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Enantioselective analysis of levetiracetam and its enantiomer *R*-α-ethyl-2-oxo-pyrrolidine acetamide using gas chromatography and ion trap mass spectrometric detection

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Abstract

A gas chromatographic–mass spectrometric method was developed for the enantioselective analysis of levetiracetam and its enantiomer (*R*)-α-ethyl-2-oxo-pyrrolidine acetamide in dog plasma and urine. A solid-phase extraction procedure was followed by gas chromatographic separation of the enantiomers on a chiral cyclodextrin capillary column and detection using ion trap mass spectrometry. The fragmentation pattern of the enantiomers was further investigated using tandem mass spectrometry. For quantitative analysis three single ions were selected from the enantiomers, enabling selected ion monitoring in detection. The calibration curves were linear from 1 μ*M* to 2 m*M* for plasma samples and from 0.5 m*M* to 38 m*M* for urine samples. In plasma and urine samples the inter-day precision, expressed as relative standard deviation was around 10% in all concentrations. Selected ion monitoring mass spectrometry is suitable for quantitative analysis of a wide concentration range of levetiracetam and its enantiomer in biological samples. The method was successfully applied to a pharmacokinetic study of levetiracetam and (*R*)-α-ethyl-2-oxo-pyrrolidine acetamide in a dog.

Keywords: Enantiomer separation; Levetiracetam; *R*-(α)-ethyl-2-oxo-pyrrolidine acetamide

1. Introduction

Chirality is a cardinal issue in modern pharmaceutical sciences. During design and development of chiral drugs one must decide between development of racemic form of a drug or an enantiospecific form [1]. Enantioselective assays are essential for drug development since isomeric impurities may have

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undesired toxicological, pharmacological or other effects.

Levetiracetam [(*S*)-α-ethyl-2-oxo-pyrrolidine acetamide, LEV, Fig. 1] is an ethyl analogue of the nootropic drug piracetam, and it is freely soluble in water, methanol and chloroform. LEV is a new antiepileptic drug (AED) [2] recently approved by the US Food and Drug Administration (FDA). LEV possesses a chiral center. The configuration of LEV and its enantiomer, (*R*)-α-ethyl-2-oxo-pyrrolidine acetamide (REV), are presented in Fig. 1. Only the (*S*) enantiomer of α-ethyl-2-oxo-pyrrolidine acetamide, levetiracetam, has anticonvulsant activity, and therefore it is administered as a single enantiomer [3]. Because the chiral center of levetiracetam is in the α position to the carbonyl group in the butyramide moiety of the molecule, chiral interconversion (enantiomerization) cannot be excluded. Consequently, the in vivo stereochemical integrity of LEV has to be assessed by an enantioselective assay. Moreover, the enantioselective analysis of levetiracetam from biological fluids is important for pharmacokinetic and pharmacodynamic studies as well as for testing the enantiomeric purity of the commercial drug preparation.

