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Short communication

Simple and sensitive fluorimetric liquid chromatography method for the determination of valproic acid in plasma

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Abstract

A simple and sensitive liquid chromatographic method is described for the analysis of valproic acid in human plasma. The method is based on the derivatization of valproic acid extracted from acidified plasma with 2-(2-naphthoxy)ethyl 2-(piperidino)ethanesulfonate. The resulting derivative is highly responsive to a fluorimetric detector (excitation at 230 nm and emission at 350 nm), giving a low detection limit of 0.6 μM (S/N = 3, 10 μl injected). The relative standard deviations of the method for intra- and inter-day analyses (n = 5) are below 3.3 and 4.1%, respectively. Toluene was used for the extraction of valproic acid from plasma and the toluene extract obtained was subjected to subsequent derivatization without solvent replacement. The simple method was applied to the analysis of valproic acid in plasma of dosed patients using only small amount of sample (10–50 μl plasma).

Keyword: Valproic acid

1. Introduction

Valproic acid (VA) is widely used for the treatment of simple and complex absence seizures [1]. The therapeutic range of VA in plasma is considered to be about 347–694 μM [2]. Monitoring of VA levels in patient plasma is essential when there were changes in VA dose, concomitant medication or clinical condition of patient.

Fig. 1 shows that VA is a carboxylic acid without chromophore for being detected in practical UV range by conventional absorption spectrophotometry. Detection of a drug at short UV wavelength (≤210 nm) tends to be interfered with sample matrix. Therefore, detection-oriented derivatization coupled with liquid chromatography [3] is used for the analysis of VA including derivatizing VA as a substituted naphthalene, benzene or coumarin for HPLC-UV [4–7] and derivatizing VA as a substituted coumarin, phenanthrene, quinoxaline or chromenyl system for HPLC-fluorimetry [8–16] with improved sensitivity. Among the fluorescent reagents used for derivatizing VA in bio-sample, 4-bromomethyl-7-methoxycoumarin (BMMC) is often applied in various approaches such as micelle-mediated derivatization of VA [8,13,14] without sample extraction, the analysis of VA with steps of sample extraction and solvent replacement [9,12] or the derivatization of VA by solid-phase catalyzed reaction for automated analysis [11]. BMMC is a light sensitive reagent and needs to be used with the exclusion of light [8]. Derivatization of VA (after converting to VA acid chloride) with 9-aminophenanthrene [10] needs multiple steps of solid phase extraction and evaporation for sample treatment, resulting in high sensitivity. Derivatization of VA with 6,7-methylenedioxy-1-methyl-2-oxo-1,2-dihydroquinoxaline-3-yl propionohydrazide [15] leads to the chromatogram of a serum blank with unfavorably high tailing; and *N*-(7-methoxy-4-methyl-2-oxo-2H-6-chromenyl)-2-bromoacetamide [16] was also used for the derivatization of VA in spiked plasma.

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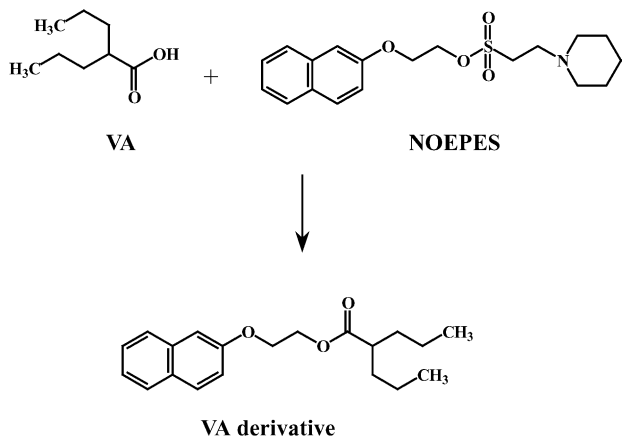

Fig. 1. A reaction scheme for the derivatization of valproic acid (VA) with NOEPES (the derivatizing reagent).

