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Method development and validation of a high-performance liquid chromatographic method for tramadol in human plasma using liquid–liquid extraction

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Abstract

An HPLC system using a simple liquid–liquid extraction and HPLC with UV detection has been validated to determine tramadol concentration in human plasma. The method developed was selective and linear for concentrations ranging from 10 to 2000 ng/ml with average recovery of 98.63%. The limit of quantitation (LOQ) was 10 ng/ml and the percentage recovery of the internal standard phenacetin was 76.51%. The intra-day accuracy ranged from 87.55 to 105.99% and the inter-day accuracy, 93.44 to 98.43% for tramadol. Good precision (5.32 and 6.67% for intra- and inter-day, respectively) was obtained at LOQ. The method has been applied to determine tramadol concentrations in human plasma samples for a pharmacokinetic study. © 2002 Elsevier Science B.V. All rights reserved.

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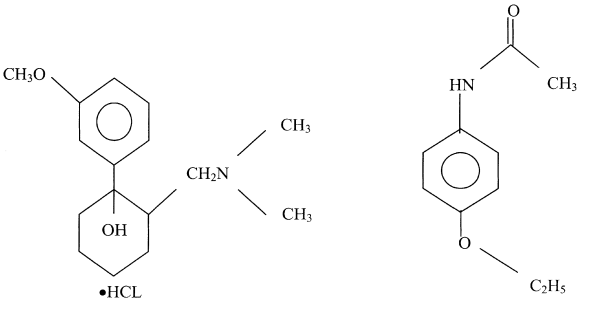
1. Introduction

Tramadol hydrochloride, (±)-*trans*-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclo-hexanol (Fig. 1a), is a μ-receptor agonist used in the treatment of mild to moderate pain [1]. Its therapeutic concentration is in the range 100–300 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].



a) Tramadol hydrochloride b) Phenacetin

Fig. 1. Chemical structures of (a) tramadol hydrochloride and (b) phenacetin (internal standard).

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The biotransformation of tramadol in man has been shown to be carried out by the isoenzyme cytochrome P4502D6 (CYP2D6). The main metabolites have been found to be *N*-desmethyltramadol and *O*-desmethyltramadol, of which only the latter is pharmacologically active [3].

The methods described for the determination of tramadol in biological samples involve gas chromatography (GC) with a nitrogen selective detector [4] and GC–mass spectrophotometry (GC–MS) [5]. These require tedious recrystallisation, synthetic and purification processes. More recent progress includes the use of a fluorescence detector [6] and capillary zone electrophoresis [7].

Recently, we reported a solid-phase extraction method for the detection of tramadol in human plasma using HPLC and UV detection [8]. Here, we describe another validated method using simple liquid–liquid extraction for the determination of tramadol in human plasma using low wavelength UV detection on an HPLC. The method developed was applied to a pharmacokinetic study of intravenous (i.v.) tramadol injections.

2. Experimental

2.1. Chemicals and reagents

Tramadol standard was a gift from Grunenthal (Aachen, Germany). The internal standard used was phenacetin (Fig. 1b) (*N*-[4-ethoxyphenyl]acetamide) purchased from Sigma (St Louis, MO, USA). Potassium dihydrogen phosphate (KH_2PO_4) was of analytical reagent-grade from Merck® (Darmstadt, Germany). Methanol, acetonitrile, ethylacetate, diethylether, dichloromethane, chloroform, hexane and triethylamine were of HPLC grade (Merck). Water was doubly distilled and purified using the Water Prodigy System (Labconco®, MO, USA).

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 analytical column was a LiChrosorb reversed-phase (RP-18) column (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, PA, USA) (5 μm: 4×4 mm I.D.). The control of the HPLC system and data collection was by an IBM compatible computer equipped with Unipoint® software (version 2.00). A 10-μl injection was used each time.

2.3. Method development and optimisation

Several parameters were varied to determine the one(s) that give the best separation. With each change in mobile phase carried out, the column was allowed to be re-equilibrated with the new solvent with at least 20 column volumes before the next separation was attempted [9].

UV spectrophotometry of both of the drugs were investigated using a Shimadzu UV–Vis Recording Spectrophotometer (Model UV-160A). From its readings, several possible wavelengths were selected to be used with the HPLC system: 273, 230, 220, 218, 210, 200 nm.

