Chromatography
Separation Techniques

El-Sayed et al., J Chromatograph Separat Techniq 2011, 2:4
http://dx.doi.org/10.4172/2157-7064.1000114

Research Article Open Access

Development and Validation of High-Performance Liquid Chromatography – Diode Array Detector Method for the Determination of Tramadol in Human Saliva

Abdel-Aziz Y. El-Sayed[1]*, Khaled M. Mohamed[2], Maha A.Hilal[3], Soheir A.Mohamed[3], Khaled E. Aboul-Hagag[3] and Ahmed Y. Nasser[4]

[1]*Chemistry Department, Faculty of Science, Al-Azhar University (Assiut Branch), Egypt*
[2]*Assiut Chemical Laboratory, Medico-Legal Department, Egypt*
[3]*Forensic Medicine and Clinical Toxicology Department, Faculty of Medicine, Sohag University, Egypt*
[4]*Biochemistry department, Faculty of Medicine, Assiut University, Egypt*

Abstract

A high-performance liquid chromatography–diode array detector method (HPLC-DAD) was developed and validated for the determination of tramadol in human saliva. Samples of saliva were prepared utilizing liquid–liquid extraction with hexane-ethyl acetate (4:1, v/v). Propranolol was used as an internal standard (IS). The linear range and average recovery of tramadol were 0.25–4.00 µg/mL and 94.70%, respectively. The intra- and inter-assay precisions at low, intermediate and high concentrations were within 1.40–9.79 % and 0.77–3.98%, respectively. Intra- and inter-assay accuracies were within 98.50–107.13% and 100.44–103.89%, respectively. The proposed method was successfully applied to determine tramadol in saliva concentrations in three healthy volunteers for 24 hours after administration of 100 mg oral doses of tramadol.

Keywords: Tramadol; Determination; Saliva; HPLC-DAD; Validation

Introduction

Screening and quantitative analysis of prescription medications as well as the more common drugs of abuse is part of any systematic toxicological analysis. Standard prescription medication drug test panels include tramadol. One of the major techniques used at present for such screening are HPLC-DAD [1-3]. While blood and urine are more commonly used for these test profiles, oral fluid (saliva) is increasing in popularity as an alternative matrix, due to its ease of collection, difficulty of adulteration and improving sensitivity of analytical techniques. This is due to saliva is less complex than blood, with fewer proteins, characteristics suggesting the possibility of successful measurement with limited sample preparation, such as dilution, protein precipitation, or centrifugation and direct injection [4].

Tramadol hydrochloride (T), (±)-*trans*-2-[(dimethyl-amino) methyl]-1-(3-methoxyphenyl)cyclohexanol (Figure 1a), is an opiate drug and centrally acting analgesic agent used in the treatment of moderate to severe pain [5]. The methods described for the determination of tramadol in biological samples involve gas chromatography (GC) with nitrogen–phosphorous detection [6], flame ionization detection [7] or mass spectrometry (MS) [4,8–17]. Methods involving liquid chromatography (LC) with ultraviolet [18-21], fluorescence [22–33], electrochemical [34], diode array [35] or MS detection [36-40] were also reported. However, there is no HPLC-DAD method for determination of tramadol in saliva. Therefore, the aim of the present work is to develop simple and sensitive analytical method for the determination of tramadol in saliva.

Experimental

Instrumentation and conditions

An Agilent technologies 1200 Series quaternary pump combined with an Agilent 1200 series photo diode array detector (USA), an Agilent 1200 series vacuum degasser (USA) and an Agilent autosampler injector. Chromatographic separation was performed on a Zorbax SB-C18 analytical column (250mm×4.6 mm, 5 µm) (USA) maintained at 25ºC. The mobile phase consisted of acetonitrile:buffer (0.01M potassium dihydrogenphosphate with the addition of 0.1% triethylamine adjusted to pH 5 with 0.1 M sodium hydroxide) (40:60, v/v) at a flow rate of 0.5 mL/min. The detector was set to scan from 200 to 800 nm and had a discrete channel set at 218 nm, which was the wavelength used for quantification.