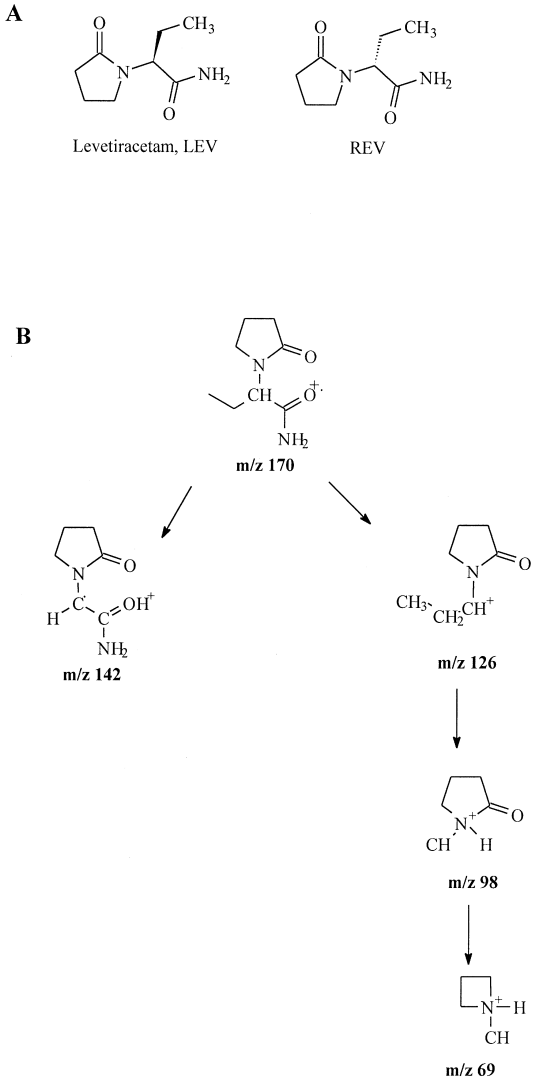

Fig. 1. (A) Chemical structures of levetiracetam (LEV) and its enantiomer (*R*)-α-ethyl-2-oxo-pyrrolidine acetamide (REV) and (B) the fragmentation pattern of LEV.

Analysis and monitoring of levetiracetam have previously been carried out using nonenantioselective high-performance liquid chromatography (HPLC) [4,5] and gas–liquid chromatography (GLC) [5]. The objective of this study was to develop an enantioselective assay capable of separating and quantifying levetiracetam and REV in biological fluids using selected ion monitoring (SIM) in ion trap mass spectrometry (MS).

2. Experimental

2.1. Materials

Methanol was purchased from Lichrosolv (gradient grade), manufactured by Merck, Germany. Double distilled ultrapure water was used throughout the study. C_{18} solid-phase extraction (SPE) cartridges (Bond Elut, 3 ml/500 mg) were obtained from Varian (Harbor City, CA, USA). *N*-dimethyl valproyl glycinamide (DIM), the internal standard, was synthesized according to a previously published method [6].

2.2. Synthesis of LEV and REV

(*S*)-α-ethyl-2-oxo-pyrrolidine acetamide was prepared according to a previously published method [7,8] starting from L-methionine. L-Methionine was esterified to its methyl ester using absolute methanol and thionyl chloride. The obtained L-methionine

methyl ester hydrochloride was amidated using gaseous ammonia to obtain L-methionine amide. This amide was treated with potassium hydroxide, tetrabutylammonium bromide and 4-chlorobutyryl chloride to yield (*S*)-α-[2-(methylthio)ethyl]-2-oxopyrrolidine acetamide). Desulfurization of the obtained acetamide using a Raney nickel-catalyst produced LEV with a specific rotation $[\alpha]_D^{25}$ −87.0 (c=1, acetone) (enantiomeric excess, ee 94%). The (*R*) enantiomer, REV, was synthesized analogously to LEV starting from D-methionine methyl ester and obtained with a specific rotation of $[\alpha]_D^{25}$ +88.9 (c=1, acetone) (ee 97%).

2.3. Gas chromatography and mass spectrometry

A Finnigan MAT GCQ equipped with an ion trap mass spectrometer was used for analysis with manual injection in the splitless mode. The gas chromatography (GC) column used was a 6-TBDMS-2,3-perme-β-cyclodextrin (20%, w/w) in SE52, 10 m×0.25 mm I.D., 0.5 μm film thickness fused-silica capillary column prepared in the laboratory.

The temperature program used for analysis was as follows: initial temperature 110°C for 2 min, temperature gradient of 10°C/min until 170°C and then hold time for 5 min. The injector was held at 210°C and the transfer line at 200°C. 99.999% pure helium, filtered through R&D separators, was used as carrier gas with a constant velocity 40 cm/s. The ion source temperature was 200°C. Automatic gain control (AGC) was used throughout the study on set value 50. The multiplier was at 1950 V and the electron energy was 70 eV. The trap offset was set at −10 V. For MS–MS experiments the excitation energy was 0.5 V. For quantification three ions were used in selected ion monitoring (SIM) mode: 69, 98 and 126 m/z for LEV and REV and 129, 157 and 186 m/z for DIM.

2.4. Preparation of standard solutions

Stock solutions of LEV, REV and DIM were prepared by dissolving each compound in methanol. The stock solution was used for recovery calculations and for mass spectrometric experiments. Working solutions of the analytes were prepared in saline and stored in a freezer at −25°C. 1 μ*M*–2 m*M* calibration samples of LEV or REV in plasma and 0.5–38 m*M* of LEV or REV in urine were prepared. The spiked samples were stored in a freezer (−25°C) up to 2 months. Pooled dog plasma and urine was used throughout the study.

