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Short communication

Improved liquid chromatographic method for the simultaneous determination of tramadol and its three main metabolites in human plasma, urine and saliva

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Abstract

Tramadol, an analgesic agent, and its main metabolites *O*-desmethyltramadol (M1), *N*-desmethyltramadol (M2) and *O,N*-didesmethyltramadol (M5) were determined simultaneously in human plasma, saliva and urine by a rapid and specific HPLC method. The sample preparation was a simple, one-step, extraction with ethyl acetate. Chromatographic separation was achieved with a Chromolith™ Performance RP-18e 100 mm × 4.6 mm column, using a mixture of methanol:water (19:81, v/v) adjusted to pH 2.5 by phosphoric acid, in an isocratic mode at flow rate of 2 ml/min. Fluorescence detection (λ_{ex} 200 nm/λ_{em} 301 nm) was used. The calibration curves were linear ($r^2 > 0.996$) in the concentration ranges in plasma, saliva and urine. The lower limit of quantification was 2.5 ng/ml for all compounds. The within- and between-day precisions in the measurement of QC samples at four tested concentrations were acceptable in all analyzed body fluids The developed procedure was applied to assess the pharmacokinetics of tramadol and its main metabolites following administration of 100 mg single oral dose of tramadol to healthy volunteers.

Keywords: Tramadol; *O*-Desmethyltramadol; *N*-Desmethyltramadol; *O,N*-Didesmethyltramadol; Plasma; Saliva; Urine; Monolithic

1. Introduction

Tramadol hydrochloride (T) is a synthetic 4-phenyl-piperidine analogue of codeine, which contains two chiral centers and thus has four stereoisomers. Trans-T, a racemates mixture of 1R, 2R-T [(+)-Trans-T] and 1S, 2S-T [(−)-Trans-T], is a centrally acting analgesic with efficacy and potency ranging between weak opioids and morphine [1]. Tramadol produces analgesia in short- and long-term pain states by synergistically combining weak μ-opioid and monoaminergically (noradrenaline and serotonin) mediated mechanisms [2]. Its therapeutic plasma concentration is in the range of 100–300 ng/ml [3]. Tramadol is rapidly and almost completely absorbed after oral administration with an absolute bioavailability of only 65–70% due to first-pass metabolism [4,5]. This analgesic is rapidly and extensively metabolized in the liver. The principal metabolic pathways, *O*- and *N*-desmethylation, involve cytochrome P-450 isoenzymes 2D6, 2B6 and 3A4, respectively. The primary metabolites *O*-desmethyltramadol (M1) and *N*-desmethyltramadol (M2) may be further metabolized to three additional secondary metabolites namely, *N,N*-didesmethyltramadol (M3), *N,N,O*-tridesmethyltramadol (M4) and *N,O*-didesmethyltramadol (M5). In phase II, the *O*-demethylated metabolites are conjugated with glucuronic acid and sulfuric acid before excretion into urine. In all species, M1 and M1 conjugates, M5 and M5 conjugates and M2 are the main metabolites, whereas M3, M4 and M4 conjugates are only formed in minor quantities (less than 1%) [6]. Only one of these metabolites, *O*-desmethyltramadol (M1 metabolite), is pharmacologically active. Approximately, 10–30% of the parent drug is excreted unchanged in the urine [7].

A number of high-performance liquid chromatographic (HPLC) methods with ultraviolet [8–10], fluorescence [11–13], electrochemical [14] and MS detection [15] have been described for determination of tramadol and its active metabolite (M1). Chiral HPLC methods [16–19] are very important for the

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determination of the enantiomeric ratios of tramadol and its metabolites. However, achiral analytical methods are still useful in some pharmacokinetic and bioequivalence studies as well as forensic investigations. So far, there is only one achiral method on simultaneous determination of tramadol and two of its major metabolites (M1 and M2) in human plasma, which is also carried out in our own lab [20].

