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Trace analysis of ethosuximide in human plasma with a chemically removable derivatizing reagent and high-performance liquid chromatography

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Abstract

A simple and sensitive liquid chromatographic method is described for the determination of ethosuximide in human plasma, as a highly sensitive derivative. Ethosuximide spiked in plasma was extracted with toluene and derivatized with a chemically removable derivatizing reagent, 2-(2-naphthoxy)ethyl 2-[1-(4-benzyl)piperazyl]ethanesulfonate, in a homogeneous system, using magnesium oxide as base catalyst. The resulting derivative was separated on a LiChrospher diol column with 1.2% isopropanol in *n*-hexane as the mobile phase and using coumarin as the internal standard. Several parameters affecting the extraction/derivatization of ethosuximide from spiked plasma were investigated. The linear range for the determination of ethosuximide in spiked plasma was over 30–700 nmol/ml. For ethosuximide in plasma, the detection limit (signal-to-noise ratio=3; sample size, 10 μl) was about 9 pmol; the relative standard deviation was 6.4% for intra-day assay (n=6) and 9.2% for inter-day assay (n=6) and the relative recovery was found greater than 94%.

Keywords: Derivatization, LC; Ethosuximide

1. Introduction

Among the succinimide antiepileptic agents, ethosuximide (3-ethyl-3-methylpyrrolidine-2,5-dione, ESM) (as shown in Fig. 1) is the most effective one and has received wide use for treatment of absence seizures [1–3]. Under usual conditions, long-term therapy with ESM is necessary. A plasma concentration of 40–100 μg/ml (about 280–700 nmol/ml) is required for satisfactory control of absence seizures in most patients. During long-term therapy, the plasma concentration of ESM averages about 2 μg/ml per daily dose of 1 mg/kg. However, because of individual variation, especially in infants and children, concentrations in plasma cannot be predicted accurately. The most common dose-related side effects of ESM include gastrointestinal complaints and CNS effects [4]. The monitoring of ESM levels in plasma is required for seizure control and reducing the chances for toxicity from overdose. Therefore, a sensitive and specific method for trace analysis of ESM in plasma is quite essential.

Several methods, including immunoassay [5–7], titration [8], gas chromatography [9–12] and high-performance liquid chromatography [13–16], have

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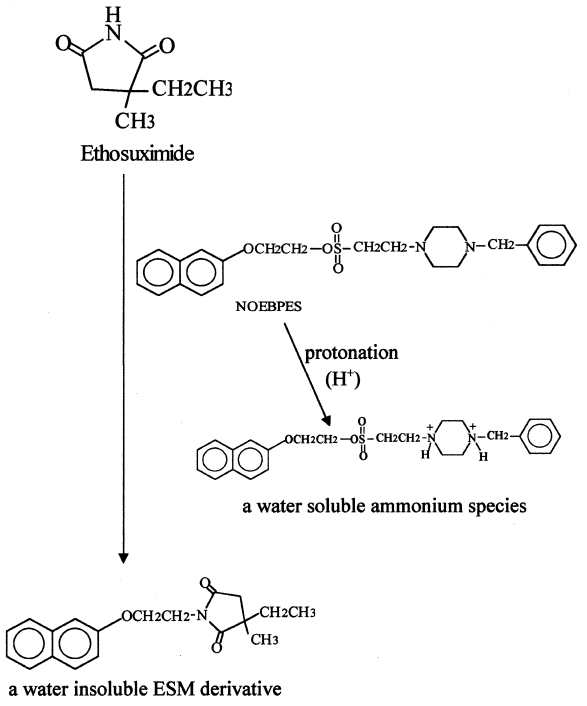

Fig. 1. Putative reaction scheme for ESM with NOEBPES and removal of the excess reagent by aqueous acid.

been applied to the analysis of ESM. Apart from the dione groups, ESM has neither a strong chromophore nor a conjugated system in the structure (Fig. 1). That has made it necessary to choose the lower UV region for detection in the direct HPLC methods [13,14] and strong background absorption of the mobile phase in this region ensued. In much the same manner as other analytes, ESM can be prepared for analysis as a detector-oriented derivative by reacting with a derivatizing reagent to enhance the detection sensitivity. Among the enormous array of widely used analytical derivatizing reagents, alkyl sulfonates, with a better leaving group than those of alkyl halides [17], display great potential for reaction with a wide range of nucleophilic analytes. In practice, however, low reactivity of alkyl sulfonates toward certain analytes makes it necessary for the reaction to be carried out under more rigorous conditions for speeding up the reaction rate. Searching for a suitable catalyst then is an alternative and valuable choice to cope with this dilemma.

