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Determination of tramadol and metabolites by HPLC-FL and HPLC–MS/MS in urine of dogs

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ABSTRACT

Tramadol is a centrally acting analgesic drug used in veterinary and human clinical practice. Its metabolism has been largely characterized in human being but is still long to be comprehended in several animal species, especially in the dog. The aim of the present study was to develop and validate a new analytical procedure to investigate HPLC the metabolization/elimination process tramadol in urine of dogs by HPLC-FL or HPLC–MS/MS. A single oral dose of tramadol (4 mg/kg) was administered to 4 male Beagle dogs and the urine was naturally collected. This matrix either hydrolyzed than un-hydrolyzed was extracted with different blends of solvents to detect the total or free form of the analytes, respectively.

The present method allowed to obtain good selectivity, accuracy, precision and recoveries without the need of time consuming or expensive clean up steps. The short chromatographic time courses allowed this analysis to be proposed for routine purposes. The HPLC–MS/MS detected in the urine two metabolites (M6 and M7) considered negligible in humans. The low LOQ showed that the method could be useful for the determination of the illegal use of this drug in race-dogs' urine.

1. Introduction

Tramadol (T) (1RS, 2RS)-2-[(dimethylamine)methyl]-1-(3-methoxyphenyl)-cyclohexanol is a centrally acting analgesic drug used for the relief of moderate to chronic pain with no clinically relevant cardiovascular or respiratory depressant activity [1]. T displays a weak affinity for the μ and δ opioids receptors, and weaker affinity for the κ-subtype. In addition, T also interferes with the neuronal release and re-uptake of serotonin and norepinephrine in the descending inhibitory pathways [1].

The metabolic fate of T is modulated by cytochrome P450 (CYP). The isozyme CYP2D6 catalyzes the reaction of *O*-demethylation forming *O*-desmethyltramadol (M1). M1 is the major active metabolite, which is 200–300 times more potent at the μ receptor than the parental drug. *N*-Demethylation reaction is conducted by CYP2B6 and CYP3A4. These isozymes form the inactive metabolite N-desmethyltramadol (M2) from T and the N,O-didesmethyltramadol (M5) from M1(Fig. 1) [2,3]. Therefore, the clinical response of T is strictly correlated to its metabolism.

Several studies have been conducted on the pharmacokinetics of T in different animal species and humans [1,2,4–14]. It has been reported that in various animal species there is a small presence of the active metabolite M1 in plasma, differently than in humans. Focusing on dogs several pharmaceutical formulations, administered by different routes have been tested: I.V. [12] and extradural [13–14] injections, oral immediate release [6] and sustained release tablets [8] and rectal suppositories [7]. These studies showed low amount in plasma concentration of M1, associated with a fast metabolization of T to M2 and M5. This phenomenon has been speculated to be due at (1) a poor presence/activity of the CYP2D6 [6,8]; (2) a flip-flop effect, characterized by an elimination of M1 as glucuronidate conjugate faster than the formation process of the active metabolite [7]. According to the above-mentioned speculations, the concentration of T and metabolites in the urine seems to play a pivotal role in the understanding of the metabolic pathway of the drug. Anyway, also other metabolites might play a significant role in this process [15].

Some studies reporting T and its metabolites in urine are present in literature [16–24]. Most of these focus their attention exclusively on T and its active metabolite M1 [18–21,23,24]. Only a HPLC-FL study reported the simultaneous determination of T, M1, M2 and M5 but no data was descripted on conjugated metabolites [22]. Even though a study on diastereoisomeric separation of some glucuronidate metabolites was published, it did not allow the quantification of the free metabolites [17].

The main objective of the present study was to develop and validate an efficient liquid-liquid extraction (LLE) applicable to an