2.5. Treatment of plasma and urine

2.5.1. Plasma

The SPE cartridges were conditioned with 2 ml of methanol followed by 2 ml of water. A 500-μl volume of plasma was applied to the cartridge. The cartridge was allowed to run dry and washed with 500 μl of water. The analytes were eluted using 1 ml of methanol and the internal standard (100 μl of 0.4 m*M* DIM) was added. The sample was evaporated to dryness, the dry residue dissolved to 250 μl of methanol and 1 μl was injected into the gas chromatograph.

2.5.2. Urine

The SPE cartridges were conditioned similarly as described for plasma samples. A 100-μl volume of urine was applied to the cartridge, the cartridge allowed to run dry and washed with 500 μl of water. The analytes were eluted with 1 ml of methanol and 400 μl of internal standard solution (0.4 m*M* DIM) was added. The samples were evaporated to dryness and reconstituted to 2 ml methanol after which 1 μl was injected into the GC system.

2.6. Method validation

The method was validated according to previously published guidelines [9,10]. Inter-assay relative standard deviations (RSDs) were calculated from 10 replicate analyses performed within 2 months at three different concentrations, 2.35 pmol, 0.2 nmol and 2 nmol/injection from plasma samples and 29 pmol, 0.5 nmol and 18.8 nmol/injection from urine samples. Recovery was calculated by comparing standard sample in methanol to a spiked sample that underwent the whole process of sample preparation described above. Recovery from the SPE cartridge was calculated by comparing a saline standard that was treated similarly to plasma and urine to a methanol sample at the same concentration.

3. Results and discussion

3.1. Treatment of plasma and urine samples

LEV has been extracted from plasma using dichloromethane [4]. A previously reported [5] SPE method of LEV from serum using C_{18} cartridges, was applied in this study to both enantiomers, LEV and REV. The recoveries of the (*S*) and (*R*) enantiomers from plasma were 86.5±4 and 91.2±4%, respectively. No decrease of the recovery was observed with increasing concentrations. The recovery of plasma samples was slightly higher than the recovery of the standards prepared in saline, indicating a possible matrix effect. The SPE of LEV and REV from urine samples, that contained approximately a 100-times higher concentrations of the analyte than reported in the literature, was also satisfactory. Even though the highest concentrations of LEV analyzed were at 38 m*M* the recoveries [86±4% and 81±4% for (*S*) and (*R*) enantiomers, respectively] were constant over the whole concentration range.

The SPE method is very effective in cleaning the samples before GC analysis. Blank chromatograms of urine and plasma illustrating lack of interference from matrix components are presented in Fig. 2. The method is also fast to perform. Twenty samples (determined by the SPE bench size) can be prepared in less than an hour. This enables analysis of large numbers of samples like required in pharmacokinetic studies.

3.2. Separation of the enantiomers

Fig. 2 represents (H) total ion chromatogram of a mixture of LEV and REV standards together with DIM, the internal standard, (D) selected ion chromatogram of plasma spiked with LEV and REV, selected ion chromatograms (C) of dog plasma (0.115 m*M* of LEV) after administration of LEV and (G) dog urine (3.26 m*M* of REV) after administration of REV. Several different temperature programs including isothermic runs were evaluated and the separation was only partially lost while moving to steeper gradients despite the changes in retention times. This shows that the separation can be considered robust and not effected with slight changes in chromatographic conditions. Also the retention time remained essentially constant. Thus, during the analysis of 100 standard samples, the retention time [8.6 min for (*S*) enantiomer and 8.8 min for (*R*) enantiomer] had a RSD of 0.7%. As demonstrated here, the chiral cyclodextrin phase can be readily used with mass-selective detection, without problems of column bleeding or other interference.

3.3. Mass spectrometry

The mass spectrum of levetiracetam is presented in Fig. 3. The fragmentation pattern of the enantiomers was identical as expected. Typically for amides the molecular ion was small and only few fragments were available for identification of the compounds. The fragmentation pathway of LEV and REV, as specified using MS–MS is presented in Fig. 1. The m/z values of the fragments obtained after MS–MS experiments from the enantiomers are presented in Table 1. Three single ions (m/z 69, 98 and 126) were selected for quantification. These ions were specific to both enantiomers but were not observed in the background. For the internal standard the three single ions were 129, 157 and 186 m/z.