For trace analysis, an excess amount of derivatizing reagent usually is used to facilitate the completion of the derivatization reaction. However, the excess of derivatizing reagent, which possesses a strong chromophore and is very responsive to the detector, may seriously interfere with the detection of the derivative of the analyte. We have solved this problem by choosing an alkyl sulfonate derivatizing reagent, which easily can be removed by washing the derivatization mixture with aqueous acid. This process removes virtually all the excess reagent after derivatization from the reaction medium. In this paper the chemically removable derivatizing reagent, 2-(2-naphthoxy)ethyl 2-[1-(4-benzyl)piperazyl]-ethanesulfonate (NOEBPES) was applied to the trace determination of ESM in human plasma in a homogeneous system.

2. Experimental

2.1. Reagents and solutions

ESM (Sigma, St. Louis MO, USA), coumarin (internal standard, I.S.) (Aldrich, Milwaukee, WI, USA), magnesium oxide, potassium carbonate, potassium bicarbonate and toluene (Merck, Darmstadt, Germany), *n*-hexane, isopropanol and other reagents were all of analytical-reagent grade. Solutions of ESM at various concentrations were prepared by dissolving a suitable amount of ESM in deionized water. The chemically removable derivatizing reagent, NOEBPES, was synthesized from 2-(2-naphthoxy)ethanol, 2-chloroethanesulfonyl chloride and *N*-benzylpiperazine according to the method described by Wu et al. [18]. Solutions of coumarin and NOEBPES were prepared in toluene.

2.2. HPLC instrumentation and conditions

A Waters-Millipore LC system with a U6K injector and a Model 486 UV-VIS detector was used. A LiChrospher diol column (250×4.0 mm I.D., 5 μm) (Merck) and a mixed solvent of 1.2% isopropanol in *n*-hexane (v/v) at a flow-rate of 2.0 ml/min at room temperature were used. The column eluate was monitored at 225 nm wavelength. The solvent was filtered with filter (Millipore, HV, 0.45 μm) under vacuum for degassing before use.

2.3. Sample preparation and derivatization procedures

A 0.28-ml volume of human plasma was pipetted into a 10-ml glass-stoppered test tube, and 20 μl of aqueous solution containing various amounts of ESM were added to each tube to prepare final ESM concentrations in plasma samples over the range of 30–700 nmol/ml. The tubes were mixed for 10 s. Toluene (0.4 ml) was added and mixed by vortexing for 30 s. The tubes were then centrifuged (1000 *g*) for 5 min. A 0.3-ml aliquot of supernatant organic layer from each tube was transferred to a 10-ml glass-stoppered test tube containing 100 mg of magnesium oxide. Then 0.3 ml of NOEBPES toluene solution (66.7 m*M*) and 0.4 ml of coumarin toluene solution (100 μ*M*) (I.S.) were added. The reaction mixture was shaken for 1.5 h at 80°C in a thermostated water bath. After cooling, 0.4 ml of the reaction solution was taken and washed with 1.5 ml of 2.0 *M* H_2SO_4 aqueous solution. A 10-μl aliquot of the toluene solution was injected into the HPLC system for analysis.

2.4. GC–MS

To elucidate the chemical structure of the ESM derivative, a Hewlett-Packard 5890 gas chromatograph-5989A mass spectrometer (GC–MS) was used in the electron impact mode at 70 eV with an ionization source temperature of 200°C. A column of 25 m×0.2 mm I.D. with a 0.33-μm film of crosslinked methyl silicone (Hewlett-Packard, Avondale, PA, USA) was used for GC separation. The temperature was set initially at 80°C for 2 min, then programmed at 30°C min^{-1} to 150°C and held there for 5 min, again programmed at 30°C min^{-1} to 250°C and finally held there for 20 min.

