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RESEARCH PAPER

Microextraction combined with microderivatization for drug monitoring and protein modification analysis from limited blood volume using mass spectrometry

Ying-Jung Wu[1] · Yi-Shan Li[1] · Wei-Lung Tseng[2] · Chi-Yu Lu[1,3,4,5]

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Abstract

In the clinic, ethosuximide is commonly used to treat generalized absence seizures but has recently been repurposed for other diseases. Because of adverse effects and drug interactions, high-throughput therapeutic drug monitoring of ethosuximide is necessary. Microextraction is a simple, effective, rapid, and low consumption of organic solvents method for sample preparation. In this study, microderivatization-increased detection (MDID)-combined microextraction was used to detect ethosuximide by mass spectrometry. Ethosuximide is a difficult to retain and ionize compound in the C18 nano-flow column and ionization interface, respectively. Hence, we developed a fast method for detecting ethosuximide in human plasma by using the MDID strategy (within 2 min). Chemical microderivatization parameters were studied and optimized to increase the sensitivity of ethosuximide detection at trace levels. The linear range for the analysis of ethosuximide in 10 μL plasma was 5–500 μg/mL with a coefficient of determination (r^2) ≥ 0.995. The precision and accuracy of intraday and interday analyses of ethosuximide were below 13.0%. Furthermore, modifications of major proteins in plasma and blood cells, induced by ethosuximide, were identified. The proposed method effectively utilizes microliter samples to detect drug plasma concentrations under suitable microextraction procedures toward the eco-friendly goal of low consumption of organic solvents.

Keywords Microextraction · Microderivatization · Ethosuximide · Chemical tag · Human plasma · Modification

Introduction

Ethosuximide is a succinimide-derived anticonvulsant. Succinimide has no anticonvulsant activity, but introduction of ethyl and methyl moieties at C-2 results in ethosuximide, which is remarkably effective in treating "petit mal" seizures, controlling absence seizures, and decreasing the frequency of seizures [1, 2]. Recent studies have indicated that ethosuximide could be repurposed to treat other diseases, as it promotes wound healing [3], prevents noise-induced hearing loss [4, 5], reduces pain [6, 7], regulates platelet functions [8], prevents stroke [9], induces hippocampal neurogenesis, and reverses cognitive deficits [10]. The adverse effects of ethosuximide include gastrointestinal (nausea, vomiting, and abdominal pain) and CNS-related (ataxia, dizziness, lethargy, and insomnia) disorders and blood dyscrasias (aplastic anemia) [11]. Hence, similar to other antiepileptic drugs, therapeutic drug monitoring (TDM) of ethosuximide is necessary to establish optimal therapy regimens for patients and avoid drug interactions [12–15].

Various methods to detect ethosuximide have been documented in recent reports [16–23]. These methods contain derivatization or non-derivatization procedures and then different instruments are utilized, including LC and GC coupled with a detector (e.g., UV detector, flame ionization detector,

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✉ Chi-Yu Lu
cylu@kmu.edu.tw

[1] Department of Biochemistry, College of Medicine, Kaohsiung Medical University, Kaohsiung 80708, Taiwan

[2] Department of Chemistry, College of Science, National Sun Yat-sen University, Kaohsiung 80424, Taiwan

[3] Research Center for Environmental Medicine, Kaohsiung Medical University, Kaohsiung 80708, Taiwan

[4] Institute of Medical Science and Technology, National Sun Yat-sen University, Kaohsiung 80424, Taiwan

[5] Department of Medical Research, Kaohsiung Medical University Hospital, Kaohsiung 80708, Taiwan

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or MS). Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) MS is an effective and a mobile phase free technique for fast, high-throughput detection of analytes [24–26]. For desired analyte detection by MALDI-TOF MS, selection of suitable target plates and matrices is necessary to obtain suitable signals at trace levels [27–30]. Nano ultra-performance liquid chromatographic system (nanoUPLC) offers enhanced sensitivity by reducing the column inner diameter and the benefits are the minimal solvent and additive volume requirements for elution [31, 32]. Reducing the usage of organic solvents is eco-friendly and one of the goals for the modern analytical chemistry.

Sample preparation is an essential and important step in the analytical process. Current efforts are being focused on the improvement of this procedure, following the current trends toward miniaturization, simplification, automation, economization, and solvent-free or environmentally friendly alternatives [33]. Microextraction takes place between several microliters of a water-immiscible solvent and the advantages of its extraction procedures are simplicity, effectiveness, rapidity, and low consumption of organic solvents [34]. Compared to conventional liquid-liquid extraction, mictroextraction reduces the sample and extraction solvent requirements [35].

Ethosuximide does not retain and ionize easily in the C18 nano-flow column and ionization interface, respectively. Therefore, we used a microderivatization-increased detection (MDID) strategy coupled with selecting different target plates and matrices to microderivatize ethosuximide with a suitable tag that would enhance ionization. However, traditional derivatization procedures proved time consuming. We tried to use a commercial microwave oven to accelerate the microderivatization reaction (within 2 min) in microscale and then established a MALDI-TOF MS/nanoUPLC MS-based method for fast analysis of ethosuximide in human plasma.

