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RAPID DETERMINATION OF VALPROIC ACID WITHOUT ESTERIFICATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract

In this communication, we have described a rapid assay system for valproic acid (VPA) using high performance liquid chromatography (HPLC) without any kinds of esterification. The assay system has been greatly simplified with regards to preparation of serum samples, that is, the samples are prepared by centrifigation at 8,000 ×g for 5 min after mixing with an equal volume of acetonitrile. The mixture of acetonitrils (AcCN) and water (30:70, %; V/V) was used as the mobile phase of HPLC.

The calibration curve was linear between 0 and 200 μg/ml of VPA and was calculated to be Y=0.9454 X. The recovery rates of VPA, estimated from standard serum Sets I and II, were calculated to be approximately 91.8% and 101.5%, respectively. Therefore, the accuracy and sensitivity were good enough to assay VPA in plasma. In addition, only 200 μl of plasma was required for the determination of VPA.

There was a good correlation between the value determined by the present method and that obtained by either gas-liquid chromatography (GLC) or enzyme immunoassay (EIA). The correlation coefficients were 0.913 and 0.955 respectively.

In a clinical study, the serum levels of valproic acid in 7 epileptic patients ranged between 50 and 120 μg/ml during the period of treatment with valproic acid.

Introduction

The rapid assay of VPA in biological fluids is very important for determining therapeutic regimens for patients with seizures disorders.

Assay systems for VPA have been reported using GLC[1)2)], HPLC[3)], and EIA[4)]. However, the assay procedures in these methods are complicated. For example, in the gas-chromatography method. VPA must be prepared as its methyl-, butyl-, propyl- and phenacyl-esters before assay[5)~13)]. Also, for these methods, a large amount of biological fluid, such as plasma, is required. However, the drug is usually administered to pediatric patients and collection of a large amount of plasma from these patients can be difficult in many cases.

In this communication, a rapid and simple assay method, not requiring the preparation of any kinds of esters of VPA, using HPLC is presented. The method does not require esterification of VPA during the assay procedure, and the assay can be carried out within 30 min by use of a 200 μl serum sample.

Materials and Methods

Blood samples were collected from patients to whom VPA had been orally administered. All patients received doses of VPA at 15~20 mg/kg of

body weight/day, devided into three equal doses to maintain plasma concentrations in the therapeutic range. Each patient underwent a minimal treatment period of one month before blood sample analysis. After centrifugation of blood sample at 6,000 ×g for 10 minutes, the serum was separated and stored at −20°C untill assay. Control serum sets I (100 μg/ml of VPA in sheep serum) and II (200 μg/ml of VPA in sheep serum) were purchased from Ortho Co., Ltd.

The HPLC apparatus, an ALC/GLC detector, was obtained from Waters Co., Ltd. A stainless steel column (15 × 150 mm) was packed with Resolve Spherical C_{18} (5 μm). As the mobile phase, a mixture of AcCN and water (30:70, %; V/V) which was adjust to pH 3.5 with 5 mM pentanesulfonic acid, was used throughout the assay procedures. The elution volume was 1 ml/min throughout the experiments.

For the standard VPA solution, Depakene R was purchased from Kyowa Hakko Co., Ltd. All other chromatographic grade reagents were purchased from Wako Chemical Co., Tokyo.

In all the assays, the mixture of 200 μl of serum and 200 μl of AcCN was centrifuged at 8,000 ×g for 5 min. A 20 μl aliquot of the upper phase was applied to HPLC. The elution pattern was detected at 214 nm using a UV M441 recorder from Waters Co., Ltd. The data module was also purchased from Waters Co., Ltd. The column was heated to 60°C, in a model III incubator obtained from Waters Co., Ltd. during the procedures. Details of the assay conditions are described in each of the figure legends.

Mathematical analysis of the elimination half-life ($T_{1/2}$) was performed as described by Chiba et al[14].

Results

Separation of authentic samples

The authentic VPA dissolved in distilled water was detected at 15.52 min on HPLC (Fig. 1a). When the sample extracted from the mixture of standard serum and authentic VPA was applied to HPLC, the retention time of VPA was 15.72 min (Fig. 1c). However, human standard serum has no distinguishable peaks in the position corresponding to the VPA peak (Fig. 1b).

