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Determination of Tramadol in human plasma by HPLC with fluorescence detection

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Abstract

Tramadol is a centrally acting analgesic, atypical opioid, and although it is generally considered as a medicinal drug with a low potential for dependence, there is growing evidence of tramadol abuse in some countries. The ultraviolet detection is not suitable for analysis of tramadol in plasma, due to the lack of sensitivity and selectivity. However, it was shown that tramadol has a weak fluorescence, and the latest techniques for determination of tramadol in plasma include liquid chromatographic methods with fluorescence detection (FL). The objective of the paper was to develop a HPLC-FL method applicable for quantification of tramadol in human plasma.The separation was achieved by reverse phase HPLC method, using as stationary phase C18 – Kromasil® column and a mobile phase consisted of acetonitrile:0.1% formic acid (20:80). The fluorescence detection has been applied with $\lambda_{ex/em}$= 280/310 nm. A solid phase extraction procedure using C18 cartridge was carried out.

The linearity of the method has been demonstrated in the range of both therapeutic and toxic plasma tramadol levels (concentrations of 0.100 – 1µg/mL). The selectivity, precision, and accuracy of the method have been demonstrated. The limit of detection (LOD= 0.010 µg/mL) and the limit of quantification (LOQ = 0.100 µg/mL) have been established.The proposed method can be used to assess tramadol levels in human plasma in pharmacokinetic studies, as well as in overdose cases. The utility of the method for the quantification of therapeutic levels of tramadol has been shown on the plasma samples from the patients with tramadol treatment as analgesic (doses ranging from 100 mg to 400 mg/day).The developed method is rapid, using simple experimental conditions and an accurate and short extraction procedure.

Keywords: tramadol, HPLC, fluorescence detection

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Introduction

The Tramadol is a central analgesic, atypical opioid, widely used in therapeutics, but frequently reported in cases of drug-related deaths (1). Therapeutic plasma concentrations, usually found after a 50-100 mg dose, can vary up to 0.3 µg/mL, while the toxic levels are generally around 1 µg/mL.

In general, HPLC methods, reported in literature, for quantification of tramadol, use the UV (at 280 nm) or fluorescence detection, and the separation is typically obtained on C-18 (egPhenomonex® Gemini C-18, Hypersil® C18) columns. The mobile phases used contain phosphate buffer-methanol-acetonitrile (11) or 35% acetonitrile and aqueous solution with 20mM sodium phosphate buffer, 30mMsodium dodecyl sulfate and 15mMtetraethylammonium bromide, pH 3.9 (2).

Although tramadol has a core structure of benzene nucleus, UV detection is not suitable for its analysis in plasma, due to the lack of sensitivity and selectivity (3).

Latest techniques for determination of tramadol include electrochemical methods (4), mass spectrometry (5), capillary electrophoresis (6) and HPLC with fluorescence detection (3, 7), the latter giving better selectivity and sensitivity.

It has been shown that tramadol has a weak fluorescence, with a maximum of emission at 300 nm, after excitation at 200 nm. In addition, the fluorescence spectrum of both parent drug and two major metabolites, O-desmethyltramadol and N-desmethyltramadol are not virtually identical. Consequently, various publications reported different sensitivities of spectrofluorimetric or HPLC with fluorescence detection methods for the quantification of tramadol.

Bahrami G. reported a HPLC method with fluorescence detection, involving pre-column derivatization with 9-fluorenylmethyl chloro formate, leading to increased fluorescence intensity of tramadoland an increase in detection and quantitation limits respectively (8).

Gu Y et al. reported a HPLC method with fluorescence detection (wavelengths of excitation and emission of 275 and 300 nm) for quantification of tramadol and its active metabolite O-desmethyltramadolul in human plasma (2). A liquid-liquid extraction procedure was carried out using dichloromethane-diethyl ether-butanol (5:3:2), followed by extraction with sulfuric acid; tramadol, O-desmethyltramadol and internal standard, sotalol, were separated on a Hypersil® C18 column.

Ebrahimzadeh H. and coworkers have developed a HPLC method with fluorescence detection (wavelengths of excitation and emission of 200 and 301nm respectively) to quantify tramadol and O-desmethyltramadol in human plasma and urine (9). Separation was achieved on a

RP-18e Chromolith® Performance column using as mobile phase methanol: water with phosphoric acid, at pH 2.5 (19:81).

