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Assay of tramadol in urine by capillary electrophoresis using laser-induced native fluorescence detection

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Abstract

Capillary electrophoresis (CE) with UV laser-induced native fluorescence detection was developed as a sensitive and selective assay for the direct determination of tramadol in human urine without extraction or preconcentration. The main problem in CE is the small inner diameter of the capillary which causes a low sensitivity with instruments equipped with a UV detector. Laser-induced native fluorescence with a frequency doubled argon ion laser at an excitation wavelength of 257 nm was used for the direct assay of tramadol in urine to enhance the limit of detection about 1000-fold compared to UV absorption detection. The detection system consists of an imaging spectrograph and an intensified CCD camera, which views an illuminated 1.5 mm section of the capillary. This set-up is able to record the whole emission spectra of the analytes to achieve additionally wavelength-resolved electropherograms. In the concentration range of 20 ng/ml–5 μg/ml in human urine coefficients of correlation were better than 0.998. Within-day variation determined on four different concentrations showed accuracies ranging from 90.2 to 108.4%. The relative standard deviation (RSD) was determined to be less than 10%. Day-to-day variation presented accuracies ranging from 90.9 to 103.1% with an RSD less than 8%.

Keywords: Tramadol

1. Introduction

Tramadol [1-(e)-(*m*-methoxyphenyl)-2-(e)-(dimethylaminomethyl)-cyclohexane-1-(a)-ol] is a centrally acting drug that possesses an analgetic action with a potency ranging between weak opioids and morphine. Clinical studies have shown that tramadol does not have a pronounced opioid side-effect profile. Little or no respiratory depression and no analgetic tolerance after repeated administration were observed with tramadol [1]. The drug has a low affinity to the opioid receptor it may exert part of its analgetic effect by activating the monoaminergic system [2]. After oral application tramadol is almost completely absorbed and extensively metabolized by *N*- and *O*-demethylation, *O*-demethyltramadol and *N*-demethyltramadol being the main phase 1 metabo-

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lites. The O-demethylated metabolites are further conjugated by phase 2 reactions [3].

Analytical methods used so far for the determination of tramadol are gas chromatography (GC) with nitrogen-selective nitrogen–phosphorus detection (NPD) [4], GC–mass spectrometry (MS) [5–8], high-performance liquid chromatography (HPLC) with UV or fluorimetric detection [9–12] or electrochemical detection [13] and capillary electrophoresis (CE) with UV absorption detection [14,15]. For these methods preconcentration or extraction of the samples had been necessary.

There is no method available for the direct quantification of tramadol in urine samples. This study describes a sensitive and highly selective CE method with laser-induced native fluorescence detection for the direct determination of tramadol in urine samples. A frequency doubled argon ion laser (λ_{em} 257 nm) was used. Lasers in the deep UV range are already available but these lasers have rarely been used for detection in CE routinely.

2. Materials and methods

2.1. Chemicals

Tramadol and *O*-demethyltramadol were a gift form Grünenthal (Stolberg, Germany). The other phase I metabolites were synthesized according to literature [16]. Naphazoline nitrate, which was used as internal standard (I.S.), was a gift from Novartis Pharma (Basel, Switzerland). All chemicals were of analytical grade purity. Sodium tetraborate (Borax) and sodium hydroxide were purchased from Merck (Darmstadt, Germany). The water was deionized and bidistilled.

2.2. Apparatus

In this work we used a modified SpectraPHORESIS 100 CE System (Thermo Separation Products, ThermoQuest Analytische Systeme, Egelsbach, Germany) and fused-silica capillaries of 75 cm (55 cm effective length)×50 μm I.D.×363 μm O.D.

The UV–LIF detector consisted of a frequency doubled argon ion laser (Lexel 95 SHG, Lexel Laser, Polytec, Waldbronn, Germany) operating at 257 nm with a power of 200 mW to provide the excitation wavelength. An on-column detection window was created by removing a 4 mm section of the polyimide coating on the fused-silica tubing. This is different to many other LIF–capillary zone electrophoresis (CZE) systems where the laser beam is focused to a spot of less than 100 μm, thus illuminating the analyte for only several milliseconds [17–20]. In the system described here, the capillary is illuminated with the laser profile at a length along the detection window of 1.5 mm and a height according to the I.D. of the capillary (50 μm) using a 40 mm focal length cylindrical quartz lens for focusing. The resulting fluorescence from this section is imaged onto a spectrograph with an attached intensified CCD camera (Flamestar 3, La Vision 2D Messtechnik, Göttingen, Germany). This set-up is used to achieve wavelength resolution of the emitted light, allowing the additional registration of the emission spectra which are read out by the CCD camera [19,21–23]. The fluorescence is collected during the entire residence time of the analyte band in the capillary section of ca. 1.5 mm enhancing significantly the sensitivity of the LIF system.