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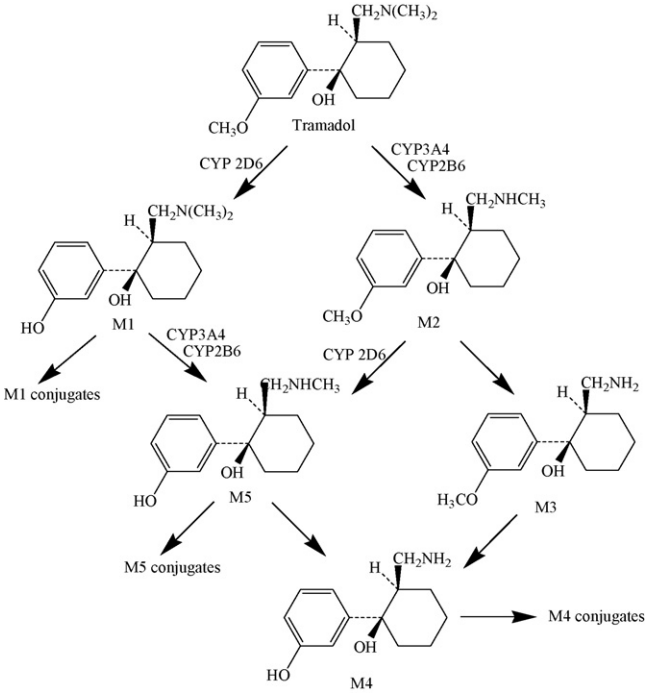

Fig. 1. Main metabolic pathways of Tramadol in human being [2].

HPLC-FL or HPLC–MS/MS analysis. Additionally, the metabolization/elimination processes of T and the presence of metabolites in urine of dogs have been investigated.

2. Experimental

2.1. Chemical and reagents

Tramadol hydrochloride (T), atenolol hydrochloride (IS) were obtained from Sigma–Aldrich (St. Louis, MO, USA), while O-desmethyl-tramadol hydrochloride (M1), N-desmethyl-tramadol hydrochloride (M2), and N,O-didesmethyl-tramadol hydrochloride (M5) pure substances, were from LGC Promochem (Milano, Italy). HPLC grade acetonitrile (ACN), methanol (MeOH), di-isopropyl ether ($[(CH_3)_2CH]_2O$) and dichloromethane (CH_2Cl_2), ethyl acetate (AcOEt), n-hexane (C_6H_{14}) were purchased from Merck (Darmstadt, Germany). Analytical grade sodium dodecyl-sulphate (SDS), sodium dihydrogen phosphate, and tetraethyl-ammonium bromide (TEA) were from BDH (Poole, UK). Potassium chloride (KCl) and ammonium acetate were purchased from Carlo Erba (Milano, Italy). Ammonium hydroxide (28%) and formic acid (96%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). β-Glucuronidase (104,800 units/mL *Helix pomatia*, K12) was obtained from Roche Biomedical (Mannheim, Germany). Deionised water was produced by a Milli-Q Millipore Water System (Millipore, MA, USA).

Stock solutions of T, M1, M2, M5 and IS in MeOH were prepared at an individual concentration of 1000 μg/mL, by using volumetric flasks, and stored at −20 °C.

Appropriate dilutions of stock standard solutions were prepared, by adding 1 mL of each solution and brought to 10 mL. A solution containing T, M1, M2 and M5 in final concentration of 10 μg/mL was then prepared. This solution has been successively diluted in glass tubes (10 mL), to reach final concentrations of 5 and 1 μg/mL, stored at −20 °C. The analytes were stable for 22 weeks [11].

The 100 μg/mL solution of IS was diluted with MeOH to obtain concentrations of 10 μg/mL. This solution was diluted to prepare a six-point calibration curve at the following concentrations: 0.05, 0.1, 0.25, 0.5, 1, and 1.5 μg/mL.

Each solution of T, M1, M2 and M5 (100 μg/mL) was diluted with MeOH, to reach the concentration of 10 μg/mL, to prepare a six-point calibration curve of the analytes at the following concentrations: 0.005, 0.025, 0.050, 0.100, 0.500, and 1 μg/mL. These solutions should be prepared fresh.

2.2. Instrumentation

2.2.1. HPLC-FL

The HPLC system was an LC Workstation Prostar (Varian, Inc., Walnut Creek, CA, USA) consisting of high pressure mixer pump (ProStar, model 230), CTO-10Avp column oven, spectrofluorometric detector (ProStar, model 363) and a loop of 20 μL. Data were processed by a Star LC Workstation (Varian, Inc.). Chromatographic separation was performed on a Luna® C18 ODS2 analytical column (150 mm × 2.1 mm inner diameter, 3-μm particle size, Phenomenex, Torrance, CA, USA) maintained at 25 °C. The mobile phase consisted of acetonitrile:buffer (20 mM sodium dihydrogen phosphate, 30 mM SDS, and 15 mM TEA, adjusted to pH 3.9 with phosphoric acid) (40:60, v/v) at a flow rate of 0.8 mL/min. Excitation and emission wavelengths were 275 and 300 nm, respectively. Validation data were previously reported [11].