In addition, we estimated the usability of our method in assaying post-translational protein modifications. Protein modifications are important indicators of physiological, pathological, and biochemical functions of the human body [36–39] and useful markers for prevention, diagnosis, and treatment of various diseases.

Our study goals were to maximize the clinical usefulness of limited volumes of biological samples and minimize the sample preparation procedures and lower consumption of organic solvents/chemicals. In the clinic, during TDM and diagnostic procedures, effective utilization of biological samples and reducing the sample volumes collected are necessary and improve patient compliance. The detection method proposed in this study could be useful for developing precision medicine [40, 41]. To the best of our knowledge, this work is the first to describe the use of MALDI-TOF MS/nanoUPLC MS for detecting ethosuximide/ethosuximide-induced protein modifications in human plasma. Figure 1 shows a diagram of the proposed method in this study for drug monitoring and protein modification analysis.

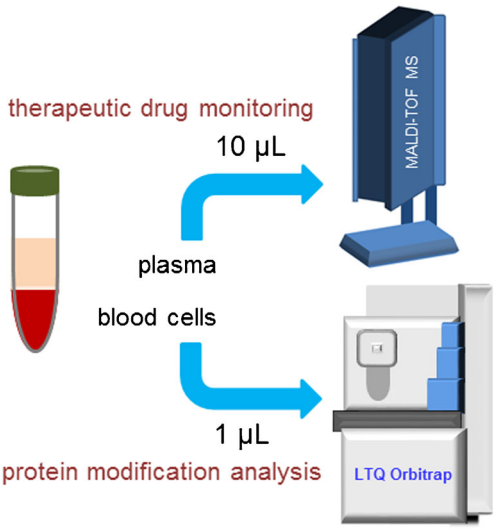

Fig. 1 The diagram of the proposed method in this study for drug monitoring and protein modification analysis

Materials and methods

Chemicals and reagents

All reagents were analytical grade. 2-(Bromomethyl)-6-methyl pyridine (BrMMP), 4-bromomethyl-6,7-dimethoxycoumarin (BrDMC), 8-(bromomethyl) quinoline (BrMQ), 9-(bromomethyl) acridine (BrMA), ethosuximide, 2,5-dihydroxybenzoic acid (25DHB), 2-mercaptobenzothiazole (MBT), 4-mercaptobenzoic acid (MBA), 7-mercapto-4-methyl coumarin (7MMC), sinapinic acid (SA), α-cyano-4-hydroxycinnamic acid (CHCA), and 18-crown-6 ether (18-crown-6) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, ethyl acetate, hexane, potassium bicarbonate ($KHCO_3$), potassium carbonate, (K_2CO_3), potassium hydroxide (KOH), toluene, and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany). Ethosuximide-d3 (internal standard, IS) was obtained from Toronto Research Chemicals (Toronto, Canada). The deionized water used was obtained from a Millipore Milli-Q® (Bedford, MA, USA) water purification system.

Working solutions

Ethosuximide and IS stock solutions (1 mg/mL) were prepared by dissolving the appropriate amounts of the compounds in acetonitrile:water (1:1, *v*/*v*). Stock solutions containing BrDMC, BrMMP, BrMQ, and BrMA (1 mM) were prepared in acetonitrile. The $KHCO_3$, K_2CO_3, and KOH aqueous stock solutions were prepared as saturated solutions in water. Trifluoroacetic acid solutions (0.1%) were prepared in water. 18-Crown-6 (100 mM) solution was prepared in acetonitrile. All matrices (10 mg/mL) used for MALDI-TOF MS analysis were prepared in acetonitrile: 0.1% trifluoroacetic acid (1:1, *v*/*v*).

Sample preparation and microderivatization procedure for analysis of ethosuximide from human plasma

Drug-free plasma samples were spiked with six different concentrations of ethosuximide (final concentration range, 5–500 μg/mL) and ethosuximide-d3 at 450 μg/mL. Aliquots (10 μL) of human plasma with different ethosuximide concentrations were pipetted into Eppendorf tubes. After adding 40 μL of toluene, the samples were vortexed for 30 s and centrifuged at 10000 rpm for 5 min. The supernatant (30 μL) was transferred to an Eppendorf tube, evaporated to dryness, and redissolved in acetonitrile:water (1:1). Two microliters of BrMA (1 mM), 2 μL of 18-crown-6 (10 mM), and 2 μL of K_2CO_3 (7 M) were then added. The solutions were radiated with microwave energy at 400 W for 2 min. After the reaction, the Eppendorf tubes were centrifuged at 10000 rpm for 1 min and 0.5 μL of the upper supernatant layer was spotted on the target plate. Finally, 0.5 μL of the matrix solution (CHCA 25DHB, 10:25 mg/mL) was added to the target plate and mixed for MALDI-TOF MS analysis.

