ARTYKUŁ ORYGINALNY/ORIGINAL PAPER
Otrzymano/Submitted: 04.02.2013 • Zaakceptowano/Accepted: 18.02.2013

Optimization and validation of a high-performance liquid chromatography method for the determination of tramadol hydrochloride in rabbit plasma

Danuta Szkutnik-Fiedler, Edmund Grześkowiak, Joanna Madziała
Department of Clinical Pharmacy and Biopharmacy, Poznań University of Medical Sciences, Poznań, Poland

Abstract

Background. A sensitive and specific high-performance liquid chromatography method with ultraviolet detection (HPLC-UV) has been validated to determine tramadol hydrochloride (TRM) in rabbit plasma. ***Material and methods.*** The analyte was extracted from plasma samples with ethyl acetate-n-hexane (1:4, v/v) and analyzed on a XTerra RP-8 column. The lower limit of quantification was 10 ng/ml and the percentage recovery of the TRM from plasma samples was greater than 95%. The calibration curves were linear within the range of 10-1000 ng/ml. ***Results.*** The results from analysis of quality-control samples at concentrations of 10, 50, 200 and 1000 ng/ml were indicative of good intra- and inter-day accuracy and precision. ***Conclusion.*** This method has been applied to determine TRM concentrations in rabbit plasma samples. *(Farm Współ 2013; 6: 3-9)*

Keywords: tramadol hydrochloride, high performance liquid chromatography, UV detection, plasma samples, rabbits

Introduction

Tramadol (TRM), (1RS, 2RS)-2-[(dimethyloamino)-methyl]-1-(3-methoxyphenyl)-cyclohexanol, is a centrally acting analgesic used to treat moderate to severe pain. This drug is a μ-opioid agonist that also inhibits the reuptake of noradrenaline and serotonin. Its therapeutic concentration in human is in the range 100-300 ng/ml [1,2].

Several methods for determination of TRM concentration in plasma samples have been reported [1-19]. High-performance liquid chromatography (HPLC)-based methods with mass spectrometry detection (HPLC-MS) [1,4,6-8,12-16] has been widely accepted in the determination of TRM and its metabolites in biological fluids. These techniques have achieved both the desired sensitivity and fast run time, however, MS equipment and detectors are very expensive and therefore not widely available in laboratories. HPLC with ultraviolet (UV) detection [3,17,18], fluorescence detection [8-11], diode array (DAD) detection [5,19], and gas chromatography [2] were also used for determining tramadol.

Most of the HPLC methods described previously have been validated in human plasma [1,3,5-7,9-18], therefore the aim of this study was an adaptation a HPLC method with UV detection and a validation the whole analytical procedure for further pharmacokinetic studies of TRM in rabbits. HPLC methods described by Gan et al. [17,18] were adapted to the conditions of our lab and validated in accordance with the published EMA guideline [20].

Materials and methods

- **Chemicals and reagents**

Tramadol hydrochloride (TRM), $C_{16}H_{25}O_2N{\cdot}HCl$, CAS: 27203-92-5 and phenacetin (internal standard), CAS: 62-44-2 were obtained from Sigma-Aldrich (Steinheim, Germany). Acetonitrile, n-hexane, methanol, ethyl acetate (HPLC grade) were purchased from Merck (Darmstadt, Germany). All other chemicals: sodium hydroxide, monopotassium phosphate, anhydrous potassium hydrogen phosphate, orthophosphoric acid 85% were of analytical-reagent grade (POCH, Gliwice, Poland).

- **Standard solutions**

Standard stock solutions of TRM (100 μg/ml) and internal standard phenacetin (100 μg/ml) were prepared by dissolving accurately weighed substances in bidistilled water dilution. Stock solutions were stored at 4°C and remained stable for at least 1 month. Working solutions of TRM (used for the purpose of validation and calibration) were prepared *ex tempore* by further dilution of the stock solution with bidistilled water to provide final concentrations of 10, 20, 30, 50, 70, 100, 200, 500, 700 and 1000 ng/ml for TRM in plasma.