Chemicals and reagents

Tramadol hydrochloride (Figure 1a) (purity >99%), propranolol hydrochloride (purity 98%) (Figure 1b) and diethyl ether (99% GC) were purchased from Fluka Chemie GmbH, Buchs (Switzerland). Acetonitrile (99.9%), Methanol (99.9%), ethyl acetate (99.7%), triethylamine (99%) and potassium dihydrogen phosphate (98-100.5%) were purchased from Sigma-aldrich (Germany). Hexane (96%) was purchased from Merck (Germany). Sodium hydroxide (99%) was purchased from Egyptian Co. for Chemicals and pharmaceuticals. Drug free saliva was obtained from human volunteers. Phosphate buffer (0.01 M) was prepared by dissolving 1.36 g (0.01 mol) of potassium dihydrogen phosphate in 1 liter deionised water.

Preparation of calibrators and controls

A stock solution of tramadol and propranolol, was prepared monthly at a concentration of 1mg/mL in methanol and kept stored

***Corresponding author:** Abdel-Aziz Y. El-Sayed, Chemistry Department, Faculty of Science, Al-Azhar University (Assiut Branch), Egypt, E-mail: ayossefelsayed@yahoo.com

Received August 29, 2011; **Accepted** October 11, 2011; **Published** October 15, 2011

Citation: El-Sayed AAY, Mohamed KM, Hilal MA, Mohamed SA, Aboul-Hagag KE, et al. (2011) Development and Validation of High-Performance Liquid Chromatography–Diode Array Detector Method for the Determination of Tramadol in Human Saliva. J Chromatograph Separat Techniq 2:114. doi:10.4172/2157-7064.1000114

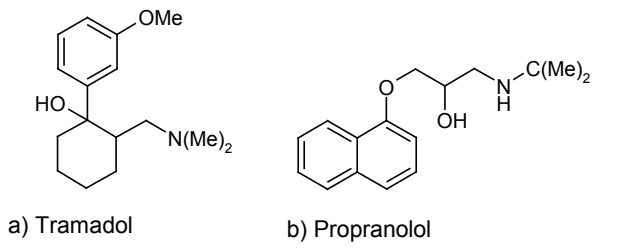

Figure 1: Chemical structures of (a) tramadol and (b) propranolol (IS).

at -20ºC. Tramadol stock solution was further diluted in methanol to get an intermediate concentration 100 µg/mL. A working standard solution of 5.00 µg/mL propranolol (used as internal standard (IS)) was prepared by diluting propranolol stock solution with distilled water.

Saliva standard solutions for the calibration curve (0.25, 0.50, 1.00, 1.50, 2.00 and 4.00 µg/mL) were made by a serial dilution of the intermediate solution with drug free human saliva. Quality control samples were prepared from a separate stock solution at concentrations of 0.75, 1.50 and 3.00 µg/mL

Extraction procedure

To 0.5 mL of saliva in a 5 mL polypropylene tube was added 50 µL of IS (5.00 µg/mL). After short, vigorous shaking 100 µL of 1M NaOH and 3 mL of hexane-ethyl acetate (4:1, v/v) were added. The tubes were then vortex mixed for 5 minutes and centrifuged at approximately 3000 rpm for 5 minutes. The organic layer was transferred to 5 mL glass tubes and evaporated to dryness in the speed vacuum concentrator. The dried extracts were reconstituted in 50 µL acetonitrile, vortex mixed for 30 seconds and 20 µL was injected into the HPLC system.

System suitability

System suitability was evaluated by equilibrate the HPLC system with the initial mobile phase composition, followed by five injections of the same standard. The system suitability parameters such as resolution (Rs), tailing factor (T) and theoretical plate number (N) were calculated by Agilent ChemStation Software for the HPLC system. The recommended values for Rs, N and T are ≥2, > 2000 and ≤ 2, respectively [41].

Calibration carve, sensitivity and specificity

Linearity of the method was investigated by evaluation of the regression line and expresses by coefficient of determination (r^2). Six calibrators in the range of 0.25–4.00 µg/mL were included in each curve and were required to meet all qualitative identification and quantification criteria. Each calibrator was calculated against the linear regression curve. Linearity was achieved with a minimal r^2 of 0.99. Negative quality control samples were analyzed after each linearity sample to evaluate potential carry-over.

Sensitivity was evaluated by determining limits of detection (LOD) and quantification (LOQ) for tramadol. The LOD was defined as signal-to-noise ratio ≥ 3 (determined by peak height) with satisfactory chromatography (peak shape and resolution) and acceptable retention time. The LOQ was defined as signal-to-noise ratio ≥ 10.

The specificity of the method was examined by analysis of blank saliva samples derived from six different human volunteers. The samples were worked up without addition of the internal standard.