Preparation of blood samples for identifying proteins and protein modifications induced by ethosuximide

Blood samples were collected and centrifuged at 4000 rpm for 20 min to separate plasma from blood cells. Blood cells fraction (1 μL) was diluted and lysed by adding 9 μL of RIPA buffer (contains protease inhibitor cocktails (Roche, Mannheim, Germany)). To collect the proteins for identification, plasma and blood cell solutions (1 μL) were transferred into separate Eppendorf tubes, mixed with acetone (10 μL), vortexed for 30 s, and centrifuged at 10000 rpm for 10 min.

After discarding the supernatant, the remaining protein pellets were evaporated to dryness. Next, 100 μL of 25 mM NH4HCO3 aqueous solution was added to re-dissolve the proteins. For protein reduction, 16 μL of the protein mixtures (~5 μg protein) was mixed with 2 μL of DTT aqueous solution in Eppendorf tubes and kept at 25 °C for 30 min. After reduction, 2 μL of IAA solution was added. The solution was kept at 25 °C for 30 min. Finally, 2.5 μL of modified trypsin solution was added into the protein mixture and kept at 37 °C for 16 h for protein digestion. To identify proteins and protein modifications, peptide solutions (2 μL) were injected into the nanoUPLC-MS/MS system.

Instrumentation and conditions for MALDI-TOF MS

For ethosuximide analysis, mass spectra were acquired in positive ion reflector mode by a MALDI-TOF MS system (model Autoflex III Smartbeam) equipped with a 355 nm Nd:YAG laser from Bruker Daltonics (Billerica, MA, USA). Acquisition parameters were listed below: mass range m/z 40–1600, digitiser sampling rate 2.00 GS/s, internal calibration with CHCA $[M + H]^+$ and CHCA $[2\ M + H]^+$ at m/z 190.05 and 379.09. Three types of target plates (stainless ground, Prespotted AnchorChip, and nanoassisted laser desorption-ionization (NALDI)) were also obtained from Bruker. After spotting 0.5 μL of sample solution on the target plate (Bruker Daltonics), 0.5 μL of matrix solution (10 mg/mL) was added (excluding the NALDI target plate) then kept plate at room temperature until dryness. Mass spectra were collected by summing 2000 laser shots, and data processing was performed with FlexAnalysis™ software (Bruker Daltonics). Spectra processing settings without smoothing were listed below: baseline subtraction, TopHat; peak detection algorithm, Snap; peak picking, Snap.

Instrumentation and conditions for ethosuximide monitoring by nanoUPLC MS

Instrumentation conditions were reported [42] and listed as follows. The nano ultra-performance liquid chromatographic system (nanoUPLC) was manufactured by Waters (Milford, MA, USA). Tandem mass spectrometry was performed with an LTQ Orbitrap Discovery hybrid Fourier Transform Mass Spectrometer (Thermo Fisher Scientific, Inc. Bremen, Germany). The LTQ Orbitrap was operated in positive ion mode with a nanospray source and at a resolution of 30,000. Voltage at the source, tube lens, and capillary was set to 2.3 kV, 80 V, and 28 V, respectively. Spray capillary temperature was set to 200 °C. Ethosuximide and its derivative were analyzed with a concentrated column (Symmetry C18, 5 μm, 180 μm × 20 mm) and a nano-flow column (BEH C18, 1.7 μm, 75 μm × 150 mm) purchased from Waters. The precursor and fragment ions obtained from LTQ Orbitrap were applied to monitor ethosuximide and its derivative. After microderivatization, ethosuximide solutions (2 μL) were injected and separated at a flow rate of 300 nL/min. To shorten the analytical time, we used a fast method to elute the ethosuximide derivative from nanoUPLC system. Mobile phase A was 0.1% formic acid, and mobile phase B was acetonitrile (containing 0.1% formic acid). The gradient conditions were $t = 0$–1 min, hold B at 1%; $t = 1$–5 min, increase B from 12 to 100%; $t = 5$–45 min, hold B at 100%; $t = 45$–60 min, decrease B from 100 to 1%.

Protein identification by nanoUPLC-MS/MS

The nanoUPLC conditions were reported [43] and listed as follows. After on-line desalting of peptide mixtures with a trapped column, peptide separation was performed with a nano-flow reverse-phase C18 column. Desalting was performed for 3 min at a flow rate of 5 μL/min with 0.1% formic acid, before the valve auto-switched to the analytical position. Tryptic peptides were separated by an analytical column at a

flow rate of 300 nL/min. The gradient conditions were $t = 0$–4 min, hold B at 1%; $t = 4$–80 min, increase B from 1 to 45%; $t = 80$–120 min, increase B from 45 to 85%; $t = 120$–140 min, hold B at 85%; $t = 140$–180 min, decrease B from 85 to 1%; $t = 180$–240 min, hold B at 1%.

