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ORIGINAL RESEARCH

High-performance liquid chromatographic determination of Levetiracetam in human plasma: comparison of different sample clean-up procedures

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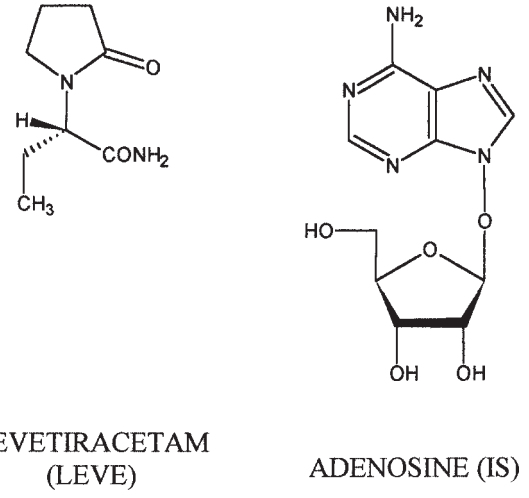
ABSTRACT: An accurate and precise high-performance liquid chromatographic method using diode array detection for the determination of the novel antiepileptic, Levetiracetam, has been developed. Three clean-up procedures for the analysis of Levetiracetam in human plasma were implemented and evaluated, namely solid-phase extraction, deproteinization by addition of organic solvents and formation of insoluble salts. Adenosine was used as the internal standard for all three sample pretreatment procedures. Among the several cartridges used for solid-phase extraction, the hydrophilic–lypophilic balance (Oasis® HLB) phase provides the best extraction yield of Levetiracetam, together with high precision. With the two other clean-up procedures involving plasma deproteinization by addition of methanol or zinc sulphate, lower sensitivity and precision of the assays were obtained. However, they are cheaper and faster when compared with the solid-phase extraction procedure. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: antiepileptic; solid-phase extraction; deproteinization; diode array detection

INTRODUCTION

Levetiracetam, the *S*-enantiomer of α-ethyl-2-oxo-1-pyrolidine acetamide (Fig. 1) is a novel antiepileptic drug (AED), structurally and mechanistically unrelated to existing antiepileptics (Hovinga, 2001). It has been shown to be effective in therapy for patients with treatment-refractory partial onset seizures with or without secondary generalization both as adjunctive treatment and as monotherapy (Dooley and Plosker, 2000; Schachter, 2000; Nash and Sangha, 2001). Levetiracetam is also useful, alone or in combination, for treatment of bipolar disorders, mania and migraine (Lamberty *et al.*, 2001).



LEVETIRACETAM (LEVE) ADENOSINE (IS)

Figure 1. Chemical structures of Levetiracetam (LEVE) and adenosine (IS).

Levetiracetam exhibits good bioavailability (Schachter, 2000), linear pharmacokinetics, insignificant protein binding, lack of hepatic metabolism and rapid achievement of steady-state concentrations (Patsalos, 2000; Perucca and Bialer, 1996). Epileptic patients generally require chronic therapy, often with two or more antiepileptic drugs at high doses. Another advantage of Levetiracetam is that it shows no interaction with

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Abbreviations used: AEDs, antiepileptic drugs; C_1, methyl cartridges; C_2, ethyl cartridges; C_8, octyl cartridges; C_{18}, octadecyl cartridges; CN, cyanopropyl cartridges; CN-N, cyanopropyl endcapped cartridges; DAD, diode array detector; EDTA, ethylenediaminetetraacetic acid; HLB, hydrophilic–lypophilic balance; HPCE, high-performance capillary electrophoresis; LEVE, Levetiracetam; PH, phenyl cartridges; PPP, plasma protein precipitation; σ, residual standard deviation; *S*, slope of the calibration curve; SPE, solid-phase extraction; TDM, therapeutic drug monitoring; USP, *United States Pharmacopeia*.

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other antiepileptic drugs even at very high dosages and it produces minimal adverse effects (Klitgaard *et al.*, 1998). The most common side effects are headache, asthenia, somnolence and dizziness. Levetiracetam has an efficacy comparable to or slightly better than other new AEDs (McAuley *et al.*, 2002) and has a wide therapeutic index, with toxic dosages being well differentiated from therapeutic dosages (Patsalos, 2000). Levetiracetam is usually well tolerated; however, sedation and respiratory depression have been reported (Barrueto *et al.*, 2002) in one case of Levetiracetam poisoning.

