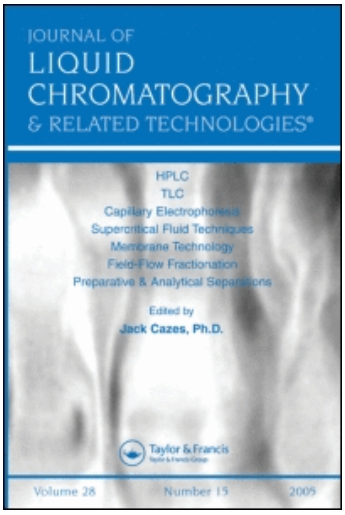


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# Determination of Ethosuximide in Plasma By Derivatization and High Performance Liquid Chromatography

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# DETERMINATION OF ETHOSUXIMIDE IN PLASMA BY DERIVATIZATION 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 plasma as a highly sensitive derivative. Ethosuximide in plasma, after separation with isopropanol extraction, was derivatized with strong chromophore reagent, 4-bromomethyl-7-methoxy-coumarin. The resulting derivatives were separated on a Nova-Pak C18 column with water-acetonitrile-methanol(60:20:20, v/v) as the mobile phase. The linear range for the determination of ethosuximide in spiked plasma was over 2-40 nmol. The limit of detection for ethosuximide was about 7.0 ± 1.2 pmol per 20 μL injection (S/N= 5). The intraday relative standard deviation (n = 6) and the interday relative standard deviation (n = 12) were all less than 2.5% for ethosuximide. The recovery for ethosuximide in plasma was greater than 96%.

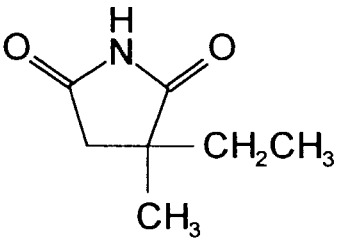


**Figure 1**. Chemical structure of ethosuximide.

## INTRODUCTION

Ethosuximide (3 - ethyl - 3- methylpyrrolidine - 2,5-dione, ESM) (Fig. 1) is an antiepileptic drug and it is widely used for treatment of absence seizures.[1-3] ESM therapy is long term therapy of at least two years.[4] Physiologic functions may change with time, especially in the infant and child. The monitoring of ESM in plasma levels is required for clinic treatment to improve the seizure control and reduce the toxicity from overdosage. Therefore, a sensitive and specific method for trace analysis of ESM in plasma is very essential.

A variety of methods including immunoassay,[5-7] titration,[8] gas chromatography,[9-12] and high performance liquid chromatography,[13-15] have been applied to the analysis of ESM. That ESM has no strong chromophore or conjugated system, except that of dione group, as shown in Fig.1, made it necessary to choose the lower UV region at 204 nm or 207 nm for detection in previous HPLC methods,[13-14] and the strong background absorption of a mobile phase in the lower UV region ensued. In order to enhance the sensitivity, we have previously reported,[15] a detector-oriented derivatization HPLC method for determination of ESM in capsules as the derivative of 4-bromomethyl-7-methoxycoumarin (BrMmC). In this paper, a sensitive and specific HPLC method is described for the trace analysis of ESM in human plasma.

## METHODS

### Reagents and Solution

ESM (Sigma, St. Louis MO, USA), BrMmC and 2-2'-dinitrobiphenyl (Aldrich, Milwaukee, WI, USA), potassium carbonate and potassium

hydroxide (E. Merck, Darmstadt, Germany), acetonitrile , isopropanol and other reagents were of analytical-reagent grade. Solutions of ESM at various concentrations were prepared by dissolving a suitable amount of ESM in deionized water. Solutions of 2-2'-dinitrobiphenyl and BrMmC were prepared in acetonitrile.

## HPLC Conditions

A Waters-Millipore LC system with a U6K injector and a Model 486 UV-VIS detector was used. A Nova-Pak C18 column(150 x 3.9 mm I.D., 4 μm) and a mixed solvent of water-acetonitrile-methanol (60:20:20, v/v) at a flow-rate of 1.3 mL/min were used. The column eluate was monitored at 320 nm. Pretreatment of the solvents with a vacuum filter for degassing was performed.



## Sample Preparation Procedures

A 0.4-mL volume of plasma was pipetted into a 10-mL glass-stoppered test tube, and 100 μL containing various amount of ethosuximide aqueous solution was added to each tube. The tubes were mixed for 10s. 1.0 mL of isopropanol was added and mixed by vortexing for 30s. The tubes were centrifuged (1800g) for 5 min. The 1.0 mL of supernatant organic layer from each tube was transferred to a 10-mL glass-stoppered test tube. Then 0.3 mL of $10^{-2}$ M NaOH aqueous solution was added and evaporated to dryness under a slow stream of nitrogen by heating in a water bath at 50°C.