2.2.2. HPLC–MS/MS

Chromatographic separation was performed by a PerkinElmer (Waltham, Ma, USA) 200 Series micro-pump system, equipped with autosampler and column oven, both 200 Series from PerkinElmer. The chromatographic separation was achieved by a Phenomenex Luna® C18 ODS2 analytical column (150 mm × 2.1 mm inner diameter, 3-μm particle size), maintained at 25 °C, and a mobile phase consisting of MeOH plus formic acid (0.1%):buffer (ammonium acetate, 5 mM, pH 4.5) (30:70, v/v) at 0.7 ml/min flow rate. MS and MS/MS experiments were carried out by an Applied Biosystems/Sciex (Foster City, CA, USA) API 4000 triple quadrupole mass spectrometer, equipped with Turbo V electrospray ionization source (ESI). Main parameters were the following: source temperature 600 °C, ion spray voltage 5.5 kV, declustering potential 30 V, and mass range 30–500 *m/z*. MS/MS parameters were: collision energy 25 V, collision gas pressure (N_2) 4.7 mPa.

2.3. Animal treatment and sampling

Urine samples were obtained from four healthy male Beagle dogs orally treated with T immediate release capsule (4 mg/kg) (Contramal® Formenti, Milano, Italy). From 2 up to 10 mL were naturally collected at assigned times (0, 0.5, 2, 4, 6, 8, 10 h) when dogs were moved out from the animal facility. The samples were then frozen quickly and stored at −20 °C. Immediately before the analysis, the samples were thawed at room temperature. The study protocol was approved by the ethics committee of the University of Pisa.

2.4. Sample extraction

Two variations of the sample preparation procedure were developed, to determine the free and the total (free + conjugated) forms of the analytes. β-Gluronidase was used to hydrolyse the bound between the glucuronic acid and the analytes, releasing these latter in the free form.

In practice, a 50 μL aliquot of urine sample was incubated at 60 °C for 4 h in 350 μL of acetate buffer (pH 5; 1 M) containing 5 μL of water (free form) or 5 μL of β-glucuronidase (total) [25]. In the incubated mixture 12.5 mg KCl, 100 μL Atenolol (MeOH solution, 10 μg/mL) as IS and 300 μL NH_4OH (28%) were added

in a 2 mL snap cap polypropylene vial and then vortexed and centrifuged at 14,000 rpm for 2 min. The supernatant was collected in a separate snap cap vial and submitted to LLE with 500 μL of a di-isopropylether:dichloromethane (1:1 v/v) solution by vertical agitation (60 rpm, 15 min). After centrifugation (5000 rpm, 2 min), the upper organic layer was moved to a conical glass tube and the aqueous phase was extracted twice again. The organic phases were then combined, evaporated under a gentle stream of nitrogen and reconstituted with 1 mL of MeOH. The prepared solutions were ready analyzed using HPLC-FL or HPLC–MS/MS.

2.5. Quantification

Calibration curves correlating peak area and concentration (μg/mL) of T, M1, M2 and M5 were plotted. Least-squares regression parameters for the calibration curve were calculated, and the concentrations of the test samples were interpolated from the regression parameters.

The concentrations of the glucuronidate metabolites were evaluated subtracting hydrolysed urine from non-hydrolysed values.

When unknown samples were assayed, a control and a fortified control were processed along with each set for quality control.