Levetiracetam may be particularly useful in patients who are non-responders to other AEDs, patients receiving drugs with a high potential for drug interactions, or those with hepatic impairment (French, 2001; Welty *et al.*, 2002). The efficacy is dose-dependent and usually the daily dosage of Levetiracetam is in the 1000–3000 mg range (Boon *et al.*, 2002; Pellock *et al.*, 2001; Perrucca *et al.*, 2001), with a therapeutic serum concentration range of about 10–50 μg/mL (Patsalos, 2000; Barrueto *et al.*, 2002).

Therapeutic drug monitoring (TDM) of AEDs is useful in various settings and particularly for new drugs. In fact, dosage regimens of AEDs should be assessed regularly, and adjusted if necessary, so that patients can derive optimal therapeutic benefits (Perrucca *et al.*, 2001). Even if Levetiracetam seems to be well tolerated, adverse effects are still possible during treatment and its recent introduction in therapy means that its pharmaco-toxicological profile is not fully understood. For this reason, TDM of patients undergoing therapy with Levetiracetam, especially children and the elderly, is advisable. Recently, we have developed methods for the separation and analysis (Mandrioli *et al.*, 2003; Bugamelli *et al.*, 2002; Raggi *et al.*, 2002; Pucci *et al.*, 2003) of some AEDs and metabolites, using liquid chromatography and capillary electrophoresis. We are now interested in developing reliable methods suitable for TDM of Levetiracetam. Only a few papers on the analysis of Levetiracetam in human plasma are reported in the literature, including high-performance liquid chromatographic (Ratnaraj *et al.*, 1996; Vermeij and Edelbroek, 1994) and gas–liquid chromatographic (Vermeij and Edelbroek, 1994; Isoherranen *et al.*, 2000) methods. In a recent work, the determination of Levetiracetam in human plasma is carried out by microemulsion electrokinetic chromatography (Ivanova *et al.*, 2003). For the pretreatment of plasma samples, the previously reported papers use solid-phase extraction procedures employing C_{18} cartridges (Vermeij and Edelbroek, 1994; Isoherranen *et al.*, 2000; Ivanova *et al.*, 2003); only one liquid–liquid extraction procedure with dichloromethane is reported (Ratnaraj *et al.*, 1996). The aim of this work is the development of alternative and feasible clean-up procedures of Levetiracetam from human plasma, which allow reliable HPLC analysis. Three pretreatments of biological samples were implemented, based on the following procedures: a novel solid-phase extraction procedure and procedures of deproteinization with methanol or zinc sulphate.

EXPERIMENTAL

Chemicals and solutions

Levetiracetam was kindly provided by UCB Pharma S.A. (Bruxel, Belgium). Adenosine (Figure 1), used as the internal standard (IS), was purchased from ACEF (Fiorenzuola d'Arda, PC, Italy).

Acetonitrile and methanol for HPLC, 85% (w/w) phosphoric acid, 37% (w/w) hydrochloric acid and 70% (w/w) perchloric acid, zinc sulphate and sodium hydroxide, pure for analysis, were purchased from Carlo Erba (Milan, Italy). Triethylamine, analytical grade, was purchased from Fluka (Buchs, Switzerland). Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus from Millipore (Milford, MA, USA).

Stock solutions (1 mg/mL) of the analyte and the IS were prepared by dissolving each compound in methanol. The stock solutions of Levetiracetam were stable for at least 2 months at –20°C. Standard solutions were prepared daily from stock solutions by dilution with the mobile phase.

Apparatus and chromatographic conditions

The chromatographic apparatus consisted of an Agilent 1100 Series (Agilent Technologies, Palo Alto, CA, USA) isocratic pump equipped with a diode array detector set at 205 nm.

A ResElut reversed phase column (C_8, 150 × 4.6 mm i.d., 5 μm; Varian, Harbor City, CA, USA) was used with a cartridge precolumn. The mobile phase (flow rate 1 mL/min) was a mixture composed of methanol, acetonitrile and a 3 mM phosphate buffer, containing 0.5 mL triethylamine (6:5:89, v/v/v) with an apparent pH of 6.0.