2.5. Precision and accuracy test

The reproducibility and reliability of the proposed method were determined by extracting the ESM from plasma spiked with three different levels of ESM (30, 300 and 700 nmol/ml), then derivatizing according to the procedure described in Section 2.3.

3. Results and discussion

The analytical derivatization of the extracted ESM from spiked plasma with the chemically removable derivatizing reagent, NOEBPES, was studied. The putative reaction scheme for ESM with NOEBPES and removal of the excess reagent by aqueous acid after derivatization is illustrated in Fig. 1. To optimize the derivatization conditions for ESM extracted from spiked plasma (700 nmol/ml), several related parameters were studied, including the extraction solvent, amount of derivatizing reagent, base catalyst and reaction temperature and time. The effect of the tested parameters on the extraction/derivatization of ESM was evaluated by the peak area ratios of the resulting derivative to the coumarin (I.S.).

3.1. Effect of organic solvents

Water-immiscible organic solvents, including toluene, dichloromethane and chloroform, were tested for their suitability as solvents for extraction/derivatization procedure. To prevent the solvents from boiling, the reaction temperatures were set below the respective boiling point of the tested solvents, that is, 80°C for toluene, 30°C for dichloromethane and 50°C for chloroform. The solvent effect on the yield of ESM derivative revealed in this way reflects the overall effect of the tested solvent on both extraction and derivatization rate. No attempt was made to distinguish between these two processes. Set as 100 for using toluene as reaction solvent, the peak–area ratios were 35 for chloroform and 14 for dichloromethane. The results indicated that toluene is the best solvent for the extraction/derivatization of ESM in human plasma.

3.2. Effect of catalysts

The imido moiety of ESM renders the molecule a weak acid in character. A base catalyst facilitates the withdrawal of the proton from the nitrogen atom of the imido group of ESM. This, in turn, speeds up the nucleophilic attack on the alkyl sulfonate derivatizing reagent. Base catalysts, including magnesium oxide, potassium carbonate and potassium bicarbonate (100 mg of each) were investigated for their

effects on the derivatization reaction. The effectiveness of the various base catalysts was evaluated by the yield of ESM derivative. The derivatization reactions catalyzed by magnesium oxide went as much as eight times faster than with potassium carbonate and 10 times faster than with potassium bicarbonate. Magnesium oxide seemed to possess the basicity sufficient to accelerate the formation of ESM derivative but not strong enough to increase the hydrolysis of the derivatization product. Therefore, magnesium oxide was chosen for the base catalyst in this study. The effect of the amount of magnesium oxide on derivatization is illustrated in Fig. 2. In the absence of the base catalyst in the reaction system, only a small amount of ESM derivative was detected. With the addition of magnesium oxide, the ESM derivative increased almost linearly. About 80 mg of magnesium oxide were needed for derivatizing the ESM extracted from spiked plasma to a plateau formation of the derivative. In this study, an amount of about 100 mg of magnesium oxide was added to the reaction mixture as base catalyst for the derivatization of the spiked ESM.

3.3. Effect of amount of derivatizing agent

The amount of NOEBPES required for derivatizing the ESM extracted from spiked plasma (700 nmol ESM) to a plateau formation of the derivative is shown in Fig. 3. 20 μmol (0.3 ml, 66.7 m*M*) of NOEBPES was needed for derivatization of the spiked ESM. The result indicates that a suitable molar ratio of NOEBPES to ESM not less than 100 was needed.