Accuracy and precision

Inter- and intra-assay accuracy and precision data for tramadol were determined with the low, medium and high quality control samples (LQC, MQC and HQC). Intra-assay data were assessed by comparing data from within one run (n = 5) and inter-assay data were determined between three separate runs (n = 15). Accuracy, expressed as a percentage, was calculated by taking the difference between mean calculated concentrations and target concentrations, dividing by the calculated mean and multiplying by 100. Precision, expressed as percent relative standard deviation (%RSD), was determined by calculating the percent ratio of the standard deviation divided by the calculated mean concentration times 100.

Recovery, dilution integrity and extract stability

The recovery of the method determined at LQC, MQC and HQC (n = 4). Extraction recovery of tramadol were calculated by comparing peak areas obtained from processed quality control saliva samples with those achieved after direct injections of standard solutions at the equivalent concentrations.

Dilution integrity was investigated by diluting quality control sample (20.00µg/mL) with blank saliva. Four replicate samples of 1/10 dilution were prepared and their concentrations were calculated by applying the dilution factor of (× 10) against the freshly prepared calibration curve of tramadol.

Stability of extracts was evaluated over 24h. Extracted quality control samples were analyzed immediately after extraction, and re-injected and analyzed after 24h.

Application of the method

Three volunteers were included in this study. The study protocol was approved by the Ethics Committee of Sohag University Egypt and written informed consent was obtained from the volunteers. Volunteers were not allowed to take any other medication for 2 weeks before and throughout the study. The volunteers received a single oral dose of 100 mg tramaldol tablets. Saliva samples were collected into glass tubes at 2, 4, 6, 12,18 and 24 hours after drug administration. The samples were stored at -20ºC until analysis.

Results and Discussion

Optimisation of the chromatographic conditions

The chromatographic conditions were aimed at getting adequate response, sharp peak shape and a short run time per analysis for the analyte and IS. This included UV spectrophotometry, mobile phase selection, pH and flow rate. With regard to UV spectra, tramadol was found to absorb strongly between 200 and 220 nm and demonstrates a smaller peak at 273 nm [21]. The propranolol was found to absorb maximally between 210 and 230 nm and demonstrates a smaller peak at 290 nm. 218 nm was selected for the UV detection, that exhibit the best peak height for tramadol.

Different volume ratios of methanol–phosphate buffer and acetonitrile–phosphate buffer combinations at pH 7 were tested as mobile phase, It was observed that acetonitrile– 0.01 M phosphate (40:60, v/v) as the most appropriate mobile phase for faster elution, peak shape and least band tailing.

The pH of the mobile phase was varied between a range of 2.0 and 7.0, where pH 6.0 or above produced band tailing and prolonged total run time. However, pH ≤ 3 the retention was dropped, hence affecting

the resolution. Therefore, pH 5.0 was selected for the mobile phase, giving the best area count and resoultion for tramadol and IS.

Parameter of system suitability, Rs, N and T are summarized in Table 1 and were within the recommended values [41].

Average retention times for tramadol and the internal standard were determined as 6.13±0.02 and 8.01±0.01 min, respectively (total run time of 10.00 min).

Propranolol has been used in the literature as internal standard for LC-MS/MS method for the extraction and quantification of tramadol and its main metabolite from plasma sample [36]. Thus, propranolol was tested as an internal standard which had similar chromatographic behavior and was easily extracted with hexane-ethyl acetate (4:1 v/v) in alkaline medium.

Specificity and sensitivity

Six different blank saliva specimens were analyzed to evaluate chromatographic interference. No interferences with analyte or internal standard peaks were detected. Representative chromatograms for blank saliva sample with no internal standard and fortified with internal standard are shown in (Figure 2). The peaks for tramadol and IS were also investigated by photo-diode array detector and found to be pure (peak purity more than 999). The LOD and LOQ for tramadol were 0.1 and 0.25 µg/mL respectively.

Linearity

The calibration curves for tramadol were linear from 0.25 to 4.00 µg/mL with correlation coefficient $r \geq 0.997$. The standard deviation values obtained for slope and intercept from three linearties was -0.004 and 0.001. Remarkably, the slopes of the three calibration curves were

Compound	Retention time (min) (mean±SD)	T (Tailing factor) (mean±SD)	N (Plate count)	Resolution (Rs) (mean±SD)
Tramadol	6.13±0.02	1.37±0.02	10852	6.58±0.12
Propranolol	8.01±0.01	1.43±0.04	8354	

Table 1: System suitability parameters.

