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Investigation of the pharmacokinetics and determination of tramadol in rabbit plasma by a high-performance liquid chromatography–diode array detector method using liquid–liquid extraction

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Abstract

An HPLC system using a new, simple and rapid liquid–liquid extraction and high-performance liquid chromatography–diode array detector method (HPLC–DAD) detection was validated to determine tramadol concentration in rabbit plasma. The method described was applied to a pharmacokinetic study of intravenous tramadol injections in rabbits. The extraction with ethylacetate yielded good response. The recovery of tramadol from plasma averaged 90.40%. Serial plasma samples were obtained prior to, during and after completion of the infusion for determination of tramadol concentrations. Tramadol concentrations were measured using reverse-phase high-performance liquid chromatography and pharmacokinetic application with intravenous tramadol in rabbits revealed that tramadol followed one-compartment open model. Maximum plasma concentration (C_{max}) and area under the plasma concentration–time curve (AUC) for tramadol were 14.3 μg mL^{-1} and 42.2 μg h mL^{-1}, respectively. The method developed was successfully applied to a simple, rapid, specific, sensitive and accurate HPLC method for investigation of the pharmacokinetics of tramadol in rabbit plasma.
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Keywords: Tramadol; Pharmacokinetics; HPLC

1. Introduction

Tramadol hydrochloride, (1*RS*,2*RS*)-2-[(dimethylamino) methyl]-1-(3-methoxyphenyl) cyclohexanol HCl (Fig. 1), is a centrally acting opioid analgesic in wide spread clinical use throughout the world [1]. It is a synthetic analogue of codeine but has a relatively low affinity for opiate receptors and has not been classified as a controlled substance [2].

The tramadol contains a weakly absorbing chromophore in its molecule and it has been determined by high-performance liquid chromatography (HPLC) with UV detection [3–8], fluorescence detection [9,10] or electrochemical detection [11] and in pharmaceutical [4], urine [8] or blood plasma [9,7]. Gas chromatography with nitrogen-selective detector [12], gas chromatography–mass spectrometry [13,14], capillary electrophoresis [15,16] or UV-spectrophotometry [17] was also used for determining tramadol. Although, the pharmacokinetics of tramadol has been described in rabbit plasma by capillary gas chromatography [18], pharmacokinetics of tramadol not has been described in rabbits by HPLC. But no HPLC method with diode array detection (DAD) system for the investigation of the pharmacokinetics and the quantitation of tramadol in rabbit plasma has been reported in the literature.

The aim of this work was to improve a method using a sensitive and specific DAD system, and to validate the whole analytical method, according to international guidelines in order to obtain an efficient tool for further pharmacokinetic studies in rabbit plasma.

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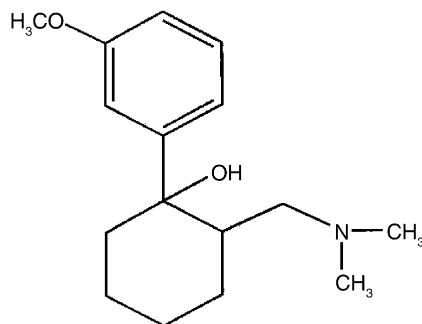

Fig. 1. Chemical structure of tramadol.

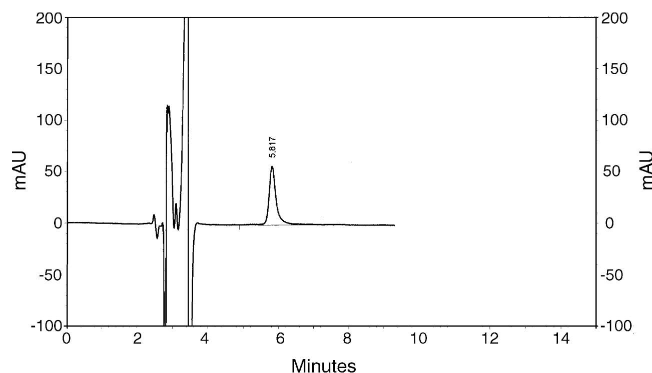

Fig. 2. Chromatogram of tramadol standard of 10 μg mL^{-1} in HPLC–DAD system.

2. Experimental

2.1. Instruments

The HPLC system consisted of a Thermoquest Spectra System P 1500 isocratic pump coupled with a Spectra System UV 6000 LP photodiode array detection system, a Spectra System AS 3000 autosampler, a SCM 1000 vacuum membrane degasser, a SN 4000 system controller. The detector was set to scan from 200 to 500 nm and had a discrete channel set at 218 nm, which was the wavelength used for quantification.