The MS conditions were as follows. The mass range in full scan was set to m/z 400–2000 with a resolution of 30,000 at m/z 400 in profile mode. Up to four of the most abundant multiple-charge ions were isolated for collision-induced dissociation. With the lock mass ion-molecular weight set to m/z 445.12, precursor ions in the linear ion trap were fragmented by applying 35 eV collision energy with helium as the collision gas to obtain MS/MS spectra. Raw data files were processed with Mascot Distiller software (Matrix Science Inc., Boston, MA, USA) to create the peak lists. The peak list files were uploaded to the Mascot server (Matrix Science Inc) for protein identification. Search settings: database, NCBIr; taxonomy, *Homo sapiens*, modifications, variable modifications; enzyme, trypsin; MS tolerance, 20 ppm; MSMS tolerance, 1 Da; missed cleavages, allow up to 1. Protein identified by protein scores greater than significant Mascot Score (above the 95% confidence level), modification confirmed by scores greater than identified scores.

Results and discussion

Instrument selection

Mictroextraction coupled with MALDI-TOF MS/nanoUPLC MS is a suitable strategy to develop an ethosuximide analytical method with high sensitivity and miniaturization of organic solvents. After ionization by the MALDI/nano electrospray interface, the ethosuximide signal should be detected by MS detector. However, we could not obtain any signal for ethosuximide using MALDI-TOF MS/nanoUPLC MS because of the poor ionization efficiency and hydrophobic interaction with C18 stationary phase. To overcome this problem, we tried to utilize MDID strategy to modify the physical/chemical properties of the ethosuximide then to enhance ethosuximide ionization in the ionization source and increase the ethosuximide retain in the C18 nano-flow column. This strategy was workable and we could obtain the ethosuximide derivative signal in MALDI-TOF MS/ nanoUPLC MS. As shown in Electronic Supplementary Material (ESM) Fig. S1, the nanoUPLC MS approach resulted in elution of the ethosuximide derivative after 13mins. Furthermore, large tailing was observed which would affect the ability to quantify effectively. Compared the analytical time and consumption of mobile phase of nanoUPLC MS with MALDI-TOF MS, we selected MALDI-TOF MS (short analytical time and mobile phase free) as the suitable instrument to detect ethosuximide.

In this study, we tried to develop a fast MALDI-TOF MS method using a MDID strategy coupled with different target plates and matrices to detect ethosuximide in human plasma by microwave-assisted microderivatization within 2 min. In order to avoid and decrease the adverse effects of ethosuximide therapy, it is necessary to monitor the concentration of the drug in human plasma.

To increase the sensitivity, MALDI-TOF MS was combined with a MDID strategy for detecting ethosuximide at trace levels. Four alkylating reagents (BrDMC, BrMMP, BrMQ, and BrMA) were used to derivatize the succinimide moiety of ethosuximide, and a matrix was selected after chemical microderivatization. Three types of target plates (stainless ground, Prespotted AnchorChip, and NALDI plates) were also tested to choose the best signals. Additional parameters of the microderivatization procedure that were studied and optimized included microderivatization reagent type and concentration, base activator type and amount, catalyst concentration, reaction volume, microwave radiation time, and microwave radiation power. Furthermore, the extraction solvent type affected the microderivatization procedure and required optimization. Figure 2 shows the block diagram of the proposed MALDI-TOF MS method for ethosuximide analysis.

Matrix and target plate selection

Ethosuximide signals were tested for MALDI-TOF MS compatibility by comparing different matrices after microderivatization, including CHCA, 25DHB, 2MBA, 2MBT, 7MMC, and SA. With these matrices, the $[M + H]^+$ signal at m/z 333 was detected for the ethosuximide derivative. The order of S/N ratios obtained in MALDI-TOF MS was CHCA >2,5-DHB > > SA, 2MBA, 2MBT, and 7MMC. When using CHCA as the matrix, the undesired signal at m/z 336 could interfere with the signal of ethosuximide-d3 (IS) (see Fig. 6), which we tried to avoid by combining different proportions of CHCA and 25DHB. CHCA:25DHB at 10:25 mg/mL was selected as the optimal matrix for detecting the derivatives of ethosuximide and ethosuximide-d3. Three types of target plates (stainless ground, Prespotted AnchorChip, and NALDI plates) were also tested to choose the best signals. Figure 3 indicates that the stainless ground type of target plate yielded a suitable signal for ethosuximide analysis after chemical microderivatization ($n = 3$). According to the manufacturer's statement (Bruker Daltonics), the NALDI plate is a disposable target for matrix-free laser desorption ionization of low mass organic molecules, and the sensitivity of AnchorChip is 10- to 100-fold higher than that of regular steel targets (reported by the manufacturer's Instructions for Use). Regardless, we found that the stainless ground plate was the most suitable plate for ethosuximide detection by MALDI-TOF MS in this study.

