Development and Validation of a Separation Method for an Analgesic Drug and its Main Metabolite from Plasma

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The objectives of this study were the development and validation of an extraction method for the simultaneous HPLC determination of Tramadol and its active metabolite o-desmethyltramadol (M1) in plasma in order to use it in bioequivalence studies. A reversed-phase mechanism with fluorescence detection and liquid/liquid extraction from basic plasma samples were chosen for the determination of Tramadol and M1. The bioequivalence study was made for 24 healthy volunteers. Fluconazol, Trimethoprim, Verapamil and Metoprolol were tested as internal standard (IS). It was chosen Metoprolol. During the validation it was studied the optimal extraction solvent (tert-butylmethylether or diisopropylether); the influence of the aqueous layer pH, ratio between phases and vortexing time to the extraction yield were also studied. The optimal extraction solvent was diisopropylether with 1:3 ratio between plasma and the organic phase; the organic layer was evaporated to dryness under N_2, at 40°C. The recovery for M1 (95-101%) and Tramadol (70-84.81%) was satisfactory to allow the determination of the lowest plasma concentration of the drug. The method was linear between 10-600 ng/mL (r=0.9924, n=8) for Tramadol and 5-300ng/mL (r=0.9957, n=8) for metabolite. The lowest limit of quantification (LLOQ) was 5ng/mL for M1 and 10ng/mL for Tramadol. The extraction procedure was applied in a new, selective and sensitive HPLC method for quantitative determination of Tramadol and M1 in a bioequivalence study.

Keywords: Tramadol separation, quantitative determination, liquid-liquid extraction, bioequivalence study

Due to its good efficacy and low addictiveness, Tramadol was administered to more and more patients to relieve pain from moderate to severe, acute and chronic pain. Tramadol hydrochloride is a centrally acting opioid analgesic agent marketed for the treatment of moderate to severe pain. Readily soluble in water and ethanol; pK_a 8.3, 9.41; partition coefficient - log P (octanol/water) = 3.01 [1].

Tramadol is rapidly and almost completely absorbed after oral or parenteral administration. The presence of food does not significantly affect the rate or extent of absorption.

Tramadol is extensively metabolized. The main metabolic reactions are *N*- and *o*-demethylation and conjugation with glucuronic acid and sulfate. The major metabolites formed are *o*-monodesmethyltramadol, *N,O*-didesmethyltramadol and their conjugates, and N-monodesmethyltramadol [1,3].

Various methods have been described for the separation of tramadol from plasma samples. Liquid/liquid extraction was used for the separation of Tramadol and O-desmethyltramadol from basic plasma samples using as solvents: tert-butylmethylether [3, 4, 9, 14], methylene chloride [7] or ethylacetate [6]. A solid-phase extraction is also used [2].

Some authors described methods for sample preparation consisted in plasma protein precipitation from 0.2 mL plasma using 0.2 mL solution of perchloric acid 7% [13]. Recovery from plasma was 88.5+/-2.1% [6], 95% [8], 86% [9], 98.63% [11], 82.6-94% for metabolite and Tramadol, respectively [2].

For the validation of the method is important to have accuracy, precision in the range of the investigated concentrations, specificity in the presence of the endogenous plasmatic components and sensibility, to allow the quantification of the drug after a period equal with 4 half-time [15, 16].

Experimental part

It was chosen the liquid/liquid extraction of tramadol and its main active metabolite *o*-desmethyltramadol from basic plasma (in the presence of 50µL Na_2CO_3 sol. 0.1M) with diisopropylether (DIPE).

The proposed HPLC method with fluorescence detection for the determination of analytes is complex, due to the extraction process and different pairs of excitation/emission wavelengths used for the quantification of the three analytes (including the internal standard Metoprolol). It was chosen the reversed phase mechanism and the fluorescence detection using as analytical wavelengths two pairs of Ex/Em wavelengths: 276/310nm, 228/310nm and Metoprolol as IS.