In this work, analytical derivatization of VA with 2-(2-naphthoxy)ethyl 2-(piperidino)ethanesulfonate (NOEPES) [17] was performed. NOEPES is a chemically removable and light stable reagent. The VA derivative from NOEPES (Fig. 1) has an auxochrome (substituted alkoxy) attached to the chromophoric (fluorophoric) naphthalene system, making it highly responsive to a fluorimetric detector. The detection limit of the proposed method is better than those reported [8,9,13–16] and the obtained chromatogram for the analysis of VA in plasma is simple and clean.

2. Experimental

2.1. Chemicals and reagent solutions

Sodium valproate (VA sodium) and nonanoic acid (used as an internal standard, IS) (Sigma, St. Louis, MO, USA), 2-(2-naphthoxy)ethyl 2-(piperidino)ethanesulfonate (NOEPES, synthesized at our laboratory) [17], 18-crown-6 ether (18-crown-6) (TCI, Tokyo, Japan), potassium bicarbonate and sulfuric acid (E. Merk, Darmstadt, Germany), and toluene (Tedia, Fairfield, OH, USA) were used without further treatment. Other chemicals were analytical reagent grade. Distilled water purified with the Ultrapure R/O water system (Millipore, MA, USA) was used for preparing related aqueous solutions. Standard solutions of VA sodium at various concentrations were prepared in deionized water; solution of IS (500 μM) was prepared in 0.5 M KOH aqueous solution; and solutions of NOEPES and 18-crown-6 were prepared in toluene.

2.2. HPLC conditions

A Waters LC system with a Model 515 HPLC pump, a Model 717 plus autosampler and a Model 2475 fluorescence detector and a Millennium chromatography manager was used. A Merck Purospher Star RP-18e column (250 mm × 4 mm i.d.: 5 μm) and a mixed solvent of methanol-water (93:7 (v/v)) at a flow rate of 1.0 ml/min were used. The column eluate was monitored at 230 nm for excitation and at 350 nm for emission. The mobile phase was filtered through a Millipore Durapore membrane filter (HVLP, 0.45 μm) under vacuum for degassing before use.

2.3. Preparation of human plasma sample

Aliquots of normal plasma (50 μl) spiked with five different levels of VA sodium over the range 10–200 μM were added with IS solution (500 μM, 20 μl) in polypropylene (PP) microcentrifuge tubes (1.5 ml); in case of patient dosed with VA sodium, a smaller aliquot of plasma (10 μl diluted with water to 50 μl) was used in this study. After mixing, the sample solution was acidified with HCl (2 M, 50 μl) and extracted with toluene (0.5 ml) by vortex mixing for 30 s. After centrifuging at 1800 g for 5 min, aliquots (200 μl) of the supernatant toluene layer were subjected to the derivatization below.

2.4. Derivatization procedure

Aliquots (200 μl) of the toluene extract were added to series of 25 ml screw capped test tubes each containing 200 μl NOEPES (15 mM) in toluene, 200 μl of 18-crown-6 in toluene (5 mM) and about 20 mg potassium bicarbonate. The reactants were shaken at 95 °C for 60 min. After cooling, aliquot (500 μl) of the solution was transferred to a PP microcentrifuge tubes tube (1.5 ml) and washed with H_2SO_4 (1 M, 1.0 ml) by vortexing for 30 s for removal of excess reagent (NOEPES). The acid-washed toluene layer (100 μl) was diluted with an equal volume of methanol for being compatible with the mobile phase in chromatographic analysis. The resulting solution was analyzed by HPLC with an injection volume of 10 μl.

3. Results and discussion

For optimizing the derivatization conditions for VA (10 nmol) and IS (10 nmol) extracted from spiked plasma, main parameters affecting the derivatization were studied, including the concentration of the derivatizing reagent, reaction time (at 95 °C) and the catalyst. The effects of the parameters on the derivatization were evaluated by peak areas of the resulting VA and IS derivatives. And then peak-area ratios of the drug analyte to the IS were used for the calibration and the analysis of VA in plasma.