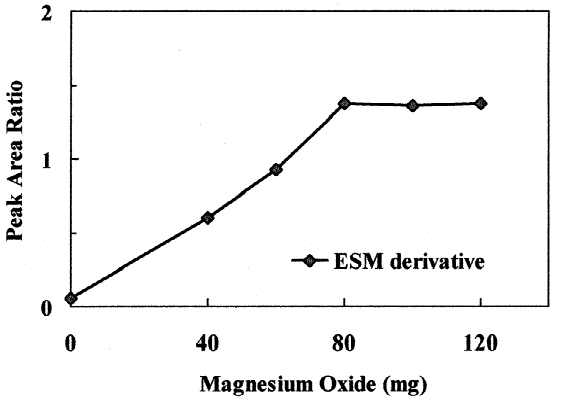

Fig. 2. Effect of the amount of magnesium oxide on the formation of the derivative of ESM extracted from human plasma. Reactions were carried out at 80°C for 1.5 h in the presence of 0–120 mg of magnesium oxide with 0.3 ml of 66.7 m*M* NOEBPES toluene solution and 0.4 ml of 100 μ*M* coumarin toluene solution as derivatizing agent and internal standard, respectively.

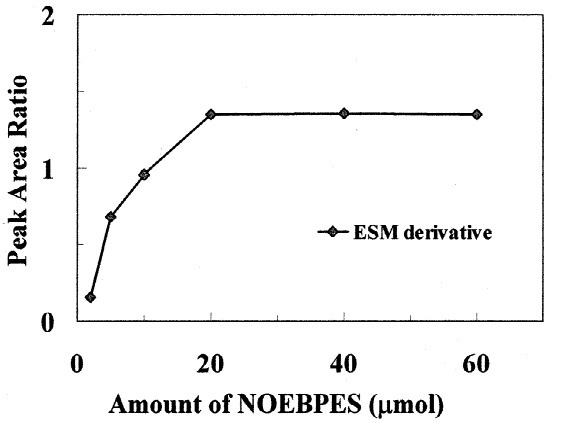

Fig. 3. Effect of the amount of NOEBPES on the formation of the derivative of ESM extracted from human plasma. Reactions were carried out at 80°C for 1.5 h in the presence of 100 mg of magnesium oxide with 0.3 ml of 6.7–200 m*M* NOEBPES toluene solution and 0.4 ml of 100 μ*M* coumarin toluene solution as derivatizing agent and internal standard, respectively.

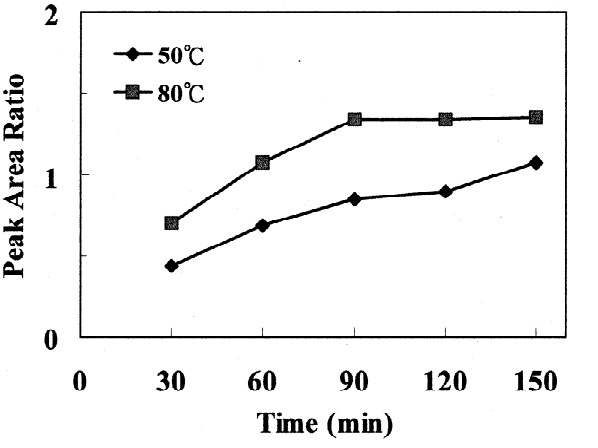

Fig. 4. Effect of reaction temperature and reaction time on the formation of the derivative of ESM extracted from human plasma. Reactions were carried out at 50 and 80°C in the presence of 100 mg of magnesium oxide with 0.3 ml of 66.7 m*M* NOEBPES toluene solution and 0.4 ml of 100 μ*M* coumarin toluene solution as derivatizing agent and internal standard, respectively.

3.4. Effect of reaction temperature and time

The effect of reaction time at 50 and 80°C on the derivatization of ESM is shown in Fig. 4. For derivatization at 80°C, the formation of the ESM derivative reached to equilibrium in 1.5 h; whereas for the reaction at 50°C, equilibrium was not attainable in 2.5 h and resulted in a lower yield of the ESM derivative. Therefore, the reaction time and temperature for determination of ESM in plasma were set at 1.5 h and 80°C, respectively.