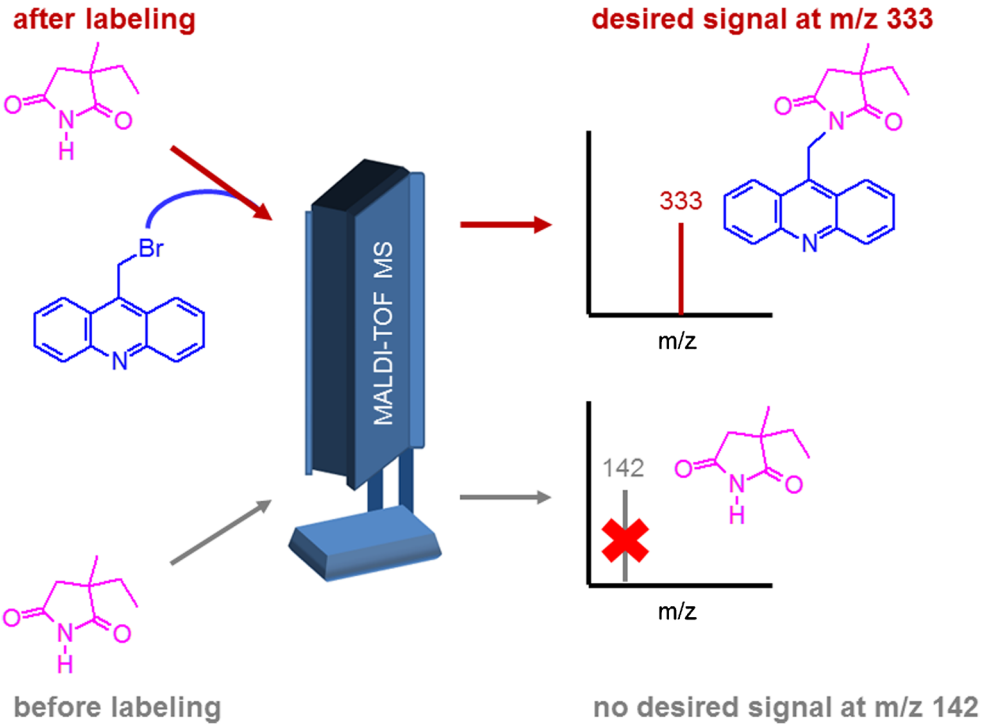

Fig. 2 Block diagram of the proposed MALDI-TOF MS methods for ethosuximide analysis before and after microderivatization. The $[M + H]^+$ signal of ethosuximide at m/z 142 could not be detected by MALDI-TOF MS directly. After microderivatization with BrMA, the $[M + H]^+$ signal of ethosuximide derivative at m/z 333 could be detected by MALDI-TOF MS easily

Optimization of the microderivatization procedure

The factors that affected the formation of ethosuximide derivatives in human plasma were studied. Figure 4 shows a simplified diagram of the reaction scheme for the microderivatization of ethosuximide. The effects of different chemical tags (BrDMC, BrMMP, BrMQ, and BrMA) on the formation of ethosuximide derivatives were compared and the results are shown in ESM Fig. S2. Using BrMA as the microderivatization reagent yielded the optimal signal for ethosuximide analysis. Figure 5 also shows that the UV-visible absorption spectra of these chemical tags are different, with only the λmax of BrDMC approximating that of the laser of the MALDI-TOF MS system (355 nm). In this study, BrMA is the most suitable reagent for ethosuximide analysis after the microderivatization reaction. The results also indicate that the λmax of these tags is not the key reason affecting the ionization of ethosuximide. In addition, the effects of different BrMA concentrations (0.25–10 mM) were compared. The data in ESM Fig. S3 show that 1 mM is the optimal BrMA

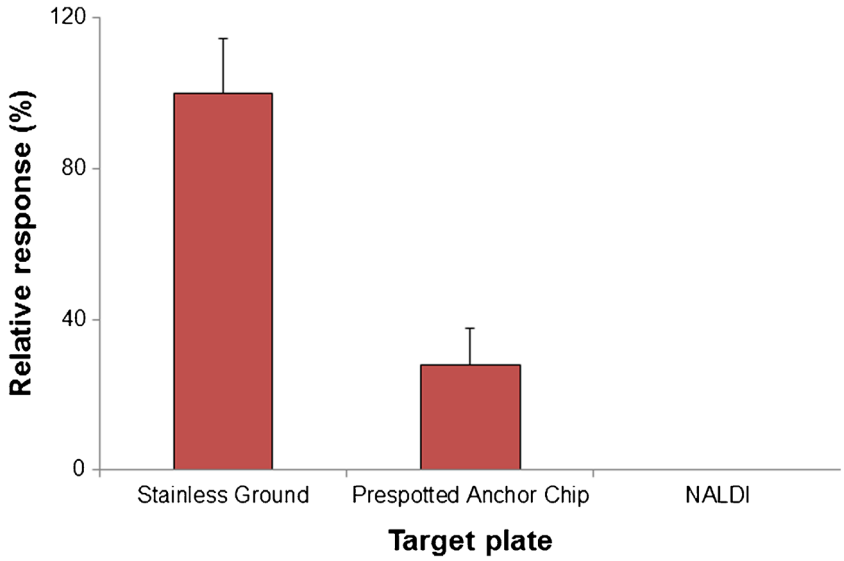

Fig. 3 Signals obtained from different target plates (stainless ground, Prespotted AnchorChip, and NALDI plates) on the detection of ethosuximide derivatives by MALDI-TOF MS