The dried residue was derivatized by adding 200 μL of BrMmC (2.5 mM) acetonitrile solution, 0.3 mL 2,2'-dinitrobiphenyl (I.S.), and 100 mg of potassium carbonate. The reaction mixture was shaken for 1.5 h at 70°C in a thermostated water bath.

At the end of the reaction, a 15μL aliquot of the solution was injected into the HPLC system.

## Precision and Accuracy Test

The reproducibility and reliability tests of ESM from spiked plasma were determined by extracting 0.5 mL aliquots of plasma sample containing ESM at three different levels (5.0, 10.0 and 40.0 nmol).

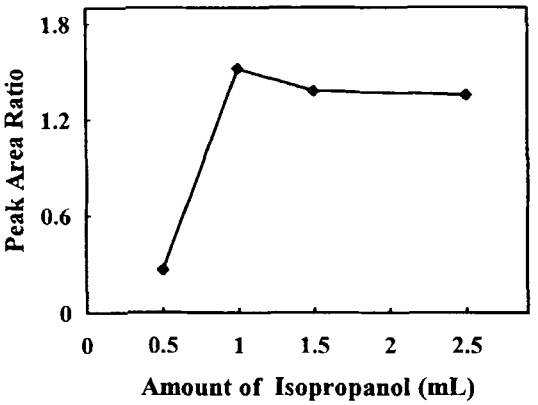


**Figure 2.** Effect of amount of isopropanol on the extraction of ESM from human plasma.



## RESULTS AND DISCUSSION

For the optimization of the conditions for liquid extraction of ESM (40 nmol) from spiked plasma and also conditions for the derivatization of the extracted ESM, several related parameters, including the volume of extraction solvent, amount of derivatizing agent, base catalyst and reaction time were studied. The effect of the tested parameters on the extraction/derivatization of ESM were evaluated by the peak area ratio of the resulting derivative to the I.S.

### Effect of the Volume of Extraction Solvent

Liquid extraction[13] and solid phase[12] methods were reported to extract ESM from human plasma, but the recoveries were not satisfied. This is likely due to its high water solubility. For increasing the extraction efficiency, the higher polar organic solvent, isopropranol, was used as an extraction solvent. The effect of various amounts of isopropanol (0.5, 1.0, 1.5 and 2.5 mL) on the liquid extraction of ESM from plasma was studied. Because of the low melting point (64-65°C) of ESM, the possible loss under purge nitrogen stream should be considered. 0.3 mL of $10^{-2}$ M NaOH solution was added to the isopropanol extract to make ESM a sodium salt before drying. The effect of the amounts of isopropanol on the extraction of ESM from human plasma is presented in Fig.2. 1 mL of isopropanol is enough for extraction of ESM from spiked plasma.

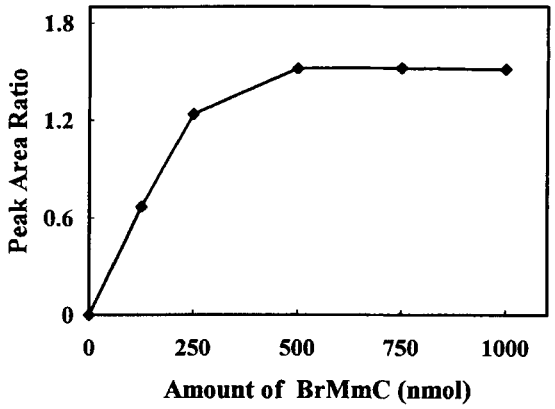


**Figure 3**. Effect of amount of BrMmC on the formation of the derivative of ESM extracted from human plasma.



### Effect of Amount of Derivatizing Agent

The amount of BrMmC required for the derivatization of ESM isolated from spiked plasma (40 nmol ESM) to a plateau formation of the derivative is shown in Fig. 3. 500 nmol of BrMmC was needed for derivatization of the ESM extracted from plasma.

### Effect of Amount of Catalyst

The effect of the amount of potassium carbonate(30-50 mesh) on derivatization is illustrated in Fig. 4. In the absence of the base catalyst in the reaction system, a small amount of ESM derivative was detected. Adding the potassium carbonate as base catalyst, the ESM derivative obviously increased. A suitable amount of potassium carbonate (about 100 mg) was selected for the derivatization of plasma ESM. After the derivatization reaction, the supernatant was directly introduced into the HPLC system.

### Effect of Reaction Time

The effect of reaction time at 70°C was examined for derivatization of ESM under the optimum conditions. The results were presented in Fig. 5. 1.5 h was needed to reach the equilibrium.

