High-performance liquid chromatographic determination of tramadol and its *O*-desmethylated metabolite in blood plasma Application to a bioequivalence study in humans

M. Nobilis[a],*, J. Kopecký[a], J. Květina[a], J. Chládek[a], Z. Svoboda[a], V. Voříšek[b], F. Perlík[c], M. Pour[d], J. Kuneš[d]

[a]*Institute of Experimental Biopharmaceutics, Joint Research Center of Academy of Sciences of the Czech Republic and PRO.MED.CS Praha a.s., Heyrovského 1207, CZ-500 02 Hradec Králové, Czech Republic*
[b]*Faculty of Medicine, Charles University, Šimkova 870, CZ-500 01 Hradec Králové, Czech Republic*
[c]*Clinical Pharmacology Unit, 1st Department of Medicine, Faculty of Medicine, Charles University, U nemocnice 2, CZ-128 08 Prague 2, Czech Republic*
[d]*Faculty of Pharmacy, Charles University, Heyrovského 1203, CZ-500 05 Hradec Králové, Czech Republic*

Abstract

Simultaneous HPLC determination of the analgetic agent tramadol, its major pharmacodynamically active metabolite (*O*-desmethyltramadol) in human plasma is described. Simple methods for the preparation of the standard of the above-mentioned tramadol metabolite and N^1,N^1-dimethylsulfanilamide (used as the internal standard) are also presented. The analytical procedure involved a simple liquid–liquid extraction of the analytes from the plasma under the conditions described previously. HPLC analysis was performed on a 250×4 mm chromatographic column with LiChrospher 60 RP-selectB 5-μm (Merck) and consists of an analytical period where the mobile phase acetonitrile–0.01 *M* phosphate buffer, pH 2.8 (3:7, v/v) was used, and of a subsequent wash-out period where the plasmatic ballast compounds were eluted from the column using acetonitrile–ultra-high-quality water (8:2, v/v). The whole analysis, including the equilibration preceding the initial analytical conditions lasted 19 min. Fluorescence detection (λ_{ex} 202 nm/λ_{em} 296 nm for tramadol and its metabolite, λ_{ex} 264 nm/λ_{em} 344 nm for N^1,N^1-dimethylsulfanilamide) was used. The validated analytical method was applied to pharmacokinetic studies of tramadol in human volunteers. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Pharmacokinetics in humans; Bioequivalence; Tramadol; *O*-desmethyltramadol

1. Introduction

Tramadol hydrochloride [rac.-1(*e*)-(*m*-methoxyphenyl)-2(*e*)-dimethylaminomethylcyclohexan-1(*a*)-ol hydrochloride, CAS 22204-88-2] is a synthetic, centrally acting analgesic, which has been used since 1977 for the relief of moderate to severe acute and chronic pain [1,2]. Two synergistic mechanisms of action are responsible for its analgetic activity, as tramadol is both a weak opioid agonist with selectivity for the μ-receptor and a weak inhibitor of monoamine neurotransmitter (noradrenaline and serotonin) reuptake. This dual mechanism of action may be attributed to the differences between the two enantiomers of racemic tramadol. The (+)-enantiomer has a higher affinity for the μ-receptor and is a

*Corresponding author.

more effective inhibitor of serotonin reuptake, whereas the (−)-enantiomer is a more effective inhibitor of noradrenaline reuptake and increases its release by autoreceptor activation [3,4].

Tramadol is rapidly and almost completely absorbed after an oral administration. However, its mean absolute bioavailability is only 65–70% due to the first-pass hepatic metabolism [5,6]. The extent of bioavailability increases to 77% after the rectal administration of tramadol suppositories [7] and to 100% after an intramuscular administration [4]. The peak plasma concentration is reached in 0.17–1.5 h following the intramuscular administration [8], in 0.5–1.7 h after the oral administration of drops [6], in 1–3 h after the oral administration of capsules [5] and in 2–6 h after the rectal administration of suppositories [7].

The biotransformation of tramadol has been investigated in mice, hamsters, rats, guinea pigs, rabbits, dogs and humans [9]. The principal metabolic pathways, *O*- and *N*-desmethylation, involve cytochrome P-450 isoenzymes 2D6 (pivotal), 2B6 and 3A4 [10]. *O*- and *N*-Desmethylated compounds undergo further sulphation or glucuronidation in phase II reactions. Only one of these metabolites, *O*-desmethyltramadol (M1 metabolite), is pharmacologically active [1,3,4,9,10]. Some new metabolites of tramadol (products of cyclohexyl oxidation in position 4, products of oxidative *N*-desalkylation and 1,6-dehydration) were discovered later in the urine of humans, rats and dogs [11,12]. Approximately 10–30% of the parent drug is excreted unmetabolised in the urine [3,9].

Tramadol plasma protein binding is shown to be 4–20% [4,13]. The drug crosses the placental barrier; its concentration in breast milk, however, is low [4]. The mean terminal half-lives of tramadol and *O*-desmethyltramadol are about 5–7 h [3–7,13–15].

Different analytical approaches were employed in the determination of tramadol and/or its metabolites in biological samples.

Capillary gas chromatography (GC) with a nitrogen-selective detector [16], GC with a flame ionization detector [17] and GC–mass spectrometry (MS) were used for the determination of tramadol [5–7,9,14,18] and its metabolites [9,14].

Capillary electrophoresis (CE) methods for the stereoselective determination of tramadol and its metabolites [19–22] have also been developed. The direct determination of tramadol in urine without extraction or preconcentration is enabled by a recently described CE method with a UV laser-induced native fluorescence detection [23].

A number of high-performance liquid chromatographic (HPLC) methods with ultra-violet [9,24,25], fluorescence [8,26–31], electrochemical [32] and MS detection [11,33] have been described. Achiral HPLC analytical methods [8,9,11,12,24–27,30,32] are routinely used in pharmacokinetic and bioequivalence studies, while chiral HPLC methods [24,28,31,33] are very important for the determination of the enantiomeric ratios of tramadol and its metabolites.