2.2. Chromatographic conditions

The analytical column was a Phenomenex Bondclone reversed-phase C_{18} column with particle size of 10 μm (300 mm × 3.90 mm i.d.). The column temperature was 30 °C. The control of the HPLC system and data collection was by a Vestel computer equipped with Chromquest software. The flow-rate was 1 mL min^{-1} and an injection volume of 25 μL was used.

The percentage of the mobile phase organic solvents was varied using different combinations of acetonitrile–0.01 M phosphate buffer (40:60, 30:70, 25:75, 20:80, 15:85 and 10:90). The 0.01 M phosphate buffer was chosen because it was sufficient in concentration to avoid peak tailing. The best combination of the mobile phase, consisting of acetonitrile–0.01 M phosphate buffer (25:75, v/v) with the addition of 0.1% triethylamine was chosen and filtered through a 0.47-μm nylon membrane filter and degassed ultrasonically before use for 20 min. Because the drug standard had a good peak shape and symmetry and plasma samples were good examples of peak tailing in the mobile phase combination. The pH of the mobile phase was adjusted to 3 and the solution was prepared fresh daily. In addition, we observed excellent base line for the mobile phase in our chromatograms. pH 3.0 was selected giving the best area count for drug with the least band tailing as the pH of the mobile phase. Besides, the washout period was used in the time interval 15 min (methanol–water mobile phase, 70:30, v/v). Adding triethylamine (TEA) reduced asymmetry and retention on the column. Retention time for tramadol was determined as 5.82 min for our HPLC–DAD system (see Fig. 2).

2.3. Reagents and standards

Tramadol HCl standard was a gift from Grünenthal (Aachen, Germany). Contramal ampoules were obtained from Department of Anaesthesia, Faculty of Medicine, Atatürk University. HPLC grade methanol, acetonitrile and triethylamine were purchased from Merck. All other chemicals were obtained from commercial sources and were of analytical grade. Buffer solution (potassium dihydrogen phosphate, KH_2PO_4) was prepared with deionised water.

2.4. Preparation of plasma standards and controls

A standard stock solution containing tramadol was prepared monthly in methanol at a concentration of 100 μg mL^{-1} and kept stored at 4 °C. The eight standard solutions from 0.5 to 40 μg mL^{-1} (0.5, 1, 3, 5, 10, 20, 30 and 40 μg mL^{-1}) in methanol were made by a serial dilution. A calibration graph was constructed in the range of 0.5–40 μg mL^{-1} for tramadol ($n = 6$). Then, plasma standard solutions were prepared by spiking into drug-free rabbit plasma with different working standard solutions, which were then further diluted to give final concentrations of between 0.5 and 40 μg mL^{-1} of tramadol for the calibration curve. Plasma control samples were prepared from a separate stock solution at concentrations of 3, 5, 20 and 40 μg mL^{-1}.

2.5. Plasma sample preparation procedure

Rabbit plasma (0.2 mL) was placed in a 12 mL capacity glass tube. The plasma was alkalinised by adding a few drops of 0.1 M NaOH. Thus, the pH of the mixture was adjusted to 11 with NaOH, followed by vortexing. Standard solution (1 mL) was added into the plasma and the solution was thoroughly vortexed. Then, 4 mL ethylacetate was added into the plasma and the solution was vortexed for 15 min. The sample was centrifuged for 20 min at 4000 rpm. The organic phase was transferred into another glass tube and evaporated to dry-

ness at 40 °C under a stream of nitrogen. The dried residue was reconstituted in 1 mL of methanol. All samples were filtered through a Phenomenex membrane of 0.45-μm pore size (25 mm filter) before injection. The sample of 25 μL was injected into the HPLC system.

2.6. Application of the method

This validated method was then applied to determination of tramadol concentrations in two rabbits after intravenous administration of 100 mg of tramadol in ampoules of Contramal. These two male New Zealand white rabbits (approved) in weighing approximately 3.2 and 3.75 kg obtained from Atatürk University, The Medical Experimental Research and Application Centre, were used for the rabbit plasma samples. After placing the animals in a restraining box, cannulations of the auricular artery and vein in the opposite ears were done. A 1 mL volume of blood was obtained from the artery at time zero and at 15, 25, 40, 60, 120, 180, 240, 300 and 480 min after 10 mg/kg intravenous administration of tramadol to rabbits [19]. However, the other rabbits were died in 30 min–1 h after 30 mg/kg i.v. administration of tramadol [18]. The blood was collected into heparinized tubes. Rabbit plasma were obtained by centrifugation at 6000 rpm for 15 min and immediately frozen at −20 °C until assay. Frozen rabbit plasma samples were left on the bench to thaw naturally and were vortexed prior to their use. A typical chromatogram of blank rabbit plasma is shown in Fig. 3a. The typical chromatograms of plasma samples collected from rabbit at 60 and 480 min after intravenous administration of tramadol is shown in Fig. 3b and c.