The percentage of the mobile phase organic solvents was varied using different combinations of acetonitrile–(0.01 *M*) phosphate buffer (35:65, 30:70, 25:75, 20:80, 18:82 and 15:85).

Using the best combination of acetonitrile/phosphate buffer determined above, phosphate buffer concentration was further varied between 0.01 and 0.05 *M*.

Triethylamine was the only suitable organic modifier present in our laboratory. It was added (v/v) to the mobile phase in varying concentrations: 0.05, 0.1, 0.5 and 1%.

The pH of the mobile phase was varied between a safe pH range of 2.5 and 7 so as not to allow the bonded phase to be stripped off [9], preserving the column life.

2.4. Preparation of stock solutions and working standard solutions

Stock solutions of tramadol (100 μg/ml) were prepared monthly by dissolving 10 mg of each drug, respectively in 100 ml methanol and storing at 4 °C. Tramadol concentrations in the working standard solutions chosen for the calibration curve were 0.01, 0.05, 0.125, 0.25, 0.5, 1.0 and 2.0 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 storing at 4 °C. From here, a 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 use. Quality control samples were prepared by spiking drug-free human plasma with the different concentrations of working standard solutions of tramadol while phenacetin was added at 1 μg/ml throughout.

2.6. Liquid–liquid extraction

Common solvents available in our laboratory were tested in various combinations for the extraction of tramadol and phenacetin. They were: (a) dichloromethane+hexane (2:3), (b) diethyl ether+hexane (2:3), (c) ethylacetate+hexane (1:4), (d) chloroform+hexane (1:4) and (e) hexane alone. Percentage recoveries of each combination was calculated to select the solvent combination that gave the best recoveries for both drugs.

The best solvent combination out of the five was further investigated to determine the effect of pH on extraction efficiency (refer to Section 3.2). Here, tramadol and phenacetin were spiked into seven clean tubes and dried. Plasma was added to dissolve the drugs, and its pH adjusted by adding a few drops of 0.1 *M* sodium hydroxide or 0.1 *M* acetic acid to make the solution more basic or acidic, respectively prior to the extraction. The solution was thoroughly vortexed and the pH checked and recorded. These solutions were then treated to the extraction conditions. Briefly, 4 ml of the solvent combinations from (a) to (e) were added, respectively into the plasma and the solutions were vortexed for 30 s. They were then subjected to centrifugation at 3500 *g* for 15 min. The organic layer was transferred into V-tubes. The tubes were then passed through a stream of nitrogen for drying and 50 μl of the mobile phase was added for reconstitution before injection into the HPLC system.

A graph of percentage recovery of tramadol and the internal standard was plotted against variation in pH. From this graph, the pH that gave the highest percentage recoveries for both drugs was selected and used for method validation (refer to Section 3.2).

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 such as tramadol; (4) ionization suppression between drug molecule and residual silanol groups on the surface of silica.

The following parameters were determined to validate the analytical method developed: selectivity, linearity, range, precision, accuracy, limit of quantitation (LOQ), recovery and ruggedness [10]. Peak purity for both drug and metabolite was further confirmed by means of a Gilson photo-diode array detector.

3. Results and discussion

3.1. Optimisation of chromatographic conditions

From the spectrophotometry, tramadol was found to absorb strongly between 200 and 220 nm and demonstrates a smaller peak at 273 nm. Phenacetin absorbs at 249 nm and with a smaller peak between 200 and 220 nm; 218 nm was selected for the UV detection.

Acetonitrile–phosphate buffer combinations of 35:65, 30:70, 25:75, 20:80, 18:82 and 15:85 demonstrated smaller area counts with decrease in the organic composition. Here, we have chosen the 30% acetonitrile and 70% phosphate buffer combination.

The choice of buffers that do not absorb at low wavelength is limited, with the inorganic phosphates (such as phosphate buffers) being the most suitable [9]. The 0.01 *M* phosphate buffer was chosen because it was sufficient in concentration to avoid band tailing.

