol and *o*-desmethyltramadol described in the present report has been demonstrated to meet all of the FDA requirements for clinical pharmacokinetic studies following single doses of tramadol. In particular, the method has satisfactory specificity, linearity, accuracy and precision range over the concentration range examined. The stability of tramadol and *o*-desmethyltramadol under conditions of storage and handling relevant to the conduct of clinical pharmacokinetic studies has been demonstrated.

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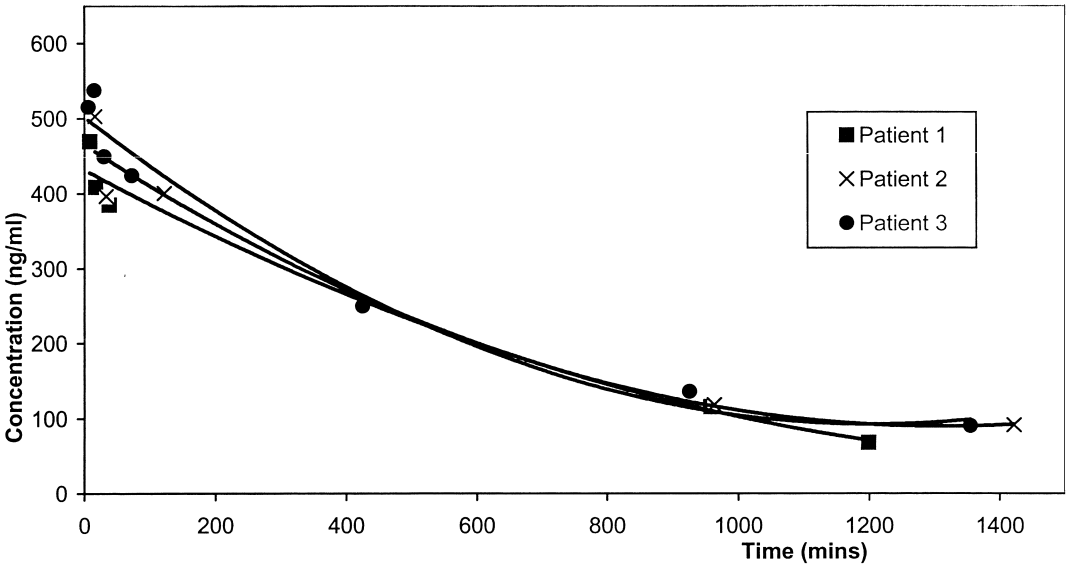

Fig. 4. Representative pharmacokinetic study of tramadol in three of the patients.

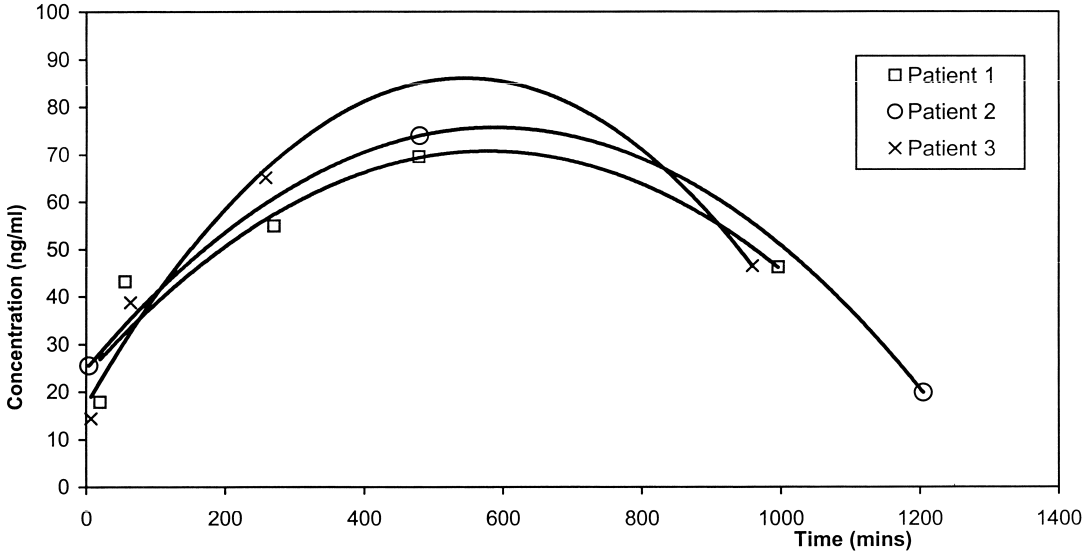

Fig. 5. Representative concentration–time profile of M1 in three of the patients.

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References

[1] R.B. Raffa, E. Friderichs, W. Reimann, R.P. Shank, E.E. Codd, J.L. Vaught, J. Pharmacol. Exp. Ther. 260 (1992) 275.

[2] K.S. Lewis, N.H. Han, Am. J. Health Syst. Pharm. 54 (1997) 643.
[3] M. Eichelbaum, B. Evert, Clin. Exp. Pharmacol. Physiol. 23 (1996) 983.
[4] R. Lee, D. McTavish, E.M. Sorkin, Drugs 46 (1993) 313.
[5] M. Nobilis, J. Pastera, P. Anzenbacher, D. Svoboda, J. Kopecky, F. Perlik, J. Chromatogr. B 681 (1996) 178.
[6] R. Becker, W. Lintz, J. Chromatogr. 397 (1986) 213.
[7] W. Lintz, H. Uragg, J. Chromatogr. 341 (1985) 65.
[8] E.C.Y. Chan, P.C. Ho, J. Chromatogr. B 707 (1998) 287.
[9] FDA guideline for submitting samples and analytical data for method validations, February 1987.
[10] R.J.M. Vervoort, F.A. Maris, H. Hindriks, J. Chromatogr. 623 (1992) 207.
[11] J.W. Dolan and L.R. Snyder, in: J.W. Dolan and L.R. Snyder (Eds.), Troubleshooting LC Systems, New Jersey, 1989, Chapter 3, p. 292.
[12] P. Hubert, P. Chiap, M. Moors, B. Bourguignon, D.L. Massart, J. Chromatogr. A 665 (1994) 87.
[13] Reviewer guidance: validation of chromatographic methods, Centre for Drug Evaluation and Research, Food and Drug Administration, November 1994.
[14] H.T. Karnes, G. Shiu, V.P. Shah, Pharmacol. Res. 8 (1991) 421.