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) ionization suppression between drug molecule and residual silanol groups on the surface of silica.

It is known that HPLC–DAD is a highly effective screening method. Criterion for idendification of the analyte is that the maximum absorption wavelength in the UV spectrum of the analyte should be the same as that of the standard material within ±2 nm. The use of the photo-DAD also confers the advantage of identifying the analyte both by retention time and UV spectrum.

The following parameters were determined for the validation of analytical method developed for tramadol in rabbit plasma: linearity, precision, accuracy, limit of quantitation, recovery and stability [20].

2.8. Linearity

Five-level calibration series with six analyses at each concentration level were measured on-line statistical processing of the calibration analyses by the least squares method was performed automatically using the Chromquest Software.

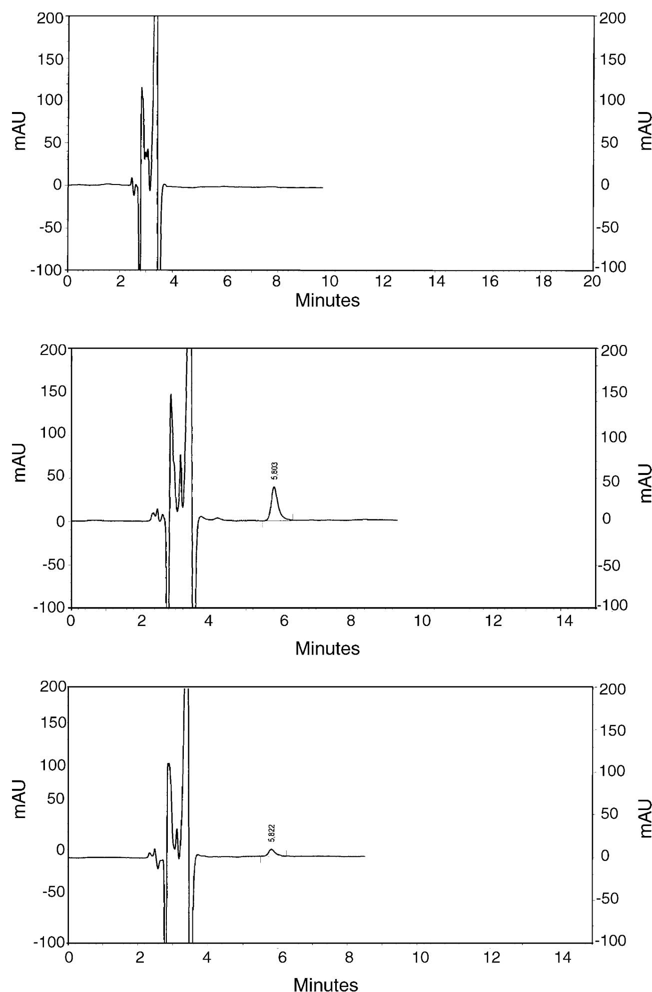

Fig. 3. Chromatograms obtained from (a) drug-free rabbit plasma; rabbit administrated an infusion of tramadol at (b) 60 min and (c) 480 min postinfusion.

2.9. Precision and accuracy

The precision of a quantitative method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings. It is measured by repeatedly injecting a ready-made sample pool and expressed as relative standard deviation of the results. The accuracy of this analytic method was determined by a method calculated as percent of mean deviation from known concentration [(concentration found − known concentration) × 100/known concentration].

3. Results and discussion

3.1. Linearity

The linearity of calibration graphs was demonstrated by the good determination coefficients (r^2) obtained for

Table 1
Summary of assay precision and accuracy data of the method for determination of tramadol in rabbit plasma ($n = 6$)

Intra-day				Inter-day		
Added concentration ($\mu g\,mL^{-1}$)	Found concentration ($\mu g\,mL^{-1}$)	R.S.D. (precision, %)	Accuracy[a] (relative error, %)	Found concentration ($\mu g\,mL^{-1}$)	R.S.D. (precision, %)	Accuracy[a] (relative error, %)
3	2.78	4.43	−7.33	2.81	8.85	−6.33
5	4.66	2.92	−6.80	4.50	3.20	−9.93
20	17.97	2.34	−10.15	18.76	4.34	−6.20
40	36.62	2.20	−8.45	36.18	1.51	−9.55

[a] Accuracy = [(found − added)/added] × 100; R.S.D.: relative standard deviation.