The laser-induced natural fluorescence signal (LINF signal) was collected in an angle of 90° to the excitation light with a spherical aluminium mirror (d. 5 cm, F/Number 1.1) to suppress chromatic aberration.

An imaging spectrograph (Multispec 77417, L.O.T.-Oriel, Darmstadt, Germany) with a 1200 lines/mm grating, holographic blazed at 250 nm is used. With the present CCD camera the covered spectral range is 105 nm wide, adjustable between 180 nm to 400 nm [24]. A CCD camera was used with readout rate of 4.5 Hz, a binning factor of 48×40 [21].

The wavelength-resolved CE–LINF data were processed with custom algorithms using Mathcad 7 (Mathsoft). Fig. 1 shows a schematic description of the experimental set-up.

2.3. Electrophoretic conditions

A 150 m*M* tetraborate buffer adjusted to pH 10.6 with 150 m*M* NaOH was used as run buffer. The applied voltage was +25 kV and the temperature was maintained at 19.5°C. The injection was performed hydrodynamically.

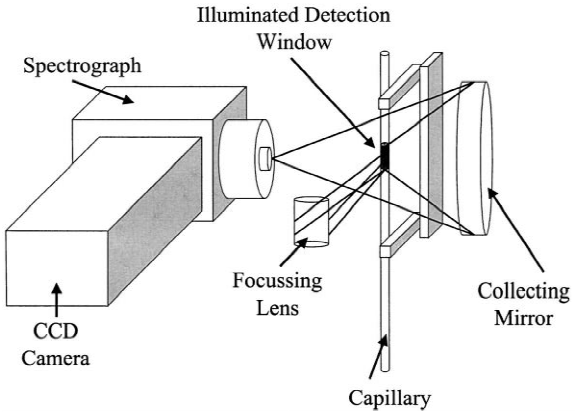

Fig. 1. Schematic description of the experimental set-up.

2.4. Internal standard solutions

For the internal standard (I.S.), stock solutions of 100 μg/ml naphazoline nitrate in water were prepared weekly and stored at 4 °C.

2.5. Standard solutions

Stock solutions of 1 mg/10 ml tramadol were prepared in deionized and bidistilled water and stored at 4°C. Standard solutions form 200 ng to 50 μg/ml were prepared daily by dilution with blank urine.

2.6. Urine samples

Pooled blank urine collected from a male healthy volunteer was used to prepare spiked urine samples. A 100-μl volume of the respective standard solution and 100 μl of I.S. were added to 800 μl blank urine in order to obtain calibration series of 20, 50, 100, 200, 500, 1000, 2000, 5000 ng/ml. Each sample, contained 10–15% water, compared to the collected urine samples which include 10% water the difference is negligible.

2.7. Validation

2.7.1. Calibration

The calibration curves were obtained by analyzing urine samples with known concentrations of tramadol. Two different calibration curves were prepared to achieve linearity. (A) Corresponds to the lowest concentrations (20–500 ng/ml), (B) corresponds to the higher concentrations (500–5000 ng/ml). Each point of the calibration curve is based on data from three separate runs. Linear regression analysis according to the I.S. method was performed by plotting the peak area ratio of tramadol/internal standard against the known concentrations of the samples.

2.7.2. Precision and accuracy

Intra-day variation was assessed by six replicated determinations of four concentrations in the tested range (50, 100 ng/ml, 2, 5 μg/ml). Intra-day accuracy was expressed as the mean of the results relative to the theoretical values (%). The intra-day precision of the method was expressed as the relative standard deviation (RSD) of the assays made for intra-day accuracy. Inter-day variation was determined by analyzing replicates of spiked urine samples with the same concentrations on 3 separate days. Inter-day accuracy was expressed as the mean of the assays relative to the theoretical values (%). The inter-day precision was expressed as the RSD of the assays made for the inter-day accuracy.