Human plasma sampling

Frozen, drug-free plasma ('blank' plasma) for calibration curves was obtained from the hospital blood bank of Bologna, stored at –20°C and thawed at room temperature before use.

Protein precipitation by adding organic solvents

Methanol and acetonitrile. The protein precipitation was carried out as follows: to 200 μL of plasma (previously spiked with the analyte and the IS) different volumes of organic solvent (200–1000 μL) were added. The mixture was vortexed for (3 min for methanol, 60 s for acetonitrile) and then centrifuged at 4°C at 3000 rpm for 15 min. Then the supernatant was filtered through a syringe filter of 0.2 μm pore size and injected into the HPLC instrument.

Protein precipitation by formation of insoluble salts

Zinc sulphate. The protein precipitation was carried out as follows: 125 µL of 1 N NaOH were added to 1000 µL of plasma previously spiked with the analyte and the IS. The mixture was vortexed for 60 s and then 125 µL of 0.7 M $ZnSO_4$ were added. The mixture was agitated again for 60 s and then centrifuged at 4°C at 3000 rpm for 15 min. Then the supernatant was filtered through a syringe filter of 0.2 µm pore size and injected into the HPLC instrument.

Perchloric acid. According to literature (Blanchard, 1981), 50 µL of 6% perchloric acid were added to 100 µL of human plasma (previously spiked with the analyte and the IS). The mixture was kept on ice for at least 5 min to ensure the complete protein precipitation. After centrifugation, the supernatant was filtered and injected into the HPLC system.

Solid-phase extraction procedure (SPE)

Waters Oasis® HLB cartridges (30 mg, 1 mL) were used. The SPE procedure was carried out on a Varian VacElut apparatus according to the following steps: (i) conditioning with 2 mL methanol; (ii) equilibration with 2 mL water; (iii) loading of a mixture of 500 µL plasma (previously spiked with the analyte and the IS) +100 µL water; (iv) washing twice with 1 mL water each; (v) cartridge drying for 30 s at −60 kPa; (vi) elution with 1 mL methanol; (vii) cartridge drying for 30 s at −60 kPa (to elute methanol as completely as possible). The eluate was then dried under vacuum (rotary evaporator), redissolved in 500 µL of the mobile phase and injected into the HPLC.

Method validation

Calibration curves. Aliquots of 25 µL analyte standard solutions at 10 different concentrations were added to blank plasma, to obtain calibration curves. The resulting plasma concentration range of Levetiracetam was 5–100 µg/mL for all plasma pretreatment procedures. A constant concentration of 10 µg/mL of IS was added to all samples. The resulting mixtures were subjected to the sample pre-treatment procedure and injected into the HPLC. The ratio between the area of Levetiracetam and that of the IS (adenosine) was plotted against the analyte concentration added to blank plasma. The calibration curves were constructed by means of the least square method.

Detection and quantitation limits were calculated according to the following equations: LOD = 3.3 σS^{-1} and LOQ = 10 σS^{-1}, where σ is the residual standard deviation and S is the slope of the calibration curve (*United States Pharmacopeia*, 2000; *ICH Quality Guidelines*, 2000).

Absolute recovery. To obtain the absolute recovery values for each sample pretreatment, three different concentrations, corresponding approximately to the upper limit, lower limit and middle point of the calibration curve were analysed. The absolute recovery values of the analytes were calculated by comparing the peak area ratio of blank plasma spiked with the analyte and subjected to the clean up procedure with the peak area ratio obtained from the corresponding standard solutions.

Precision. The assays described under 'Absolute recovery' were repeated six times within the same day to obtain the repeatability (intraday precision) and six times over different days to obtain the intermediate precision (interday precision) of the method, both expressed as RSD% values.