3.4. Linearity and quantification limit

The calibration curves were prepared so that both extremes of expected concentrations in plasma and urine after administration of LEV or REV to a dog or a human were covered. The levetiracetam concentrations in the majority of clinical samples are expected to be in the middle of the range analyzed. Peak area ratios between the test compounds and the internal standard were plotted as a function of the concentration of the analyte in the sample, and the calibration curves were constructed by method of least-squares linear regression. The details of the calibration curves are characterized in Table 2. The calibration curves were linear between 2.3 pmol and 2.35 nmol injected for plasma samples and between 15 pmol and 18.8 nmol injected for urine samples. A slight deviation from linearity (less than 5% in the slope) was observed between 1.3 nmol and 2.35 nmol injected from plasma samples but quantification was still possible. The most likely reason for this deviation is an interference of the analyte ions

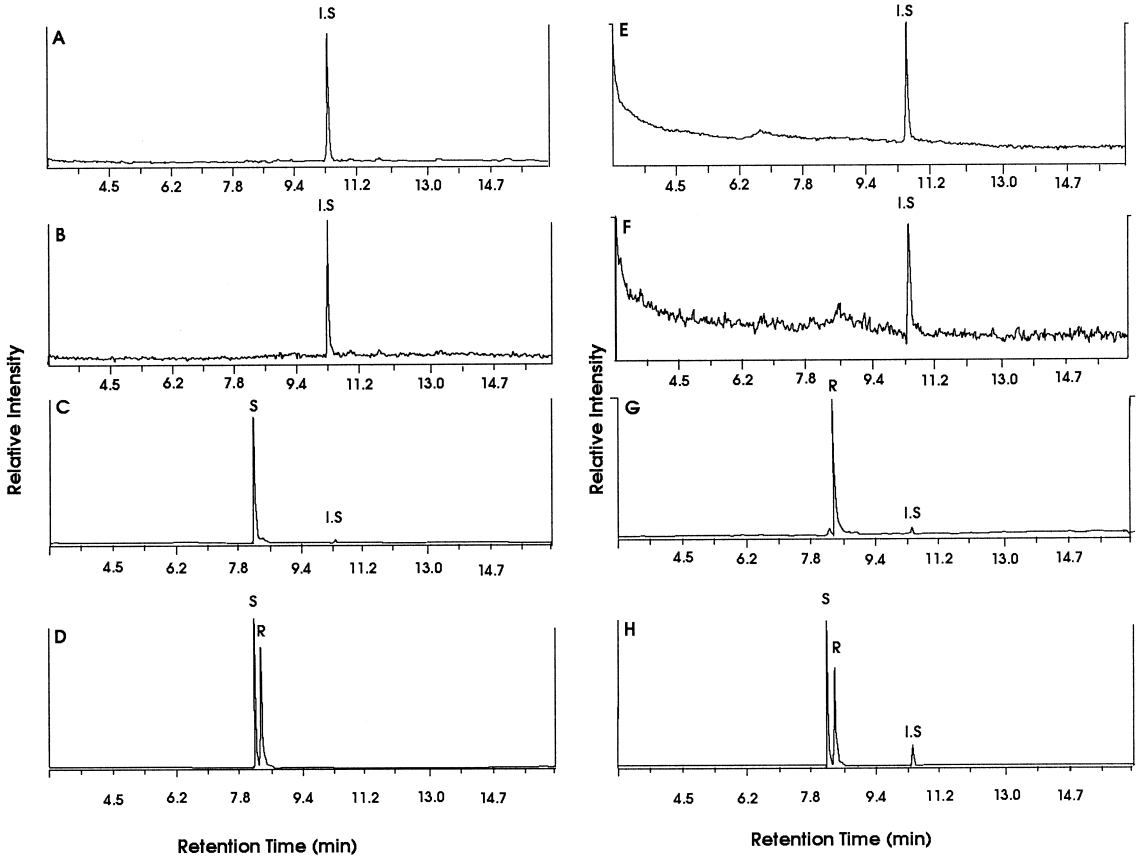

Fig. 2. Chromatograms of total ion (A) and selected ion (B) monitoring of blank dog plasma, an ion chromatogram of dog plasma obtained 4 h after administration of levetiracetam (20 mg/kg) (C) and selected ion chromatogram of dog plasma spiked with LEV and REV (D). Chromatograms of total ion (E) and selected ion (F) monitoring of blank dog urine, ion chromatogram of dog urine 10 h after administration of REV (20 mg/kg) (G) and a total ion chromatogram of the standard mixture of the enantiomers of α-ethyl-2-oxo-pyrrolidine acetamide and the internal standard, *N*-dimethyl valproyl glycinamide (H). The selected ions are 69, 98 and 126 *m/z*. I.S.=internal standard, R=REV and S=LEV.

with the internal standard peak. However, the linear range from urine samples demonstrates, that the linear range of the method can be expanded when dilution is used. It should be emphasized that ion trap mass spectrometry was used for the quantification and it showed satisfactory linearity over a wide concentration range.