3.1. Optimization of the derivatization

The main parameters in the derivatization procedure (Section 2.4) were varied to evaluate their effects on the derivatization of VA in toluene extract. The effects of NOEPES at varied concentrations (1–30 mM) on the derivatization of VA and IS show that plateau formation of both

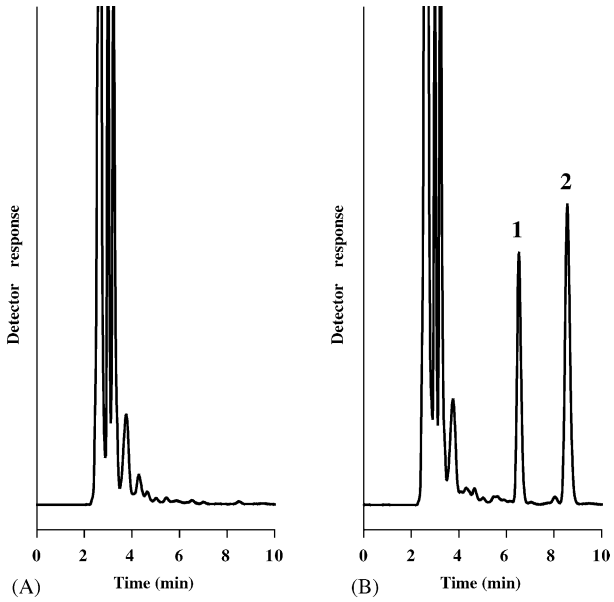

Fig. 2. Liquid chromatograms for (A) normal plasma blank; (B) VA in patient plasma (patient B in Table 2). Peak 1, the VA derivative and peak 2, the IS derivative; see Section 2 for LC conditions.

derivatives is attainable using NOEPES at concentrations ≥15 mM. The effect of derivatization time (0.25–3.0 h) at 95 °C on the formation of the derivatives indicates that equilibrium formation of the derivatives can attain in 1 h; whereas derivatization by reacting at 95 °C for 0.5 h results in ≥90% yield of the VA derivative obtained from reacting for 1 h. Therefore, derivatization at 95 °C for a shorter time can be used if the sensitivity is sufficient for an analysis. Optimum amounts of potassium bicarbonate (0–35 mg) and 18-crown-6 (0–30 mM) used as reaction activator and catalyst [19] for the derivatization of VA were ≥20 mg and 5 mM, respectively. The derivatization of fatty acids with NOEPES related reagents in toluene gives favorable results in our previous studies [17,18] and VA is similar to fatty acid in property. In consideration of using a solvent both for the extraction of VA from plasma and for its subsequent derivatization, we tried toluene and proved it feasible. Based on the optimization conditions obtained, the derivatization procedure was formulated in Section 2.4.

Fig. 2 shows the typical chromatograms for the analysis of VA in plasma, indicating that the plasma blank does not interfere with the derivatives of VA and the IS. The chromatogram is simple and clean due partly to the removal of excess reagent as water soluble ammonium species (Fig. 1) after derivatization by the acid treatment (protonation of the basic nitrogen on NOEPES).

3.2. Stability of the derivative

The stabilities of the derivatives of VA and IS were studied by observing the peak-area of each analyte for 24 h after derivatization. No significant changes of the peak-areas of the analytes were found, indicating that the derivatives are sufficiently stable for the time required for their analysis.