RESULTS AND DISCUSSION

Chromatographic conditions

The experiments were first carried out using the same conditions described in our recent paper (Bugamelli *et al.*, 2002) on the simultaneous determination of antiepileptic drugs using a C_{18} column and a 15 mM pH 3.0 phosphate buffer/methanol/acetonitrile (64.0:19.2:16.8, v/v/v) mixture as the mobile phase. Under these conditions, due to the high hydrophilicity of the drug, the peak of Levetiracetam was not detected, thus a C_8 column was used as the stationary phase and the composition of the mobile phase studied, in order to obtain good efficiency. The effect of chromatographic parameters such as organic modifier amount and buffer concentration and pH, on the retention time of Levetiracetam was investigated. Satisfactory results were obtained with a mobile phase composed of a 3 mM pH 3.0 phosphate buffer/methanol/acetonitrile (89:6:5, v/v/v) mixture with an apparent pH* of 6.0. Under these conditions Levetiracetam gives a neat chromatographic peak with a retention time of 7.3 min, and detection at λ = 205 nm. In order to select the appropriate internal standard, several substances were tested, namely tryptophan, melatonin and adenosine. Adenosine was chosen as the internal standard because it elutes before Levetiracetam and is well separated from the analyte (Fig. 2). In addition to the retention time, the use of the IS and the comparison of the UV spectra (obtained by a photodiode array detector) with those of standard solutions allowed for the unambiguous identification of the analyte peaks in plasma samples without the need to add analyte standard solutions.

HPLC analysis of standard solutions of Levetiracetam

The selected methodology was applied to the analysis of aqueous standard solutions of Levetiracetam. A standard calibration curve was established by plotting the ratio between the area of Levetiracetam and that of the IS against the analyte concentration. Linearity was obtained in the 5–100 µg/mL Levetiracetam concentration range. The regression equation obtained by means

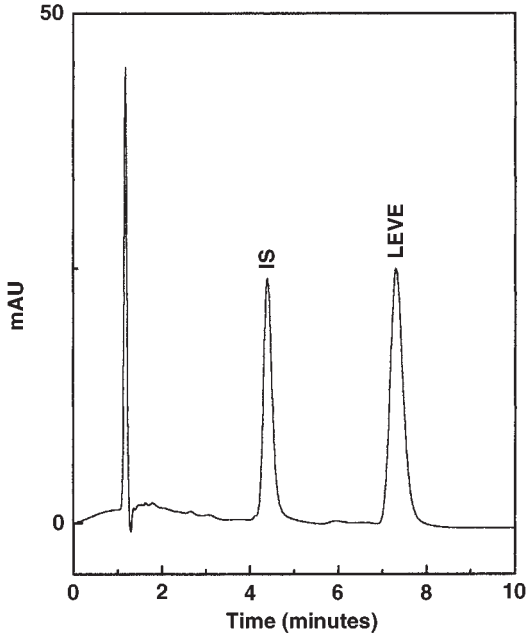

Figure 2. Chromatogram of a standard solution containing Levetiracetam (LEVE) (10 μg/mL) and IS (10 μg/mL). Chromatographic conditions: stationary phase, ResElut C_8 column (150 × 4.6 mm i.d., 5 μm); mobile phase composed of 89% 3 mM phosphate buffer, containing 0.56% triethylamine, 5% of acetonitrile and 6% of methanol, at pH* 6.0; flow rate, 1.0 mL/min; detection: λ = 205 nm.

of the least square method was $y = 0.0154 + 0.0346x$, where y is the ratio between the area of Levetiracetam and that of the IS, and x is the concentration of Levetiracetam, expressed as μg/mL. The linear correlation coefficient was 0.9999.

Precision expressed by the mean relative standard deviation (RSD%) values ranged from 1.1 to 1.9 for repeatability and from 1.8 to 2.3 for intermediate precision. The limit of detection (LOD) was 150 ng/mL, while the limit of quantitation (LOQ) was 500 ng/mL.

Plasma protein precipitation (PPP)

As Levetiracetam is minimally bound to plasma protein (10%; Nash and Sangha, 2001), PPP was investigated as a suitable pretreatment procedure of plasma samples. The preparation of protein-free solutions is particularly important for the liquid chromatographic analysis of substances in blood and in tissue extracts. The deproteinization of plasma samples can occur by addition of organic solvents, which are miscible with water, or by the formation of insoluble salts.