To our knowledge, there is no validated achiral HPLC method capable of simultaneous quantification of tramadol and its three main metabolites (M1, M2 and M5) in plasma, urine or saliva. Among these metabolites, M2 and M5 are not pharmacologically active. The M5 metabolite may be formed either by *N*-demethylation of M1 or *O*-demethylation of M2. A metabolic switch in favor of enhanced *N*-demethylation of T has been suggested which consequently may affect the M5 concentration [21]. M5 is very similar in structure and lipophilicity to M1 and consists a considerable fraction of this metabolite (~30%). Therefore, inability to separate M5 causes significant error in measurement of the active metabolite (M1). Therefore, M5 – though inactive – is a very important metabolite in bioanalysis of tramadol and its metabolites.

The objective of this study was to improve our previously published HPLC method for simultaneous determination of tramadol and its three main metabolites in human plasma. The method is also tested and modified to be used in other biological samples (urine and saliva).

2. Experimental

2.1. Materials

The pure substances of tramadol, M1, M2, M5 and *cis*-tramadol (Fig. 1) as internal standard were kindly supplied by GrÜnenthal (Stolberg, Germany). HPLC-grade acetonitrile and methanol and analytical grade ethyl acetate and phosphoric acid (85%) were supplied by Merck (Darmstadt, Germany). Blank plasma, saliva and urine samples were obtained from healthy volunteers.

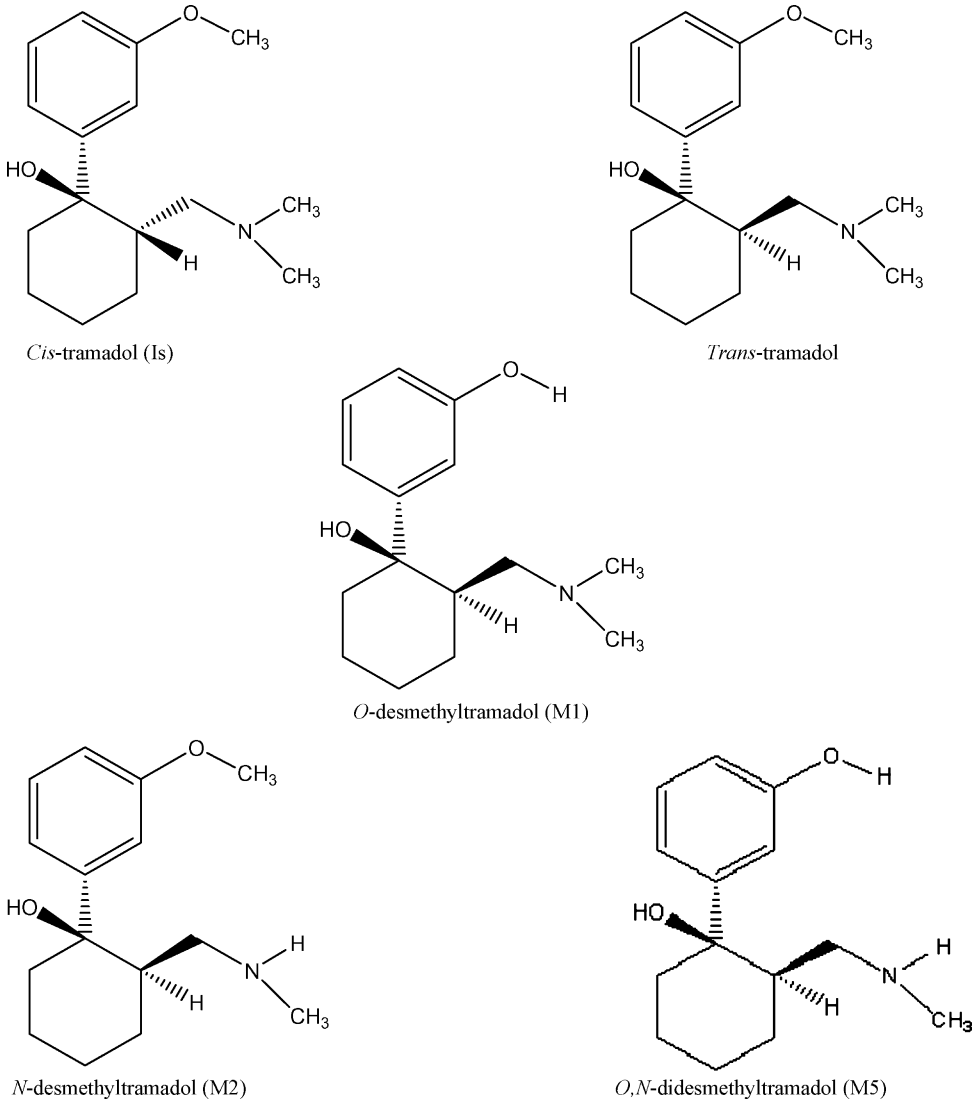

Fig. 1. Chemical structures of *cis*-tramadol (IS), trans-tramadol and its main metabolites.