A HPLC method with fluorescence detection (excitation and emission wavelength of 200 nm and 295 nm) for determination of tramadol and O-desmethyltramadol in plasma was proposed by Curticapean A. et al. working on a RP18column with C18 pre-column, with elution gradient over 26 minutes, with the mobile phase consisting of phosphoric acid, triethylamine, methanol, acetonitrile (10). Liquid-liquid extraction with ethyl acetate and sotalol as internal standard were used.

Based on the literature data review regarding the methods of quantification of tramadol, mainly in biological samples, we have proposed the development of a HPLC method with sensitive fluorescence detection, rapid and suitable for routine clinical laboratories and forensic toxicology everyday use, in order to determine acute intoxication with tramadol.

Materials and method

Chemicals and apparatus:

Reference standards and active substances:

- Tramadol hydrochloride (Sigma Aldrich)

Reagents:

- Methanol Chromosolv for HPLC (Merck)
- Acetonitrile Chromosolv for HPLC (Merck)
- Formic acid (Merck)
- Water for chromatogtaphic use (obtained with Barnstead®EasypureRoDi system)

Materials

- Solid phase extraction cartridges, DSC 18 (Supelco)
- Chromatographic column: Kromasil®, 150 mm x 4.6 mm, 5 μm; C_{18}stationary phase

Apparatus:

- Liquid-chromatograph Surveyor Plus (Thermo Electron Corp.), with the following modules:

- DAD (diode array detector), with a specialized program for the determination of the spectral purity for eluted compounds
- Fluorescence detector
- Quaternary pump with vacuum degasser
- Autosampler with thermostat (Peltier)
- Column compartment with thermostat (Peltier)

- Ultrasonic bath (Elmasonic S60 /Hmodel, Elma)
- Analytical balance (Ohaus)
- Evaporator under nitrogen stream (Techne Dry-Block DB-3Dmodel, Bibby ScientíficInc.)
- Solid phase extraction system (Visiprep Manifold model, Supelco)

- Refrigerated centrifuge Sigma 2-15 K (Sigma)

Biological material

- Human plasma obtained from peripheral venous blood provided by a Hematology Institute

Methodology

Chromatographic conditions

- Mobile phase: 0.1% formic acid: acetonitrile (20:80)
- Flow rate: 1 mL/min
- Column temperature: 22°C
- Sample temperature: 20°C
- Fluorescence detector: λ ex= 280 nm and λem= 310 nm
- Elution: isocratic
- Injection volume: 20 µL
- Analysis time : 6.5 minutes

Preparation of solutions

Tramadol stock solution: 10 mg of tramadol hydrochloride were quantitatively transferred into a 10 mL volumetric flask, dissolved in methanol and diluted to volume with the same solvent. Tramadol working solution (10 µg/mL) was prepared by dilution with mobile phase, from the stock solution.

Control samples

The „simulated" (spiked) human plasma controls were prepared at the following concentrations: 0.1 µg/mL, 0.5 µg/mL and 1 µg/mL.

Tramadol extraction procedure from plasma

A solid phase extraction procedure was applied: at 1 mL plasma add 200 µLNaOH 0.1 mol/L; the mixture is subject to vortex stirring for 2 minutes, then it is slowly passed in the solid phase extraction system, over DSC 18cartridge, previously wetted with methanol (2 mL). Subsequently, the analyze is eluted from the cartridge with methanol (2 mL); the extract is centrifuged at 3000 rpm for 10 minutes, then it is evaporated to dryness under nitrogen stream. The residue was sampled in with 500 µL mobile phase, sonicated for 1-2 minute and then the samples obtained were injected into the HPLC system.

Analytical method validation

The method validation was carried out in accordance with FDA Guidance for Industry-Bioanalytical Method Validation, DRAFT guidance, 2013 (11).

The experimental data was analyzed with Chromquest 4.2 data processing software.

Results

Development and validation of the method

In the elaboration of HPLC tramadol dosing method we start from the literature data, indicating the use of C18 chromatographic columns. Recommended mobile phases contain mostly acetonitrile or methanol, mixed with various buffer solutions (phosphate, triethylamine, acetate, formic acid) with pH between 2.5 and 7.3.

The wavelengths of excitation and emission were typically 200/295, 301 or 275/300. Since the excitation wavelength of 200 nm is likely to be subject of much interference, and based on the absorption spectrum of tramadol with a peak at 280 nm (12), we established the following experimental conditions for the fluorescence detection used for HPLC determination of tramadol: λ_{ex} = 280 nm, λ_{em} = 310 nm.