The limit of detection, determined as the concentration in which the signal-to-noise ratio was three, was 0.94 pmol of LEV or REV injected from plasma samples. The limit of quantitation (LOQ), determined as the concentration where the signal-to-noise ratio was at least ten, was 2.35 pmol of LEV or REV injected from plasma and 15 pmol injected from urine samples. The LOQs could easily be lowered if smaller final dilution volume was used and separate calibration curves were prepared. However, lower concentrations are not relevant in studies concerning levetiracetam and therefore the range was not expanded.

3.5. Method validation

The method was validated to concentrations that are relevant in clinical and preclinical research. The method validation results are presented in Tables 3 and 4. Both, peak area and peak height ratios were evaluated for quantification. Peak area gave superior

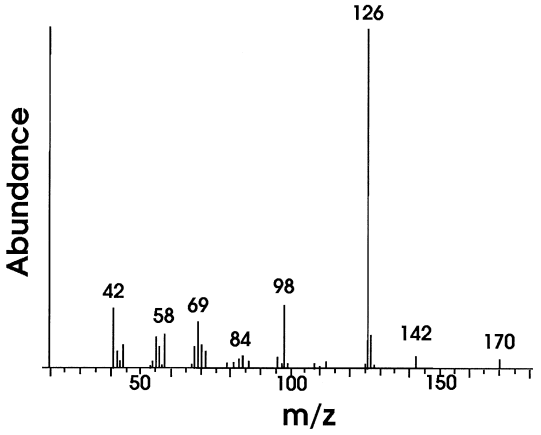

Fig. 3. Mass spectrum of levetiracetam.

Table 3
Validation results for LEV and REV in dog plasma[a]

Sample	Precision (% RSD)	Accuracy (%)
LEV plasma	14.6, 11.8, 8.1	105.5, 104.5, 101.3
REV plasma	15.0, 6.0, 9.7	108.9, 106.6, 98.3

[a] Concentrations of 2.35 pmol, 0.2 nmol and 2 nmol per injection used, respectively.

Table 4
Validation results for LEV and REV in dog urine[a]

Sample	Precision (% RSD)	Accuracy (%)
LEV urine	10.8, 11.3, 8.4	95.0, 106.8, 103.3
REV urine	11.7, 9.4, 6.6	100.4, 97.4, 106.0

[a] Concentrations 29 pmol, 0.5 nmol and 18.8 mmol per injection used, respectively.

results in terms of robustness and thus it was chosen for validation. The results illustrate that ion trap mass spectrometry is suitable for quantitative analysis in terms of repeatability.

Due to the generally high concentrations of levetiracetam in urine, the validation was performed in sample concentrations generally higher than samples analyzed with MS instruments. Dilution of urine samples was necessary in order to achieve small enough concentrations suitable for injection to the MS. Therefore, it was not attempted to evaluate how low concentrations could be analyzed with the developed method.

Several different aspects were evaluated in the selection of an internal standard. An internal standard that elutes after the analytes was preferred.

Table 1
Tandem mass spectrometric data obtained from the enantiomers

Parent ion m/z	Fragments from LEV (relative intensity)	Fragments from REV (relative intensity)
126	126 (77), 108 (5), 98 (100), 83 (11), 69 (30), 58 (28), 55 (31)	126 (76), 108 (5), 98 (100), 83 (9), 69 (30), 58 (27), 55 (30)
98	98 (86), 81 (5), 70 (100), 68 (15), 56 (12)	98 (85), 81 (7), 70 (100), 68 (12), 56 (10)
69	69 (7), 41 (100), 32 (2)	69 (6), 41 (100), 32 (2)

Table 2
Calibration curve data in dog plasma and urine for LEV and REV

Sample	Linear regression equation $y=a+bx$		
	Slope b (SD)[a]	Intercept a (SD)[a]	No. of calibration points
LEV plasma	8.41 (0.17)	0.04 (0.02)	7
REV plasma	12.49 (0.36)	−0.09 (0.03)	7
LEV urine	0.775 (0.0077)	0.0395 (0.03)	7
REV urine	0.655 (0.010)	−0.299 (0.05)	7

[a] $n=4$.