2.2. Preparation of standard solutions

Tramadol, M1, M2, M5 and *cis*-tramadol in concentrations of 1 mg/ml were prepared separately by dissolving 10 mg of each compound in methanol. The standard solutions of *cis*-tramadol (10 μg/ml) and (1 μg/ml) for urine and plasma/saliva were similarly prepared. Standard and stock solutions of all compounds were stored at 4 °C. All stock and standard solutions were stable for 3 months at this temperature.

2.3. Apparatus and chromatographic condition

The chromatographic apparatus consisted of a low-pressure gradient HPLC pump, a fluorescence detector [excitation wavelength (λ_{ex}) 200 nm/emission wavelength (λ_{em}) 301 nm] and an online degasser, all from Knauer (Berlin, Germany). A Rheodyne model 7725i injector with a 100 μl loop was used.

The data was acquired and processed by means of ChromGate chromatography software (Knauer). Chromatographic separation was achieved by a Chromolith™ Performance RP-18e 100 mm × 4.6 mm column (Merck, Darmastadt, Germany) protected by a Chromolith™ Guard Cartridge RP-18e 5 mm × 4.6 mm. For the mobile phase, a mixture of methanol:water (19:81, v/v) adjusted to pH 2.5 by phosphoric acid (final acid concentration of about 1.5 mM), was delivered in isocratic mode at 2 ml/min flow rate.

2.4. Sample preparation

The preparation of biologic samples was a modification of our previously published method [20]. The conditions consisted of mixing 120 μl of plasma with 50 μl *cis*-tramadol as internal standard (1 μg/ml) and 50 μl NaOH (2N) in a 2 ml Eppendorf polypropylene tube and then extracting with 1.25 ml of ethyl acetate. After vertical agitation (10 min) and centrifugation (10,000 × *g*, 2 min), the upper organic layer was transferred into a conical glass tube. The organic phase was then evaporated under a gentle stream of air and reconstituted in 120 μl of the mobile phase. To apply the extraction for saliva, samples were first diluted two times with distilled water, and then 100 μl of diluted saliva was extracted and reconstituted in 200 μl of mobile phase. For urine sample, 50 μl of urine was directly used for extraction and was reconstituted in 2 ml of mobile phase. A 100 μ1 aliquot of each sample was then injected on to the HPLC system.

2.5. Preparation of calibration standards

Each calibration curve consisted of 10 calibration points, covering 2–500 ng/ml for plasma, 100–6000 ng/ml for saliva and 2–100 μg/ml for urine. Calibration curves were determined by least square linear regression analysis. Peak area ratio of each analyte to *cis*-tramadol versus the corresponding concentration was plotted.

2.6. Accuracy, precision, limit of quantification (LOQ) and recovery determination

Accuracy, between- and within-day precisions of the method were determined for each compound according to FDA guidance for bioanalytical method validation [22]. Five replicate spiked samples were assayed between- and within-day at four different concentrations in plasma (10, 50, 100 and 300 ng/ml), saliva (100, 500, 1000, 2000 ng/ml) and urine (10, 30, 50, 100 μg/ml) for each analyte. Accuracy was calculated as deviation of the mean from the nominal concentration. Between- and within-day precision were expressed as the relative standard deviation of each calculated concentration. For the concentration to be accepted as LOQ, the percent deviation from the nominal concentration (accuracy) and the relative standard deviation has to be less than 20%, respectively. The recoveries of all analytes over the QC sample ranges were determined by comparing peak areas obtained from each body fluid sample and those found by direct injection of an aqueous standard solution at the same concentration, using the same loop.