Materials

Analytes and reagents used for the quantitative determination of Tramadol were: Tramadol Hydrochloride (TR) and *o*-desmethyltramadol (M1) – standards provided by Labormed S.A. (Romania); methanol HPLC grade purchased from Merck; triethylamine (TEA) supplied by Fluka; phosphoric acid 85% (pro analysi), diisopropylether HPLC grade, *tert*-butylmethylether HPLC grade, KH_2PO_4, Na_2CO_3, were supplied by Merck (Darmstadt, Germany); Fluconazol, Trimethoprim, Verapamil and Metoprolol were all purchased from Merck; ultrapure deionized water was locally produced by NANOpure Diamond ultrapure water system. The human blank plasma was supplied by the Local Blood Center – Bucharest. All other reagents were of analytical grades.

Preparation of standard solutions

Stock standard solutions of Tramadol and *o*-monodesmethyltramadol (M1) (metabolite purity is 90%) were prepared in water at a concentration of 200µg/mL

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and 110μg/mL, respectively. Metoprolol (IS) stock standard solution was prepared in methanol, concentration of 545 μg/mL. This solution was further diluted in water to obtain appropriate working solution of 2.18μg/mL internal standard, used to prepare the calibration solutions. Metoprolol solution in water is not stable more than one week. All stock solutions were stored at 2-8°C.

Preparation of spiked calibration standards and quality control samples

Starting from pooled stock solutions of TR and M1 in water, standards were prepared using pooled human drug free plasma, obtained from healthy volunteers as diluent. It were diluted apropriate volumes of each standard solution, in order to achieve concentration ranges of 600-10ng/mL for TR and 297-4.95ng/mL for M1 respectively. It was defined the work range considering the normal therapeutic doses (300-600mg/day) and the time to achieve the maximum plasmatic levels (1-3h after dose) making 8 spiked plasma samples.

Pools of quality control samples (QC) were prepared by spiking human plasma with the standard solutions of all compounds. The concentrations in QC plasma samples were: 320, 80, 40ng/mL for TR and 158, 39.6 and 19.8ng/mL for M1. All samples were treated and analyzed as it is described in section 2.4.

Equipment and chromatografic conditions

For samples preparation, it was used a centrifuge-Hettich Universal, a solvent evaporation (under N2 stream) system, Sartorius BP 121S analytical balance, Ultrasonic Cleaner BRANSON - 2510E, vortex. The Hewlett Packard High Performance Liquid Chromatograph, series 1100 equipped with a thermostatted auto sampler ALS G1329A, binary pump G1312A and vacuum degasser G1322A. Compounds were screened for, identified, and quantified in plasma using a fluorescence detector – FLD G1321A using PMT- Gain 14. For the optimization of the measurements, the FLD was programmed as following: Ex/Em wavelengths were adjusted to 276/310nm for 8 min and to 228/310nm for 6 min. Chromatographic separations were carried out by a 5μm particle size Microsorb-MV 100 C18 column (150x4.6mm id., Varian) whose temperature was maintained at 40°C. Samples were eluted with a methanolic mobile phase with buffer phosphate 10mM KH_2PO_4 adjusted at *p*H=5.9 delivered at a flow – rate of 0.7mL/min. The injection volume was 100 μL.

Extraction procedure from biological samples

Procedure designed to determine the optimal extraction method required for TR, M1 and IS isolation from plasma consists in the following steps: 500 μL plasma samples were transfered into a 15 mL glass tube and 100μL of internal standard solution - IS - (metoprolol 2.18 μg/mL in water) was added. The samples were agitated for at least 30 s and then 50μL of Na_2CO_3 solution 0.1M were added (pKa 8.3, 9.41). An aliquot - 1.5mL- of diisopropyl-ether (DIPE) was added in all samples, which was followed by strong shaken for 30 min. The organic layer was separated after centrifugation for 5 min at 4000rpm. An aliquot of 1mL supernatant was transfered into an appropriate test tube and evaporated to dryness at 40^{0}C under a stream of nitrogen. The dried residue was finally reconstituted in 200μL mobile phase and shaken at least 30s. A 100 mL aliquot was injected into the chromatographic HPLC system.

Results and discussion

The choice of the detection system

The UV absorbtion spectra for the analytes: *Tr*amadol, *o*-desmethyltramadol (Metabolite M1) and internal standard (metoprolol) are presented in figure 1. Absorbtion of UV radiation is much weaker than the property of fluorescence emission, for both analytes and internal standard figure 2.

