High-performance liquid chromatographic determination of tramadol in human plasma

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Abstract

Tramadol has been determined in human plasma samples using a sensitive high-performance liquid chromatographic method. The plasma samples were extracted with *tert.*-butylmethyl ether in one-step liquid–liquid extraction (recovery 86%) and analyses of the extracts were performed on reversed-phase silica gel using ion-pair chromatography (verapamil as an internal standard) and fluorescence detection. The method was applied to the determination of tramadol levels in twelve healthy volunteers after oral administration of 100 mg of tramadol in capsules of Protradon and Tramal.

Keywords: Tramadol

1. Introduction

Tramadol (see Fig. 1a and Section 2.1 is a centrally acting, analgesic agent with high oral bioavailability [1–3]. Molecular modelling showed that the (±)-*trans*-isomer with both large substituents (3-methoxy-phenyl- and dimethylamino-methyl-) in equatorial positions on a cyclohexane ring is most similar to the geometrical structure of morphine and the tests on mice confirmed the best analgesic activity of this isomer from all tested ones [2]. Tramadol and its metabolites are widely distributed in tissues, the binding to human serum proteins is only 4% [3].

The biotransformation of tramadol has been investigated in animals and man [4–7]. In all species,

the main metabolic pathways are N- and O-demethylation and conjugation of O-demethylated compounds (to glucuronides and sulfates) [5]. The principal metabolite, O-demethyl-tramadol, is pharmacologically active (compare with codeine and its
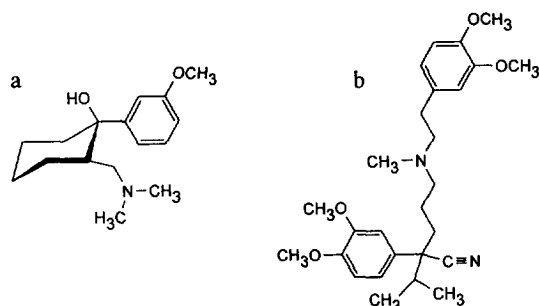

Fig. 1. Chemical structures of tramadol (a) and verapamil (b). Both compounds are displayed as bases.

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O-demethyl metabolite, morphine) and shows a 2–4 fold higher analgesic potency than tramadol in animals [1]. Apart from the O-demethylated metabolite, all the other metabolites are pharmacologically inactive. Biological half-life of tramadol in man is around 6 h. More than 90% of tramadol in man is eliminated in urine (70% in the form of its metabolites), the rest is excreted in faeces [5].

Tramadol contains a weakly absorbing chromophore in its molecule [8]. This is the reason why UV detection is not suitable for the determination of low tramadol concentrations in plasma samples. Recent methods for determination of tramadol in biological samples use gas chromatography with a nitrogen-selective detector [9], gas chromatography–mass spectrometry [5,10], or (in the case of labelled tramadol) radiochromatography [5]. HPLC methods with UV detection were used for determination of higher concentrations of tramadol and its metabolites in urine [5,7] and chiral high-performance liquid chromatography has been used for determination of enantiomeric ratios of the compounds mentioned [7].

Usually, biological samples have been extracted using multi-step pH-dependent procedures, with recoveries of the extractions being 50–87% [5,7,9,10]. A higher homologue of tramadol, (±)-*trans*-2-dimethylaminomethyl-1-(3-ethoxyphenyl)-cyclohexanol hydrochloride has been used as an internal standard in GC and HPLC determinations [7,9].

The present communication describes a newly developed high-performance liquid chromatographic determination with fluorescence detection. Human plasma samples containing tramadol and spiked by the internal standard were extracted with *tert.*-butylmethyl ether with 86% recovery. Determination of tramadol was performed on a reversed-phase C_{18} column using ion-pair chromatography. Fluorescence detection gave a response that was more than two orders of magnitude higher than that obtained with UV detection (dependent on excitation and emission wavelength used).

The developed method was applied to a bioequivalence study of two oral forms of tramadol (Protradon, cps. 50 mg, PRO.MED.CS, Czech Republic, versus Tramal cps. 50 mg, Grünenthal, Germany). The open, randomized, cross-over study

performed on a group of twelve healthy volunteers confirmed the bioequivalence of both preparations.