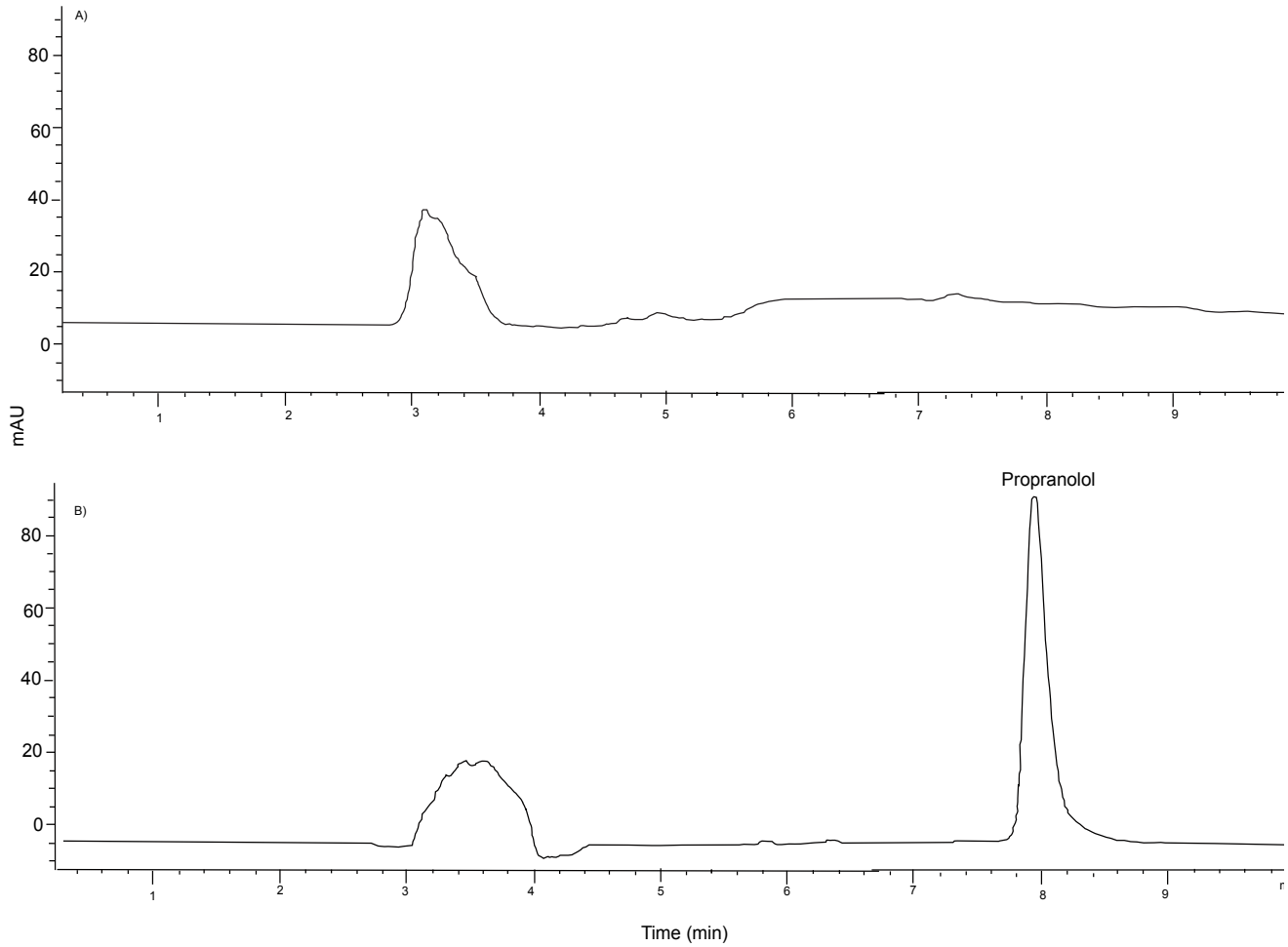

Figure 2: Chromatograms of human blank saliva with a) no internal standard and b) with internal standard.

very similar. Their observed mean back calculated concentration with accuracy (%) and precision (% RSD) of three linearties (c.f. Table 2).