Since the SPE had good repeatability and involved minimum amount of pipetting steps, it was decided to use an internal standard just to correct the error in the final dilution volume and the injection volume. Also, there was no isotopically labeled compound available and thus a standard that could be assumed to have identical performance in the SPE with the analyte was not readily available. If it will be shown that no chiral inversion (enantiomerization) occurs in vivo, the second enantiomer can be used as an internal standard for the analyzed enantiomer [11]. Piracetam, 2-oxo-1-pyrrolidine acetamide, was evaluated as a first choice of internal standard. However in the column used, piracetam eluted too close to levetiracetam and had unacceptable chromatographic performance.

No interference was observed from any urine or plasma samples analyzed including samples of six different dogs that were administered with levetiracetam. Stability of the prepared samples was evaluated and the results showed that after the sample preparation the dried samples could be saved at least for a week before injection without loss of analyte. The stability of the biological samples during freezing, thawing and storage was assessed by analysis of spiked samples after subsequent freezing and storage of 1–4 months. Plasma and urine samples could be thawed and refrozen repeatedly without effect on the analyte concentration.

3.6. Application

Fig. 4 shows a representative graph of a plasma concentration vs. time profile for LEV and REV after intravenous (i.v.) administration of 20 mg/kg of each one of the enantiomers to a dog and the profiles of cumulative amount of LEV and REV excreted in urine of the same dog.

4. Conclusions

A sensitive enantioselective assay for analysis of levetiracetam and its pharmacologically inactive enantiomer (*R*)-α-ethyl-2-oxo-pyrrolidine acetamide in plasma and urine was developed. This new assay was validated in concentration ranges relevant for clinical and preclinical research and can be applied in pharmacokinetic studies of levetiracetam. The mass spectra of the enantiomers were determined together with tandem mass spectrometric experiments of the main fragment ions. Three ions were selected for determination of the enantiomers. The study demonstrated the applicability of GC–ion trap

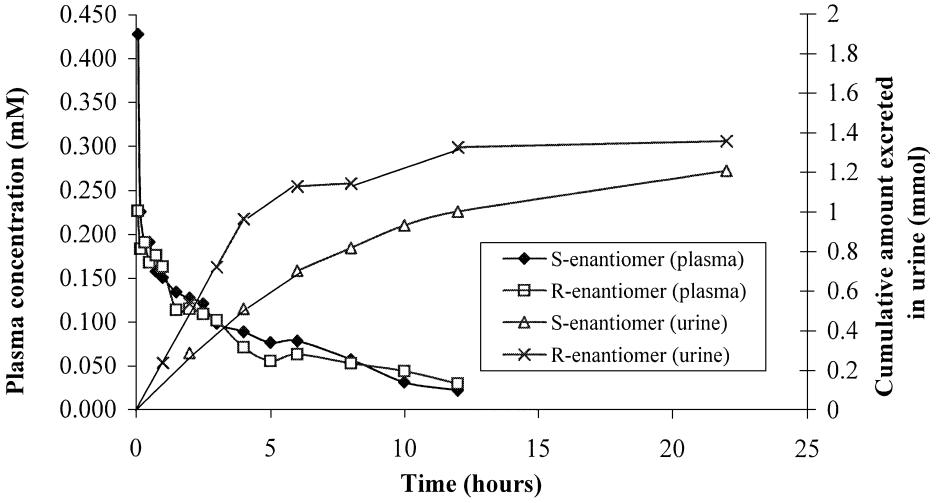

Fig. 4. Plasma concentration (m*M*) versus time profiles of LEV and REV and cumulative amount excreted in urine (mmol) of the two enantiomers after i.v. administration of 20 mg/kg of each enantiomer to a dog.

MS instruments to quantitative studies in wide concentration range. The method has successfully been applied in a pharmacokinetic study of levetiracetam and (*R*)-α-ethyl-2-oxo-pyrrolidine acetamide in a dog.

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