3. Results and discussion

3.1. Optimization of the extraction method

The urine extraction procedure was derived from a previously published method [22]. The influence of kind of solvent and number of extraction cycles on the extraction efficiency was studied in order to find the most suitable conditions for T, M1, M2 and M5. In this frame, several organic solvents (as CH_2Cl_2, AcOEt, $[(CH_3)_2CH]_2O$, C_6H_{14}) and also their mixtures were evaluated in terms of recovery and selectivity. The extraction solvent must be able to extract the analytes of interest, minimizing the co-extraction of other matrix components. The compatibility of the solvent with the later analytical steps (e.g. extract cleanup, pre-concentration, or analysis), as well as the solvent volatility when an extract concentration is necessary, have to be taken into account. Once the most suitable solvent was found, the effect of the number of extraction cycles was also evaluated. All the pure solvents provided poor results in the extraction of T, M1, M2 and M5 from the urine samples, while their mixtures, such as CH_2Cl_2:AcOEt, C_6H_{14}:CH_2Cl_2, C_6H_{14}:AcOEt, and $[(CH_3)_2CH]_2O$:CH_2Cl_2, were more effective. T and M2 were not valuable when the blend CH_2Cl_2:AcOEt (2:3, v/v) was used, because of the presence of interfering peaks (Table 1). The blend $[(CH_3)_2CH]_2O$:CH_2Cl_2 (1:1, v/v) produced an emulsion phase, easily destroyed by adding KCl to increase the ionic strength. This adjustment allowed to get comparable recoveries in the extraction of all the analytes. Finally, $[(CH_3)_2CH]_2O$:CH_2Cl_2 was selected as extraction solvent because it provided cleaner extracts and better analytical precision.

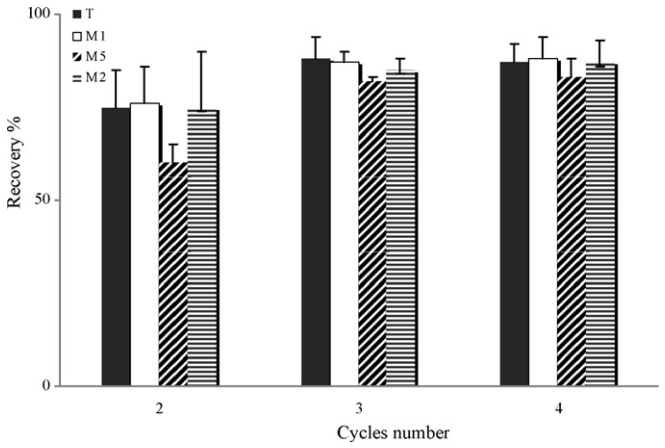

Fig. 2. Effect of the number of cycles on extraction of T, M1, M2 and M5 from urine samples.

To investigate the effect of this parameter on the extraction efficiency of T and metabolites from the urine, the extraction time was set at 15 min and the number of extraction cycles was varied from two to four (Fig. 2). In general, an increment in of the number of extraction cycles allows the exposure of the matrix to fresh solvent and favours the solvent/sample equilibrium, improving partitioning into the liquid phase and thus, increasing analyte recoveries. On the other hand, a high number of extraction cycles is time consuming and it is advisable to keep them as lower as possible. Finally, three extraction cycles were selected to ensure an efficient extraction of T, M1, M2 and M5.

3.2. Quality parameters

Analyte recoveries were measured by comparing the peak areas after the extraction procedure with the peak areas obtained by direct injection of pure standard solutions. The average recoveries of T, M1, M2 and M5 spiked at 1 and 5 μg/mL were 82 ± 6%, 88 ± 3%, 85 ± 1% and 79 ± 3%, respectively. The mean recoveries of the IS at the spiked level (10 μg/mL) were 72 ± 9%.

Accuracy and precision were evaluated using quality control (QC) urine samples spiked at three concentration levels (0.1, 0.5 and 1 μg/mL) using the optimized analytical method. Five replicates of QC samples at each concentration were analyzed in a single sequence to evaluate intra-day variation. For the evaluation of inter-day variation four replicate QC samples at each concentration were analyzed on three different days. Accuracy values were within acceptable limit for T, M1, M2 and M5. The RSD% values of intra- and inter-day precision were below 8% (Table 2).

According to EMEA guideline [26] limits of detection (LODs) and of quantification (LOQs) were calculated basing on signal-to-noise approach. They were performed by comparing measured signals from samples with known low concentrations of analyte with those

Table 1
Influence of the solvent extraction on the recoveries (%) of T, M1, M2 and M5 from urine samples. Spiking level 0.5 μg/mL ($n = 3$), 2 cycles (15 min-each).