The chromatogram of a spiked plasma sample monitored in FLD also eliminates the interference of endogenous components from plasma pattern, unlike the chromatogram monitored in UV, λ= 220 or 270 nm. With these values of excitation and emission wavelengths, the fluorescence monitoring during the chromatographic elution reaches the lowest detection limit and decreases the injection volume in the analytical column. Moreover, the sensitivity of fluorescence measurements can be increased by the increase of the PMT-gain of the detector.

Selection of optimal Internal Standard

Fluconazol, Trimethoprim, Verapamil and Metoprolol were tested as internal standard (IS). Results are shown in figure 3. Metoprolol it was the choice as IS.

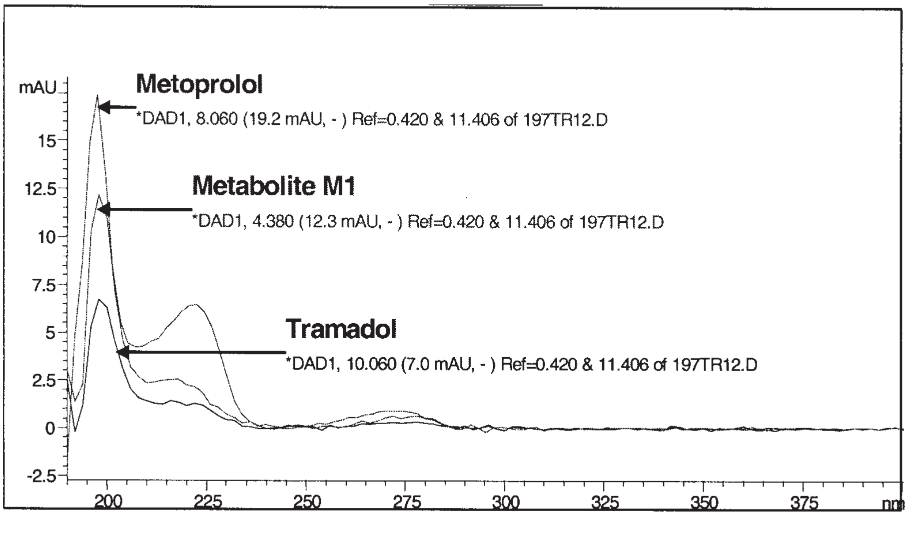

Fig. 1. UV spectra of Metoprolol, metabolite M1 and Tramadol

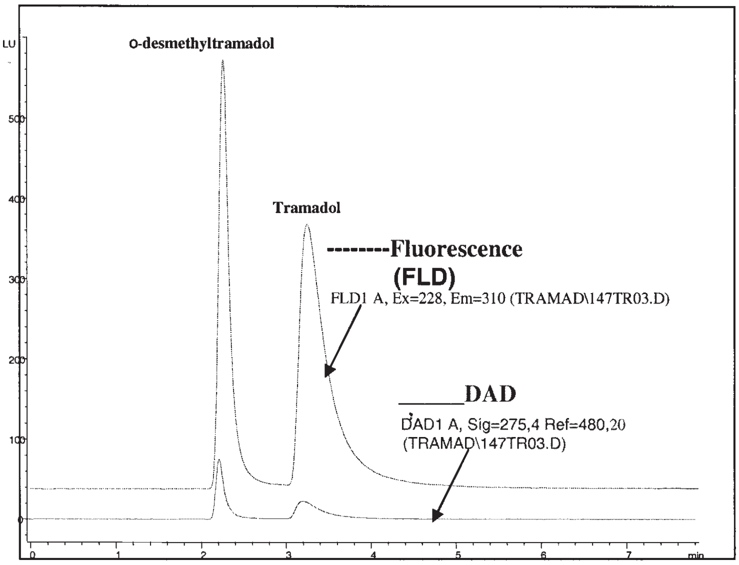

Fig. 2. Differences between UV spectrum and fluorescence emision for TR and M1

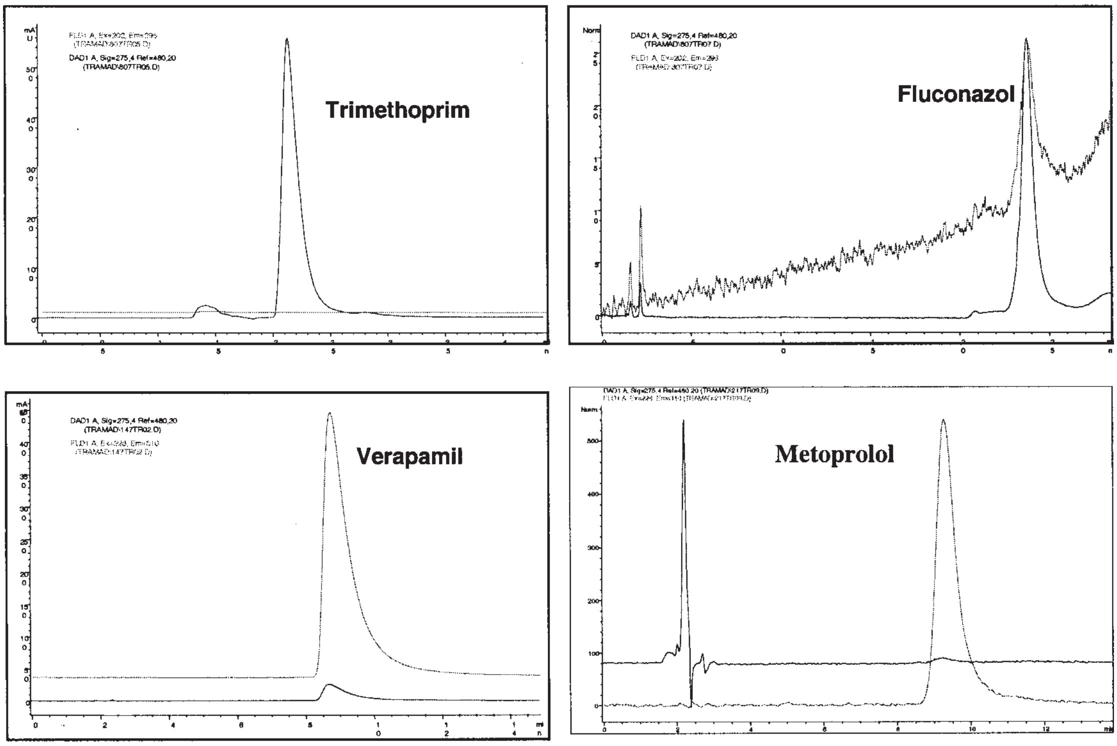

Fig. 3. Selection of optimal Internal Standard

Optimization of extraction conditions

Optimization process started with some reported organic mixtures but most of them proved to be unefficient due to low extraction of the analytes or even lack of reproductibility. The optimal extraction solvent (tert-butylmethylether and diisopropylether) - figure 4, the effects of *p*H – figure 5, ratio between phases and vortexing time, which influence the quality of the separations of the analytes, IS and the extraction yields were also studied.

During the validation procedure it was also studied the posibility of deproteinisation of plasma samples using methanol (fig. 4). The optimal extraction solvent for TR, M1 was DIPE with 1:3 ratio between plasma and organic phase, because, after plasma extraction, no coeluting peak was detectable in control samples at the retention time of analysed compounds.

Liquid-liquid extraction was used because of the short duration of the clean-up procedure. The proposed sample preparation procedure was optimised to offer the most reliable, effective and rapid approach for the routine analysis, as well as to permit elimination of time-comsuming purification steps, including expensive solid-phase extraction (SPE). Preparation of biological samples was simple and cheap in comparison with SPE method, based only on single liquid-liquid extraction.

The best *p*H for extraction from human plasma, giving the highest percentage recovery for both analytes was a basic one (fig. 5).

The variation of extraction yield with vortexing time of samples, during the sample preparation procedure, shows that the best results are obtained at 30 min for both analytes. After this point the extraction yield shows a decrease from 85-95% to 65-73% for analytes (fig. 6).