Standard curve for the estimation of VPA in human serum

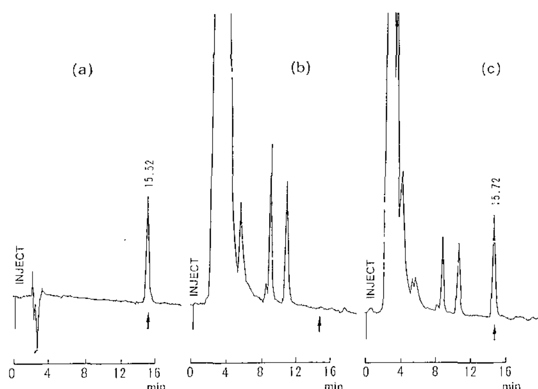

Fig. 1 *Chromatograms of authentic valproic acid and standard serum*
(a) VPA in distilled water, (b) serum only, (c) VPA in serum. The conditions of assay of VPA by HPLC as follows, the elution volume was 1 ml/min and AUFS was 0.001. The detection were length was 214 nm throughout the experiments.

Table 1 Reproducibility and daily equation reproducibility

	Reproducibility			Daily equation reproducibility
	Control serum (50μg/ml)	Standard solution (100μg/ml)		Control serum (60μg/ml)
N	10	10	N	6
X̄	49.2	100.3	X̄	58.63
SD	0.97	2.58	SD	3.57
CV (%)	1.97	2.57	CV (%)	6.09

The calibration curve was obtained using the mean of the peak heights of triplicate determinations for each concentration of the standard solution. The curve was linear between 0 and 200 μg/ml of VPA and was calculated to be Y=0.9454X.

Recoveries estimated from standard serum Sets I and II were calculated to be approximately 91.8% and 101.5%, respectively. The minimum concentration of VPA was 25 μg/ml with this method (Fig. 2).

On the other hand, the correlation curve between HPLC and EIA value was calculated to be Y=0.929X + 0.865 with a correlation coefficient of 0.995 (n=26) (Fig. 3).

Reproducibility and daily equation reproducibility

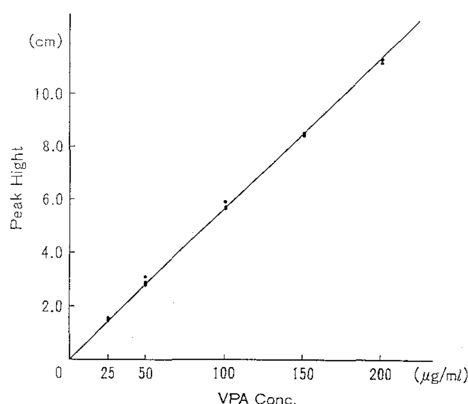

Fig. 2 Standard curve for VPA obtained by HPLC

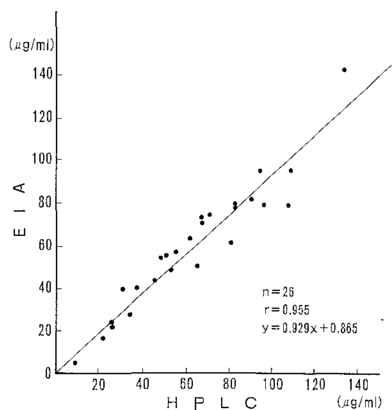

Fig. 3 Correlation between EIA and HPLC

As an index of precision, CV=1.97% for simultaneous reproducibility and CV=6.09% for daily equation reproducibility were calculated after assay of VPA 10 times at the different concentrations of VPA (Table 1).

Separation of 5 anticonvulsants on HPLC

A mixture of primidone (3.0 μg/ml), phenobarbital (15.0 μg/ml), phenyltoin (7.5 μg/ml), carbamazepine (6.0 μ/ml), and VPA (80 μg/ml) in standard serum was extracted by the above method, and applied to HPLC. The retention times of each of the drugs were 3.77 min, 5.02 min, 8.30 min, 10.10 min and 16.62 min, respectively (Fig. 4).

Correlation of VPA assayed by HPLC with GLC or EIA

Authentic samples of VPA at concentrations of 20, 40, 60, 80, 100, 120, 140 μg/ml were simultaneously assayed by HPLC, gas-liquid chromatography (GLC)[15] and enzyme immunoassay (EIA)[4].