Table 1
System suitability parameters

Compound	Retention time (min)	Capacity factor (k')	Resolution (R_s)	N (plate count)	T (Tailing factor)
Tramadol	11.80	2.12	3.23	12 548.36	1.78
Phenacetin	16.50	3.07	1.80	52 134.40	1.69

Adding triethylamine (TEA) reduced asymmetry and retention on the column. However, there was no improvement in peak symmetry for TEA concentrations greater than 0.1%.

The area count for tramadol increased with increasing pH. However, a pH of 6.0 or above produced band tailing. Therefore, pH 3.0 was selected giving the best area count for both drugs with the least band tailing.

Average retention times for tramadol and the internal standard were 11.80 and 16.50 min, respectively (total run time of 19.00 min). There were no interfering peaks from the plasma matrix in the analysis. The peaks for both drug and metabolite were found to be pure (peak purity more than 99%) further determined by a Gilson photo-diode array detector.

Using the parameters optimized, system suitability parameters were calculated (Table 1) and compared against that recommended by the Centre for Drug Evaluation and Research (CDER) [11].

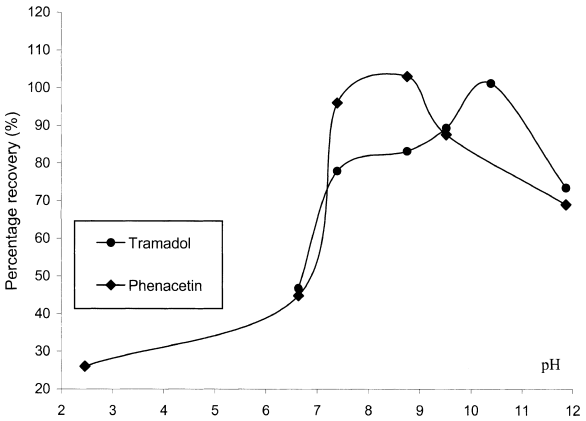

Fig. 2. Percentage recoveries of tramadol and phenacetin at different plasma pH values using ethylacetate/hexane (1:4) as the extracting solvents.

3.2. Liquid–liquid extraction

Table 2 shows that ethylacetate/hexane (1:4) combination is the best for extracting both drugs simultaneously. This solvent combination was thus selected for our liquid–liquid extraction method. Ethylacetate is a very popular extracting solvent because of its high polarity, cheapness and volatility [12].

When the pH of the plasma was varied, it was discovered that the best pH for the ethylacetate–hexane combination that gives the highest percentage recovery for both drugs was 10.6 (Fig. 2). At this pH, an excellent percentage recovery of approximately 102% was obtained for tramadol supporting the findings of Lintz and Uragg [5] who showed that extraction recovery was almost 100% if alkaline

Table 2
Percentage recoveries of tramadol and phenacetin by liquid–liquid extraction method using different solvents

Solvent combination	Percentage recovery of tramadol	Percentage recovery of phenacetin
1. Dichloromethane+hexane (2:3)	77.9	75.8
2. Diethyl ether+hexane (2:3)	102.0	65.0
3. Ethylacetate+hexane (1:4)	103.0	75.0
4. Chloroform+hexane (1:4)	88.0	68.0
5. Hexane alone	63.0	13.0

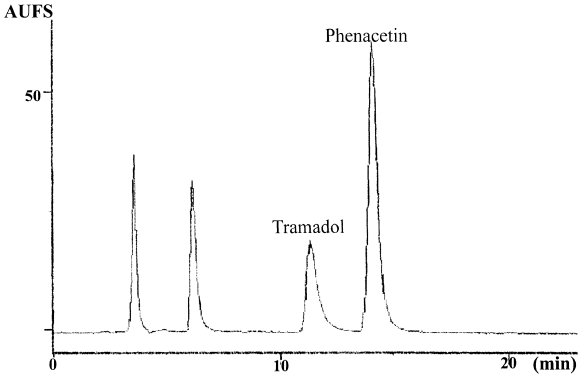

Fig. 3. Representative chromatogram of human plasma spiked with tramadol (0.5 μg/ml) and internal standard (1.0 μg/ml).

aqueous phases of pH value of more than 9 were extracted with hexane. As a result, the plasma was alkalinised to pH 10.6 in our method.

Our HPLC assay was found to be selective and free from other possible interferences (Fig. 3).