2.7.3. Detectability

The limit of detection (LOD) was defined as the analyte concentration which leads to a S/N ratio of 3:1. The limit of quantification (LOQ) was defined as the analyte concentration that could be analyzed with acceptable precision and accuracy. It was the same as the lowest of the tested concentrations described in Section 2.7.2.

2.7.4. Selectivity

Selectivity was investigated by comparing a blank urine sample with an urine sample spiked with tramadol, internal standard and the metabolites *N*-demethyltramadol and *O*-demethyltramadol.

2.8. Application

The method was applied to samples taken from one healthy volunteer. Urine samples were collected during 0–1, 1–2, 2–3, 3–4, 4–5, 5–6, 6–7, 7–8, 8–14 and 14–24 h after oral administration of 150 mg tramadol hydrochloride (three tablets of Tramal each containing 50 mg tramadol hydrochloride). The concentration of tramadol in the collected intervals

was calculated from the calibration equations obtained in Section 2.7.1. All collected intervals except for the 12–24 interval were diluted with blank urine because the concentration of tramadol exceeded the calibration range.

3. Results and discussion

A sensitive and specific method for the determination of tramadol in urine without extraction is described. The combination of the characteristic fluorescence emission spectra and the retention time of the analytes in CE represents a method for simultaneous identification and determination of the amount of the natively fluorescent analytes which would not be possible with filter-based, single-channel fluorescence detectors. The characteristic spectral fingerprint of the analytes allows a more complete identification compared to the migration time alone. Fig. 2 shows the wavelength-resolved detection of tramadol, *N*-demethyltramadol, *N*,*O*-didemethyltramadol and the internal standard. When excited at 257 nm for tramadol and *N*-de-