Table 2
Summary of assay recovery of tramadol in rabbit plasma ($n = 6$)

Added concentration ($\mu g\,mL^{-1}$)	Found concentration ($\mu g\,mL^{-1}$)	Recovery ratio[a] (%)	Accuracy relative error (%)	R.S.D. (%)
3	2.69	89.67	−10.33	7.50
5	4.51	90.20	−9.80	3.26
20	17.97	89.85	−10.15	4.70
40	36.74	91.86	−8.15	1.94

[a] Mean values.

the regression line. The mean regression equation is $y = 9 \times 10^{-6}x - 1.757$ (0.9909). The regression equations were calculated from the calibration graphs, along with the standard deviations of the slope (S_b) and intercept (S_a) on the ordinate. S_a, standard deviation of intercept of regression line is 1.6×10^{-2} and S_b, standard deviation of slope of regression line is 5.2×10^{-7}.

3.2. Precision and accuracy

Precision and accuracy were determined on spiked rabbit plasma samples at eight concentrations with respect to a calibration graph prepared every day ($n = 6$). The precision of the method was evaluated as the intra- and inter-day R.S.D. of the measured peak areas by assaying spiked plasma samples at four different concentrations. All samples for these purposes were freshly prepared including preparing the standard solution from the same stock solution (100 $\mu g\,mL^{-1}$). Six replicates from each pool were assayed on each of 3 days so that both intra- and inter-day precision and accuracy could be determined. The results for tramadol in rabbit plasma are shown in Table 1. Precision and accuracy studies in plasma showed an acceptable the R.S.D. values and the relative errors were ≤10% and high accuracy for both intra- and inter-day ($n = 6$) studies (≤10%).

3.3. Limit of quantification and detection

The limit of quantification (LOQ), defined in the presented experiment as the lowest plasma concentration in the calibration curve that could be measured routinely with acceptable precision (R.S.D. < 20%) and for all concentrations of compounds the accuracy was higher than 90%. The limit of quantitation for tramadol determination in rabbit plasma was 0.4 $\mu g\,mL^{-1}$. The limit of detection for tramadol defined, as a signal-to-noise ratio of 3, was 0.25 $\mu g\,mL^{-1}$ of plasma.

3.4. Recovery

The extraction recovery of tramadol in rabbit plasma was determined at all levels of the calibration graph by comparing the data obtained by the direct injection of standard aqueous solutions to those obtained after the whole extraction procedure. The one-step extraction procedure was fairly rapid. Ethylacetate solvent was selected for our liquid–liquid extraction method. Ethylacetate is a very popular extracting solvent because of its high polarity and volatility. The solvent ethylacetate gave good recovery and the absolute recoveries of tramadol from plasma were between 89 and 92% as shown in Table 2. No interfering peak and ghost peak were detected in the blank plasma and plasma samples from the animals in analysis.

3.5. Stability

The stock solution was stable for at least 4 weeks when stored at 4 °C. No change in the stability of the stock solution over one month was observed. The stability of tramadol at different temperatures indicated that it was stable at 4 and 25 °C for at least 4 weeks (on day 0, 7, 14, 21 and 28). The results indicated that no significant degradation occurred at 4 weeks as shown in Table 3.

3.6. Pharmacokinetic study

Pharmacokinetic parameters such as half-life, apparent distribution volume, clearance and area under the plasma concentration–time graph were calculated by standard formulas [21]. Tramadol elimination after a single intravenous

Table 3
Stability of tramadol stock solution under different storage conditions (at $10\,\mu g\,mL^{-1}$)

Temperature (°C)	Day				
	0	7	14	21	28
4	98	101	99	102	94
25	102	104	97	99	96

The data represents the recovery (%).

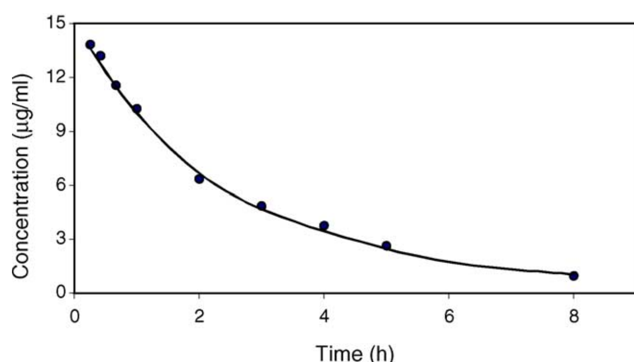

Fig. 4. Plasma concentration–time profile of tramadol for the mean of two rabbits receiving intravenous tramadol.