- **Chromatographic system**

Plasma concentrations of TRM were analyzed by high-performance liquid chromatography with UV detection. The HPLC system was constructed from the following components: HPLC Waters 2695 Separations Module with autosampler, Waters 2487 Dual λ Absorbance Detector, the wavelength 218 nm, an analytical column XTerra RP-8, 250 mm × 4.6 mm, 5 μm from Waters; temperature of column 25°C, mobile phase: acetonitrile (200 ml) – 0.01 M phosphate buffer (800 ml) with addition of orthophosphoric acid 85% (5.0 ml) to obtain pH of mobile phase 3.0, flow rate of mobile phase 1.0 ml/min, isocratic analysis, volume of each injection 60 μl. Data collection and processing were carried out using Empower Pro software, v. 1154.

- **Sample preparation and extraction procedure**

For the analyses, frozen rabbit plasma samples were left on the bench to thaw naturally and were vortexed prior to use. Plasma extraction was accomplished with liquid-liquid extraction. Briefly, to 900 μl of plasma, 100 μl of *aqua pro injectione*, 5 μl of internal standard (phenacetine working solution 100 μg/ml), 0.5 ml of 0.1 M sodium hydroxide solution were added consecutively. Thus, the pH of the mixture was adjusted to 10.6 with NaOH, followed by vortexing. Then 4 ml of ethyl acetate-n-hexane solution were added in a ratio 1:4 (v/v), vortexed for 30 s, centrifuged at 2880 g for 15 min. The organic phase containing TRM and internal standard was transferred into another glass tube and evaporated to dryness at 50°C under a stream of nitrogen. The dried residue was reconstituted in 140 μl of the mobile phase and a 60 μl sample was injected into the HPLC system.

For preparing a calibration curves, a volume of 100 μl of an adequate working solution of TRM and 5 μl of internal standard (phenacetine working solution 100 μg/ml), were spiked to 0.9 ml of blank heparinised plasma, and then extracted, according to the procedure described above. Calibration curves (concentration of TRM range from 10 to 1000 ng/ml) and quality control samples were prepared fresh for each quantitative assay. The concentration of TRM in each sample was determined by the internal standard method using the peak area ratio and linear regression analysis.

- **Method validation**

➢ ***Specificity***

The specificity of the method and the probable interferences from endogenous substances was evaluated by analyzing blank drug-free plasma samples obtained from six healthy rabbits and spiked plasma samples, as well as samples from these rabbits after intravenous administration of TRM at a dose 10 mg/kg. Carry-over was assessed by injecting blank plasma sample after a high concentration of TRM plasma sample.

➢ ***Linearity***

Calibration curves (n = 9) were prepared as described above. Three spiked plasma samples were analysed at each concentration level.

The linearity of the standard curves was assessed with the slope, intercept, determination coefficient (r^2) and correlation coefficient (r).

➢ ***Recovery***

The recovery of TRM was assessed by comparing the response of five replicates of extracted quality control (QC) samples (10, 50, 200 and 1000 ng/ml) to the response of the pure standard at the same concentration level.

➢ ***Precision and accuracy***

Blank plasma samples were spiked with TRM at four concentration levels QC (10, 50, 200 and 1000 ng/ml). The intra-day accuracy and precision were determined using five replicates of QC samples on the same day; the inter-day accuracy and precision were determined by analyzing three calibration curves with five replicates of QC samples on three separate days. Intra- and inter-day precision was expressed as coefficient of variation value: $CV = [SD/C] \times 100\%$, where: SD is the standard deviation of the mean concentration of TRM and C is the mean concentration of TRM. The accuracy (Bias) for TRM was expressed

as the percentage deviation of the observed concentration from theoretical concentration. Bias = [(concentration found – concentration added)/concentration added] ×100%.

➢ ***Sensitivity: the lower limit of quantification (LLOQ) and limit of detection (LOD)***

The lower limit of quantification (LLOQ) of TRM was defined as the lowest concentration that could be measured with accuracy and precision, i.e. within ± 20% of the actual value. The lowest amount of TRM in the sample, which can be detected but not necessarily quantitated under stated experimental conditions (limit of detection, LOD), was based on SD of response and slope: LOD = 3.3 × (δ/ S) where S is the slope of the calibration curve and d is SD of blank response.