Table 1
Precision and accuracy for the determination of valproic acid spiked in human plasma

Concentration known[a] (μM)	Concentration found (μM)	R.S.D. (%)	R.E.[b] (%)
Intra-day			
30.0	30.0 ± 0.98	3.3	−0.1
120.0	119.6 ± 0.99	0.8	−0.3
180.0	179.6 ± 1.65	0.9	−0.2
Inter-day			
30.0	30.0 ± 1.21	4.0	−0.1
120.0	120.4 ± 1.54	1.3	0.4
180.0	180.3 ± 2.38	1.3	0.2

[a] Intra-day assay variance from triplicate analysis of VA at five intervals on a single day and inter-day assay variance from triplicate analysis of the drug spiked in human plasma on five consecutive days.
[b] R.E. calculated from (value found-value known)/value known.

3.3. Structural analysis of the VA derivative

The derivative of VA was prepared by scaling up the amount of VA (0.12 mmol) with similar derivatization procedure indicated in Section 2.4. The purified derivative was examined by electron impact MS (JEOL-SX102A mass spectrometer with an ionization energy of 70 eV). The mass spectrum obtained exhibited a molecular ion peak at *m/z* 314 (M) for the derivative of VA and a base ion peak at *m/z* 171, corresponding to the naphthoxyethyl fragment ($C_{10}H_7OCH_2CH_2$).

3.4. Analytical calibration and precision

Quantitative applicability of the method for the analysis of VA in spiked plasma was evaluated at five different concentrations over the range 10–200 μM. Calibration graph was established with y for the peak-area ratios of VA to the IS (nonanoic acid,) and x for the concentration (μM) of VA. The linear regression equation obtained is as follow: $y = (0.0060 \pm 0.0001)x - (0.0091 \pm 0.0058)$ with a correlation coefficient $r = 0.999$ ($n = 5$). The detection limit (S/N = 3; sample size, 10 μl) of VA is 0.6 μM.

The intra- and inter-day precisions (relative standard deviations, R.S.D.) of the method were studied based on the peak-area ratios for the analysis of VA at three levels of 30, 120 and 180 μM. The analytical results (Table 1) indicate that the relative standard deviations (R.S.D.) are below 4.0% for intra- and inter-day assay ($n = 5$) and the relative errors (R.E.) are below 0.4%. The relative recoveries of VA spiked in plasma at three levels (50, 100 and 150 μM) by the method are in the range of 98.0–104.0% (data not shown).

3.5. Application

The method was demonstrated to the analysis of VA in plasma of patients at the Department of Psychiatry,

Table 2
Analytical results of valproic acid in patient plasma

Patient[a]	Concentration found[b] (μM)	R.S.D. (%)
A	633.0 ± 10.5	1.7
B	502.2 ± 5.9	1.2
C	320.0 ± 12.3	3.8

[a] Patients received various oral doses of VA sodium (Depakine® tablet, 500 mg VA sodium per tablet) for 1 month at Department of Psychiatry, Kaohsiung Medical University Hospital. Patient A (male, 34 years, 82 kg) with daily dose of 2000 mg VA sodium (500 mg at 9 a.m. and 1500 mg at 9 p.m.), patient B (female, 26 years, 50 kg) with daily dose of 1000 mg VA sodium (1000 mg at 9 p.m.), and patient C (male, 22 years, 72 kg) with daily dose of 1250 mg VA sodium (500 mg at 9 a.m. and 750 mg at 9 p.m.); blood samples were collected from patients at 9 a.m. for VA monitoring after 1 month treatment.
[b] Mean ± S.D. (n = 3).

Kaohsiung Medical University Hospital. The results (Table 2) indicate that the VA concentrations in plasma of patients treated with various daily dose of VA sodium for 1 month are in the range of 320–633 μM. The therapeutic level of VA in plasma is considered to be in the range of 347–694 μM [2].

In conclusion, a sensitive fluorimetric HPLC was developed for the analysis of VA in plasma. The method is simple, using the same solvent (toluene) for the extraction and derivatization of VA from the plasma without the lengthy step of solvent replacement. The method is effective for monitoring of VA levels in patient plasma for therapeutic purpose.

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