Tramadol and its metabolites were profiled, characterized, identified and determined in different biomatrices — in urine [9,11,12,20,21,23,24,29], faeces [9,12], bile [9] and in the microsomal fraction of the hepatocytes [10,27,29]. For the pharmacokinetic and bioequivalence studies, the determination of tramadol and its biotransformation products in the serum [5–7,14,16], plasma [8,14,17,18,22,25,26, 28,30–33] or in the whole blood [14] were used.

A sample handling step is usually necessary prior to the instrumental analysis of xenobiotics in the biomatrices in order to remove interfering compounds and to increase the selectivity and sensitivity of the analytical method.

In case of tramadol derivatives, a pH-dependent liquid–liquid extraction (LLE) [8,9,16,17,20–22,24–27,30–32] and a solid-phase extraction (SPE) [11,12,18,20,28,29,33] were usually employed.

N-Acetylprocainamide [8], [1-(*m*-hydroxyphenyl)-2-(*N*-ethyl-*N*-methylaminomethyl)-cycloheptan-1-ol hydrochloride [27], *O*-ethoxy derivative of tramadol [16,24,32,33], fluconazole [30], ketamine [31], metoprolol [25], meperidin [17], nefopam [18], verapamil [26] and $^2H_2$,^{15}N tramadol [14] were used as internal standards for the determination of tramadol and its metabolites in biological fluids.

A number of the above-mentioned bioanalytical methods were employed in the pharmacokinetic studies after oral [5,6,25,26,33], rectal [7,18], intravenous [14,17,30,32] and intramuscular [8] administration of tramadol hydrochloride.

This communication describes a bioanalytical method involving a simple LLE of plasma samples

[26] and subsequent HPLC determination of tramadol and its principal metabolite, *O*-desmethyltramadol, using fluorescence detection. The chromatographic conditions used enable a selective separation of tramadol and its two metabolites, *O*- and *N*-desmethyltramadol. A simple and unambiguous degradation method for *O*-desmethylation of tramadol was proposed and the synthetic standard of the principal pharmacodynamically active metabolite was prepared in this way. Also, a suitable 'tailor-made' internal standard for the HPLC determination of tramadol was synthesized and employed in this bioanalytical method.

The developed and validated analytical method was applied to pharmacokinetic studies of tramadol and its *O*-desmethylated metabolite in humans. As an example of the use, the results of a bioequivalence study of two rectal forms of tramadol (Protradon supp. 100 mg vs. Tramal supp. 100 mg), performed on a group of twenty-four healthy volunteers, are presented in this communication.

2. Experimental

2.1. Chemicals, preparations, solutions, materials

Tramadol hydrochloride [(rac.-1(*e*)-(*m*-methoxyphenyl)-2(*e*)-dimethylaminomethylcyclohexan-1(*a*)-ol hydrochloride, $C_{16}H_{25}O_2N \cdot HCl$, formula molecular mass=299.84 g mol^{-1}) and Protradon[®] (tramadol hydrochloride 100-mg suppository) were from PRO.MED.CS Praha (Prague, Czech Republic). Tramal[®] (tramadol hydrochloride 100-mg suppository) was from Léčiva (Prague, Czech Republic, in collaboration with Grünenthal, Stolberg, Germany). The standards of the tramadol metabolite (*O*-desmethyltramadol) and N^1,N^1-dimethylsulfanilamide [diMeSA, internal standard (I.S.)] were synthesized in our laboratory (see Section 2.3.). Acetonitrile and *tert.*-butylmethyl ether (both HPLC grade, Merck, Darmstadt, Germany), ammonium hydroxide (26% water solution of NH_3), phosphoric acid (85%), methanol, sodium hydrogenphosphate dodecahydrate (all analytical grade, Lachema, Brno, Czech Republic), ultra-high quality (UHQ) water (prepared using Elgastat UHQ PS apparatus, Elga, Bucks, UK) were used for the chromatography. Boron tribromide

(1 *M* solution in dichloromethane, Sigma–Aldrich, Prague, Czech Republic) was used for the preparation of the *O*-desmethyl derivative of tramadol. *N*-Acetylsulfanilyl chloride 98%, 2 *M* dimethylamine in tetrahydrofuran and *N,N*-dimethylformamide 99.9% (all from Sigma–Aldrich) were used for the preparation of N^1,N^1-dimethylsulfanilamide (internal standard).

Phosphate buffer (0.01 *M*, pH 2.8) was prepared by the dissolution of sodium hydrogenphosphate dodecahydrate (3.58 g) in UHQ water (990 ml), adjusting to pH 2.8 with a solution of phosphoric acid (2 *M*) and filling to a total volume of 1000 ml with UHQ water.

2.2. NMR and GC–MS analyses

A Varian Mercury-Vx BB 300 NMR spectrometer was used for the NMR analyses of the synthetic standard of *O*-desmethyltramadol and the I.S.. The NMR spectra were recorded at 300 MHz for ^{1}H, and 75 MHz for ^{13}C. Chemical shifts are given as δ values in ppm, the coupling constants are given in Hz.

A GC–MS instrument with an ion-trap detector from ThermoFinnigan (formerly Finnigan MAT, type Magnum™) in combination with a gas chromatograph Varian 3300 was used for the analyses of tramadol and its metabolites (*O*- and *N*-desmethyltramadol) in biomatrices as well as for the structural analysis of the standards. The instrument was equipped with a DB-5 column, 30 m×0.25 mm I.D., 0.25 μm film thickness. Helium at a flow-rate of 1 ml/min was used as the carrier gas. The injection mode was split/splitless 45 s). The dry extracts from plasma samples were dissolved in ethyl acetate before injection.

2.3. Syntheses of the analytical standards

2.3.1. O-Desmethylation of tramadol

Tramadol hydrochloride (2.4 g, 0.008 mol) was converted into the free amine using a pH-dependent extraction. The amine was dissolved in dichloromethane (10 ml) and cooled to −50 °C (a bath of solid carbon dioxide in methanol). A solution of boron tribromide (20 ml of 1*M* solution in dichlorome-

thane, 0.02 mol) was subsequently added in several portions and the reaction mixture was stirred for 50 min. The mixture was then allowed to warm up to room temperature and was poured onto ice. pH was increased to 10 and the resultant mixture was extracted with *tert.*-butylmethyl ether. The organic phase was dried over Na_2SO_4 and dry HCl (gas) was passed through the ethereal extract until the crystals of *O*-desmethyltramadol hydrochloride ($C_{15}H_{24}O_2NCl$, formula molecular mass = 285.81 g/mol, 1.6 g, 70% yield) precipitated out.