2.7. Application of the method

Twenty-four volunteers were included in this study. The study protocol was approved by the Ethics Committee of Tehran University of Medical Sciences and written informed consent was obtained from the subjects. Volunteers were not allowed to take any other medication for 2 weeks before and throughout the study. The volunteers received two 50 mg Tradolan tablets (Lannach, Austria) after an overnight fasting. Intake of food was delayed for 3 h after medication. Peripheral venous blood, saliva and urine samples were taken at predetermined intervals. Samples were stored at −20 °C until analysis.

3. Results

3.1. Selectivity

The separation achieved using the experimental conditions of the present assay for tramadol and its main metabolites are presented in Fig. 2. The absence of interfering endogenous components at the retention times of all analytes in blank body fluids extracts, shows the high selectivity of the developed chromatographic method. Mainly, the fluorescence properties of the analytes are responsible for this effect, since at excitation (λ_{ex}) and emission (λ_{em}) wavelength of 200 and 301 nm, the responses of endogenous plasma, saliva or urine components are negligible. Retention times for tramadol, M1, M2, M5 and IS in plasma, saliva and urine were (4.9, 0.2), (2.1, 0.15), (6.4, 0.22), (2.5, 0.16) and (3.6, 0.16) min, respectively.

3.2. Linearity

The linearity of the relationship between peak area ratios and corresponding concentrations (which were needed in this study) were demonstrated by the correlation coefficients obtained for the regression lines. The correlation coefficients of all standard

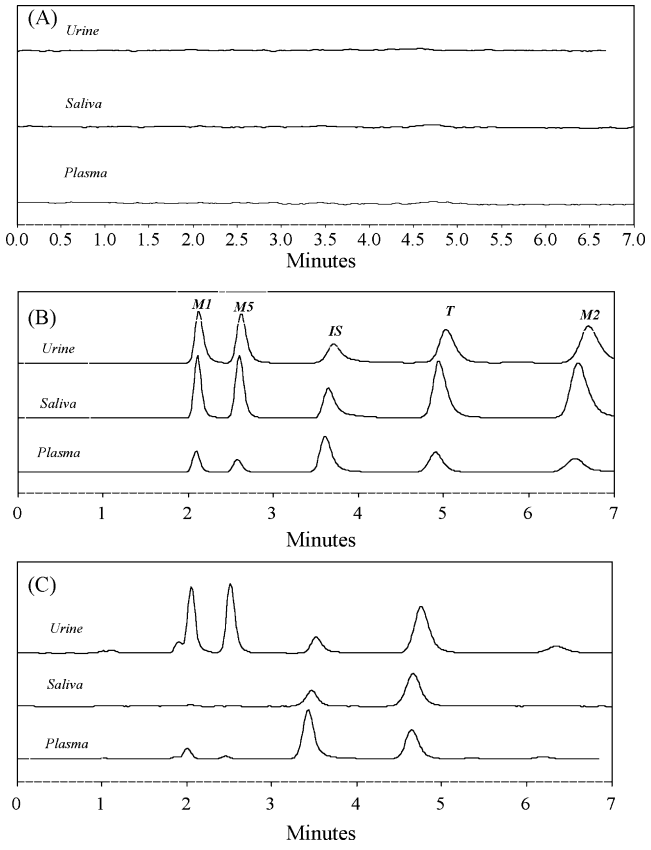

Fig. 2. Chromatograms of tramadol (T) and its metabolites (M1), (M2), (M5) and *cis*-tramadol (IS) in human plasma, saliva and urine. (A) Blank human plasma, saliva and urine spiked with IS. (B) Plasma, saliva and urine spiked with T, M1, M2, M5 and IS. (C) Plasma, saliva and urine of a volunteer 2 h after single oral administration of 100 mg tramadol.

curves were more than 0.998, 0.995 and 0.996 for plasma, saliva and urine, respectively.

3.3. Limit of quantification

LOQs as defined previously were 2.5 ng/ml for all analytes in plasma, saliva and urine.