2. Experimental

2.1. Chemicals

Tramadol hydrochloride [(±)-*trans*-2-dimethylaminomethyl-1-(3-methoxyphenyl)-cyclohexanol hydrochloride, $C_{16}H_{25}O_2N \cdot HCl$, F.W.=299.84 g mol^{-1}, subst. lot T-90001] and Protradon (tramadol hydrochloride 50 mg capsules) were from PRO.MED.CS. (Prague, Czech Republic). Tramal (tramadol hydrochloride 50 mg capsules) was from Grünenthal (Stolberg, Germany). Verapamil hydrochloride {2-(3,4-dimethoxyphenyl)-2-isopropyl-5-[N-2-(3,4-dimethoxyphenyl)-ethyl-N-methylamino]valeronitrile hydrochloride, $C_{27}H_{38}O_4N_2 \cdot HCl$, F.W.= 491.1 g mol^{-1}, subst.} was from Sigma-Aldrich (Prague, Czech Republic). Acetonitrile and *tert.*-butylmethyl ether (both HPLC grade, Merck, Darmstadt, Germany), 1-hexanesulfonic acid sodium salt monohydrate (for ion-pair chromatography, Fluka, Buchs, Switzerland), ammonium hydroxide (26% water solution of NH_3), phosphoric acid (85%), methanol (all of analytical grade, Lachema, Brno, Czech Republic) and demineralised water (Fotochema, Hradec Králové, Czech Republic) were used.

2.2. Volunteers

The pharmacokinetics after administration of two oral forms of tramadol, Protradon (cps. 50 mg, PRO.MED.CS) and Tramal (cps. 50 mg, Grünenthal) was evaluated. The open, randomized, cross-over study was licensed by the regulatory governmental agency and the regional ethical committee.

Twelve healthy volunteers [six males and six females, mean age 23 ± 3 years, mean weight 69 kg (range 58–85 kg) and mean height 176 cm (range 160–185 cm)] were examined clinically and biochemically.

A single oral dose of 100 mg (two capsules of one of the preparations, i.e., Protradon or Tramal) was administered after five days restriction of fat, alcohol and caffeine intake in the diet and smoking prohibi-

tion. After two weeks, the second dose of Tramal or Protradon was administered under the same conditions. The sequence of the preparations was given randomly (chosen by computer).

Blood samples were taken from the cubital vein into a heparinized syringe at 0–24 h (−10, 30, 45, 60, 90, 120, 150, 180, 240, 480, 720, 1440 min). Plasma was obtained by centrifugation at 3000 g for 10 min. Plasma specimens (2 ml) were stored at −20°C until analysis.

2.3. Sample preparation

The frozen plasma samples were thawed, turbid samples were centrifuged (2100 g for 8 min). To 0.5 ml (or 1 ml) of human plasma in a 9-ml glass tube equipped with a ground-glass stopper, 60 μl of $10^{-5} M$ aqueous verapamil (internal standard) and 60 μl (or 120 μl when 1 ml of plasma was used) of 26% aqueous ammonium hydroxide were added and plasma was shaken vigorously for 5 s. tert.-Butylmethyl ether (3 ml) was added and the content of the tube was vortex-mixed for 1 min and then gently shaken for another 30 min. After centrifugation (2600 g, 10 min) the tubes containing lower water and upper organic layers were stored in a freezer (−20°C for 6 h) until the water layer was frozen. Then the organic layer containing analytes was decanted into another clean 3-ml tube and tert.-butylmethyl ether was evaporated (water bath 40°C, stream of nitrogen). The dry extract in the glass tube was reconstituted in 600 μl of the mobile phase (300 μl in the case of manual dosing using a Rheodyne injector on the first chromatographic system (see Section 2.4) and transferred into the microvial of the autosampler and 100 μl of the sample were injected onto the column.

2.4. Chromatography

Two Spectra-Physics (now Thermo Separation Products) chromatographic systems were used. The first chromatographic system consisted of an SP 8800 ternary HPLC pump, a Spectra 100 UV–Vis detector, a FL2000 fluorescence detector (connected behind the UV detector), an SP4400 ChromJet integrator with two independent channels (for data acquisition from both UV and fluorescence detectors) and an AT-computer (DRV, Dreieich, Germany), equipped with a Labnet interactive communication network controlled by WINner/286 software. A Rheodyne Model 7125 injection valve with a 50-μl injection loop was used. This chromatographic system was used for development of the chromatographic method and for measurement of the excitation and emission spectra of tramadol and verapamil in the mobile phase used.