^1H NMR (^2H_2O) δ: 7.33 t $J = 7.78$ Hz (H5), 7.09–7.01 m 2H (H2, H4), 6.88–6.80 m 1H (H6), 2.92 dd $J = 13.05$ $J = 10.25$ Hz (N–CH_3), 2.70s 3H CH_3, 2.61s 3H CH_3, 2.18– 1.37 m 9H (CH_2, CH).

^{13}C NMR (^2H_2O) δ: 23.407, 26.897, 28.279, 42.613, 43.592, 43.990, 47.819, 62.521, 78.017, 114.506, 116.611, 119.597, 132.908, 151.476, 158.446.

GC–MS, electron impact ionisation (EI) mode: molecular ion at m/z 249, further characteristic ions at m/z 218, 206, 188, 189.

2.3.2. Synthesis of N^1,N^1-dimethylsulfanilamide (internal standard)

N-Acetylsulfanilyl chloride (10 g, 0.043 mol) in 20 ml of *N,N*-dimethylformamide was heated under reflux with 25 ml of a solution of dimethylamine. The reaction mixture was then treated with 6 *M* hydrochloric acid in order to remove the acetate protective group from the N^4-amino function. N^1,N^1-Dimethylsulfanilamide was extracted into ethyl acetate and the solution was washed with 2% aqueous sodium carbonate. After recrystallization from ethanol, N^1,N^1-dimethylsulfanilamide ($C_8H_{12}O_2SN_2$, formula molecular mass = 200.26 g/mol, 4.7 g, 55% yield) was obtained.

^1H NMR ($C^2H_3O^2H$) δ: 2.60 (6H, s, N$(CH_3)_2$), 6.75–6.68 (2H, m AA'BB', H2, H6), 7.47–7.40 (2H, m AA'BB', H3, H5). ^{13}C NMR ($C^2H_3O^2H$) δ: 38.4, 114.4, 121.7, 130.9, 154.5.

2.4. Volunteers, study design and biological material

Twenty-four healthy volunteers (twelve males and twelve females), 20–29 years old (mean 23.5 year), weighing 45–85 kg (mean 66 kg) entered the study.

All the subjects were healthy according to their medical history, physical examination, haematology, clinical chemistry and urinalysis. The study was approved by the state authority and the institutional ethics committee.

Two rectal tramadol preparations (both containing 100 mg of tramadol hydrochloride in one suppository) were compared in the study: a test preparation Protradon (PRO.MED.CS Praha) and a reference preparation Tramal (Léčiva).

The study was of open, single dose, randomized, two-way, crossover design with a 2-week washout period between treatments. Smoking, medication and alcohol, methylxanthine and chinine containing beverages were restricted 5 days before and 32 h after dosing.

Blood samples of 5-ml volume were taken in the following time intervals: 0 (predose), 0.5, 1.0, 1.5, 2.0, 2.5, 3, 4, 6, 8, 12, 24 and 32 h after the rectal administration of one suppository (a dose of 100 mg of tramadol hydrochloride). The samples were withdrawn from the cubital vein into the heparinized Monovette syringes (Sarstedt, Germany) and centrifuged immediately at 3000 rpm for 10 min. The plasma was separated and stored in polypropylene tubes at -60 °C until the analysis. According to the stability studies, tramadol and *O*-desmethyltramadol in plasma did not decompose significantly for at least 49 days.

The plasma samples from beagle dog (*Canis familiaris* var. beagle, male, 11.5 kg, 2 h after the oral administration of Protradon, caps., 50 mg of tramadol hydrochloride) containing higher amounts of two tramadol metabolites (*O*- and *N*-desmethyltramadol) were used for comparison (GC–MS and HPLC studies).

2.5. Sample preparation

The frozen plasma samples were thawed out and centrifuged (2000 *g* for 10 min at 20 °C). To 1 ml of human plasma in a 9-ml glass tube equipped with a ground-glass stopper, 216 μl of $10^{-5}M$ diMeSA (internal standard) and 120 μl of 26% aqueous ammonium hydroxide were added and the plasma was shortly vigorously shaken. *tert.*-Butylmethyl ether (3 ml) was added and the content of the tube was vortexed-mixed for 1 min and then gently

shaken for another 30 min. After centrifugation (2000 *g*, 12 min), the tubes were stored in a deep freezer (−60 °C for 30 min) until the water layer froze to ice. The organic layer containing the analytes was decanted into another clean 3-ml tube and the solvent 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 and transferred into a vial of the autosampler. A 100-µl volume of the sample was injected into the chromatographic column. Animal blood plasma was treated in an analogous way.

2.6. Chromatography

Chromatographic analyses were performed using a Thermo Separation Products (formerly Spectra Physics) chromatograph. The chromatographic system was composed of a SCM400 solvent degasser, P4000 quaternary gradient pump, AS 3500 autosampler with a 100-µl sample loop, FL3000 fluorescence detector, SpectraFOCUS high-speed scanning UV–vis detector, SN4000 system controller and a data station (Intel-Pentium 166 MMX, RAM 64 MB, HDD 2GB) with the analytical software CHROMQUEST 2.1 (ThermoQuest, San Jose, CA, USA) working under the operating system Windows NT Workstation 4.0 (Microsoft). A LiChroCART® 250×4 mm analytical column packed with LiChrospher 60 RP-selectB, 5 µm and a precolumn LiChroCART 4×4 mm with the same stationary phase (Merck, Darmstadt, Germany) were used for the analyses. The mobile phase composition in the analytical period (in the time interval 0–8 min; mobile phase A: acetonitrile–0.01*M* phosphate buffer, pH 2.8, 3:7, v/v) was different from that in the washout period (in the time interval 8–14 min; mobile phase B: acetonitrile–UHQ water, 8:2, v/v). After an equilibration (the time interval 14–19 min; mobile phase A) preceding the initial chromatographic conditions, the next sample in the autosampler queue could be analyzed. The flow-rate was 1 ml min^{-1}.