3.4. Recovery, accuracy and precision

The results from the validation of the method in human plasma, saliva and urine are listed in Table 1. The method proved to be accurate and precise. Accuracy values were within acceptable limits for tramadol, M1, M2 and M5. The values of within- and between-day precision were below 13.6% for all samples. The absolute recoveries ranged from 86.2% to 92.9%, 76.9% to 87.3%, 80.1% to 90.5% and 88.7% to 91.3% for tramadol, M1, M2, M5 and IS, respectively.

3.5. Application of the method

The applicability of this method has been demonstrated by determination of the of tramadol and its main phase I metabolites in plasma, saliva and urine samples from healthy volunteers

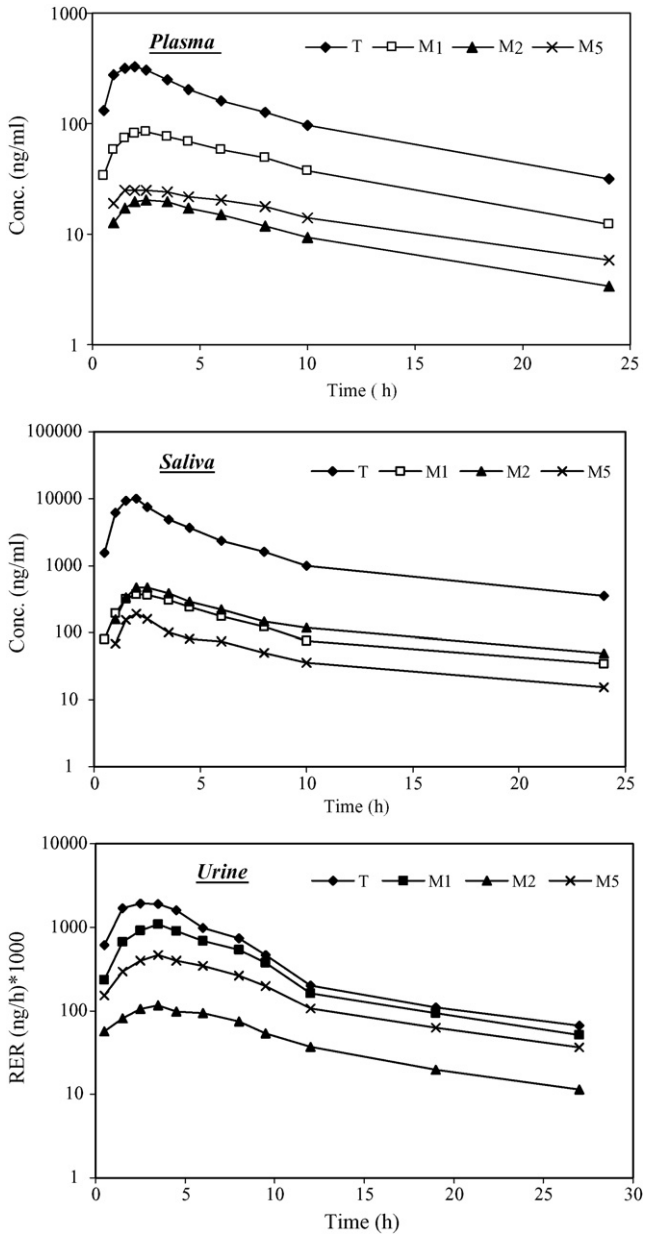

Fig. 3. Mean concentration–time profile in plasma and saliva and renal excretion rate of T, M1, M2 and M5 after administration of 100 mg single oral dose of tramadol.

receiving a single oral dose of 100 mg tramadol tablet. Mean plasma and saliva concentration–time curves and renal excretion rate of tramadol, M1, M2 and M5 are depicted in Fig. 3.

4. Discussion

The limitation of flow rate applied to routine HPLC columns could be resolved by using newly developed Chromolith™, as a monolithic HPLC column (http://www.chromolith.com/).