Analyte	C_6H_{14}:AcOEt (1:1)		C_6H_{14}:CH_2Cl_2 (1:1)		AcOEt:CH_2Cl_2 (3:2)		AcOEt:CH_2Cl_2 (2:3)		$[(CH_3)_2CH]_2O$:CH_2Cl_2[a] (1:1)	
	R%	RSD%	R%	RSD%	R%	RSD%	R%	RSD%	R%	RSD%
T	13	15	67	11	35	12	ND	–	75	8
M1	73	4	45	6	80	7	76	10	76	10
M2	40	9	20	13	58	9	ND	–	78	15
M5	80	6	80	7	78	3	75	7	60	5

ND: not determinated because of the presence of interference peaks.
[a] Extraction conducted following the addition of KCl. RDS%: relative standard deviation. R%: recovery. Spiking level 0.5 μg/mL ($n = 3$), 2 cycles (15 min-each).

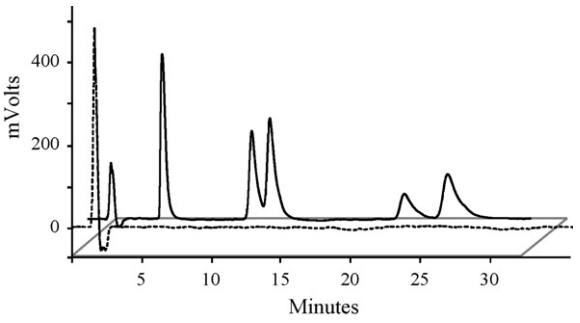

Fig. 3. Chromatograms of T, M1, M2, M5 and IS in dogs' urine. Blank urine (dot line) compared to urine spiked (1 μg/mL) with T, M1, M2, M5 and IS (solid line).

of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified (LOQ) or detected (LOD). The typical signal-to-noise ratios were 10:1 and 3:1 for LOQ and LOD respectively. In accordance with the above-mentioned rules, the resulting LODs and LOQs are those shown in Table 3.

Selectivity was assessed by comparing the chromatograms of different batches of blank matrices to those from spiked urine solutions. No impurities from the sample matrix or additives should interfere with the peaks of analytes. Typical retention times for IS, M1, M5, T and M2 were 5.4, 11.8, 13.1, 22.8 and 26.0 min, respectively (Fig. 3).

A six-point matrix matched calibration curves were prepared by fortification of control urine extracts with T, M1, M2 and M5, within the range 0.01–1 μg/mL, thus assuring a perfect match between analyzed samples and standard curves (blank sample; urine drug free). Under the instrumental conditions reported in the experimental section, the calibration curves presented good linearity (Table 3).

3.3. Application of the method

The application of this method has been demonstrated by determining T and its main metabolites in free and glucuronide forms in dogs' urine. Samples were collected from 0.5 up to 10 h after drug administration. By comparing samples before and after enzymatic hydrolysis, the presence of T and several metabolites was observed. HPLC analysis of the un-hydrolysed urine confirmed the presence of free T, M2 and M5 in different amounts, but not free M1 (Fig. 4). This latter metabolite was detected only as glucuronide conjugate form. The amount of M5, obtained from urine samples untreated with β-glucuronidase, was very low compared to enzymatically hydrolysed urine samples. The differences in hydrolysed/un-hydrolysed urine amount of T and M2 were not significant (Fig. 4A and C), proving that M2-glucuronate complex is not widely produced in dogs' urine according to a previous study on rat and dogs [15]. On the contrary, Elzagawi et al. [9] reported that after basic hydrolysis, the amount of M2 raised consistently in urine samples of camels; such unselective basic hydrolysis could have broken the bound with both glucuronide and sulphate.

3.4. Characterization of some unknown metabolites

In order to better understand the metabolism of T in dogs, the nature of some peaks relative to unknown compounds detected by HPLC-FL was investigated by HPLC-ESI-MS and HPLC-ESI-MS/MS. With a view to do that, it was necessary to modify the HPLC method developed for HPLC-FL, in order to make it compatible with the electrospray ionization process [27]. The resulting method was less efficient in terms of chromatographic separation, but still good enough for a mass spectrometric study (Fig. 5). Despite that, it was demonstrated by several experiments that the analytes of interest maintained the elution order of the HPLC-FL method. The HPLC-ESI-MS acquisitions allowed to detect the molecular ions, which are related to the molecular weight, of some compounds different from those of main interest. A further investigation on these ions carried out by HPLC–MS/MS allowed to formulate some