The fluorescence detector was programmed as follows: excitation/emission wavelengths were adjusted to 202/296 nm for the time interval 0.0–6.5 min (the detection of tramadol and its metabolites) and to 264/344 nm for the time interval 6.5–10.0 min (the detection of N^1,N^1-dimethylsulfanilamide

as the I.S.). During the washout and equilibration periods of the analysis (in the time interval 10–19 min), the fluorescence detector could be switched off. The lamp flash rate was adjusted to 100 Hz, photomultiplier tube (PMT) voltage 600 V.

The UV detection, which was used together with NMR analyses for the evaluation of the purity of the standard of *O*-desmethyltramadol and the internal standard, was performed either in a single wavelength mode (275 nm for tramadol and its metabolites and 264 nm for N^1,N^1-dimethylsulfanilamide) or in a high-speed scanning mode (the range 195–365 nm with a 1-nm distance, used for the collection of the spectra).

2.7. Calibration

Standard $10^{-3}M$ stock solutions (29.98 mg of tramadol hydrochloride, 28.58 mg of *O*-desmethyltramadol hydrochloride, both in 100 ml of UHQ water and 20.02 mg of diMeSA in 100 ml of methanol) were prepared. Lower concentrations ($10^{-4}M$, $10^{-5}M$) of each compound were obtained by dilution with UHQ water. A calibration series of tramadol + *O*-desmethyltramadol/diMeSA (I.S.) mixtures with tramadol and *O*-desmethyltramadol concentration of 10, 20, 40, 60, 100, 200, 300 and 400 pmol/ml and with the same diMeSA concentration (2160 pmol/ml) was made. Six individual samples were prepared at each calibration level. The same concentrations were used to make a calibration curve with a drug-free human plasma spiked with tramadol, *O*-desmethyltramadol and diMeSA (using appropriate concentrations in order to keep the volumes at minimum). The extraction procedure was the same as described in Section *2.5*.

2.8. Validation of the analytical procedure

Eight-level calibration series with six analyses at each concentration level were measured. On-line statistical processing of the calibration analyses by the least-squares method was performed automatically using the CHROMQUEST software. The linearity of the calibration curve from aqueous solutions of tramadol, *O*-desmethyltramadol and diMeSA (I.S.) and from extracts of a drug-free human plasma spiked with the above-mentioned analytes was tested

and evaluated [$y = kx + q$, where x is the concentration ratio of tramadol (*O*-desmethyltramadol, respectively) to diMeSA (I.S.) and y is the corresponding peak-area ratio tramadol (*O*-desmethyltramadol) to diMeSA (I.S.)] and the correlation coefficient (r) was expressed. The accuracy was determined as a relative error (%) found on the standard curve and was calculated from the following equation: Accuracy (%) $= 100 \cdot (C_{real} - C_{determined})/C_{real}$ [32]. The precision of the method, expressed as the relative standard deviation (RSD = 100 SD/mean), was also assessed. Both statistical parameters were calculated for each concentration level. The limit of quantification (LOQ) was determined as the lowest concentration on the standard calibration curve which was measured with a precision of 20% and accuracy of 80 or 120% [34]. The limits of detection (LOD) for tramadol and *O*-desmethyltramadol were also calculated [34,35].

3. Results and discussion

3.1. Identification of tramadol metabolites in plasma samples

A search for tramadol metabolites (see Fig. 1) in human plasma after the rectal administration of the parent compound in the form of a suppository was undertaken. The results of HPLC and GC–MS analyses revealed the presence of just one of all the described biotransformation products in the plasma extracts.

EI-MS analysis of plasma extract samples gives several typical ions, such as m/z 58, 121, 135 and 263 (M$^{+\cdot}$), which corresponds to the molecule of
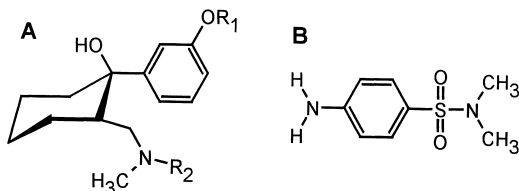

Fig. 1. Structures of compounds under study: (A) tramadol (R$_1$ = CH$_3$, R$_2$ = CH$_3$) *O*-desmethyltramadol (R$_1$ = H, R$_2$ = CH$_3$) *N*-desmethyltramadol (R$_1$ = CH$_3$, R$_2$ = H) (B) N^1,N^1-dimethylsulfanilamide (diMeSA, I.S.).

tramadol. Besides the parent compound, *O*-desmethyltramadol was confirmed in the plasma extracts. *O*-Desmethyltramadol is characterized by a significant loss of water corresponding to the ion with m/z 231. Due to this phenomenon, the compound (having the same molecular ion m/z 249 as *N*-desmethyltramadol) is distinguishable from its *N*-desmethyl analogue under the conditions of GC–MS in the EI mode. *N*-Desmethyltramadol, which was found in the dog plasma extracts, gives typical intensive ions at m/z 121 and 135. These ions serve as evidence of the presence of the methoxy group in these fragments. In addition, the higher intensity of the ions at m/z 202 and 201 indicates the presence of a quinone-like oxo group after the cleavage of original methoxy group attached to the phenyl ring in comparison with the *O*-desmethyl analogue.

3.2. Syntheses of the tramadol metabolites and the 'tailor made' internal standard

Our attempts to obtain the synthetic standard of *O*-desmethyltramadol from a commercial source were unsuccessfull. Consequently, a simple method for the preparation of the *O*-desmethylated product from tramadol was proposed and developed.

Total syntheses of tramadol derivatives (including the metabolites) have been described by Flick et al. [1]. Generally, the syntheses employed a Mannich condensation followed by a Grignard reaction furnishing 1-aryl-2-dialkylaminomethyl-1-cyclohexanol derivatives. For our purposes, more straightforward ways to prepare *O*-desmethyltramadol needed to be found. Hence, the degradation reactions starting from tramadol were tested.