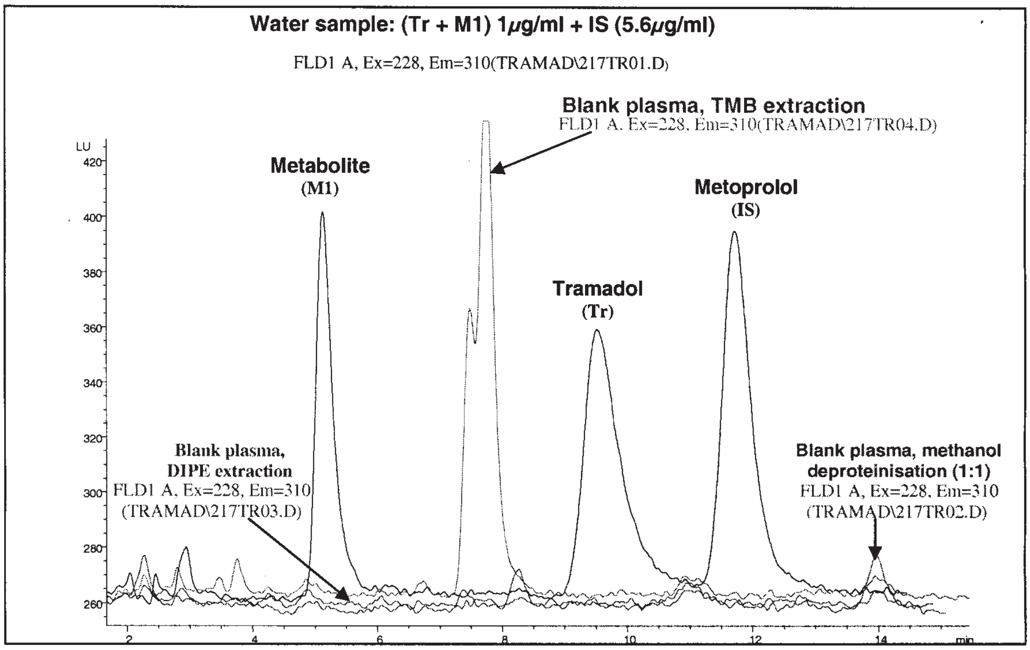

Fig. 4. Optimization of extraction solvent

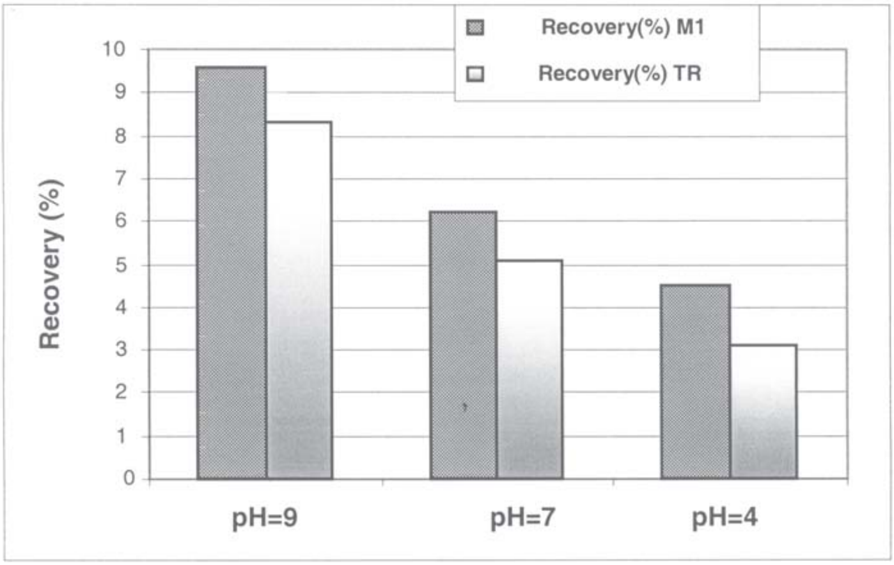

Fig. 5. Percentage recoveries variation at different plasma *p*H values for TR and M1

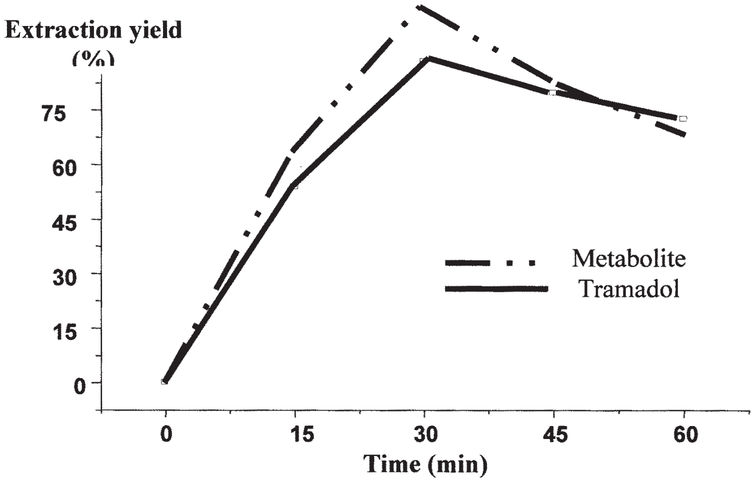

Fig. 6. Extraction yield variation with vortexing samples time

Method validation

Assay performance of the present method was assessed by all the following criteria: linearity, accuracy, precision, LOD, LOQ, stability and applicability to pharmacokinetic studies.