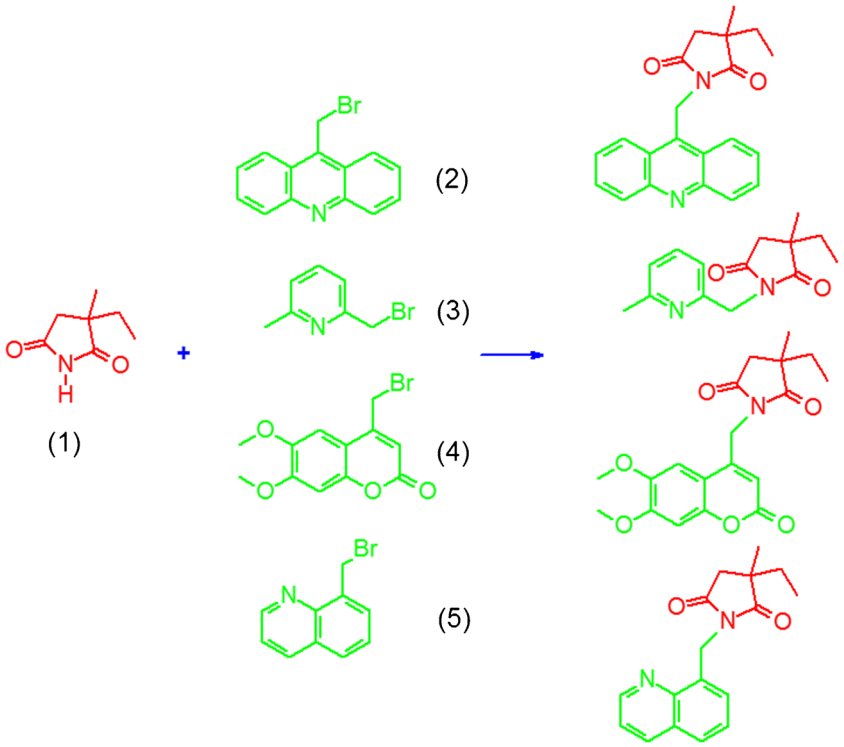

Fig. 4 Simplified reaction scheme for microderivatization of ethosuximide by alkylating agents. Compounds 1 to 5 are ethosuximide, BrMA, BrMMP, BrDMC, and BrMQ

concentration for formation of the ethosuximide derivative, higher concentration of BrMA might have the effect of competitive ionization with ethosuximide derivative.

In the microderivatization process, an alkaline reaction environment was beneficial to form the ethosuximide derivative. Three different base activators, saturated solutions of KOH, $KHCO_3$, and K_2CO_3, were used and compared. ESM Fig. S4 shows that K_2CO_3 was the optimal base activator in the microderivatization reaction. ESM Fig. S5 shows that, in further comparisons of the effects of K_2CO_3 concentration (from 1 M to saturated solution) on the microderivatization reaction, a 7 M solution of K_2CO_3 was the optimal concentration to use. In addition, the effect of 18-crown-6 catalyst (0 to 100 mM) was tested, and ESM Fig. S6 shows that 10 mM 18-crown-6 was the most suitable concentration for the microderivatization reaction. In the presence of 10 mM crown ether, the microderivatization reaction could be accelerated and the yield of ethosuximide derivative could be increased by 50%. The reaction volume (2, 5, and 10 μL) of the reagents (BrMA, 18-crown-6, and K_2CO_3) also affected the formation of ethosuximide derivatives, with 2 μL providing optimal signals for detection (ESM Fig. S7). These results indicate that reducing the reaction volume increases the microderivatization product formation. The optimal microwave conditions were a power setting of 400 W and a radiation time of 2 min, with ranges of 100–1000 W and 1–10 min studied (ESM Fig. S8 and S9, respectively). The results indicate that higher radiation powers and times can generate less microderivatization product because of the effect of competitive ionization with ethosuximide derivative might occur after many compounds in plasma react with BrMA to form undesired products.