For the cleavage of arylalkylethers (*O*-desalkylation), the treatment of the ether with hydrobromic or hydroiodic acid in acetic acid at reflux has usually been used. However, the presence of the tertiary alcoholic group in tramadol might have led to a pinacoline-like rearrangement under strongly acidic conditions. Thus, the *O*-desmethylation of tramadol was carried out with boron tribromide in dichloromethane at −50 °C [36]. This method was extremely mild and led to a single product.

The preparation of N^1,N^1-dimethylsulfanilamide (diMeSA, I.S., see Fig. 1) consisted in a simple amidation of *N*-acetyl-4-amino-sulfanilyl chloride

followed by the deprotection of N^4 (desacetylation). The reasons for the use of diMeSA as the I.S. are further discussed in Section 3.3.

3.3. Chromatography

In our previous paper [26], the conditions for the chromatographic separation of tramadol and verapamil (used as the internal standard) were described. An acceptable separation efficiency, peak width and peak symmetry on a non-endcapped C_{18} column (Tessek, Czech Republic) was achieved upon using ion-pair chromatography. A Merck column (250×4 mm) containing LiChrospher RP-select B, was chosen for the present pharmacokinetic studies of tramadol and its metabolites.

LiChrospher RP-select B is a spherical carrier built on a porous silica with a 6-nm pore size, having chemically bound octylsilane on the surface. The starting silica material is optimised in order to prevent any secondary interactions with basic substances. Thus, basic compounds would be eluted as symmetric substance peaks from these columns. The addition of an alkanesulfonic acid into the mobile phase, which has been used in the ion-pair chromatography of amines (tramadol and verapamil), should not be necessary in this case.

We have tested tramadol and verapamil separation on an RP-select B column using a mobile phase acetonitrile–phosphate buffer (0.01 M, pH 2.8; 3:7, v/v) without the addition of hexanesulfonic acid. The chromatographic parameters of the tramadol peak were reasonable (t_R =4.2 min and the peak symmetry was excellent), but the peak of the tertiary amine verapamil was extremely wide, asymmetric and had a very long retention time (t_R =24.8 min). From this point of view, verapamil was not a suitable I.S. for the HPLC determination of tramadol under the above-mentioned conditions. For this reason, a new I.S. had to be identified.

We have accumulated a reasonable amount of experience with N^1-alkyl- or N^1,N^1-dialkylsulfanil-amides accessible by simple syntheses (see Sections 2.3.2 and 3.2). These compounds give narrow and symmetric peaks in reversed-phase chromatography, are well extractable from different biomatrices and yield a good response in both UV and fluorescence detectors. Their lipophilicity and, consequently, also

their retention times in different chromatographic systems depend on the nature of the substituents on the amide nitrogen (N^1) and/or on the aniline nitrogen (N^4). From this group of compounds, diMeSA has been shown to be the most convenient internal standard for the HPLC determination of tramadol. The retention time of diMeSA was sufficiently close to that of tramadol (see Fig. 2).

The UV spectra of tramadol and its metabolite are practically identical (maxima at 210 and 275 nm), while diMeSA (I.S.) possesses a different type of chromophore and absorbs at 265 nm. The fluorescence spectral properties of the analytes are demonstrated in Fig. 3. The fluorescence detection is much more sensitive than the UV detection [26] and thus enabled the measurement of low analyte concentrations in the blood plasma. The emission maximum of O-desmethyltramadol is shifted up to 306 nm [37] in comparison with tramadol and N-desmethyltramadol (both 296 nm). Based on the excitation and emission fluorescence spectra (Fig. 3), the fluorescence detection was performed at λ_{ex} 202 nm/λ_{em} 296 nm for tramadol and its metabolites and at λ_{ex} 264 nm/λ_{em} 344 nm for N^1,N^1-dimethylsulfanilamide.

In the chromatograms of the blood plasma extracts, several peaks of ballast compounds appeared at retention times 40–125 min. These compounds could interfere in the next analyses of the next samples in the queue. Two approaches could in principle be applied to resolve this problem. The first one is based on the liquid–liquid reextraction of the basic analytes (tramadol and its metabolites) into the acidic water layer [14,16]. In our case, this approach could not be used, because the I.S. (diMeSA) was not basic and hence its pH-dependent reextraction was impossible. Thus, we used another approach. In order to accelerate the elution of the ballasts from the chromatographic column, a washout period had to be applied after every analysis of the plasma extract. The washing of the column with 80% acetonitrile after the analytical period was necessary for the elution of the ballast compounds native to the plasma (see the peaks in the range 11–17 min shown in Fig. 2). The equilibration preceding the initial analytical conditions lasted 5 min and the whole chromatographic analysis was 19 min long.