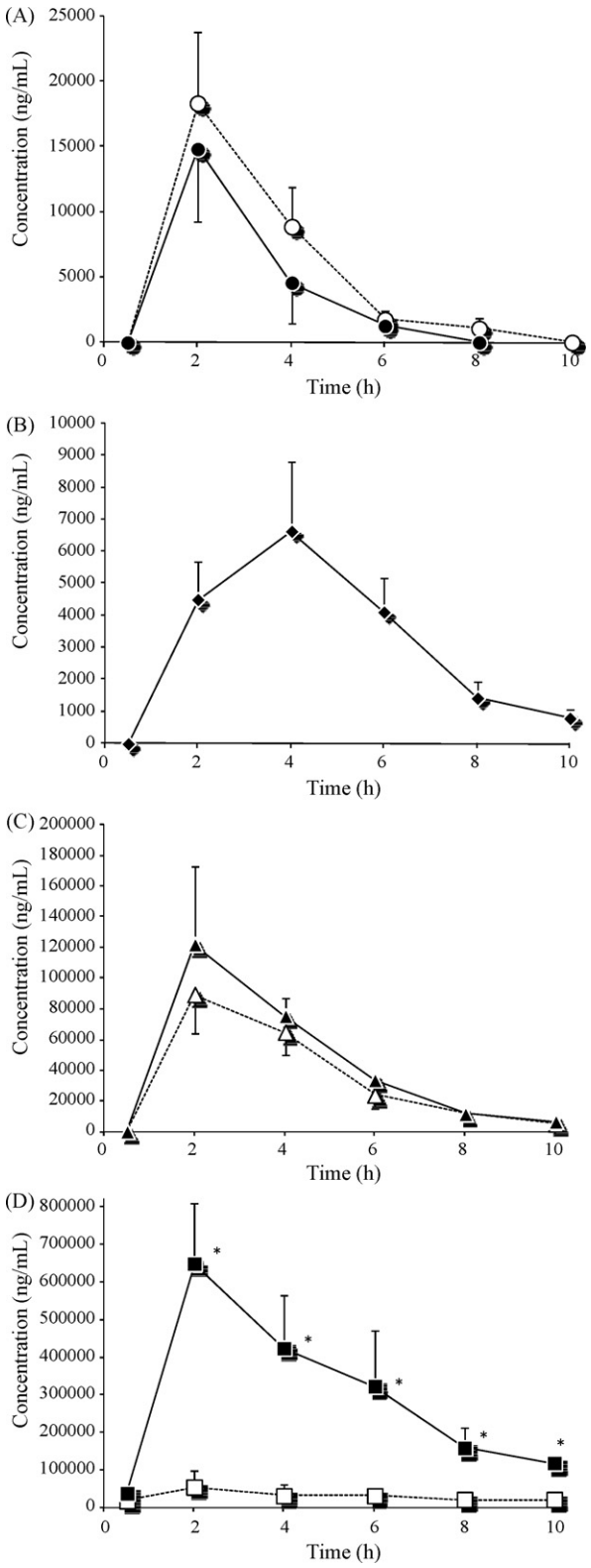

Fig. 4. Observed mean concentrations of T, M1, M2, and M5 metabolites in urine following single oral administration of immediate release capsules of Tramadol (4 mg/kg) in four Beagle dogs.

Table 2
Intra-day and inter-day precision (RSD%) obtained for the analyzed T, M1, M2 and M5 after extraction of urine samples ($n = 5$) spiked at different concentration levels.

Analyte	Intra-day RDS%			Inter-day RDS%		
	0.1 μg/mL	0.5 μg/mL	1 μg/mL	0.1 μg/mL	0.5 μg/mL	1 μg/mL
T	6.1	8.0	2.2	6.5	8.3	2.7
M1	7.2	3.2	1.0	6.9	3.4	1.6
M2	4.4	2.3	8.3	5.3	3.3	7.2
M5	7.9	2.9	0.8	6.8	3.3	2.2

RDS%: relative standard deviation.

Table 3
Results from linearity experiments for T, M1, M2, M5 and IS.