The second chromatographic system consisted of a SCM400 solvent degasser, a P4000 quaternary gradient pump, an AS3500 autosampler with a 100-μl sample loop, a FL2000 fluorescence detector, a SN4000 system controller and a Spectra 386 E computer, with PC1000 analytical software working under the operating system OS-2. This chromatographic system was used for serial determinations of tramadol in plasma extracts and for measurement of the UV spectra of tramadol and verapamil.

A compact glass cartridge (CGC) analytical column (150 × 3.3 mm I.D.) and a CGC guard column (30 × 3.3 mm I.D.), both packed with Separon SGX C_{18}, 5 μm (Tessek, Prague, Czech Republic), were used. An HPLC column LiChroCART 250 × 4 mm I.D. with a LiChroCART (4 × 4 mm I.D.) precolumn, containing LiChrospherÒ 100 RP-18 (5 μm, endcapped, Merck) was also tested for comparison.

The mobile phase for the Tessek column was prepared by dissolving hexanesulfonic acid sodium salt monohydrate (4 g) in 400 ml of 0.02 M phosphoric acid (1.28 ml of 85% phosphoric acid in 1000 ml of water) and this solution was mixed with 600 ml of acetonitrile. The mixture was degassed ultrasonically before use. The mobile phase for the Merck column mentioned had to be changed (400 ml of acetonitrile plus 600 ml of 0.02 M phosphoric acid with 4 g of hexanesulfonic acid sodium salt monohydrate). The flow-rate was 0.9 ml min^{-1} in both cases (the pressures for both columns were different being −25 MPa on the Tessek column and 13 MPa on the Merck one).

UV detection was carried out at 275 nm and the rise time was 1 s.

The fluorescence detector was programmed as follows: Excitation wavelength was 202 nm for the

whole analysis, the emission wavelength was 296 nm for the time interval 0.0–6.5 min (detection of tramadol) and 314 nm for the time interval 6.5–10.0 min (detection of verapamil as internal standard). The integration inhibition was switched on in the time interval of change of the emission wavelength (6.4–6.6 min). Lamp flash-rate was adjusted at 100 Hz, PMT voltage was 600 V.

2.5. Calibration

The standard stock solutions (29.98 mg of tramadol hydrochloride in 100 ml and 49.11 mg of verapamil hydrochloride in 100 ml, both $10^{-3}M$) were diluted with water to a calibration series of tramadol/verapamil mixtures with tramadol concentrations of $5\cdot10^{-7}M$, $1\cdot10^{-6}M$, $2\cdot10^{-6}M$, $3\cdot10^{-6}M$ and $4\cdot10^{-6}M$ and with the same verapamil concentration ($1\cdot10^{-6}M$) at each calibration level. The same concentrations were used to make a calibration curve with drug-free human plasma spiked with tramadol and verapamil. The extraction procedure was the same as described in Section 2.3.

2.6. Testing and statistical evaluation of the analytical procedure

Five-level calibrations with six analyses at each concentration were performed. The on-line statistical processing of calibration analyses by the least-squares method (normal and weighted linear regression) was performed automatically by the PC1000 software directly on computer which was connected on-line with the detector.

The linearity of the calibration curve from aqueous solutions of tramadol and verapamil (line A: $y = k_A x + q_A$) and from extracts of drug-free human plasma spiked with the same amount of verapamil (internal standard) and with different amounts of tramadol (line B: $y = k_B x + q_B$) was tested (x is the concentration ratio of tramadol to verapamil, y is the corresponding peak-area ratio tramadol/verapamil and the coefficients q_A and q_B should approach zero). The accuracy was calculated as the percentage found on the standard curve (line B). The precision of the method, expressed as the relative standard deviation (R.S.D. = 100 S.D./mean) for line B, was also calculated (see Section 3).

The recovery was determined as the ratio of the slopes of line B' and line A' (calculated in this case using the method of external standard for tramadol), multiplied by 100: recovery (%) = 100 (k_B'/k_A').

The quantification limit (at a tramadol signal-to-noise ratio of 10) was calculated from six chromatograms.