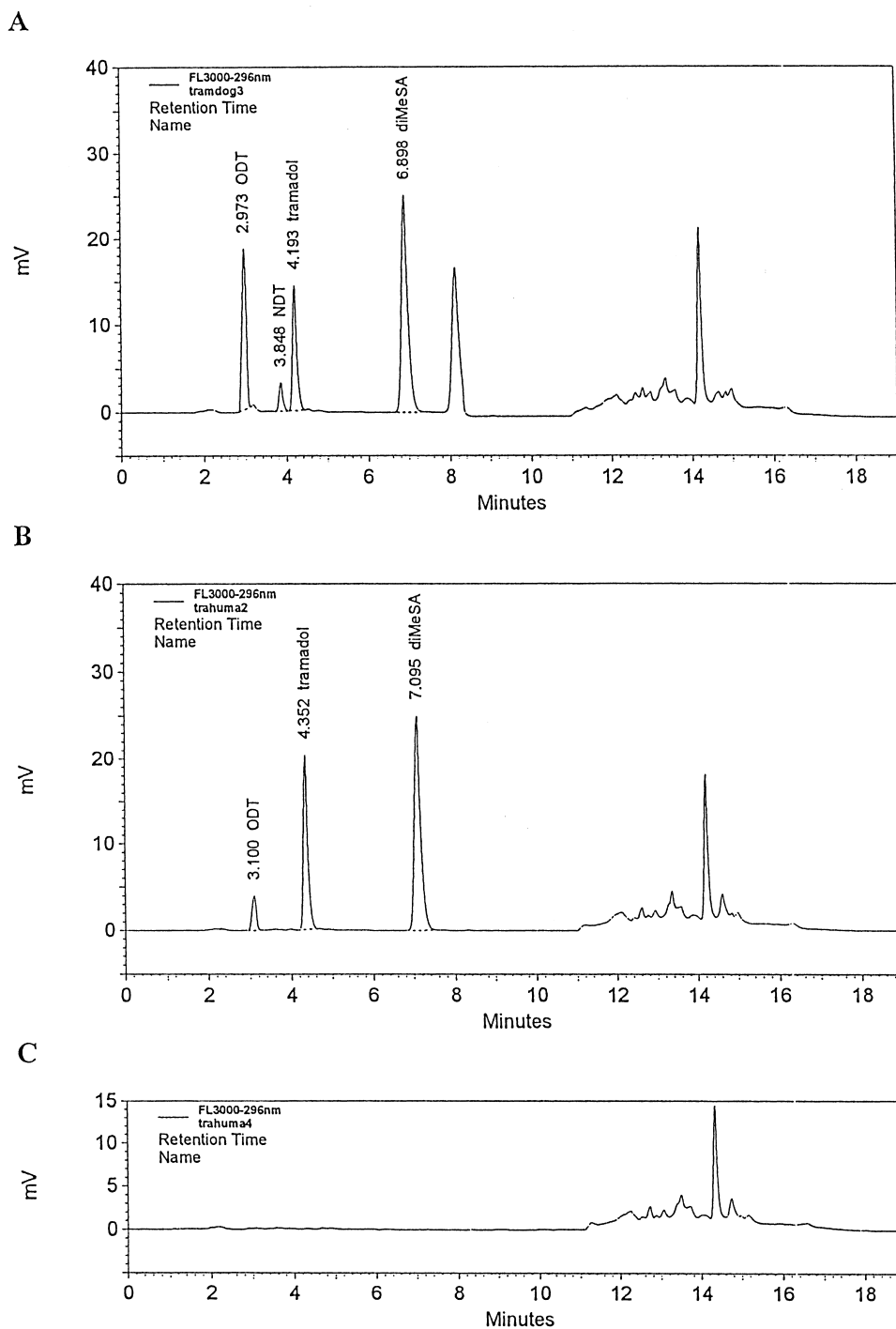The advantages of *tert*.-butyl methyl ether as the

Fig. 2. Typical chromatograms of the extracts from a dog plasma after oral administration of tramadol (A), human plasma after rectal administration of tramadol (B) and blank human plasma (C). ODT = O-desmethyltramadol, NDT = N-desmethyltramadol. Chromatographic and detection conditions — see Section 2.6.
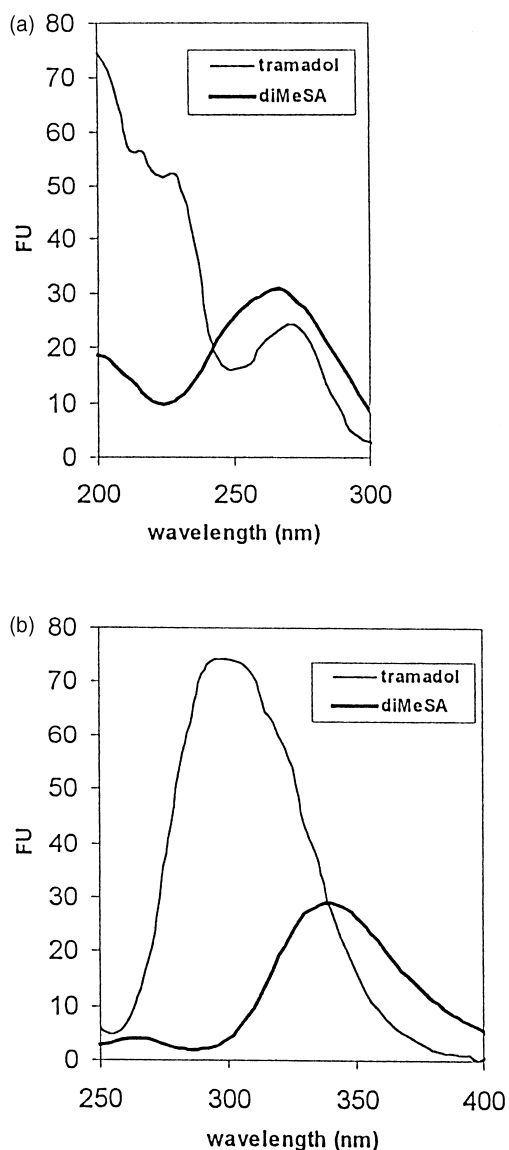
Fig. 3. (a) Excitation spectra for tramadol and diMeSA (measured at the emission wavelength of 296 nm for tramadol and 344 nm for diMeSA. (b) Emission spectra for tramadol and diMeSA (measured at the excitation wavelength of 202 nm for tramadol and 264 nm for diMeSA).

extraction medium have been discussed in our previous paper [26] and also confirmed by other authors [30,31].