Recovery from plasma

The extraction recovery of TR and its main metabolite from plasma was calculated for water and plasma samples by comparing the obtained data for aqueous solutions to those obtained from plasma samples after the extraction procedure.

This procedure was designated to estimate the recovery of TR and M1 from plasma matrix. To calculate the absolute recovery of L/L extraction procedure, six replicates of spiked plasma samples at three different concentration levels (404.4, 202.4 and 50ng/mL TR and 374.4, 187.2, 46.8ng/mL M1) were used.

Sample level (ng/ml)		Average peak area for water samples (Lu*s)			Average peak area for plasma samples (Lu*s)			Recovery from plasma (%)
M1	IS	M1	IS	Ratio	M1	IS	Ratio	
374.4	2180	5708.5	3145.6	1.815	6978.2	3844.7	1.8150	100
187.2	2180	2295.8	2882	0.7966	3278.6	4074.5	0.8046	101
46.8	2180	750	2783	0.2695	972.6	3765	0.2583	95.84
TR	IS	TR	IS	Ratio	TR	IS	Ratio	
404.8	2180	8827	3145.6	2.8065	8767	3844.7	2.3802	84.81
202.4	2180	4595.7	2882	1.5945	3945.1	4074.5	0.9682	81.3
50	2180	1110.4	2783	0.3989	1033	3765	0.2743	68.76

Fig. 7. Recovery from plasma for M1 and TR

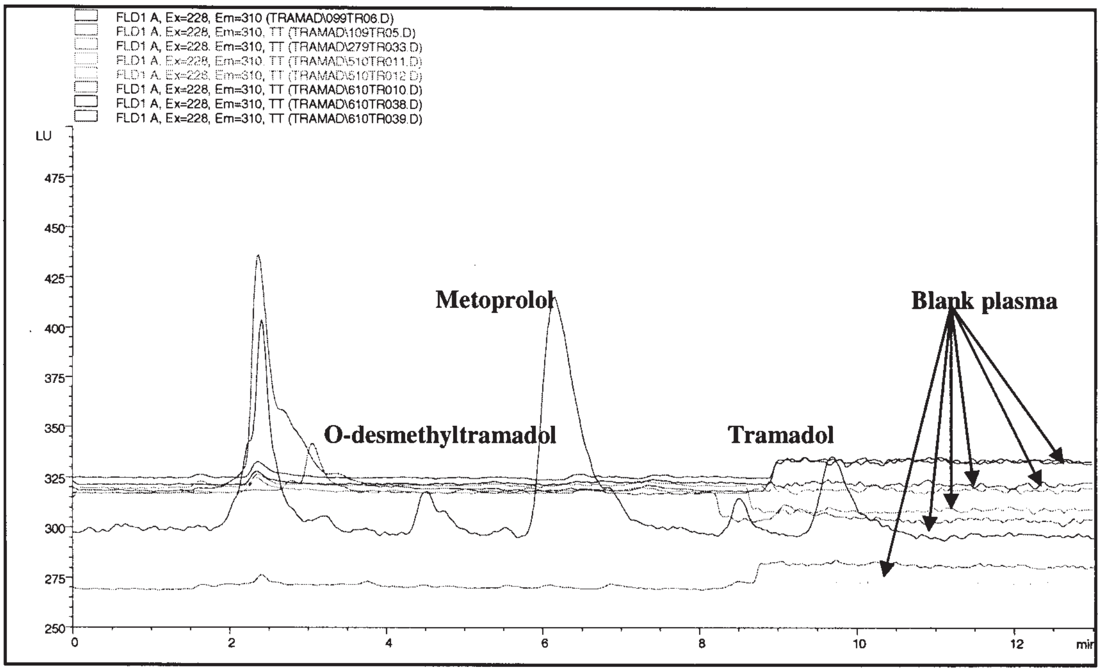

Fig. 8. Overlaid chromatograms resulting after the injections samples for selectivity determination

Once samples prepared, they were injected into the analytical column, using operational parameters and method described in section 2.4. The results are given in figure 7.