3. Results and discussion

3.1. Chromatography

When developing a new method for HPLC determination, one of the principal questions to be solved is the choice of the internal standard. In the literature [7,9], the use of a higher homologue of tramadol (an ethoxy-derivative) was described. However, this derivative was not readily available; another, commercially available, standard had to be found. In general, such a compound should possess a similar structure to its chromophore (i.e. should exhibit similar spectral characteristics), should be of a similar polarity (to have the retention time sufficiently close to that of the compound which is to be determined and to exhibit similar behaviour during the extraction). In this work, papaverine, pethidine, pentazocine and verapamil were tested. For analyses with Tessek HPLC columns, verapamil was found to be the most suitable (t_R of verapamil = 7.34 min, t_R of tramadol = 4.34 min, see Fig. 4). For spectral detection, the similarity of both the UV absorption and fluorescence spectra of tramadol and verapamil are important (see Figs. 2 and 3). The suitability of the UV and fluorescence detection methods was compared. For excitation of the fluorescence, two wavelengths were chosen based on the spectral data (Fig. 3), i.e. 202 and 232 nm, respectively; emission was followed at 296 nm for tramadol and at 314 nm for verapamil. Comparison of the UV (at 275 nm) and fluorescence detection (at 296 nm) of tramadol documents clearly the advantages of the latter, as the response of the UV detector is less than 0.5% of the fluorescence response for excitation of the tramadol spectrum at 202 nm. Excitation of tramadol fluorescence at 232 nm and detection at 296 nm is 2.6 times less sensitive than with excitation at 202 nm. In other
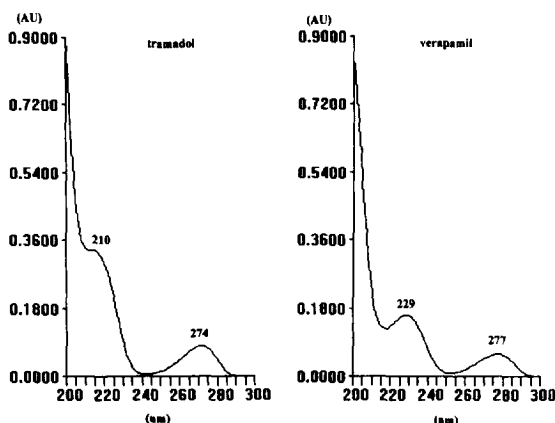
Fig. 2. UV spectra of tramadol and verapamil. Spectra obtained using Spectra-FOCUS UV-detector in the course of HPLC analysis.

Fig. 4. Typical chromatogram of a human plasma extract. The peak at t_R =4.34 min corresponds to 89.94 ng of tramadol, the peak at t_R =7.34 min represents 49.1 ng of verapamil (as internal standard).

words, the fluorescence response is more than two orders of magnitude stronger than the response of the UV detector (275 nm), when the appropriate wavelengths are chosen (excitation at 202 nm and emission at 296 nm for tramadol).

Taking into account that the Tessek columns (which were used routinely for tramadol determination in our laboratory) are not commercially available in all geographical areas, a comparison with Merck columns has been performed also. To get

similar tramadol retention times with the Merck columns, the mobile phase had to be more polar (see Section 2.4). Under these conditions, the retention time of tramadol was 4.4 min. The retention time of verapamil was upshifted by 11 min, so its determination did not interfere with that of tramadol; however, this shift makes verapamil a less optimal internal standard for Merck columns, as the time of analysis is relatively long (17 min).

The tert.-butylmethyl ether has been proven to be a suitable medium for extraction of tramadol and verapamil from blood plasma. The one-step extraction method with 86% recovery of tramadol is quick and reliable; a separation of the aqueous and organic phases by freezing the aqueous phase is an elegant
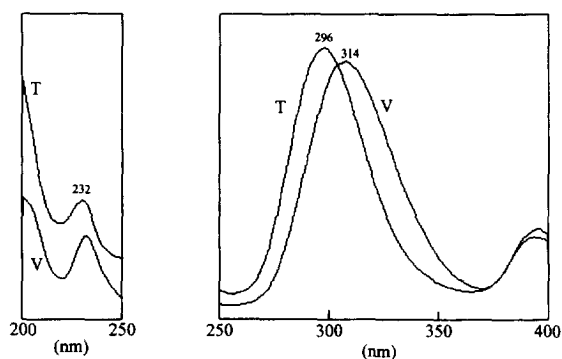