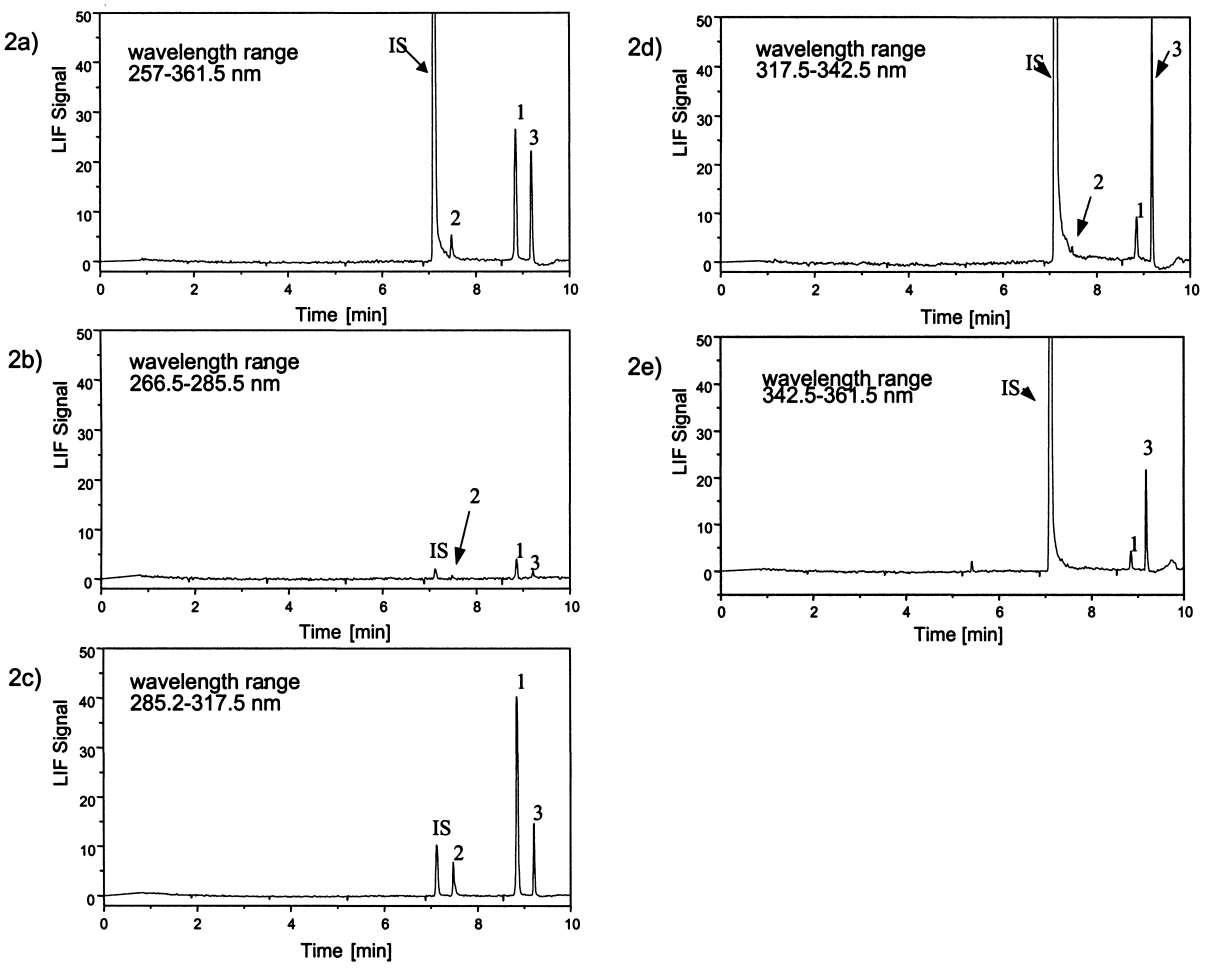

Fig. 2. Wavelength-resolved detection of *N*-demethyltramadol, internal standard, tramadol and *N*,*O*-didemethyltramadol. (a) Emitted light collected in the total range of 257–361.5 nm. (b) Emitted light collected in the range of 266.5–285.5 nm. (c) Emitted light collected in the range of 285.5–317.5 nm optimized for tramadol (b) and for *N*-demethyltramadol (a). (d) Emitted light collected in the range of 317.5–342.5 nm optimized for *N*,*O*-didemethyltramadol (c). (e) Emitted light collected in the range of 342.5–361.5 nm. Experimental conditions: 150 m*M* borax buffer, pH 10.6, applied voltage: +25 kV. Concentration of the sample: 500 ng/ml tramadol and metabolites.

methyltramadol a maximum of emission is found at 295 nm. To get the optimum S/N ratio for these analytes the signal is collected from 285.5 nm to 317.5 nm as shown in Fig. 2c. For metabolites with a different emission wavelength different collection intervals would give the optimum sensitivity and selectivity. For *N*,*O*-didemethyltramadol the maximum of emission is found at 320 nm. Fig. 2d shows the wavelengths optimized electropherogram for *N*,*O*-didemethyltramadol. The signal is collected from 317.5 nm to 342.5 nm. In contrast to Fig. 2c the selectivity between the I.S. and *N*-demethyltramadol (a) is lost, but there is a three-fold improvement of sensitivity for the *N*,*O*-didemethyltramadol (c), while the sensitivity for tramadol (b) is decreased by a factor of 4. Because of the different emission spectra it is necessary to choose different wavelength intervals from the entirely collected spectral range for the data processing to achieve the best S/N ratio for a certain analyte in the sample mixture. The main advantage is the capability for direct determinations and quantification of tramadol in urine samples due to the extremely high sensitivity. Several other methods have been described in the literature for determination of tramadol in different biological fluids [4,6,7,10,11], but there is no technique for the determination of tramadol without extraction available. Fig. 3 shows the electropherogram of an urine sample compared to blank urine. No interference of the tramadol peak with peaks of the metabolites was observed. The approximate retention times of *N*-demethyltramadol, tramadol, *N*,*O*-didemethyltramadol and the internal standard were 7.6, 9.0, 9.6 and 7.1 min, respectively. The total analysis time was 10 min. Additional metabolites like *O*-demethyltramadol and *N*,*N*,*O*-tridemethyltramadol were identified but not quantified. No interference of endogenous substances with the studied compounds was observed because the emission spectra were recorded and appropriate spectral ranges were individually selected for tramadol and the metabolites in data processing. The calibration curves of tramadol were linear over the whole concentration range. Correlation coefficients were higher than 0.998.

Calibration curve a: $y=0.06699+x0.00143$, $r=0.999$.