We selected the chromatographic column after testing three types of chromatography column with octadecylsilyl stationary phase 5 mm (C18): Symmetry® 3.9 mm x 150 mm, 4.6 mm x 150 mm, Kromasil®4.6 mm x 150 mm and Hypersil Gold® 4.6 mm x 100 mm. Kromasil column provided suitable results in terms of analysis time. The retention time of tramadol is approximately 4.7 min, in the working conditions, using a mixture of acetonitrile - 0.1% formic acid (20: 80), as mobile phase. We checked the possibility of using a simple mobile phase, containing methanol and 0.2% formic acid solution in a 65:35 ratio; in these experimental conditions we cannot achieve an appropriate symmetry peak, and the retention time is very short (about 1,2 min.) and susceptible to interference with unretained compounds and plasma separated compounds.

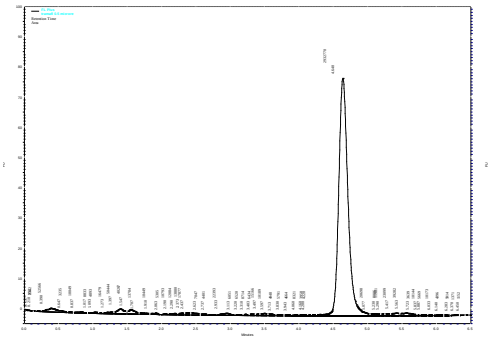

Figure 1.Chromatogram of tramadol hydrochloride standard solution of 0.1 µg/ mL

Given these results, we resorted to a mobile phase of acetonitrile and 0.1% formic acid, the proportions being determined by successive trials: 30:70 (T_R = 2.25 min.), 25:75 (T_R = 2 , 5 min.), 20:80 (T_R = 4.65 min.). Based on these determinations, we selected a mobile phase of acetonitrile and 0.1% formic acid 20:80 (Figure1).

We established the calibration curve using 5 concentrations of tramadol hydrochloride solution in the concentration range of 0.1 µg / mL - 1 µg / mL (0.1 µg / mL, 0.25 µg / mL, 0.5 µg / mL; 0.75 µg / mL and 1 µg / mL). This concentrations

domain coversboth therapeutic (from 0.1 to 0.3 µg / mL) and toxic/lethal (1 µg / mL and 2 µg / mL) levelsof tramadol (13). Three replicate injections from each solution were performed. The experimental data well fitthe model of linear equations y = A + Bx, as we obtained aregression equation y = 1.70311e-007x - 0.00338394 with a correlation coefficient of 0.997414.

Discussions

The precision of the method was determined as repeatability in the linearity study of the method. Based on the results (analysis report provided by the software Chromquest 4.1, data not shown), the method proved to be precise (RSD ≤ 2%). In addition, the intermediate precision was examined at threeconcentration levels, by determination carried out on different days; the experimental data are summarized in Table. 1.

Table 1HPLC method for determination of tramadol hydrochloride - intermediate precision

Tested concentration (µg/mL)	Average concentration found (µg/mL)	RSD (%)
0.1	0.092	5.2
0.5	0.45	3.78
1.0	0.96	4.56
Average	-	**4.51**

The detection and quantification limits were established based on the signal – noise ratio, by recording chromatograms and measuring the signals corresponding to reference solutions tramadol hydrochloride at concentrations of 0.01 µg/mL - 1µg/mL.The value obtained for the limit of detection (DL) was of 10 ng / mL, while the limit of quantification (QL)was set at 100 ng/mL, a level at which both precision and accuracy have been determined.

The specificity/selectivity of the method were determined by recording and comparing of the chromatograms of solutions obtained by processing plasma (blank), and plasma with the addition of tramadolhydrochloride (spiked plasma).

The extraction from plasma samples was performed by solid phase technique (SPE), at alkaline pH, using DSC C18 extraction cartridges (Supelco). The chromatograms shown in the Figure 2 demonstrate the selectivity of the method; no other compound from plasma elutesat the retention time of analyze. Thus, the extraction procedure is appropriate regarding tramadol peak symmetry and separation from other plasma generated peaks.

The method accuracy, as average recovery, is within acceptance limits according to FDA guidelines. In our experiments we followed the possibility of a simple procedure of processing samples, involving only deproteinization with acetonitrile; however, this isolation method,although shorter regardinganalysis time,cannot be applied, as many interferenceswith the tramadol peak are obtained (possible endogenous compounds).

Tramadol plasma dosing method in patients treated with tramadol

Weverified the applicability of the HPLC method with fluorescence detection for the determination of tramadol in patients receiving tramadol, hospitalized at an Oncology Clinic from Bucharest. Samples were obtained under the informed consent of the patients.