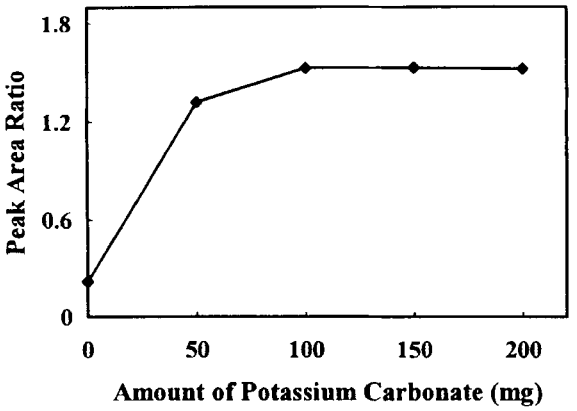


**Figure 4**. Effect of the amount of potassium carbonate on the formation of the derivative of ESM extracted from human piasma.



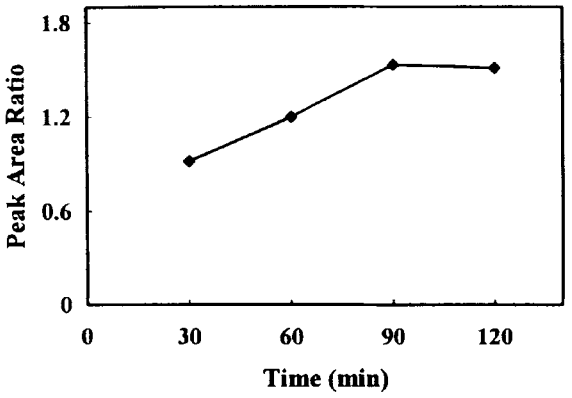


**Figure 5**. Effect of reaction time on the formation of the derivative of ESM extracted from human plasma.

## Analytical Calibration

To evaluate the quantitative applicability of the method, five different amounts of ESM spiked in plasma over the range of 2-40 nmol were analyzed. The linear regression equation obtained were y = (-0.052 ± 0.003) + (0.040 ± 0.001) x for intraday assay (n = 6, r = 0.999); y = (-0.036 ± 0.009) + (0.038

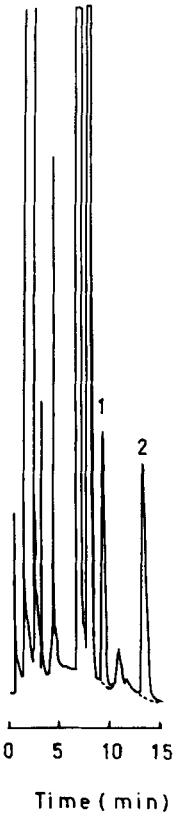


**Figure 6**. Composite liquid chromatogram of reagent blank (dotted line) and the derivative of ESM extracted from spiked human plasma (solid line).

± 0.002)x for interday assay (n = 12, r = 0.999); y is the peak-area ratio of ESM to I.S., x is the amount of ESM (in nanomoles), and r is the correlation coefficient. The data indicate good linearity of the method. The detection limit (signal to noise ratio = 5) of ESM was 7.0 ± 1.2 pmol in 20 μL of injection sample. A typical chromatogram for the analysis of ESM extracted from plasma is illustrated in Fig 6. Peak 1 and peak 2 represented the ESM derivative and the I.S., respectively. There was no interference in the resolution of the peak of ESM derivative from that of the reagent blank. The structure of the ESM derivative was performed by JEOL JMS-HX mass spectrometry with fast atom bombardment of xenon as the ionization mode and an acceleration energy of 10 kV . The quasi-molecular ion of the derivative of

**Table 1**

**Precision and Accuracy of the Analysis of ESM Spiked in Human Plasma**

| Amount Range (nmol) | Amount Found (nmol) | R.S.D. (%) | Recovery (%) |
|---|---|---|---|
| Intraday* | | | |
| 40 | 40.69 ± 0.34 | 0.85 | 101.7 |
| 10 | 9.94 ± 0.19 | 1.87 | 99.4 |
| 5 | 4.89 ± 0.09 | 1.84 | 97.8 |
| Interday** | | | |
| 40 | 40.11 ± 0.42 | 1.05 | 100.3 |
| 10 | 9.87 ± 0.23 | 2.32 | 98.7 |
| 5 | 4.95 ± 0.12 | 2.51 | 99.0 |

* Intraday assay variance was calculated from the assay values of prepared standards on a single day (n = 6).

**Interday assay variance was calculated from the assay values of prepared standards on twelve consecutive days (n = 12).