Precipitation by organic solvents

Proteins may be removed by precipitation with organic solvents, which are miscible with water, e.g. acetone, acetonitrile, methanol and ethanol, since the organic solvents lower the solubility of proteins. Among the solvents used for protein removal, methanol and acetonitrile were chosen due their high efficiency and, moreover, because they are constituents of the mobile phase.

Different acetonitrile volumes, (ranging from 200 to 1000 μL) were added to 200 μL of spiked plasma. Even though a 1:2 plasma–acetonitrile ratio (which is reported to be sufficient to remove 99.4% of the proteins; Blanchard, 1981) was used, the absolute recovery of Levetiracetam from the plasma samples was very low, ranging from 12 to 23%. Thus this procedure was discarded.

Better results were obtained using protein precipitation with methanol. Accordingly, different volumes of methanol were added to 200 μL of spiked plasma. By increasing the methanol percentage (from 200 to 1000 μL), the absolute recovery significantly increased from 47 to 98%, and almost no disturbance from the biological matrix was observed. An excellent mean recovery of Levetiracetam (98%) was achieved when 500 μL of methanol were added to 200 μL of plasma. With this procedure, mixing methanol and plasma for 3 min was sufficient for the precipitation of the proteins.

Figure 3(a) shows the chromatogram of a blank plasma sample spiked with 10 μg/mL of IS, and 10 μg/mL of Levetiracetam. Some peaks from the biological matrix are present but not at the retention times of the Levetiracetam and IS.

Absolute recovery values were evaluated for plasma spiked with different concentrations of Levetiracetam. Table 1 shows that the mean absolute recovery values ranged from 94 to 101%, indicating that the procedure is suitable for the determination of the drug in human plasma.

Validation of the method in terms of linearity and precision was carried out. The calibration curve was measured in the 5–100 μg/mL concentration range in blank spiked plasma. The IS concentration was always 10 μg/mL. The linear regression equation was: $y = 0.0164 + 0.0369x$ where y is the ratio of the area of the analyte and that of the IS, and x is the concentration of the analyte in μg/mL. Linearity, expressed by the linear correlation coefficient, r, was 0.9998. The relative standard deviations range from 1.6 to 2.5% with 100 and 5 mg/mL Levetiracetam, respectively (as detailed in Table 1). Thus, sufficient precision for the quantitative determination from biological matrices is ensured, since the acceptable deviation of 20% is not exceeded in any case. The LOD and the LOQ of Levetiracetam were 375 and 1250 ng/mL, respectively.

The results show that this simple and fast sample preparation technique can be applied to the plasma determination of Levetiracetam with satisfactory reproducibility and efficiency.