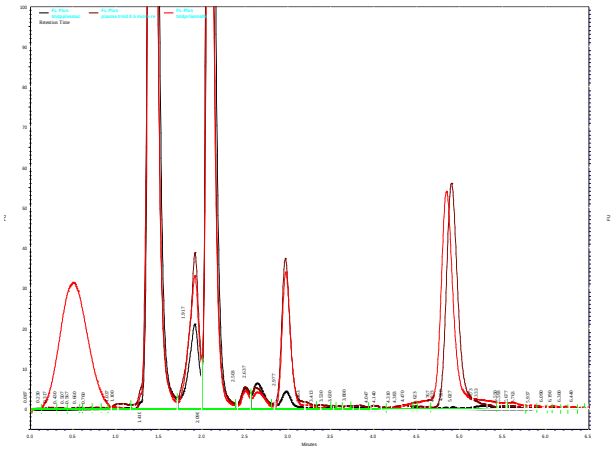

Figure 2. The selectivity of the HPLC method for determination of tramadol(blank plasma and plasma spiked with tramadol 0.5 µg/mL - 2 samples, SPE extraction procedure)

Table 2. **The accuracy (as percentage of recovery after extraction) of HPLC method for the determination of tramadol in plasma**

Concentration (µg/mL)	**0.1**	**0.5**	**1.0**
Average recoveypercentage(%)	**87.56**	**86.37**	**91.2**
Standard error	0.92	6.20	1.84
Standard deviation	1.83	12.41	3.69
CV	0,97%	6,98%	1,96%
Averagerecovery: 88.37%			

Plasma samples were analyzed in 6 patients (5 women and one man) with cancer, treated with tramadol as an analgesic in doses ranging from 100 mg to 400 mg/day, as tablets, capsules or injection.

Experimental data indicate that tramadol has been identified in plasma in 5 of the 6 patients;the plasma levels determined were in therapeutic range, 0.212 µg/mL, respectively 0.325 µg/mL in 2 patients (Figure3); these patients received tramadol dose of 200 mg/day orally or 300 mg / day injection (14).

In samples obtained from three patients, the quantification of tramadol could not be achieved as interferences were observed. There were other compounds extracted from the sample plasma that co-eluted with tramadol (data not shown). The results suggested a similar therapeutic scheme in the treatment of these three patients.

The experimental results show that the developed HPLC method with fluorescence detection can be applied for determination of tramadol at therapeutic levels in plasma. In addition, the proposed extraction method - solid phase extraction, is simple, accurate and requires a shorter analysis time than liquid-liquid processes.

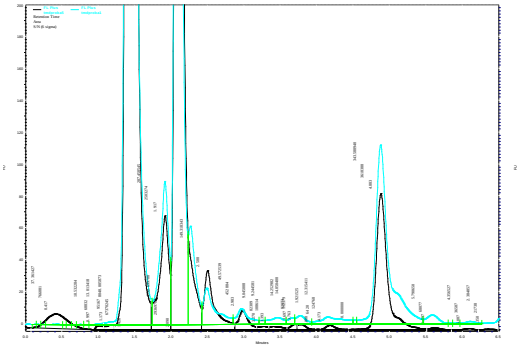

Figure 3.*Chromatograms obtained from plasma tramadol dosage in patients receiving tramadol (pacients 1 and 5)*

Conclusions

- ✓ Considering the native fluorescence of tramadol, and based on the literature data, we developed and validated a new HPLC method with fluorescence detection for the determination of tramadol in human plasma.
- ✓ The method uses simple experimental conditions: Kromasil column (C18 bound phase), mobile phase acetonitrile: 0.1% formic acid, the fluorescence detection, $\lambda_{ex}/_{em}$ = 280/310 nm for 6.5 minutes analysis time, applying SPE procedure from plasma on C18 cartridge.

- ✓ The developed method is selective, is linear on concentration range of 100 ng - 1 μg/ mL, corresponding to both therapeutic and the toxic/lethal tramadol levels, and has the detection/quantification limitsof 0.010 μg/mL and 0. 100μg/mL respectively. In addition, the useof the fluorescence detector contributes, in itself, to increasing the selectivity of the method.
- ✓ The applicability of HPLC-FL method was verified for determination of tramadol in plasma in patients treated with tramadol. Thus,the utility of the method for quantification of therapeutic levels of tramadol has been demonstrated.

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