3.5. Structural identification of the derivative

A typical chromatogram for the analysis of ESM extracted from plasma is illustrated in Fig. 5. Peak a and peak b represent the ESM derivative and the I.S., respectively. There was no interference from the

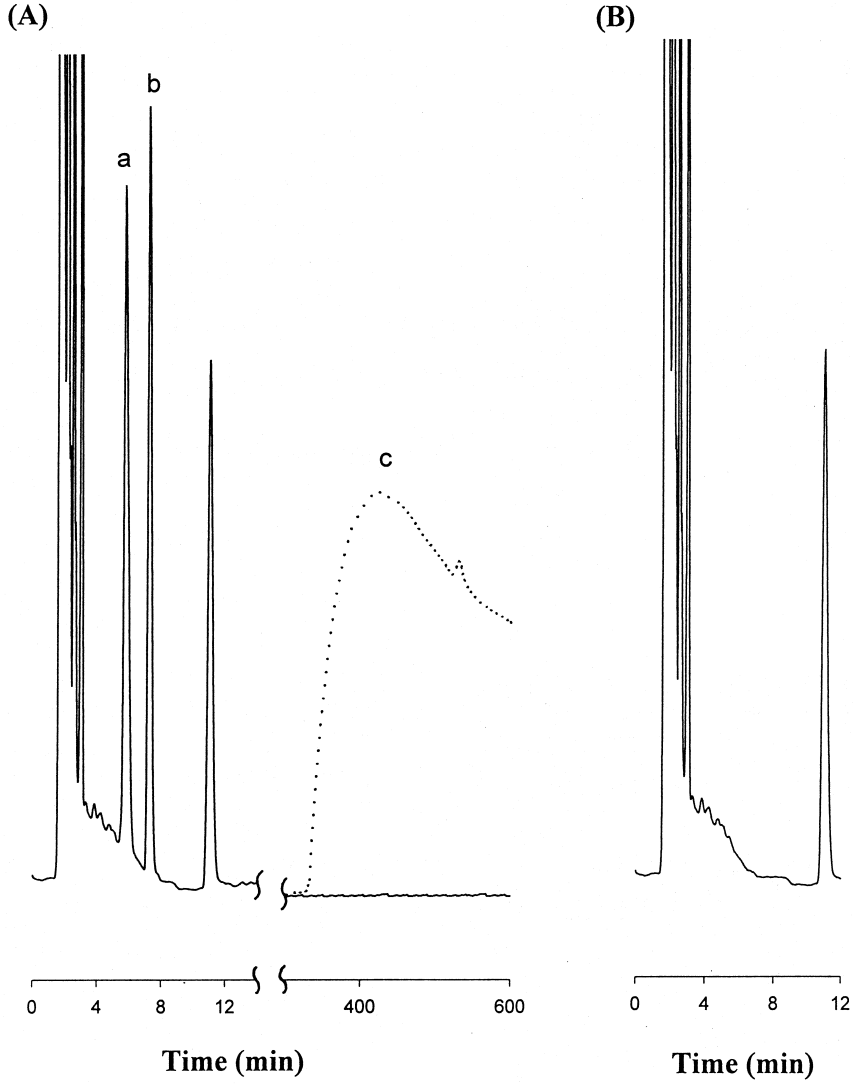

Fig. 5. HPLC chromatograms for determination of ESM in human plasma. (A) ESM in human plasma, with (solid line) and without (dashed line) acid treatment, after derivatization. (B) Plasma blank. Peaks: (a) ESM derivative; (b) coumarin (I.S.). HPLC conditions: LiChrospher diol column (250×4 mm I.D.; 5 μm); mobile phase, 1.2% isopropanol in *n*-hexane; flow-rate, 2.0 ml/min; detection, 225 nm.

reagent blank with the resolution of the peak of ESM derivative. However, a broad and tailing peak from the excess NOEBPES, with a retention time more than 400 min, was found when acid treatment of the reaction mixture after derivatization was omitted.

GC–MS was used to examine the ESM derivative, peak a in Fig. 5. The mass spectrum exhibited a molecular ion at m/z 311 and a basal ion at m/z 168, equivalent to the naphthoxy fragment. This suggests that the resulting derivative of peak a in Fig. 5 is 1-[2-(2-naphthoxy)ethyl]-3-ethyl-3-methylpyrrolidine-2,5-dione (as shown in Fig. 1).