Calibration curve b: $y=0.44174+x0.00064$, $r=0.998$.

Only calibration curve b was used for the analysis of the urine samples.

Table 1 lists the results obtained from the inter- and intra-day precision and accuracy studies. For all samples inter- and intra-day precision was better than 90%. The inter- and intra-day accuracy ranged between 90 and 110%. These results meet the

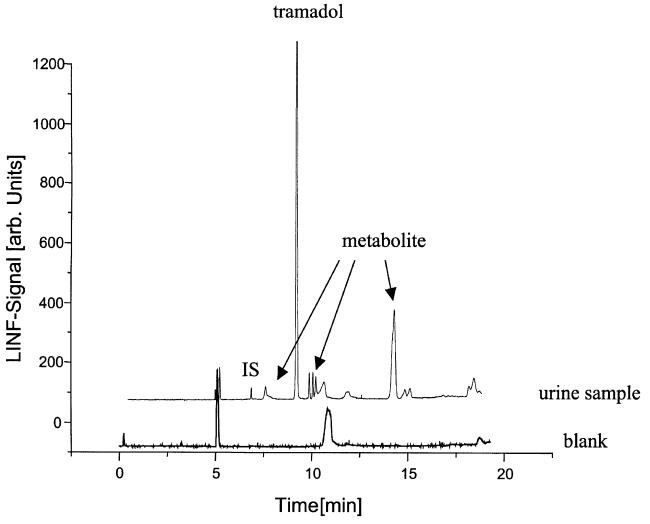

Fig. 3. Collection interval of 7–8 h compared to blank urine. Electrophoretic conditions: 150 m*M* borax buffer, pH 10.6, applied voltage: +25 kV.

Table 1
Results of the validation – direct determination of tramadol in urine

	Theoretical concentration (ng/ml)	Concentration found (ng/ml)	Mean accuracy (%)	Mean precision (%)
Day 1 (n=6)	50	45.1	90.2	7.7
Day 2 (n=6)		46.2	92.3	6.4
Day 3 (n=6)		45.5	90.9	10.6
Days 1–3 (n=18)		45.5	90.9	7.9
Day 1 (n=6)	100	93.0	93.0	6.7
Day 2 (n=6)		96.2	96.2	6.9
Day 3 (n=6)		96.5	96.5	4.5
Days 1–3 (n=18)		95.1	95.1	5.9
Day 1 (n=6)	2000	1952	97.6	9.2
Day 2 (n=6)		1836	91.8	5.6
Day 3 (n=6)		1818	90.9	3.4
Days 1–3 (n=18)		1843	92.2	6.6
Day 1 (n=6)	5000	4748	95.0	4.1
Day 2 (n=6)		5307	106.1	8.1
Day 3 (n=6)		5418	108.4	3.2
Days 1–3 (n=18)		5157	103.1	7.4

requirements for the validation of biological samples. Figs. 4 and 5 show the precision and accuracy of the method.

The LOD given by the S/N ratio of 3:1 was determined to be 1 ng/ml. Thus the LOD is about 1000-fold higher compared to CE methods using instruments equipped with a UV detector. The LOQ was determined to be 50 ng/ml. Fig. 6 gives the comparison between LIF and UV absorption detection.

The concentration of the analyte in the different collected intervals taken from one healthy volunteer is shown in Fig. 7. In Fig. 8 the cumulative excretion as a function of time is given. 27.7% of the adminis-

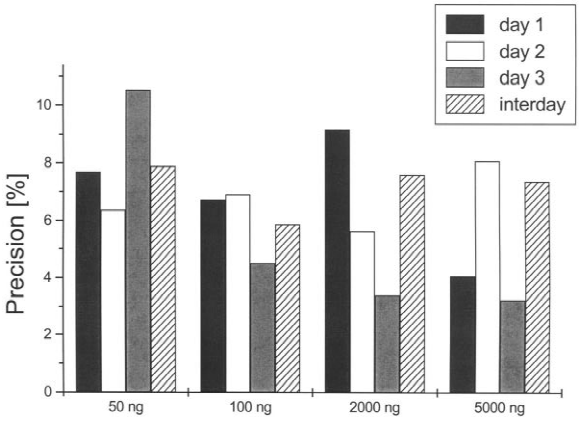

Fig. 4. Precision of the method. Direct determination of tramadol in urine.