injection was regarded a first-order reaction kinetic following the equation $C = C_0e^{-kt}$, where C represents tramadol concentration in any time points, C_0 is the concentration when time (t) equals zero and k_{el} is the first-order rate constant expressed in units of concentration per hour. The elimination rate constant k_{el} and half-life $t_{1/2}$ were calculated from the slope of the linear regression line in the elimination phase of the semi-logarithmic plot of plasma concentration versus time as $\log C = \log C_0 - k_{el}\,t/2.3$. Results were expressed as V_d, Cl, k_{el}, $t_{1/2}$ and AUC as they apply to one-compartment open linear model. Half-life $t_{1/2}$ was calculated as $0.693/k_{el}$. The area under the plasma concentration–time curve $AUC_{0-8\,h}$ was calculated on the experimental values (trapezoidal rule) with extrapolation to infinity, obtained by the elimination rate constant. Distribution volume V_d and clearance Cl were calculated as D/C_0 and V_dk_{el}, respectively.

Table 4
Pharmacokinetic parameters of tramadol in rabbit after intravenous tramadol injection

Parameter	Result	Unit
C_{max}	14.3	$\mu g\,mL^{-1}$
$t_{1/2}$	2.04	h
$AUC_{0-8\,h}$	42.2	$\mu g\,h\,mL^{-1}$
Cl	824	$mL\,h^{-1}$
k_{el}	0.339	h^{-1}
V_d	2430	mL

C_{max}: maximum plasma concentration; $t_{1/2}$: half-live; $AUC_{0-8\,h}$: the area under the plasma concentration–time curve; Cl: clearance; k_{el}: elimination rate constant; V_d: distribution volume.

4. Conclusions

It has good precision and accuracy that this sensitive method has been studied in plasma. Plasma samples were extracted with ethylacetate in a one-step liquid–liquid extraction. And only one-step extraction procedure was fairly rapid and advantage. In the developed method, 0.2 mL of plasma was used in the sample preparation. With regard to plasma samples, the extraction method is simpler and in smaller volume of plasma sample than others reported previously [3,6,11–13]. Only 25 µL of the reconstituted solution (1 mL) was injected into the system. This method has been utilized in the analysis of plasma samples collected from rabbits. The new method had been successfully applied to the analysis of samples from a pharmacokinetic study consisting of two rabbits. In particular, the data from rabbits were satisfactorily fitted to a linear one-compartment open model. Fig. 4 represents the mean concentration–time profile of two rabbits.

In the developed method, the maximum plasma concentration C_{max} for tramadol was $14.3\,\mu g\,mL^{-1}$. Tramadol was well tolerated after a single intravenous infusion. Plasma tramadol concentration declined rapidly after the end of the 1 h tramadol infusion. Tramadol began to disappear from plasma after 8 h for plasma concentration–time profile fitting in previous report, but a longer half-life was obtained. The $AUC_{0-8\,h}$ and $t_{1/2}$ were $42.2\,\mu g\,h\,mL^{-1}$ and 2.04 h, as different from that of previous report [18]. The major pharmacokinetic parameters are reported in Table 4.

In previous report, the capillary gas chromatographic assay with flame ionisation detection was developed for the determination of tramadol in human, rabbit, pig and dog plasma. Pharmacokinetic application with i.v. tramadol in humans and rabbits revealed that tramadol followed a two-compartment open model with one distribution phase and one elimination phase. The distribution and elimination half-lives in humans were 1.02 and 141.9 min. The distribution and elimination half-lives in rabbits were 7.31 and 63.2 min, respectively [18].

The other method was developed for simultaneous determination of tramadol and its main active metabolite *o*-desmethyltramadol by high-performance liquid chromatography with electrochemical detection after a 5-min i.v. infusion administration of tramadol to one rat. Pharmacokinetic analysis of the data indicated a mean C_{max} of $10\,\mu g\,mL^{-1}$ for tramadol and of $850\,ng\,mL^{-1}$ for the metabolite. The mean AUCs of tramadol and ODMT were 493 and $119\,\mu g\,mL^{-1}$ min, respectively [11].

We have developed a rapid, sensitive, precise and accurate HPLC–DAD method for determination of tramadol in rabbits. To our knowledge, this is the first description of tramadol pharmacokinetics in rabbit plasma by HPLC–DAD method in the literature. It can be very useful and an alternate to performing pharmacokinetic studies in determination of tramadol for clinical use.

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