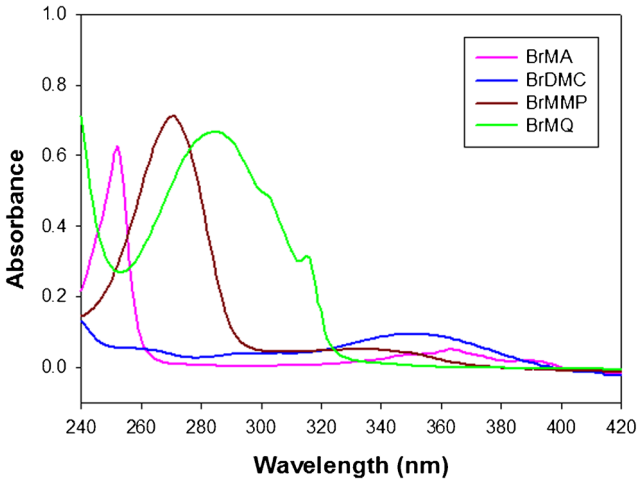

Fig. 5 The UV-visible absorption spectra of the chemical tags, BrDMC, BrMMP, BrMQ, and BrMA

For analysis of ethosuximide in human plasma, the drug should be isolated from plasma before microderivatization. To extract ethosuximide from plasma, three different water immiscible solvents (toluene, ethyl acetate, and hexane) were tested. ESM Fig. S10 shows that toluene was the optimal solvent for extracting ethosuximide. Figure 6 shows the MALDI-TOF MS spectra for ethosuximide derivatives in human plasma, which were obtained using the optimal chemical microderivatization procedure.

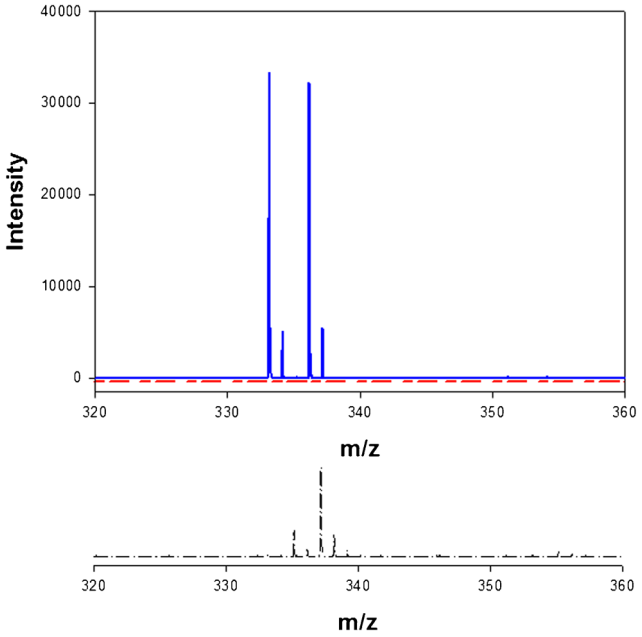

Fig. 6 Typical mass spectra obtained in MALDI-TOF MS of reagent blank (red line) and ethosuximide derivatives (blue line) in human plasma samples under optimal microderivatization conditions. Peaks 1 and 2 are the ethosuximide (m/z 333) and ethosuximide-d3 (m/z 336) derivatives, respectively. When blank using CHCA alone as the matrix (black dash line in the bottom), the undesired signal at m/z 336 could interfere with the signal of ethosuximide-d3 derivative

Ethosuximide analysis in human plasma

Under the optimal microderivatization procedure, the linear range for the analysis of ethosuximide in plasma was 5–500 μg/mL (ESM Fig. S11 shows the calibration curve). The analysis showed high linearity $y = (2.191 \pm 0.108)x + (0.020 \pm 0.007)$ with a coefficient of determination (r^2) ≥ 0.995 ($n = 5$). Furthermore, the recovery was 98–111%, the limit of quantitation (LOQ) was 5 μg/mL, and the limit of detection (LOD) was 0.5 μg/mL. The precision and accuracy of intraday and interday analyses of ethosuximide in human plasma were tested at 10, 80, and 400 μg/mL. Table 1 shows that the RSD and relative error (RE) were below 13.0% in intraday ($n = 5$) and interday assays ($n = 5$). The proposed MALDI-TOF MS method was further used to monitor plasma ethosuximide in a healthy volunteer. Plasma samples were collected before and after a single oral dose of ethosuximide (500 mg) in tablet form. After 2 h, the peak plasma concentration of ethosuximide was 16.95 ± 0.53 μg/mL, consistent with the ranges reported earlier by Giaccone et al. [44]. These results demonstrate that the proposed MALDI-TOF MS method can be used for microscale quantitation of ethosuximide in human plasma samples with volumes as low as 10 μL.

Table 1 Precision and accuracy of analyses of ethosuximide in human plasma

Concentration known (μg/mL)	Concentration found (μg/mL)	RSD[a] (%)	RE[b] (%)
Intra-day ($n = 5$)			
400	393.07 ± 14.42	3.7	− 1.7
80	79.67 ± 4.05	5.1	− 0.4
10	11.09 ± 1.38	12.4	+ 10.9
Inter-day ($n = 5$)			
400	394.39 ± 15.86	4.0	− 1.4
80	84.58 ± 5.55	6.6	+ 5.7
10	10.49 ± 1.17	11.2	+ 4.9

[a] Relative standard deviation (RSD) = (SD/mean) × 100

[b] Relative error (RE) = (concentration found − concentration known/concentration known) × 100

Stability of ethosuximide in human plasma

In order to examine ethosuximide stability, the ratio of the stability of the ethosuximide derivative to that of the IS was evaluated. Plasma samples were stored at − 20 °C and the stability of the ethosuximide derivative, relative to that of IS, was examined over 14 days. No obvious change in ethosuximide derivative/IS ratio was observed, indicating that the ethosuximide derivative is sufficiently stable for MALDI-TOF MS analysis.