Precision, accuracy and recovery

The intra-assay precision and accuracy were evaluated in five replicate analyses for tramadol at three concentration levels LQC, MQC and HQC each on the same analytical run as described. The intra- and inter-assay precision was less than 10%. The proposed method was found to be reproducible. The detailed results for intra- and inter-assay accuracy and precision are given in Table 3. Mean extraction recovery of tramadol and internal standard were 94.70 ±7.21 and 80.60 ±4.20 %, respectively.

Dilution integrity and extract stability

Six calibrators in the range of 0.25–4.00 µg/mL were included for calibration curve in the present study. With increasing the concentration of calibrators upper 4.00 µg/mL., the bis % was ≥ ±20%. Dilution integrity was tested to evaluate saliva concentration upper the high limit of quantification. Because some saliva samples collected from participants during first 2h were in the concentration of 5.00 µg/mL, for this reason dilution integrity was evaluated. The mean back-calculated concentration for 1/10 dilution samples for tramadol was 106.00±0.13% of its nominal value and RSD was 6.06%. Stability of tramadol after extraction also was examined. Tramadol was stable, differing from sample injected immediately by less than 3% after 24 h.

Application of the method

The applicability of this method has been demonstrated by determination of the tramadol in saliva samples from three healthy volunteers receiving a single oral dose of 100 mg tramadol tablet. The

ID no.	Concentration (µg/mL)							Regression Parameters		
	0.25	0.50	1.00	1.50	2.00	4.00		Intercept	Slope	r^2
1	0.24	0.56	0.95	1.53	2.08	3.93		0.0154	0.0005	0.9971
2	0.27	0.52	1.02	1.50	2.02	3.92		0.0267	0.0006	0.9954
3	0.23	0.53	0.96	1.44	2.11	4.20		-0.0532	0.0010	0.9975
Mean	0.24	0.53	0.98	1.49	2.07	4.01		-0.0037	0.0007	0.9967
SD	0.02	0.02	0.06	0.07	0.13	0.15		0.0432	0.0003	0.0011
% RSD	8.96	4.29	6.30	4.68	6.07	3.76				
% Bias	-3.20	6.40	-2.33	-0.78	3.33	0.29				

Table 2: Summary of calibration curves for tramadol with back calculated concentrations.

Nominal concentration (µg/mL)	Found concentration (µg/mL)	Precision[a] (RSD)	Accuracy (%)
Intra-assay (n = 5)			
LQC (0.75)			
Day 1	0.74	6.06	99.20
Day 2	0.75	5.75	100.53
Day 3	0.75	9.79	100.27
MQC (1.50)			
Day 1	1.53	4.30	101.67
Day 2	1.60	7.41	106.83
Day 3	1.48	1.40	98.50
HQC (3.00)			
Day 1	3.14	3.11	104.53
Day 2	3.21	1.95	107.13
Day 3	3.00	7.25	99.92
Inter-assay (n = 15)			
LQC	0.75	0.77	100.44
MQC	1.53	3.98	102.22
HQC	3.12	3.43	103.89

[a]RSD: relative standard deviation

Table 3: Intra- and inter-assay precision and accuracy for tramadol in human saliva.