3.6. Analytical calibration

To evaluate the quantitative applicability of the method, five different amounts of ESM spiked in plasma over the range of 30–700 nmol/ml were analyzed. The linear regression equations were as follows: $y=(-0.0383\pm0.0048)+(0.0089\pm0.0001)x$ for intra-day assay ($n=6$, $r=0.999$); $y=(-0.0417\pm0.0151)+(0.0091\pm0.0001)x$ for inter-day assay ($n=6$, $r=0.999$). Here y represents the peak area ratio of ESM derivative to I.S.; x, the amount of ESM (in nanomoles) and r, the correlation coefficient. The data indicate good linearity of the method. The detection limit (signal to noise ratio=3) of ESM was 9 pmol in 10 μl of injection. The stability of the ESM derivative was studied over a period of 48 h after derivatization at room temperature; no significant change of the peak area ratio was found. This indicates favorable stability of the derivative for ESM analysis. The stability of ESM in spiked plasma under storage was also examined. Three different concentrations of ESM at 90, 300 and 700 nmol/ml in spiked plasma were studied to assess the stability of the ESM stored at -70 ± 2°C. For each sample, determination of plasma ESM was performed on days 0, 7 and 14. Statistical analysis of the results did not show any significant difference; therefore, ESM is stable in plasma samples stored at -70 ± 2°C for periods up to 14 days.

3.7. Precision and accuracy

The reproducibility and reliability of the method for the determination of ESM were evaluated as relative standard deviation (RSD) and relative recovery, respectively. As shown in Table 1, the precision of the method for ESM analyses were below 6.4% (RSD) and 9.2% (RSD) for intra-day and inter-day, respectively. The relative recovery of the ESM, as shown in Table 2, is more than 94%, which was obtained from the calibration graph constructed from plasma spiked with different amounts of ESM over the range of 30–700 nmol/ml.

Table 1
Precision and accuracy of the analysis of ESM spiked in human plasma[a]

Amount range (nmol/ml)	Amount found (nmol/ml)	RSD (%)	R.E. (%)
Intra-day ($n=6$)			
700.0	706.2±7.2	1.0	0.9
100.0	94.1±1.2	1.2	−5.9
30.0	32.5±2.1	6.4	8.3
Inter-day ($n=6$)			
700.0	699.9±11.6	1.7	−0.0
100.0	98.4±2.9	3.0	−1.6
30.0	30.9±2.8	9.2	3.0

[a] Intraday data were based on six replicate analyses and inter-day data were from six different days.

3.8. Selectivity of the method

The selectivity of the method was studied by spiking standard ESM with other anticonvulsants, including valproic acid, carbamazepine, primidone and acetazolamide, each at 700 nmol/ml in plasma. The spiked plasma was derivatized according to

Table 2
Relative recoveries of ESM extracted from human plasma

Sample	Amount spiked (nmol/ml)	Amount found[a] (nmol/ml)	Recovery (%)
1	–	–	–
	100.0	94.5±0.8	94.5
	300.0	294.7±4.8	n98.2
	700.0	707.8±5.8	101.1
2	–	–	–
	100.0	94.2±0.7	94.2
	300.0	293.2±4.2	97.7
	700.0	700.8±4.9	100.1
3	–	–	–
	100.0	96.8±0.83	96.8
	300.0	283.8±2.2	94.6
	700.0	712.3±5.3	101.7

[a] Mean±SD of triplicate analyses.

procedure described in Section 2.3. The ESM derivative could be resolved from those of the other drugs, indicating that other anticonvulsants did not interfere with the HPLC analysis of ESM in this study. As a consequence, the proposed method is specific and feasible for the analysis of ESM in plasma for biological study or therapeutic drug monitor.

4. Conclusion

A simple and sensitive method has been established by which the ESM extracted from plasma can be readily derivatized by chemically removable reagent, NOEBPES; the resulting derivative is then analyzed using the HPLC system. The proposed method is specific and feasible for the analysis of ESM in plasma for the biological or clinical reasons.

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