A calibration graph was constructed in duplicate in the range of 10–2000 ng/ml for tramadol (Table 3) ($n=7$). The linearity of the calibration graph was demonstrated by the good determination coefficient (r^2) obtained for the regression line.

The precision of the test was evaluated by determining the inter-day and intra-day relative standard deviation (RSD) of the measured peak area ratios for different concentrations. Accuracy was expressed as the mean percentage of analyte recovered in the assay.

The LOQ, defined in the presented experiment as the lowest plasma concentration in the calibration curve that can be measured routinely with acceptable precision (RSD<20%) and accuracy (80–120%) was 10 ng/ml (Table 4). In this experiment, LOD and LOQ were not defined in terms of 2, 3 or 10 times noise level which is no longer practical as noise levels vary from detector to detector [11]. The LOD was found to be similar to the LOQ (10 ng/ml).

Recovery was quantified by finding the ratio of the slopes of the calibration curves for extracted to non-extracted samples (Table 5). For phenacetin, its area count in the extract was taken against that in the standard.

The formal ruggedness test was conducted when the method was validated on another HPLC system (Waters®, CA, USA) by another analyst (results not shown). Using the optimised parameters, the method was found to be equally robust.

The stability study of tramadol has been carried out extensively before [8] and was not repeated here.

Table 3
Linearity data for tramadol—calibration standard response values

Calibration	Set 1 ($n=7$)	Set 2 ($n=7$)	SD	SE	Mean
Day 1					
Intercept	−0.0297	−0.0624	0.0231	0.0164	−0.0460
Slope	0.0080	0.0086	0.0004	0.0003	0.0083
Correlation coefficient (r^2)	0.9997	0.9997			0.9997
Day 2					
Intercept	−0.9017	−0.2848	0.4362	0.3084	−0.5932
Slope	0.0120	0.0106	0.0010	0.0007	0.0113
Correlation coefficient (r^2)	0.9907	0.9994			0.9924
Day 3					
Intercept	−0.0502	0.0428	0.0658	0.0465	−0.0037
Slope	0.0063	0.0062	0.0000	0.0000	0.0062
Correlation coefficient (r^2)	0.9998	0.9980			0.9989

SD, standard deviation; SE, standard error.

Table 4
Precision and accuracy of the method for the determination of tramadol in human plasma

Concentration (ng/ml)	Mean	SD	Precision RSD (%)	Accuracy (%)	Percentage difference
Inter-day (between batch) ($n=5$)					
10	9.84	0.66	6.67	98.43	−1.57
30	28.95	2.64	9.14	96.49	−3.51
200	186.88	8.60	4.60	93.44	−6.56
1500	1448.56	24.95	1.72	96.57	−3.43
Intra-day (within batch) ($n=2$)					
10	9.10	0.48	5.32	87.55	−12.45
30	31.39	0.57	1.81	105.99	5.99
200	187.13	12.05	6.44	89.31	−10.69
1500	1432.05	4.92	0.34	95.24	−4.76

Table 5
Recovery study for Tramadol (by ratio of slopes)

	Combined standard curve	Combined extraction curve
Intercept	−0.0266	−0.0676
Slope	0.0073	0.0072
Correlation coefficient (r)	0.9995	0.9919
Recovery by ratio of slopes	=0.0072/0.0073=98.63%	

The method has been successfully applied to the analysis of samples from a pharmacokinetic study consisting of 67 patients. Fig. 4 represents the concentration–time profile (fitted by a log-linear relationship) in three of the subjects.

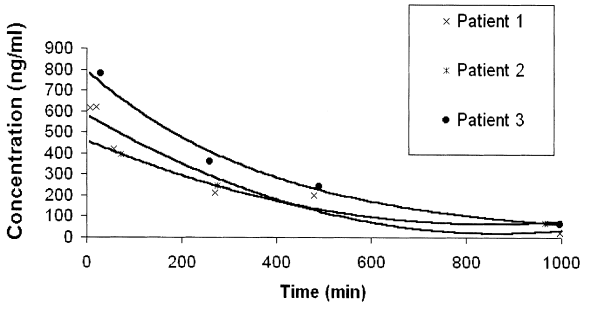

Fig. 4. Representative concentration–time profile for tramadol in three of the patients following a single 100 mg i.v. bolus dose of the drug.

4. Conclusions

The assay for tramadol 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.

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