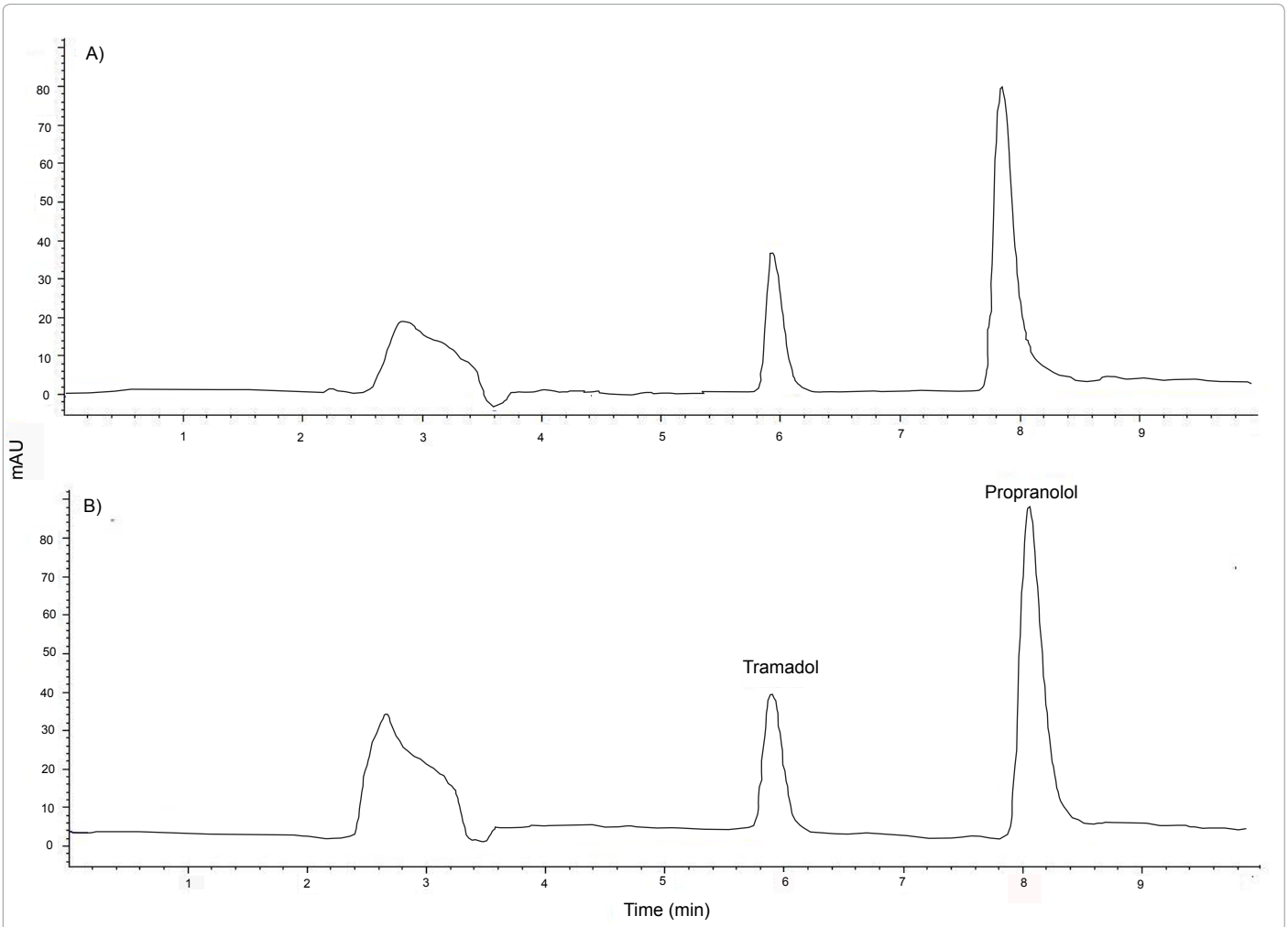

Figure 3: Chromatograms of tramadol and propranolol (IS) at a) low quality control (0.75 µg/mL) and b) saliva sample collected from volunteer after 12 h.

method was sensitive enough to monitor their saliva concentration up to 24 h. Figure 3 represents the chromatogram of tramadol for the analysis of saliva sample obtained at 12 h from a volunteer who received a 100-mg tramadol tablet. The mean saliva concentration-time profile of tramadol for all participants is presented in (Figure 4).

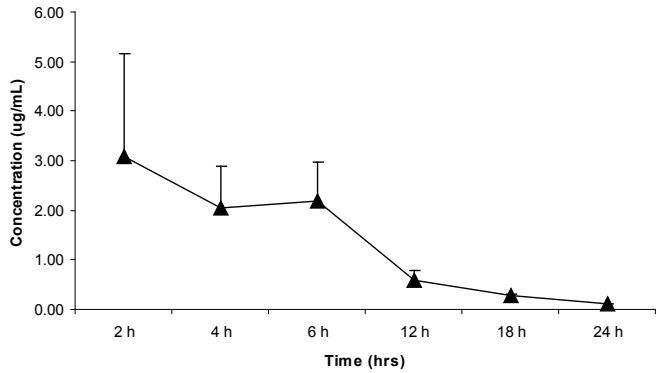

Figure 4: Saliva concentration–time courses of tramadol in a three healthy participants after oral administration of 100-mg doses of tramadol.

Conclusion

A validated method for the detection and quantification of tramadol in human saliva is presented. Good linearity, LOQ, accuracy, precision, and recovery were demonstrated. The method is useful for the analysis of tramadol in saliva.