Fig. 3. Excitation (left panel, range 200–250 nm) of tramadol (T, at emission wavelength 296 nm) and verapamil (V, at emission wavelength 314 nm). Emission spectra (right panel, range 250–400 nm) of tramadol (T) and verapamil (V), both recorded with an excitation wavelength of 202 nm. Spectra were obtained using FL2000 detector in the course of HPLC analysis.
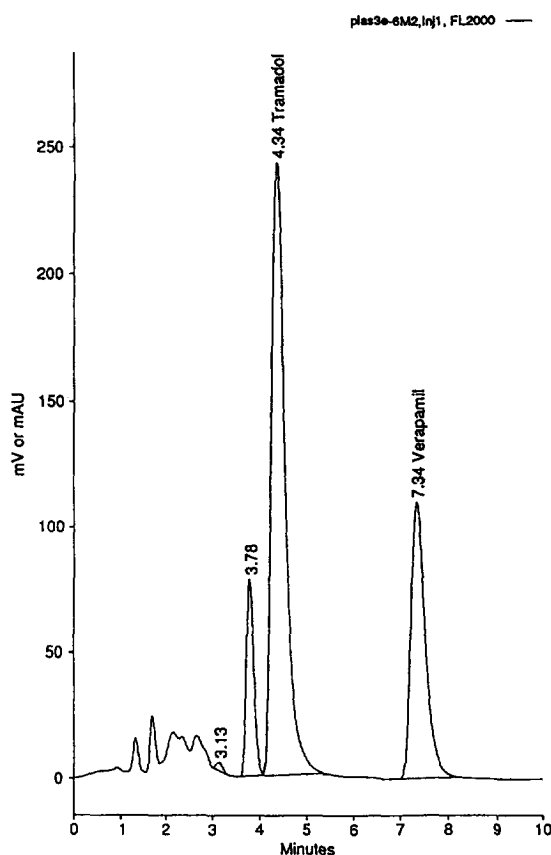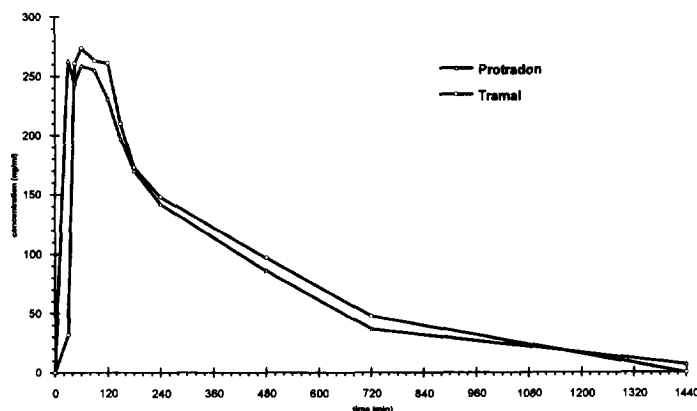
Fig. 5. Representative comparative pharmacokinetic study of tramadol in Tramal® and Protradon® preparations at the first volunteer (male, 185 cm, 80 kg).

and effective method as the upper organic phase is easily removable.

The analytical procedure is accurate and precise. Two calibration curves (A, samples from aqueous solutions of tramadol and verapamil; B, samples from spiked human plasma) were found to be linear; weighted linear regression expressed both curves as linear relationships in the form of the following equations (for A: $y = 0.942x + 0.135$, $r=0.998$; for B: $y = 0.923x + 0.195$, $r=0.994$).

The accuracy and precision of the tramadol determination (using verapamil as the internal standard) in spiked human plasma samples were found to be 96.53% (range 91.7–99.4%) and 5.2% (range 4.5–5.6%), respectively.

The total recovery of tramadol (calculated from calibration curves) was 86%, and the quantification limit was 17 ng/ml of plasma.

3.2. Pharmacokinetics

The area under the curve from $t = 0$ to 24 h (AUC_{0-24}) was determined via the linear trapezoidal rule (see Fig. 5). The extrapolation to infinity which is necessary for $AUC_{0-\infty}$ evaluation was calculated using a linear regression model from the last three data points that had been log transformed. Maximal concentration achieved (C_{max}) was obtained directly from the measured concentrations (without interpolation).

Assuming the multiplicative model, expected medians of these parameters of test (Protradon) and reference (Tramal) formulation were computed and are presented in Table 1, as are their ratios. These confidence intervals are suitable for bioequivalence testing. They are well within the bioequivalence range 0.8–1.25 adopted in a recent CPMP guide on bioequivalence studies and that is why one can conclude that Protradon and Tramal are bioequivalent.

Table 1
Pharmacokinetic data of tramadol samples

	Protradon	Tramal	Ratio	90% Confidence interval (%)
AUC_{0-24} (ng.h/ml)	2933	2985	0.983	90.3–106.9
$AUC_{0-\infty}$ (ng.h/ml)	3223	3450	0.934	82.9–105.3
C_{max} (ng/ml)	387	376	1.029	86.2–123.1

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