In this method, we modified our pervious study [20] to be able to determine M5 metabolite besides tramadol, M1 and M2. To achieve a suitable resolution between M1 and M5, a 100 mm column was used instead of 50 mm column used in our previous report. This method also enables us to determine all four above-

Table 1
Between- and within-day variability and accuracy for determination of tramadol, M1, M2 and M5 ($n = 5$)

Concentration	T		M1		M2		M5	
	R.S.D.	Accuracy	R.S.D.	Accuracy	R.S.D.	Accuracy	R.S.D.	Accuracy
Between-day ($n = 5$)								
Plasma (ng/ml)								
10	1.1	88.4	1.9	87.8	9.5	92.9	1.6	86.9
50	3.6	102.5	3.0	101.1	10.1	102.1	2.5	95.9
100	5.2	107.1	1.3	101.8	5.1	106.9	3.5	102.2
300	2.5	102.4	1.0	103.9	1.9	104.2	4.0	103.5
Saliva (ng/ml)								
100	3.3	97.3	3.7	93.9	4.6	107.6	6.2	101.3
500	6.4	98.8	2.2	97.7	2.0	101.3	7.1	104.9
1000	3.9	96.9	1.4	100.5	4.6	93.6	3.4	98.2
2000	3.7	98.3	3.5	103.1	2.5	97.3	3.7	97.4
Urine (μg/ml)								
10	3.7	96.5	1.3	99.9	2.9	100.0	3.1	95.9
30	5.3	98.4	1.4	95.9	3.1	103.0	2.3	94.5
50	11.3	104.3	4.5	101.1	1.7	98.7	4.0	101.2
100	1.4	100.6	2.3	100.3	3.9	99.4	1.2	99.2
Within-day ($n = 5$)								
Plasma (ng/ml)								
10	2.1	89.8	2.9	90.2	8.4	104.6	5.2	88.9
50	9.4	100.3	10.2	98.1	7.3	110.5	9.3	97.3
100	6.2	107.3	10.3	101.4	3.5	111.9	7.3	100.7
300	4.3	102.2	6	102.8	7.9	107.2	2.5	102.9
Saliva (ng/ml)								
100	11.4	93.9	3.3	91.3	1.6	106.6	5.6	94.2
500	11.5	84.9	9.5	90.5	2.2	100.4	9.2	108.7
1000	11.6	100.8	9.0	92.5	5.1	91.4	8.5	102.2
2000	7.1	96.4	1.6	95.3	8.5	98.5	9.0	96.4
Urine (μg/ml)								
10	10.6	97.1	6.6	100.3	9.0	98.5	6.9	99.1
30	4.6	95.9	6.2	95.5	7.7	103.5	9.5	92.1
50	6.9	90.6	5.6	98.4	7.6	99.1	13.6	91.9
100	8.7	94.3	2.5	100.7	5.0	101.7	9.6	98.5

mentioned analytes (tramadol, M1, M2 and M5) in saliva and urine in addition to plasma samples.

The method also involves one-step LLE instead of two-step extraction that was described in our pervious study. Therefore, the current method is proved to be less time consuming. Also, 120 μl plasma was used in the present study instead of 250 μl reported previously. This in turn helps the researchers with less sample sizes needed.

As the urine and saliva concentrations of T, M1, M2 and M5 are far above their plasma concentrations, the calibration range for these analytes in urine and saliva were constructed accordingly by first or last dilution of samples. However, the LOQ determined for plasma could be extended to these matrices if samples were not diluted.

Recently, the use of biological specimens other than blood for the detection and quantitation of drugs has been the focus of substantial scientific attention and research [23].

Several drugs have been found to be excreted largely into the saliva and urine. These biological samples have several advantages such as noninvasive, easy and a large number of samples can be obtained and are particularly useful in ambulatory pediatric and geriatric patients. Urine is also the preferred specimen in polymorphic studies of drugs.

The simultaneous achiral separation of the later compounds in different human body fluids had not been reported yet. The parameters of the assay obtained in the course of validation processes presented above in the results section were considered satisfactory for its clinical application.

5. Conclusion

A sensitive, accurate and precise bioanalytical method involving a simple liquid–liquid extraction of samples and high-performance liquid chromatographic determination of tramadol and its principal metabolites (M1, M2 and M5) was developed and validated to meet the requirements of the pharmacokinetic investigations of these compounds. This procedure may be fully recommended for pharmacokinetic studies as well as for therapeutic drug monitoring. Determination of tramadol and its metabolites in saliva or urine could also be an alternative method in forensic and TDM studies.

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