References

1. Bogusz M, Wu M (1991) Standardized HPLC/DAD system, based on retention indices and spectral library, application for systematic toxicological screening. J Anal Toxicol 15: 188-197.
2. Tracqui A, Kintz P, Mangin P (1995) Systematic toxicological analysis using HPLC/DAD. J Forensic Sci 40: 254-262.
3. Elliott SP, Hale KA (1998) Development of a high-performance liquid chromatography retention index scale for toxicological drug screening. J Anal Toxicol 22: 279-289.
4. Moore C, Rana S, Coulter C (2007) Determination of meperidine, tramadol and oxycodone in human oral fluid using solid phase extraction and gas chromatography–mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 850: 370-375.
5. Raffa RB, Friderichs E, Reimann W, Shank RP, Codd EE, et al. (1992) Opioid and nonopioid components independently contribute to the mechanism of action of tramadol, an 'atypical' opioid analgesic. J Pharmacol Exp Ther 260: 275-285.

6. Tao Q, Stone DJ, Borenstein MR, Jean-Bart V, Codd EE, et al. (2001) Gas chromatographic method using 425 nitrogen-phosphorus detection for the measurement of tramadol and its *O*-desmethyl metabolite in plasma and brain tissue of mice and rats. J Chromatogr B 763: 165-171.

7. Ho ST, Wan JJ, Liaw WJ, Ho CM, Li JH (1999) Determination of tramadol by capillary gas chromatography with flame ionization detection, application to human and rabbit pharmacokinetic studies. J Chromatogr B 736: 89-96.

8. Lintz W, Uragg H (1985) Quantitative determination of tramadol in human serum by gas chromatography-mass spectrometry. J Chromatogr 341: 65-79.

9. Xu YX, Xu YQ, Zhang CJ, Shen L (1993) Analysis of tramadol and its metabolites in human urine. Acta Pharmaceutica Sinica 28: 379-383.

10. Merslavic M, Zupancic-Kralj L (1997) Determination of tramadol in human plasma by capillary gas chromatography-mass spectrometry using solid-phase extraction. J Chromatogr B 693: 222-227.

11. Staerk U, Külpmann WR (2000) High-temperature solid-phase microextraction procedure for the detection of drugs by gas chromatography–mass spectrometry. J Chromatogr *B* 745: 399-401.

12. Hadidi KA, Almasad JK, Al-Nsour T, Abu-Ragheib S (2003) Determination of tramadol in hair using solid phase extraction and GC–MS. Forensic Sci Int 135: 129-136.

13. Gambaro V, Benvenuti C, De Ferrari L, DellAcqua L, Fare F (2003) Validation of a GC/MS method for the determination of tramadol in human plasma after intravenous bolus. Farmaco 58: 947-950.

14. Leis HJ, Fauler G, Windischhofer W J (2004) Synthesis of d_1-*N*-ethyltramadol as an internal standard for the quantitative determination of tramadol in human plasma by gas chromatography–mass spectrometry. Chromatogr B Analyt Technol Biomed Life Sci 804: 369-374.

15. Sha YF, Shen S, Duan GL (2005) Rapid determination of tramadol in human plasma by headspace solid phase microextraction and capillary gas chromatography–mass spectrometry. J Pharm Biomed Anal 37: 143-147.

16. Cheng P, Lee C, Liu C, Chien C (2008) Simultaneous determination of ketamine, tramadol, methadone, and their metabolites in urine by gas chromatography–mass spectrometry. J Anal Toxicol 32: 253-259.

17. Chytil L, Stichac M, Matouskovab O, Perlik F, Slanar O (2009) Enatiomeric determination of tramadol and *O*-desmethyltramadol in human urine by gas chromatography–mass spectrometry. J Chromatogr B 877: 1937-1942.

18. Lintz W, Erlac S, Frankus E, Uragg H (1981) Metabolism of tramadol in man and animals. Arzneim-Forsch /Drug Res. 31: 1932-1943.

19. Elsing B, Blaschke G (1993) Achiral and chiral high-performance liquid chromatographic determination of tramadol and its major metabolites in urine after oral administration of racemic tramadol. J Chromatogr 612: 223-230.

20. Gan SH, Ismail R (2001) Validation of a high-performance liquid chromatography method for tramadol and *o*-desmethyltramadol in human plasma using solid-phase extraction. J Chromatogr B 759: 325–335.

21. Gan SH, Ismail R, Wan-Adnan WA, Wan Z (2002) Method development and validation of a high-performance liquid chromatographic method for tramadol in human plasma using liquid–liquid extraction. J Chromatogr B 772: 123–129.