The recoveries of tramadol, *O*-desmethyltramadol and diMeSA were found to be 86, 70 and 89%, respectively). The plasma calibration curves for

tramadol and its metabolite were found to be linear ($y = 2.5518x + 0.0573$, $r = 0.9992$ for tramadol; $y = 1.3076x + 0.0323$, $r = 0.9991$ for *O*-desmethyltramadol) in the range of the tramadol and *O*-desmethyltramadol concentrations of 10–400 pmol/ml. The concentrations over 400 pmol/ml caused a non-linear response of the fluorescence detector. For this reason, the plasma sample extracts containing higher concentrations of tramadol were diluted in order to reach a linear range of the calibration curve. The analytical procedure involving the liquid–liquid extraction and HPLC analysis of tramadol, *O*-desmethyltramadol and diMeSA (I.S.) is accurate and precise. The accuracy of tramadol and *O*-desmethyltramadol determination in spiked human plasma samples was found to be 101.22% (range of 95.97–113.45% for tramadol) and 99.8% (range 93.04–103.52% for *O*-desmethyltramadol), the precision was calculated 3.04% (range 0.77–11.47% for tramadol) and 4.74% (range 2.47–7.73% for *O*-desmethyltramadol). Sensitivity of the method is documented with LOQ and LOD values. The LOQ value was calculated at 39 pmol of tramadol/ml of plasma and 35 pmol of *O*-desmethyltramadol/ml of plasma, the LOD value at 5.9 pmol of tramadol/ml of plasma (5.3 pmol of *O*-desmethyltramadol/ml of plasma). The selectivity of the HPLC method for tramadol, *N*-desmethyltramadol and *O*-desmethyltramadol is demonstrated in Fig. 2.

3.4. Pharmacokinetics

The above-described assay was applied to plasma samples from a bioequivalence study of two suppository formulations for rectal administration. The geometric mean±exp[mean(ln)±SD (ln)] of tramadol and *O*-desmethyltramadol concentrations in the plasma after the administration of 100 mg tramadol hydrochloride (333.51 μmol) are shown in Fig. 4 and the corresponding pharmacokinetic variables are summarized in Table 1. The sensitivity of the analytical method for tramadol in the plasma was adequate. The mean half-life of tramadol in the plasma was approximately 3.5-times lower than the total sampling period of 32 h. The results of the statistical analysis of bioequivalence obtained by parametric (two one-sided test procedure [38]) and nonparametric (Mann–Whitney–Wilcoxon tests
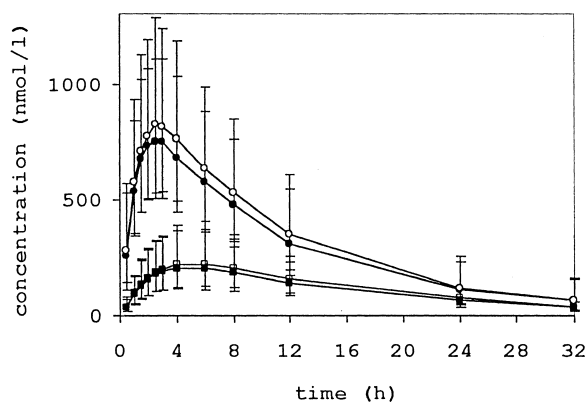
Fig. 4. Average pharmacokinetics of tramadol ($\bullet$,$\circ$) and its principal metabolite O-desmethyltramadol ($\blacksquare$,$\square$) in 24 human volunteers after the rectal administration of Protradon 100 mg supp. ($\bullet$) vs. Tramal 100 mg supp. ($\circ$).

Table 2
Point estimates and 90% confidence intervals (CI) for the ratios (test/reference) of the pharmacokinetic variables (C_{max}, $AUC_{0-\infty}$) and for the difference (test−reference) in T_{max} of tramadol in plasma

	Parametric estimates		Nonparametric estimates	
	Median	90%-CI	Median	91%-CI
$AUC_{0-\infty}$ ratio (%)	94	87–101		
C_{max} ratio (%)	91	84–98		
t_{max} difference (h)	–	–	−0.25	−0.75–0.0

ously published pharmacokinetic data [7,18]. In these reports, however, only the pharmacokinetics of the parent compound was studied. In this paper, the pharmacokinetic data for both tramadol and its pharmacodynamically active metabolite, O-desmethyltramadol, are available.

Enantiospecific analytical methods are an important tool in studying pharmacokinetic–pharmacodynamic relationships as well as mutual interactions of racemic drugs. In comparative bioavailability studies, however, their use is not so beneficial unless

[39]) procedures are given in Table 2. They give evidence for the bioequivalence of the two compared tramadol formulations.

Our pharmacokinetic results for tramadol after the rectal administration are comparable with the previ-

Table 1
Pharmacokinetic variables of tramadol and O-desmethyltramadol in plasma after a single rectal dose of 100 mg of tramadol HCl

		Geom. mean	Exp.		Minimum	Maximum
			(mean−SD**)	(mean+SD**)		
Tramadol						
C_{max} (nM)	T	796	540	1170	300	1480
	R	879	572	1350	319	1680
t_{max} (h)	T	2.5*			1.5	4.0
	R	2.7*			1.5	6.0
AUC_{0-32} (nmol h/ml)	T	9510	5880	15 400	2900	24 500
	R	10 410	6390	17 000	3200	25 800
$AUC_{0-\infty}$ (nmol h/ml)	T	10 700	6220	18 400	3000	37 300
	R	11 450	6670	19 700	3300	32 850
$t_{1/2}$ (h)	T	9.1	6.5	12.8	5.1	20.1
	R	8.5	6.3	11.4	5.6	15.8
O-Desmethyltramadol						
C_{max} (nM)	T	214	121	381	48.2	489
	R	230	136	397	62.2	407
t_{max} (h)	T	4.0*			1.5	12.0
	R	4.0*			2.5	12.0
AUC_{0-32} (nmol h/ml)	T	3590	2180	5910	806	7100
	R	3960	2510	6240	1240	7230

T, test formulation (Protradon supp.); R, reference formulation (Tramal supp.).
*, median, **, ln-data.

the drug or metabolite S:R ratios significantly vary in relationship to the route of administration (mostly after an oral administration) [40]. In a recent study, it has been demonstrated that, after an oral administration, enantioselection in the kinetics of tramadol and its active O-desmethyl metabolite is relatively insignificant [33]. Thus, tramadol is the category III drug (high first pass metabolism of the active enantiomer and a low variability in the S:R ratios) according to the classification system suggested by Karim [40].