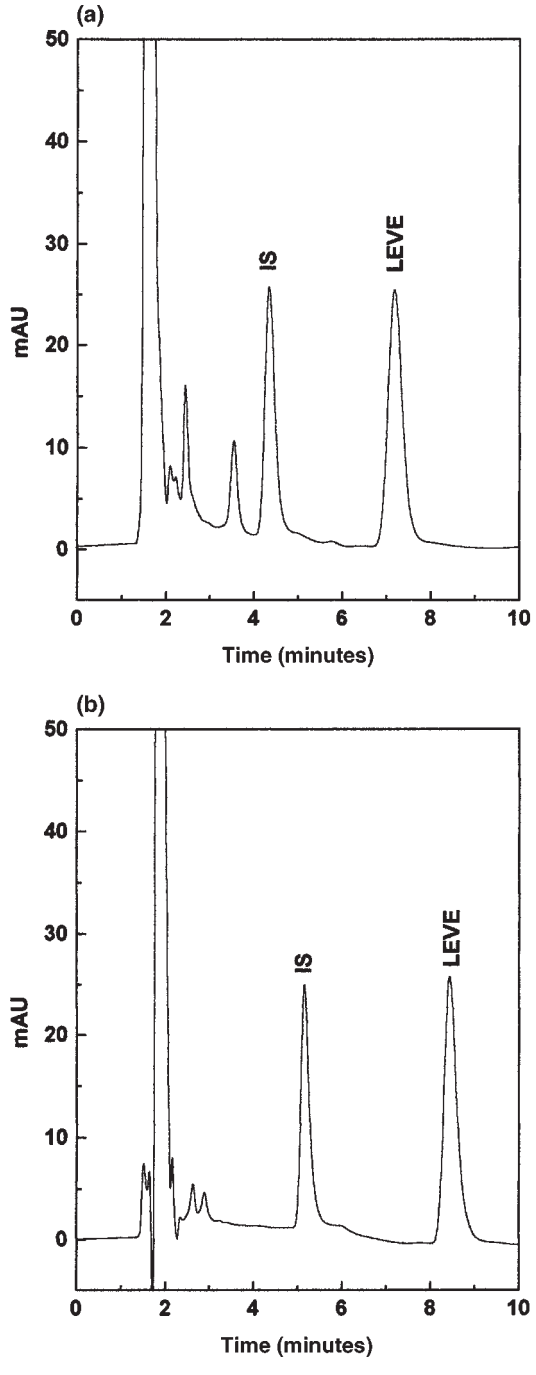

Figure 3. (a) Chromatogram of a blank plasma sample spiked with Levetiracetam (LEVE) (10 μg/mL) and IS (10 μg/mL), after methanol plasma protein precipitation; (b) after zinc sulphate plasma protein precipitation. Chromatographic conditions as in Fig. 2.

Precipitation by formation of insoluble salts

Plasma proteins can be precipitated from biological samples using cationic or anionic precipitants. The proteins are positively charged in acidic solutions and negatively charged in basic solutions.

The efficacy of perchloric acid was tested following the procedure already reported in the experimental section. As a very low absolute recovery of Levetiracetam was found (35%), this pretreatment was discarded. Protein precipitation using zinc sulfate–1 N NaOH, however, was very effective, as the metal-ion precipitation yielded supernatants free of any particulate matter, and the precipitation with zinc sulphate gave satisfactory absolute recovery values even though such a low volume of precipitant was used. These results show the high efficacy of zinc sulphate as a precipitant agent and convalidate the data reported in the literature (Iyer and Przybycien, 1995; Polson *et al.*, 2003). The chromatogram of a blank plasma sample spiked with 10 μg/mL of IS, and 10 μg/mL of Levetiracetam (subjected to protein precipitation with zinc sulphate) is shown in Fig. 3(b). As one can see, the peak of endogenous substances, present with the methanol deproteinization method, do not appear; Thus, zinc sulphate has a higher deproteinization efficacy than methanol. The mean absolute recovery of Levetiracetam from spiked human plasma samples using this method was within 90–97% and RSD ≤3.9% (mean absolute recovery within 80–90% and RSD ≤5.6% at the LOQ). Table 2 details the mean absolute recovery and precision.

Calibration samples were prepared at six different concentrations in triplicate in the range 5–100 μg/mL for Levetiracetam. The calibration curve, represented by the plot of the peak area ratio Levetiracetam/IS vs Levetiracetam concentration in the calibration sample, was generated using the least square method weighted linear regression. The linear calibration equation was $y = 0.0146 + 0.0353x$ with a linear correlation coefficient of 0.9998. The LOD and the LOQ values were found as 225 and 750 ng/mL, respectively.

It can be concluded that protein precipitation with zinc sulphate followed by direct injection into the HPLC system is a rapid, simple and effective sample pretreatment procedure.

Solid-phase extraction (SPE)

The use of plasma protein precipitation as the sample pretreatment has the disadvantage of diluting the sample and lowering the LOQ of the procedure. This might be a disadvantage for certain methods such as capillary electrophoresis, which has restricted sensitivity due to its small injection volume and short pathway of the optical detector. For this reason, a novel SPE procedure was developed as an alternative sample pretreatment procedure for the analysis of Levetiracetam in plasma. The efficiency of several polymeric sorbents for the extraction of Levetiracetam was investigated, namely: C_1, C_2, C_8, C_{18}, PH, CN, CN-N and Oasis HLB.

As one could predict, extraction with the lypophilic sorbents was difficult (most of all with the PH

Table 1. Mean absolute recovery of Levetiracetam and precision data, after protein precipitation with methanol

Substance	Nominal concentration (μg/mL)	Absolute recovery, %[a]	Repeatability, RSD%	Intermediate precision, RSD%
Levetiracetam	5	101	2.5	3.1
	20	99	2.2	2.5
	100	94	1.6	2.2
IS	10	92	1.8	2.9

[a] Absolute recovery: (amount found/amount added) × 100. Six replicates were made at each concentration.