Analyte	Linearity range (μg/mL)	Correlation factor (R^2)	Calibration equation	LOD (ng/mL)	LOQ (ng/mL)
T	0.001–1	0.999	$y = 7500x - 15$	5	10
M1	0.001–1	0.998	$y = 7200x - 12$	5	10
M2	0.001–1	0.999	$y = 7500x - 18$	5	10
M5	0.001–1	0.999	$y = 7700x - 9$	5	10
IS	0.05–1.5	0.999	$y = 4802x - 30$	15	30

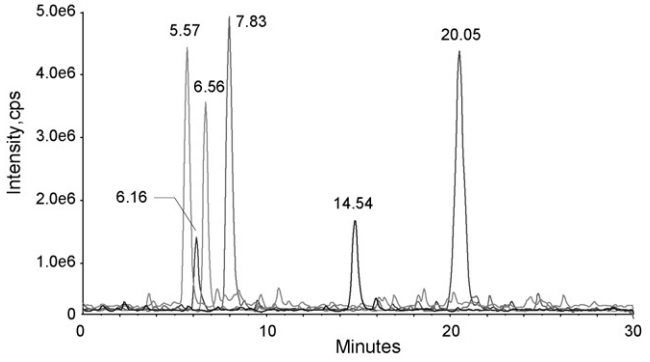

Fig. 5. Extracted ion chromatograms of the ions at 236, 250, 264, 266, and 280 m/z in a dog's urine sample, acquired by HPLC–MS. The overlapped plots show peaks at the following retention times: 5.57 (m/z 280), 6.16 (m/z 250), 6.57 (m/z 266), 7.83 (m/z 236), 14.54 (m/z 264), and 20.05 min (m/z 250).

hypothesis on their structures. Actually, the MS/MS spectra were interpreted taking advantage just of the data available in the literature, as it was not possible to confirm them by a comparison with authentic standard compounds, which were not available [28]. Up to date, it was possible to speculate the presence of 4-hydroxy-ciclohexyl tramadol (M6) and 4-hydroxy-ciclohexyl-N-desmethyl tramadol (M7) (Figs. 6 and 7). These results are consistent with other researches present in the literature dealing with metabolic studies carried out on rats, dogs and humans [15,28].

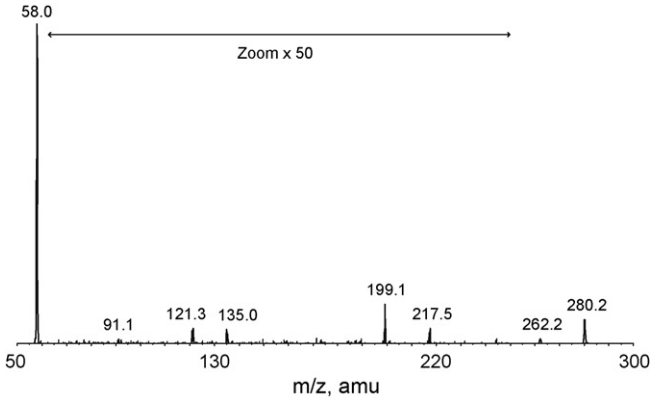

Fig. 6. Product ion spectra of the ion at m/z 280.

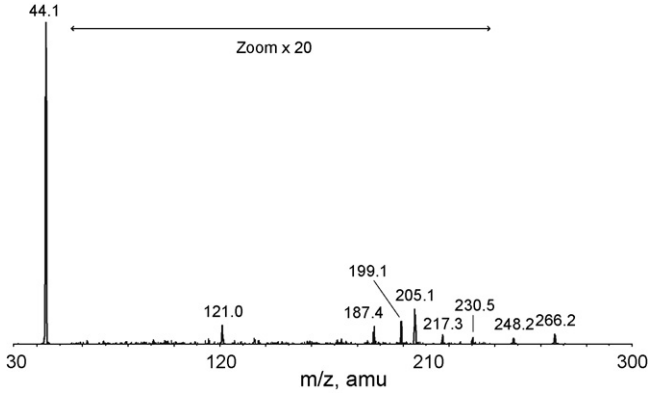

Fig. 7. Product ion spectra of the ion at m/z 266.

4. Conclusion

The analytical method described in this work allows the selective and accurate analysis of T and its metabolites without the need of time consuming or expensive clean up steps. The short chromatographic time courses make this method appropriate for routine analysis. The low LOQ shows that the method could be useful for the determination of the illegal use of this drug in race-dogs' urine. The present investigation allowed to find out in dogs' urine two metabolites considered negligible in humans, suggesting the co-existence of a possible alternative metabolic pathway. However, the metabolic pattern of T in this animal species, owing to several other metabolites not characterized in the present study, is extremely intricate, and further studies (concerning both metabolism and metabolites characterization) will be necessary to fully elucidate the metabolic pathways.

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