ESM was found at m/z = 330 ($MH^+$). Furthermore, by comparing the $^1H$-NMR($CDCl_3$) spectrum of the ESM derivative with that of underivatized ESM, a chemical shift about $\delta$ 8.96 of imide proton disappears. It is due to the alkylation of ESM on imide nitrogen atom. The structure of the ESM derivative of peak 1 in Fig 6 is briefly identified as 4-[1-(3-ethyl-3-methyl 2,5-dioxo)pyrrolidinyl]methyl-7-methoxy coumarin.

## Precision and Accuracy

The reproducibility and reliability of the method for determination of ESM were studied on the plasma spiked at three different levels of the ESM and evaluated as relative standard deviation (R.S.D.) and relative recovery, respectively. As shown in Table 1, the precision of the method for ESM is less than 3% (R.S.D.) for both intraday and interday analyses. Based on a calibration graph constructed from plasma spiked with different amounts of ESM over the range of 2-40 nmol, as shown in Table 2, the relative recovery of the method for ESM is more than 95%.

**Table 2**

**The Recoveries of ESM Extracted from Human Plasma**

| Sample | Amount Spiked (nmol) | Amount Found* (nmol) | Recovery (%) |
|---|---|---|---|
| 1 | ---- | ---- | ---- |
| | 5.00 | 4.85 ± 0.03 | 96.93 |
| | 15.00 | 14.95 ± 0.25 | 99.68 |
| | 30.00 | 30.01 ± 0.22 | 100.05 |
| 2 | ---- | ---- | ---- |
| | 5.00 | 4.83 ± 0.16 | 96.60 |
| | 15.00 | 14.59 ± 0.11 | 97.27 |
| | 30.00 | 29.18 ± 0.46 | 97.27 |
| 3 | ---- | ---- | ---- |
| | 5.00 | 4.86 ± 0.22 | 97.20 |
| | 15.00 | 14.41 ± 0.10 | 96.07 |
| | 30.00 | 29.05 ± 0.14 | 96.83 |

*Mean ± S.D. of triplicate analyses.



In our previous paper,[15] the specificity of the method was studied by spiking ESM with other anticonvulsants including valproic acid, carbamazepine, primidone, acetazolamide and phenobarbital. 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. As a consequence, the proposed method is specific and feasible for the analysis of ESM in plasma for biological study or therapeutic drug monitor.

In conclusion, the present study showed that ESM extracted from plasma may be readily derivatized by 4-bromomethyl-7-methoxy coumarin. The proposed method is specific and feasible for analysis of ESM in plasma for biological or clinic interest.

## ACKNOWLEDGMENT

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## REFERENCES

1. R. Musolino, G. Gallitto, P. D. Domenico, M. M. Bonazinga, R. Sturniolo, C. Labate, R. D. Perri, J. Int. Med. Res., **19**, 55-62 (1991).

2. T. Villen, L. Bertilsson, F. Sjoqvist, Ther. Drug Monit., **12**, 514-516 (1990).

3. A. Pellegrini, R. C. Dossi, F. D. Pos, M. Ermani, L. Zanotto, G. Testa, Brain Res., **497**, 344-360 (1989).

4. J. McCormack, G. Brown, M. Levine R. Rangno J. Ruedy, **Drug Therapy Decision Making Guide**, W.B. Saunders, Inc., Pennsylvania, 1996, p. 310.

5. M. Cosgrove, S. Pople, S. J. Wallace, Dev. Med. Child Neurol., **32**, 796-799 (1990).

6. K. Bachmamm, C. A.. Chu, V. Greear, Pharmacology, **45**, 121-128 (1992).

7. E. D. Marquardt, D. Y. Ishisaka, K. K. Batra, B. Chin, Clin. Pharm., **11**, 1030-1031 (1992).

8. U.S. Pharmacopeia XXIII Revision, United States Pharmacopeial Convention, Rockville, MD, 1995, p. 642.

9. E. B. Solow, J. B. Green, Clin. Chim. Acta., **33**, 87-90 (1971).

10. E. B. Solow, J. M. Metaxas, T. R. Summers, J. Chromatogr. Sci., **12**, 256-260 (1974).

11. E. B. Solow, N. L. Tupper, C. P. Kenfield, J. Anal. Toxicol., **2**, 39-40 (1978).

12. J. Volmut, E. Matisova, P. T. Ha, J. Chromatogr., **527**, 428-435 (1990).

13. N. Wad, J. Chromatogr., **305**, 127-133 (1984).

14. N. Wad, J. Chromatogr., **338**, 460-462 (1985).

15. J. K. Wu, S. H. Chen, H. S. Kou, S. M. Wu, H. L. Wu. Chin. Pharm. J., **46,** 413-421 (1994).

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