Table 2. Mean absolute recovery of Levetiracetam and precision data obtained following protein precipitation with $ZnSO_4$ in NaOH

Substance	Nominal concentration (μg/mL)	Absolute recovery, %[a]	Repeatability, RSD%	Intermediate precision, RSD%
Levetiracetam	5	90	3.0	3.9
	20	92	2.4	3.4
	100	97	1.9	2.3
IS	10	91	2.1	2.5

[a] Absolute recovery: (amount found/amount added) × 100. Six replicates were made at each concentration.

Table 3. Mean extraction yield and intra- and inter-day precision data of Levetiracetam after solid-phase extraction

Substance	Nominal concentration (μg/mL)	Extraction yield, %[a]	Repeatability, RSD%	Intermediate precision, RSD%
Levetiracetam	5	100	1.9	2.3
	20	99	1.8	1.9
	100	98	1.2	1.4
IS	10	96	1.5	1.6

[a] Extraction yield: (amount found/amount added) × 100. Six replicates were made at each concentration.

cartridges), due to the low molecular mass and the chemical structure of the analyte; nevertheless, satisfactory extraction yields were obtained, namely 91 ± 5.5% using C_{18} cartridges. Low repeatability of the measurements was, however, also found (RSD ≥ 6.0%). It was also observed that the CN cartridges were not retaining Levetiracetam; in fact, it was found that about the 83% of the analyte was lost upon loading the sample through the cartridge.

Good results were found, on the other hand, using Oasis HLB (hydrophilic–lipophilic balanced copolymer) cartridges. The procedure was very feasible: 500 μL of blank plasma samples spiked with the analyte and IS were loaded onto suitably conditioned cartridges and the elution was carried out with methanol. The eluate was then evaporated, and redissolved in 500 μL of mobile phase. The extraction yield data obtained with the Oasis HLB cartridges are reported in Table 3. Very high and reproducible values of absolute recovery were obtained. The mean extraction yield of the analyte was 99%. Figure 4 shows the chromatogram of a blank plasma sample spiked with the IS and Levetiracetam (10 μg/mL, respectively) and subjected to the SPE procedure. As can be seen, the analyte is detected as a symmetrical chromatographic peak at a retention time of 7.4 min, no interference is present, and the baseline is neater than those obtained by plasma protein precipitation.

Calibration curves were measured for spiked blank plasma samples in the 5–100 μg/mL concentration range for Levetiracetam. The regression equation was $y = 0.0294 + 0.0355x$, where y is the ratio between the area of Levetiracetam and that of the IS, and x is the concentration of Levetiracetam, expressed as μg/mL. The linear correlation coefficient was $r = 0.9996$. The LOQ was 500 ng/mL and the LOD value was 150 ng/mL.

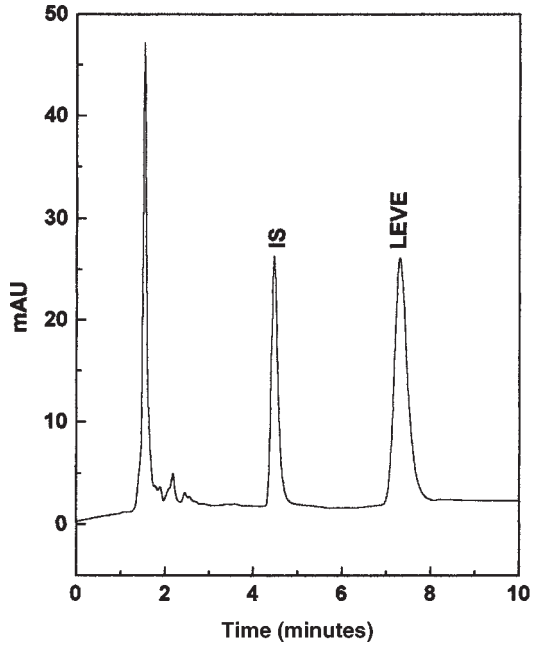

Figure 4. Chromatogram of a blank plasma sample spiked with Levetiracetam (LEVE) (10 µg/mL) and IS (10 µg/mL), after solid-phase extraction procedure. Chromatographic conditions as in Fig. 2.