22. Nobilis M, Pastera J, Anzenbacher P, Svoboda D, Kopecky J, et al. (1996) High-performance liquid chromatographic determination of tramadol in human plasma. J. Chromatogr B 681: 177-183.

23. Paar WD, Frankus P, Dengler HJ (1996) High-performance liquid chromatographic assay for the simultaneous determination of tramadol and its metabolites in microsomal fractions of human liver. J Chromatogr B 686: 221-227.

24. Ceccato A, Chiap P, Hubert P, Crommen J (1997) Automated determination of tramadol enantiomers in human plasma using solid-phase extraction in combination with chiral liquid chromatography. J Chromatogr B 698: 161-171.

25. Campanero MA, Calahorra B, Garcia-Quetglas E, Escolar M, Honorato J (1998) High-performance liquid chromatographic assay for simultaneous determination of tramadol and its active metabolite in human plasma. application to pharmacokinetic studies. Chromatographia 48: 555-560.

26. Campanero MA, Garcia-Quetglas E, Sadaba B, Azanza JR (2004) Simultaneous stereoselective analysis of tramadol and its primary phase I metabolites in plasma by liquid chromatography Application to a pharmacokinetic study in humans. J Chromatogr A 1031: 219-228.

27. Gu Y, Fawcett JP (2005) Improved HPLC method for the simultaneous determination of tramadol and *O*-desmethyltramadol in human plasma. J Chromatogr B 821: 240-243.

28. Rouini M, Ardakani YH, Soltani F, Aboul-Enein HY, Foroumadi A (2006) Development and validation of a rapid HPLC method for simultaneous determination of tramadol, and its two main metabolites in human plasma. J Chromatogr B 830: 207-211.

29. Ardakani YH, Rouini M (2007) 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 44: 1168-1173.

30. Mehvar R, Elliott K, Parasrampuria R, Eradiri O (2007) Stereospecific high-performance liquid chromatographic analysis of tramadol and its *O*-demethylated (M1) and *N*,*O*-demethylated (M5) metabolites in human plasma. J Chromatogr B 852: 152-159.

31. Giorgi M, Soldani G, Manera C, Ferrarini PL, Sgorbini M, et, al. (2007) Pharmacokinetics of tramadol and its metabolites M1, M2 and M5 in horses following intravenous, immediate release (fasted/fed) and sustained release single dose administration. J Equine Vet Sci 27: 481- 488.

32. Ebrahimzadeh H, Yamini Y, Sedighi A, Rouini MR (2008) 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 863: 229-234.

33. Curticapean A, Muntean D, Curticapean M, Dogaru M, Vari C (2008) Optimized HPLC method for tramadol and O-desmethyl tramadol determination in human plasma. J Biochem Biophys Methods 70: 1304-1312.

34. Valle M, Pavon JM, Calvo R, Campanero MA, Troconiz IF (1999) Simultaneous determination of tramadol and its major active metabolite *O* demethyltramadol by high-performance liquid chromatography with electrochemical detection. J Chromatogr 5: 83-89.

35. Kucuk A, Kadioglu Y, Celebi F (2005) 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 816: 203-208.

36. Bogusz MJ, Maier RD, Kruger KD, Kohls U (1998) Determination of common drugs of abuse in body fluids using one isolation procedure and liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. J Anal Toxicol 22: 549-558.

37. Ceccato A, Vanderbist F, Pabst JY, Streel B (2000) Enantiomeric determination of tramadol and its main metabolite *O*-desmethyltramadol in human plasma by liquid chromatography–tandem mass spectrometry. J Chromatogr B 748: 65-76.

38. Patel BN, Sharma N, Sanyal M, Shrivastav PS (2009) An accurate, rapid and sensitive determination of tramadol and its active metabolite *O*-desmethyltramadol in human plasma by LC–MS/MS. J Pharm Biomed Anal 49: 354-366.

39. Gergov M, Nokua P, Vuori E, Ojanpera I (2009) Simultaneous screening and quantification of 25 opioid drugs in post-mortem blood and urine by liquid chromatography–tandem mass spectrometry. Forensic Sci Int 186: 36-43.

40. De Leo M, Giorgi M, Saccomanni G, Manera C, Braca A (2009) 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 23: 228-236.

41. Reviewer guidance: validation of chromatographic methods, Centre for Drug Evaluation and Research (CDER), Food and Drug Administration, November 1994.