➢ ***Stability***

The stability of plasma samples (20, 90 and 400 ng/ml of TRM) under different conditions was evaluated. The short-term and long-term stability of plasma samples were assessed by determining the concentration of the samples kept at room temperature for 2, 4, 24 h, and at -30°C for 30 days, respectively. The stability of extracted plasma samples was evaluated by keeping samples at room temperature for 2, 7, 13, and 18 h. Freeze-thaw stability was determined by following three freeze-thaw cycles from -30°C to room temperature for every 24 h. The stabilities under different conditions were obtained from comparing the measured concentration with the concentration of sample at 0 h.

▪ **Animals**

Six adult healthy New Zealand White rabbits (mean weight ± SD, 3.4 ± 0.1 kg) were used in this study. Food was withheld from all animals for 12 h before drug administration and during this time free access to fresh water was provided. Eight hours after drug administration, the animals were allowed access to feed. All animals were given TRM intravenously at a dose 10 mg/kg (Poltram 100 mg/2 ml, batch No. 510804; Polpharma, Poland), between 8 a.m. and 9 a.m. Blood samples (1.5 ml) were obtained from the catheter remaining in the ear vein, prior to TRM administration (sample 0) and 1, 5, 10, 15, 30, 45, 60, 120 min and 4, 6, 8 h following administration. Blood samples were transferred into collection tubes containing lithium heparin, immediately centrifuged at 2880 g for 10 min, than the plasma was frozen at -30°C until analysis. The study was performed according to a protocol approved by the Local Ethical Committee at the University of Life Sciences in Poznan (agreement No. 71/2008), and was in accordance with the rules and guidelines concerning the care and the use for laboratory animal experiments [21].

Results and discussion

The requirements for specificity determination of TRM in rabbit plasma were fulfilled. There were no interfering peaks in blank plasma at the retention time of TRM and the internal standard. No carry-over problem was also observed. Retention time of TRM and the internal standard were 7.5 and 15.0 min, respectively. The total run time for each sample injection was 17.0 min. The chromatograms of TRM and the internal standard obtained under this assay conditions, were sharp and symmetrical. Representative chromatogram is presented in figure 1.

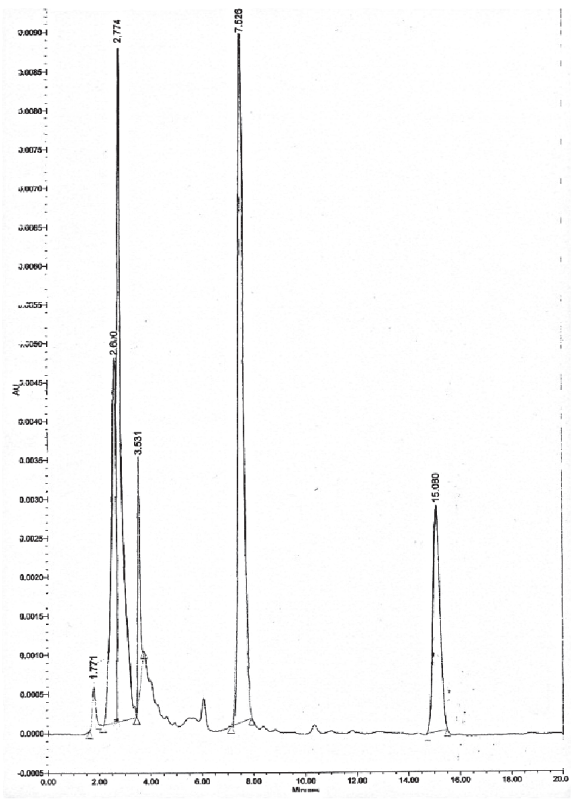

Figure 1. Representative chromatogram of rabbit plasma sample spiked with TRM and the internal standard phenacetine (retention times 7.5 and 15.0 min, respectively)

The linearity of calibration graphs was demonstrated by the good determination coefficients (r^2) of 0.999 or higher. Assay linearity in the range of the TRM concentrations to be expected was concluded from mean correlation coefficient of r = 0.9995 ± 0.00017 (n = 9); the coefficient of variation (CV) was 0.0173%. The residuals and the coefficient of variation were within ± 20% at the LLOQ and ± 15% for the rest of the tested concentrations.