Intra- and inter-day precision assays were carried out to evaluate the precision of the method (extraction and chromatography) and gave the data reported in Table 3. The relative standard deviation values were lower than 1.9 for repeatability (RSD% intraday) and lower than 2.3 for the intermediate precision (RSD% interday).

Comparison of the purification procedures

The comparison of the performance of the HPLC method using the three purification procedures is shown in Fig. 5, which summarizes mean absolute recovery, procedure time, sample dilution factor, LOD and precision data. The mean absolute recovery of the analyte from plasma are as high as 97, 93 and 99% for methanol–PPP, $ZnSO_4$–PPP and SPE. Precision data were satisfactory, as the intra- and inter-day relative standard deviation (RSD) values were within 3.9%, with the best results obtained using the SPE procedure (RSD% = 2.3). Comparing the chromatograms reported in Figs 3(a) and (b) and 4, it can be concluded that the SPE procedure allows the best clean-up of the human plasma. On the contrary, the two PPP procedures are simpler to carry out, require less expensive equipment, and could be useful alternatives when the number of samples to be analysed is very high. In particular, for the two precipitation procedures methanol is preferable to zinc sulphate as it gives higher absolute recovery and precision. Although the precipitation methods are more rapid and feasible (in fact, the sample pretreatment is carried out within 25 min), the accompanying sample dilution affects method sensitivity disadvantageously, as shown by the corresponding LOD values (Fig. 5).

CONCLUSION

In this paper, the development of a reliable HPLC method for the analysis of Levetiracetam in human plasma is described. Compared with that reported in

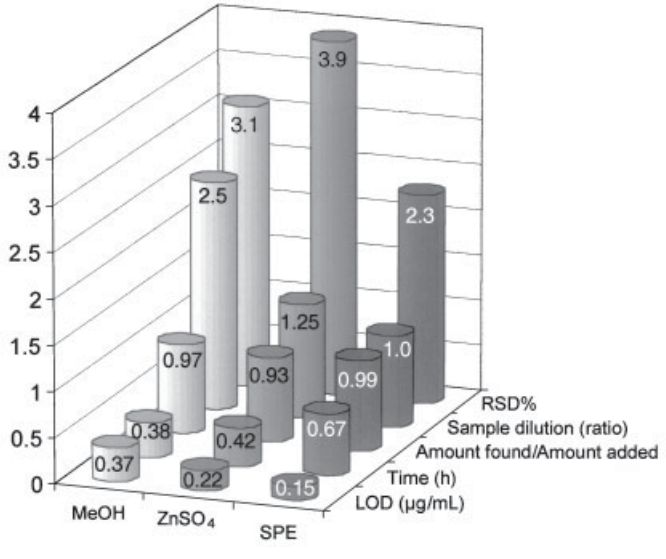

Figure 5. Bar graphs of the performances of the three extraction procedures. For details, see text.

the literature the chromatographic conditions developed herein allow the analysis to be carried out in shorter run times (Vermeij and Edelbroek, 1994) and with lower amounts of organic modifier (Ratnaraj *et al.*, 1996). As particular attention is devoted to plasma sample pretreatment, three different purification procedures were implemented, namely methanol–PPP, $ZnSO_4$–PPP, and SPE. Sample preparation by methanol PPP or $ZnSO_4$ PPP was approximately twice as fast as SPE. The performance of all clean-up methods was satisfactory, as evidenced by the absolute recovery and precision data. In particular, the SPE method proved to be the most accurate and precise, however, all three clean-up procedures seem to be suitable for the TDM of Levetiracetam. Compared with other reported extraction procedures (Ratnaraj *et al.*, 1996; Isoherranen *et al.*, 2000; Ivanova *et al.*, 2003), the proposed methods allow to obtain higher recovery results within comparable (SPE) or shorter (PPP) times and with better reproducibility.

It follows from our results that the SPE procedure is best suited for analysis of Levetiracetam and pharmacokinetic studies when high sensitivity is required. If, on the other hand, the number of plasma samples to analyse is very high, it is preferable to use the PPP with zinc sulphate or methanol, because they are faster and simpler.

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