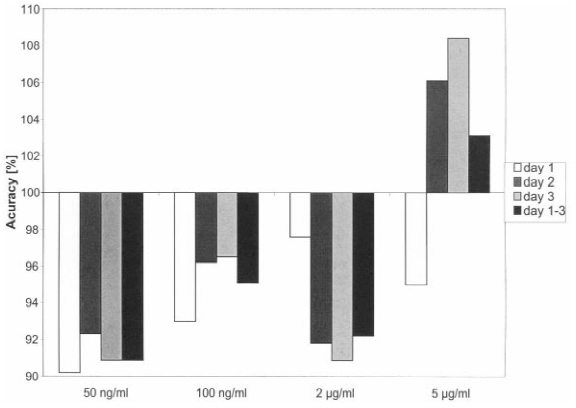

Fig. 5. Accuracy of the method. Direct determination of tramadol in urine.

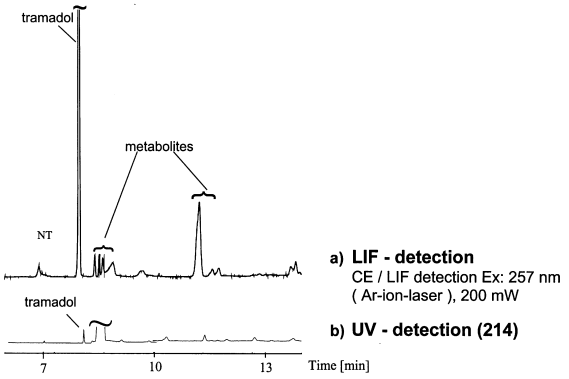

Fig. 6. Comparison of UV and LIF detection. Tramadol and metabolites. Urine sample after oral administration of tramadol. ~1 nl injected directly.

tered dose was finally excreted as unmetabolized tramadol.

4. Conclusion

CE–UV–LINF detection was used for direct determination of tramadol from urine.

CE–LINF detection is a rapid, specific, sensitive and accurate method for quantification of tramadol in urine samples and with great potential for sensitive assay of other fluorescent drugs.

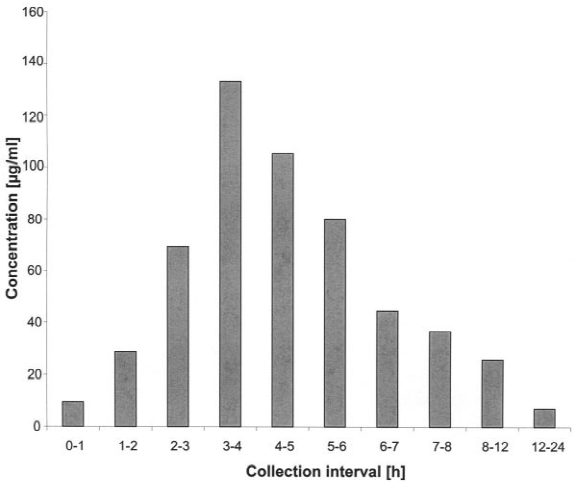

Fig. 7. Concentration of tramadol (μg/ml) in the collection intervals after oral administration of 150 mg tramadol hydrochloride.

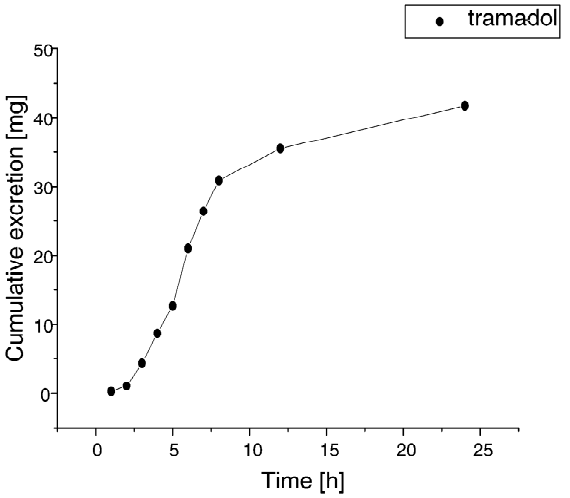

Fig. 8. Cumulative excretion curve of tramadol as a function of time.

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