The recovery for o-desmethyltramadol (95 - 101%) and Tramadol (68.76 - 84.81%) is satisfactory to allow the determination of the lowest plasma level concentration of the analyte.

Selectivity of the method

The ability of the analytical method to differentiate and quantify the analyte in the presence of other components in the sample.

The procedure was designed to demonstrate the capability of the method to separate Tramadol, Metabolite and internal standard (Metoprolol) against the components of the plasma matrix. The method has been performed by comparing chromatograms obtained from 6 blanks of human plasma of different origins, with the chromatograms of QC3 sample (with nominal concentration 19.8ng/ml of Metabolite, 40ng/mL of Tramadol and IS). The results of this assay confirmed that there were no endogenous peaks interfering with peaks of analyzed substances and internal standard (fig. 8).

Linearity

Each calibration curve consisted of eight calibration points (10-600ng/mL for Tramadol and 4.95-297ng/mL for its main metabolite M1). From the chromatograms resulting after injection of samples belonging to the same analyte concentration, it was calculated the mean peak area and the corresponding standard deviation (SD). Calculations should always be performed on Tramadol and Metabolite peak areas, normalizing the values to peak areas of the Metoprolol. The calibration curve and the statistical parameters are given in figure 9 (TR) and figure 10 (M1).

The linearity of the method was statistically confirmed by comparing the slopes, the intercepts of calibration curves with zero and the correlation coefficients with 1.

Accuracy and Precision

The accuracy and the precision were determined by replicate analysis of samples containing known amounts of the analyte. Between and within-day accuracy and precisions of the method were determined for each compound according to the GLP and FDA guidance for bioanalytical method validation. In this work the precision was calculated for three concentration levels: 158, 39.6 and 19.8 ng/mL for M1 and 320, 80 and 40 ng/mL for TR. Precision was measured using five spiked plasma samples for each concentration level and each day.

Accuracy was calculated as deviation of the mean from the nominal concentration. Precision was expressed as the relative standard deviation of each calculated concentration. The between- and within-day assay validation data are reported.

$$\text{Accuracy } \% = C_{exp}/C_{th} \text{ x } 100$$

Precision and accuracy showed an acceptable RDS values for M1(2-8%), and TR(4-7%). The within-day

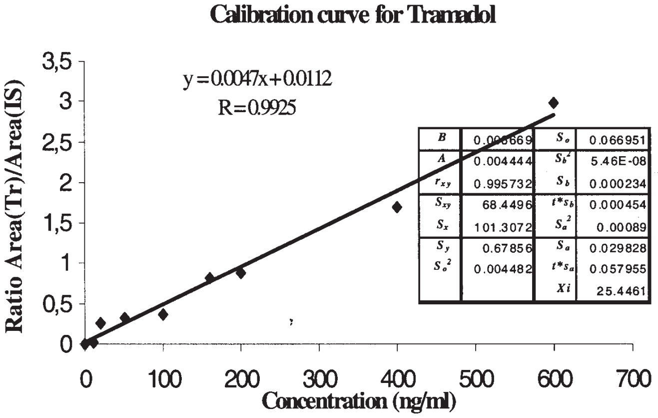

Fig. 9. Calibration curve and statistical parameters for Tramadol

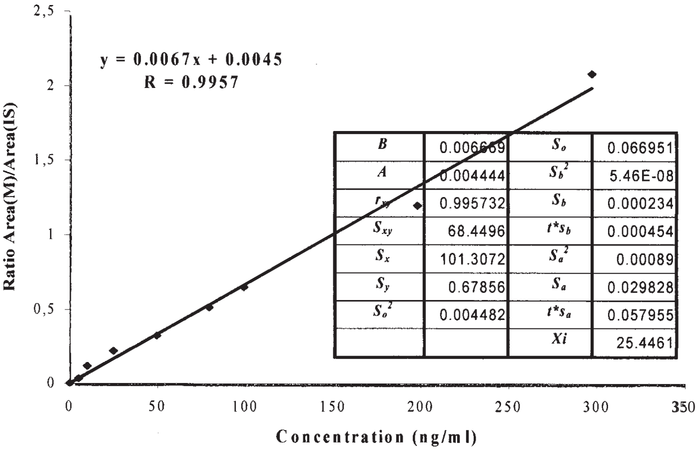

Fig. 10. Calibration curve and statistical parameters for o-desmethyltramadol

accuracy and precision of the chromatographic method meet requirements according to the acceptance criteria.