Extraction recoveries for TRM was consistent, precise and reproducible throughout the validation experiments and were within the acceptance criteria [20]. The one-step extraction procedure was fairly rapid. The recovery of TRM for QC samples (10, 50, 200 and 1000 ng/ml) was greater than 99% (table 1). The recovery for the rest of the tested TRM concentrations was greater than 95%.

Precision and accuracy studies in rabbit plasma showed an acceptable the CV values. Intra-day and inter-day precision of TRM in rabbit plasma was less than 8%, and accuracy was less than 6%. The results are shown in table 1.

The lower limit of quantification (LLOQ) and limit of detection (LOD) were 10 ng/ml and 5 ng/ml, respectively. The LLOQ and LOD values obtained in our study show an optimized method for determining of TRM in plasma samples, compared to the e.g. HPLC with DAD detection [19] or gas chromatography [2].

TRM in rabbit plasma samples was stable during the storage, freeze-thaw cycles, processing and analysis. Stability experiments showed that no significant degradation occurred at room temperature for 24 h, 30 days and during the three freeze-thaw cycles for morphine plasma samples, and for 18 h for extracted plasma samples (table 2).

The method has been successfully applied to the analysis of plasma samples from a pharmacokinetic study in rabbits (n = 6, single intravenous administration of TRM at a dose 10 mg/kg). TRM began to disappear from plasma after 8 h. Mean plasma concentration-time profile of TRM in rabbits is similar to those obtained by Küçük et al. [19] (figure 2).

Summing up, the method has satisfactory specificity, linearity, accuracy and precision. The main advantage of this analysis is low LLOQ for TRM (10 ng/ml), a relative short time of analysis per sample (17 min) and high recovery of TRM from plasma samples (greater than 95%). However, by using e.g. molecularly

Table 1. Results of intra-day and inter-day validation of TRM in rabbit plasma

Spiked TRM plasma concentration (ng/ml)	Mean TRM plasma concentration found (ng/ml) ± SD	CV (%)	Bias (%)	Recovery (%)	n
Intra-day					
10	10.50 ± 0.80	7.61	5.00	110.33	5
50	52.30 ± 3.00	5.62	4.60	101.60	5
200	207.30 ± 3.80	1.83	3.65	100.69	5
1000	1008.57 ± 1.19	0.18	0.86	105.29	5
Inter-day					
10	10.48 ± 0.68	6.47	4.79	106.92	15
50	50.99 ± 3.24	6.35	1.98	104.06	15
200	206.83 ± 10.18	4.92	3.41	100.99	15
1000	1005.28 ± 3.33	0.33	0.53	101.63	15

TRM – tramadol hydrochloride, SD – standard deviation, CV – coefficient of variation defined as the ratio of the SD to the mean $CV = [SD/mean] \times 100\%$,

Table 2. The stability of TRM under different conditions.

	Short-term stability (24 h)		
Spiked TRM plasma concentration (ng/ml), n = 3	20	90	400
Recovery (%)	92.8	98.94	95.46
CV (%)	3.08	0.82	0.54
	Long-term stability (30 days)		
Recovery (%)	73.3	90.1	92.3
CV (%)	4.32	4.89	8.23
	Freeze-thaw stability after three cycles (-30°C)		
Recovery (%)	92.8	98.9	97.8
CV (%)	1.38	3.63	0.49
	Extracted sample stability (18 h)		
Recovery (%)	93.8	102.0	99.6
CV (%)	9.02	1.45	2.25

The determined concentrations at 0 h were defined as 100%. The ratio of determined concentration each time versus concentration at 0 h was calculated.

n – number of replicates of each concentration level, TRM – tramadol hydrochloride, SD – standard deviation, CV – coefficient of variation defined as the ratio of the SD to the mean $CV = [SD/mean] \times 100\%$,