Comparison to other methods

Table 2 compares the proposed method and other methods reported in the literature for determining ethosuximide by LC or GC coupled with different detectors. Recent trends in sample preparation include miniaturization and low-cost operation through extremely low, solventless, or no solvent consumption, microextraction has these advantages [45, 46]. Microextraction represents a key step in analytical methodologies by providing samples in the suitable volumes and purification levels necessary for the characterization of the target analytes [47]. The optimized method in our study can utilize smaller sample volumes of human plasma and shorter analytical times to detect the ethosuximide by using MDID without the need for a mobile phase (or carrier gas). Furthermore, analytical and separatory columns, solid-phase extraction (SPE) cartridges- and 0.22-mm syringe filters were unnecessary because a microextraction was utilized to eliminate interfering compounds in human plasma. Without this extraction step, no ethosuximide signal could be detected by MALDI-TOF MS after microderivatization.

Identification of ethosuximide-induced major protein modifications

Major protein modifications are useful diagnostic indicators and adverse effects markers in the clinic. Hence, in this

Table 2 Comparison of current methods for analysis of ethosuximide

Analytical method	Sample	Sample volume (μL)	Sample preparation	Labeling reagent	Reaction time (min)	Analytical time (min)	LOD (μg/mL)	Mobile phase/carrier gas	Ref.
HPLC-UV	Human serum or plasma	25	Protein precipitation	–	–	15	1	Acetonitrile, phosphate buffer	[16]
GC-FID	Human serum	25–50	LLE	–	–	9.5	1.4	Nitrogen	[17]
HPLC-UV	Human serum	200	LLE	–	–	8	–	Acetonitrile, methanol, phosphate buffer	[18]
HPLC-UV	Human plasma	300	LLE	NOEBPES	90	12	1.3	Isopropanol in n-hexane	[19]
GC-MS	Whole blood	200	SPE	Butyl iodide	5	20	–	Helium	[20]
GC-MS	Human plasma	50	LLE	–	–	16	0.76	Helium	[21]
UPLC-MS/MS	Human plasma	250	SPE	–	–	1.8	0.25	Acetonitrile, ammoniumacetate	[22]
UPLC-MS/MS	Human plasma	50	Protein precipitation	–	–	6.5	–	Methanol, ammoniumacetate	[23]
MALDI-TOF MS	Human plasma	10	Micro-LLE	BrMA	2	<1	0.5	–	This work

UV, ultraviolet; *FID*, flame ionization detector; *MS*, mass spectrometry; *UPLC*, ultra performance liquid chromatography; *LLE*, liquid-liquid extraction; *SPE*, solid-phase extraction; 2-(2-naphthoxy)ethyl 2-[1- (4-benzyl)piperazyl] ethanesulfonate (NOEBPES); *BrMA*, 9-(bromomethyl)acridine; *LOD*, limit of detection; *Ref.*, reference

study, we tried to develop a simple model for drug monitoring and protein modification analysis simultaneously from limited blood volume at the same sample. Blood samples before and after a single oral dose of ethosuximide were tested as the control and ethosuximide-induced samples, respectively. To survey ethosuximide-induced major protein modifications, blood samples were separated into plasma and blood cells compartments using centrifugation. A number of protein modifications in major proteins were identified, including acetylation, nitration, nitrosylation, oxidation, phosphorylation, and other protein adducts (acrolein, crotonaldehyde, 4-hydroxynonenal, and hexanoyl-lysine adduct). ESM Table S1 shows all protein modifications observed in this study. Oxidation was the most common modification observed in major proteins. The protein modifications observed are associated with signal transduction, reactive nitrogen and oxygen species, and lipid peroxidation. This study used a simple strategy to identify the major protein modifications present in the blood samples tested. These modifications could be used as diagnostic indicators in the clinic.

Conclusions

This work establishes a fast method for analysis of a difficult to ionize drug (ethosuximide) in human plasma. The results indicate that, even with low sample volumes (10 μL for plasma, 1 μL for the blood cells compartment), the method effectively detects ethosuximide in human plasma and ethosuximide-induced protein modifications in the blood. After simple microextraction, the sensitivity of ethosuximide detection was increased by using MDID, coupled with selecting a suitable matrix and ground target plate. The proposed method efficiently analyzes limited clinical samples with MALDI-TOF MS/nanoUPLC MS to monitor drug plasma concentrations/drug-induced protein modifications simultaneously and appears feasible as a new clinical method.

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Compliance with ethical standards

The experiments were approved by the Institutional Review Board of Kaohsiung Medical University Chung-Ho Memorial Hospital. The method was performed in accordance with the approved guidelines and written informed consent was obtained from all participants.

Conflict of interest The authors declare that they have no conflict of interest.

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