Lower limit of quantification and the limit of detection

The limit of detection (LOD) and the lower limit of quantification (LLOQ) were determined as analyte concentrations giving signal-to-noise ratios of 3 and 10 respectively.

The stability of stock solutions of the drug and the internal standard was also studied in processed samples, left at room temperature (20 ± 3^0C) over 24h.

Application of the method

The applicability of this method has been demonstrated by determination of the Tramadol and its main phase I metabolite in plasma samples from healthy volunteers in a pharmacokinetic study. The concentrations of TR and M1 were measured in more than 700 plasma samples. The study was appproved by the state authority. In the study, 24 healthy volunteers (males and females) received a single oral dose of Tramadol. Plasma concentration-time profiles for TR and M1 were determined over 32h after drug administration.

Conclusions

A sensitive, accurate and precise liquid/liquid method for separation of Tramadol and its main active metabolite from the plasma samples was developed. The method is also rapid, simple and suitable for routine analysis [16].

The method was validated over a concentration range of 10-600 ng/ml for Tramadol and 5-300ng/mL for metabolite and it offers good accuracy and precision requirements of the pharmacokinetic investigations.

The lowest limit of quantification (LLOQ) was 5ng/ml for M1 and 10ng/mL for Tramadol. The procedure was applied to bioequivalence studies of Tramadol and its metabolite, *o*-desmethyltramadol.

References

1.MOFFAT, C.A., OSSELTON, M.D. AND WIDDOP, B, “Clarke’s Analysis of Drugs and Poisons”, Third edition, vol. **2,** Edited by Pharmaceutical Press, 2004, p.1655

2.GAN, S.H., ISMAIL, R., J. Chromatogr. B., **759,** 2001, p.325

3.NOBILIS M., KOPECKY J., KVETINA J., CHLADEK J., SVOBODA Z., VORISEK V., PERLIK, F., POU,R M., KUNES, J., J. Chromatogr. A., **949,** 2002, p.11

4.GARRIDO, M. J., JPET, **295,** 2000, p.352

5.MEDVEDOVICI A., ALBU F., FARCA A., DAVID V., J. Pharm. Biomed. Anal., **34,** 2004, p.67

6.YEH, G.C., J. Chromatogr. B. Biomed. Sci. Appl., **725, nr.** 1-2, 1999, p. 247

7.PAAR W.D., FRANKUS, P., DENGLER, H.J., J. Chromatogr. B. Biomed. Sci. Appl, **686,** nr. 2, 1996, p. 221

8.CAMPANERO, M. A., QUETGLAS, E. G., SADABA, B., AZANA, J. R., J. Chomatogr. A, **1031**, 2004, p. 219

9.NOBILIS M., PASTERA J., ANZENBACHER P., SVOBODA D., KOPECKY J., J. Chromatogr. B. Biomed. Appl., **681,** nr. 1, 1996, p. 177

10.PAAR, W.D., FRANKUS, P., DENGLER, H.J., J. Chromatogr. B. Biomed. Appl., **686,** nr. 2, 1996, p. 221

11.GAN, S.H., ISMAIL, R., WAN ADNAN, W.A., WAN, Z., J. Chromatogr. B. Anal. Tech. Biomed. Life Sci., **772,** nr. 1, 2002, 123-129

12.WHITE, S., WONG, S., Clin. Chem., **44,** nr. 5, 1998, p. 1110

13.VLASE L., LEUCUTA S. E., IMRE S., Talanta, **75**, 4, 2008, 1104

14.MEHVAR R., ELLIOTT K., PARASRAMPURIA R., ERADIRI O., J. of Chromatography B, **852,** 2007, p. 152

15. VLASE, L., LEUCUTA, S., IMRE, S., Rev. Chim (Bucuresti),**58,** nr 1, 2007, 3

16.KISS, B., POPA, D.S., BOJITA, M., LOGHIN, F., Rev.Chim (Bucuresti), **59,** 12, 2008, p. 1301

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