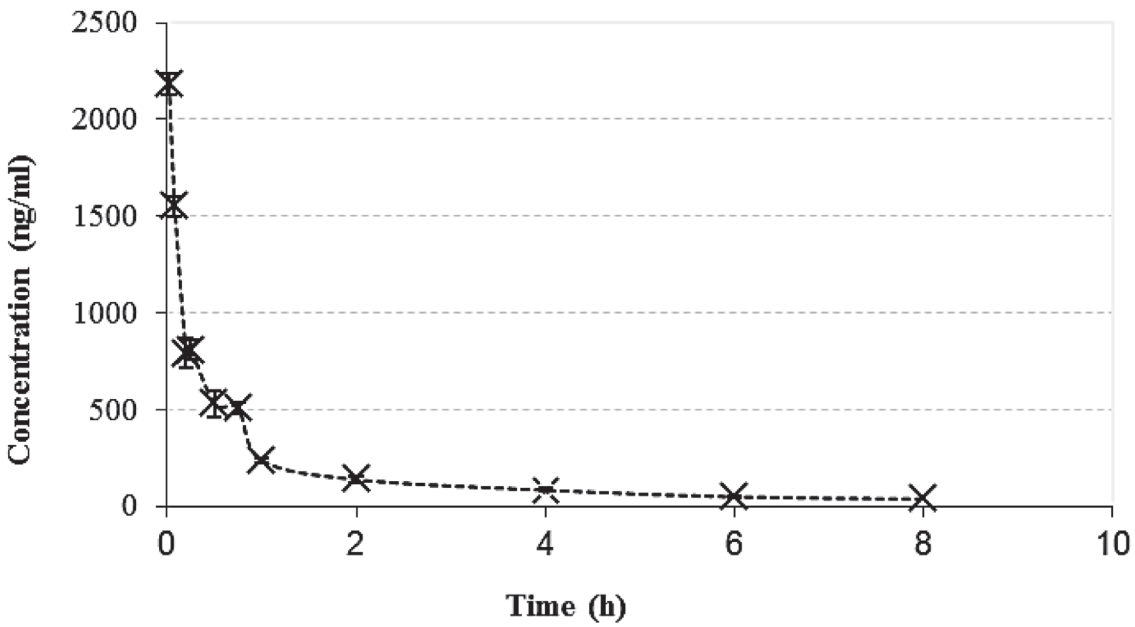

Figure 2. Mean plasma concentration-time profile of TRM after a single intravenous administration (10 mg/kg) in rabbits (n = 6)

imprinted monolithic column coupling with HPLC-UV [3] the lower limit of quantification for TRM in human plasma samples was only 1.0 ng/ml. An analytical column XTerra RP-8, 250 mm × 4.6 mm, 5 μm used in our study, allowed to obtain a shorter retention times, compared to the LiChrosorb RP-18, 250 mm × 4.6 mm, 5 μm used by Gan et al. [18] (7.5 and 15.0 min *vs.* 11.80 and 16.50 min, for TRM and the internal standard, respectively).

The study is not free of limitations: for financial reasons the active metabolite of TRM, *O*-desmethyltramadol, was not determined in this study and this method is less sensitive than e.g. HPLC-MS analysis [1,4,6-8,12-16]. However, MS detection is far more expensive than the UV method. The reagents used in our assay are inexpensive and readily available. The procedure does not involve any critical experimental conditions and the extraction procedure included only one step.

Conclusion

The procedure is sensitive and selective as well as suitable for quantification of TRM in rabbit plasma samples in pharmacokinetic studies.

Konflikt interesów / Conflict of interest
Brak/None

Correspondence address:
Danuta Szkutnik-Fiedler
Department of Clinical Pharmacy and Biopharmacy, Poznan University of Medical Sciences
14, Św. Marii Magdaleny Str.; 61-861 Poznań, Poland
☎ (+48 61) 668 78 53
d.szkutnik@wp.pl