4. Conclusions

A new bioanalytical method involving a simple liquid–liquid extraction of plasma samples and subsequent high-performance liquid chromatographic determination of tramadol and its principal metabolite, O-desmethyltramadol, using a fluorescence detection was developed and validated. The method is selective for the separation of tramadol, O-desmethyltramadol and N-desmethyltramadol. The identity of these two metabolites was confirmed using GC–MS, NMR and HPLC in plasma of two species. Simple methods for the preparation of the standards (O-desmethyltramadol and N^1,N^1-dimethylsulfanilamide, an internal standard) were proposed and used. The validated bioanalytical method was applied to the bioequivalence study of tramadol and O-desmethyltramadol in 24 healthy volunteers after the administration of two rectal formulations containing 100 mg of tramadol hydrochloride, each.

Acknowledgements

References

[1] K. Flick, E. Frankus, E. Friderichs, Arzneim. Forsch. 28 (1978) 107.

[2] E. Frankus, E. Friderichs, S.M. Kim, G. Osterloh, Arzneim. Forsch. 28 (1978) 114.

[3] C.R. Lee, D. McTavish, E.M. Sorkin, Drugs 46 (1993) 313.

[4] L.J. Scott, C.M. Perry, Drugs 60 (2000) 139.

[5] W. Lintz, H. Barth, G. Osterloh, E. Schmidt-Böthelt, Arzneim. Forsch. 36 (1986) 1278.

[6] W. Lintz, H. Barth, R. Becker, E. Frankus, E. Schmidt-Böthelt, Arzneim. Forsch. 48 (1998) 436.

[7] W. Lintz, H. Barth, G. Osterloh, E. Schmidt-Böthelt, Arzneim. Forsch. 48 (1998) 889.

[8] C.K. Chao, L.L. Yu, L.L. Su, C.M. Liu, T.H. Yang, C.M. Chen, Arzneim. Forsch. 50 (2000) 636.

[9] W. Lintz, S. Erlacin, E. Frankus, H. Uragg, Arzneim. Forsch. 31 (1981) 1932.

[10] V. Subrahmanyam, A.B. Renwick, D.G. Walters, P.G. Young, R.J. Price, A.P. Tonelli, B.G. Lake, Drug Metab. Dispos. 29 (8) (2001) 1146.

[11] W.N. Wu, S. Liao, L.A. McKown, A.R. Takacs, ISSX Proc. 11 (1997) 159.

[12] W.N. Wu, L.A. McKown, A.D. Gauthier, W.J. Jones, R.B. Raffa, Xenobiotica 31 (7) (2001) 423.

[13] W. Lintz, Naunyn-Schmiedeberg's Arch. Pharmacol. 313 (Suppl.) (1980) R53.

[14] W. Lintz, H. Uragg, J. Chromatogr. 341 (1985) 65.

[15] S. Liao, J.F. Hill, R.K. Nayak, Pharm. Res. 9 (Suppl.) (1992) 308.

[16] R. Becker, W. Lintz, J. Chromatogr. 377 (1986) 213.

[17] S.-T. Ho, J.-J. Wang, W.-J. Liaw, Ch.-M. Ho, J.-H. Li, J. Chromatogr. B 736 (1999) 89.

[18] M. Merslavic, L. Zupancic-Krajl, J. Chromatogr. B 693 (1997) 222.

[19] E.C.Y. Chan, P.C. Ho, J. Chromatogr. B 707 (1998) 287.

[20] B. Kurth, G. Blaschke, Electrophoresis 20 (1999) 555.

[21] S. Rudaz, J.-L. Veuthey, C. Desiderio, S. Fanali, J. Chromatogr. A 846 (1999) 227.

[22] S. Rudaz, S. Cherkaoui, P. Dayer, S. Fanali, J.-L. Veuthey, J. Chromatogr. A 868 (2000) 295.

[23] U.B. Soetebeer, M.-O. Schierenberg, H. Schulz, G. Grünefeld, P. Andresen, G. Blaschke, J. Chromatogr. B 745 (2000) 271.

[24] B. Elsing, G. Blaschke, J. Chromatogr. 612 (1993) 223.

[25] G.C. Yeh, M.T. Sheu, C.L. Yen, Y.W. Wang, C.H. Liu, H.O. Ho, J. Chromatogr. B 723 (1999) 247.

[26] M. Nobilis, J. Pastera, P. Anzenbacher, D. Svoboda, J. Kopecký, F. Perlík, J. Chromatogr. B 681 (1996) 177.

[27] W.D. Paar, P. Frankus, H.J. Dengler, J. Chromatogr. B 686 (1996) 221.

[28] A. Ceccato, P. Chiap, Ph. Hubert, J. Crommen, J. Chromatogr. B 698 (1997) 161.

[29] P. Overbeck, G. Blaschke, J. Chromatogr. B 732 (1999) 185.

[30] M.A. Campanero, B. Calahorra, E. Garcia-Quetglass, M. Escolar, J. Honorato, Chromatographia 48 (1998) 555.

[31] M.A. Campanero, B. Calahorra, M. Valle, I.F. Troconiz, J. Honorato, Chirality 11 (1999) 272.

[32] M. Valle, J.M. Pavón, R. Calvo, M.A. Campanero, I.F. Trocóniz, J. Chromatogr. B 724 (1999) 83.

[33] A. Ceccato, F. Vanderbist, J.-Y. Pabst, B. Streel, J. Chromatogr. B 748 (2000) 65.

[34] V.P. Shah, K.K. Midha, S.V. Dighe et al., Int. J. Pharm 82 (1992) 1.

[35] CDER Reviewer Guidance for Validation of Chromatographic Methods, November 1994, CFDA

[36] J.F.W. McOmie, D.E. West, Org. Synth. Collect. Vol. V (1973) 412.

[37] U.B. Soetebeer, M.-O. Schierenberg, J.-G. Moller, H. Schulz, G. Grünefeld, P. Andresen, G. Blaschke, G. Ahr, J. Chromatogr. A 895 (2000) 147.

[38] D.J. Schuirmann, J. Pharmacokin. Biopharm. 15 (1987) 657.

[39] D. Hauschke, V.W. Steinijans, E. Diletti, Int. J. Clin. Pharmacol. Ther. Toxicol. 28 (1990) 72.

[40] A. Karim, J. Clin. Pharmacol. 36 (1996) 490.