References

1. Vlase L, Leucuta SE, Imre S. Determination of tramadol and O-desmethyltramadol in human plasma by high-performance liquid chromatography with mass spectrometry detection. Talanta 2008;75(4):1104-9.
2. Ho ST, Wan JJ, Liaw WJ, et al. Determination of tramadol by capillary gas chromatography with flame ionization detection. Application to human and rabbit pharmacokinetic studies. J Chromatogr B Biomed Sci Appl 1999;736:89-96.
3. Javanbakht M, Moein MM, Akbari-Adergani B. On-line clean-up and determination of tramadol in human plasma and urine samples using molecularly imprinted monolithic column coupling with HPLC. J Chromatogr B Analyt Technol Biomed Life Sci 2012;911:49-54.
4. Godoy AL, De Moraes NV, Martinez EZ, et al. Simultaneous analysis of tramadol, O-desmethyltramadol, and N-desmethyltramadol enantiomers in rat plasma by high-performance liquid chromatography-tandem mass spectrometry: application to pharmacokinetics. Chirality 2011;23(4):287-93.
5. Javanbakht M, Attaran AM, Namjumanesh MH, et al. Solid-phase extraction of tramadol from plasma and urine samples using a novel water-compatible molecularly imprinted polymer. J Chromatogr B Analyt Technol Biomed Life Sci 2010;878(20):1700-6.
6. Liu P, Liang S, Wang BJ, Guo RC. Development and validation of a sensitive LC-MS method for the determination of tramadol in human plasma and urine. Eur J Drug Metab Pharmacokinet 2009;34(3-4):185-92.
7. Lintz W, Uragg H. Quantitative determination of tramadol in human serum by gas chromatography-mass spectrometry. J Chromatogr 1985;341(1):65-79.
8. De Leo M, Giorgi M, Saccomanni G, et al. Evaluation of tramadol and its main metabolites in horse plasma by high-performance liquid chromatography/fluorescence and liquid chromatography/electrospray ionization tandem mass spectrometry techniques. Rapid Commun Mass Spectrom 2009;23(2):228-36.
9. Curticapean A, Muntean D, Curticapean M, et al. Optimized HPLC method for tramadol and O-desmethyl tramadol determination in human plasma. J Biochem Biophys Methods 2008;70(6):1304-12.
10. Ardakani YH, Mehvar R, Foroumadi A, Rouini MR. Enantioselective determination of tramadol and its main phase I metabolites in human plasma by high-performance liquid chromatography. Chromatogr B Analyt Technol Biomed Life Sci 2008;864(1-2):109-15.
11. Ebrahimzadeh H, Yamini Y, Sedighi A, Rouini MR. Determination of tramadol in human plasma and urine samples using liquid phase microextraction with back extraction combined with high performance liquid chromatography. J Chromatogr B Analyt Technol Biomed Life Sci 2008;863(2):229-34.
12. Musshoff F, Trafkowski F, Kuepper U, Madea B. An automated and fully validated LC-MS/MS procedure for the simultaneous determination of 11 opioids used in palliative care, with 5 of their metabolites. J Mass Spectrom 2006;41(5):633-40.
13. Zhao LM, Chen XY, Cui JJ, Sunita M, et al. Determination of tramadol and its active metabolite O-desmethyltramadol in plasma and amniotic fluid using LC/MS/MS. Yao xue xue bao = Acta Pharmaceutica Sinica 2004;39(6):458-62. Chinese.

14. Malonne H, Sonet B, Streel B, et al. Pharmacokinetic evaluation of a new oral sustained release dosage form of tramadol. Br J Clin Pharmacol 2004;57(3):270-8.
15. Ceccato A, Vanderbist F, Pabst JY, Streel B. Enantiomeric determination of tramadol and its main metabolite O-desmethyltramadol in human plasma by liquid chromatography-tandem mass spectrometry. J Chromatogr B Biomed Sci Appl 2000;748(1):65-76.
16. Ardakani YH, Rouini MR. Improved liquid chromatographic method for the simultaneous determination of tramadol and its three main metabolites in human plasma, urine and saliva. J Pharm Biomed Anal 2007;44(5):1168-73.
17. Gan SH, Ismail R. Validation of a high-performance liquid chromatography method for tramadol and o-desmethyltramadol in human plasma using solid-phase extraction. J Chromatogr B Biomed Sci Appl 2001;759(2):325-35.
18. Gan SH, Ismail R, Wan Adnan WA, Wan Z. Method development and validation of a high–performance chromatographic metod for tramadol in human plasma using liquid–liquid extraction. J Chromatogr B Analyt Technol Biomed Life Sci 2002;772(1): 123-9.
19. Küçük A, Kadıoğlu Y, Çelebi F. 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. J Chromatogr B 2005;816:203-8.
20. European Medicines Agency. Guideline on bioanalytical method validation. Committee for Medicinal Products for Human Use (CHMP) EMEA/CHMP/EWP/192217/2009, 21 July 2011.
21. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal of the European Union L276/33, 20.10.2010.