The correlation between the values determined by HPLC and GLC is shown in Fig. 5. The

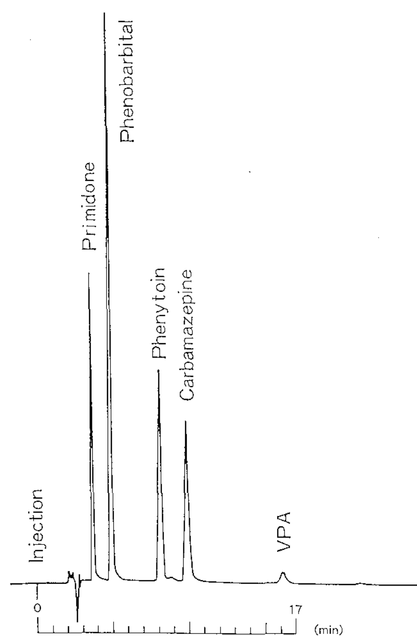

Fig. 4 Separation of 5 anticonvalsants by HPLC
Five anticonvalsants were dissolved in standard serum and extracted by the method described in the text.

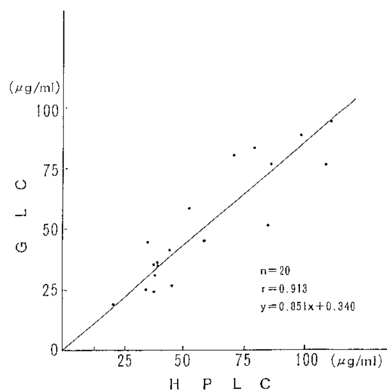

Fig. 5 Correlation between GLC and HPLC

correlation curve has been calculated to be Y=0.815X + 0.340 with a correlation coefficient of 0.913 (n=20).

VPA levels in the plasma of patients with seizure disorders

Using this assay procedure, the plasma levels of VPA were estimated in 7 patients with seizures due to brain tumors. The plasma samples were collected in the early morning before drug administration and 4 and 8 hours after drug administration. The results are summarized in Fig. 6. The mean value of the elimination half-life ($T_{1/2}$) was 15.46 ± 5.69 hours.

Discussion

Many assays for VPA have been reported using HPLC, GLC, EIA and other methods. However, for determining clinical dosages of VPA, the assay method should be simple and rapid. For this purpose, assay procedures using HPLC, but not requring esterification of VPA, have been developed.

The method described here was simplified in that only one step was required for preparation of the blood sample before application to to HPLC, and the retention time of VPA was estimated to be 15~16 min. Therefore, the result was obtained within approximately 30 min from the time collection of the blood sample, making it easy to determine the drug regimen in a clinical field.

In addition, the recovery of VPA from standard serum I and II has been calculated to be 91.8% and

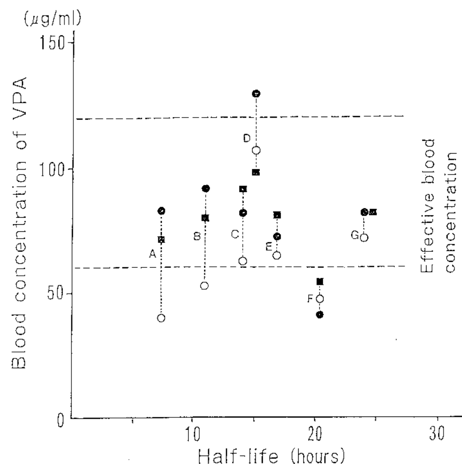

Fig. 6 Concentration and half-life of VPA in the patients ○: the concentration before drug administration, ●: the concentration 4 hours after drug administration, ■: the concentration 8 hours after drug administration.

101.5%, respectively, and the correlation coefficients between the concentration obtained from HPLC and either EIA or GLC have been estimated to be 0.955 (n=26) or 0.913 (n=20), respectively.

On the other hand, for therapeutic purposes, a combination of anticonvulsants is generally applied to the treatment of patients with seizures. With this method, primidone, phenobarbital, phenytoin, carbamazepine and VPA were completely separated on HPLC as shown in Fig. 4. Therefore, the method described in this communication is sufficient for assay of VPA in serum of speed, specificity and accuracy.

In addition, the clinical trials in 7 patients with seizures conformed that the drug concentration was ranged in the therapeutic concentration of VPA during the administration of this drug.

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エステル化を行わない血中バルプロ酸の HPLC による迅速測定法

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抗てんかん薬である，バルプロ酸（VPA）の血中濃度の測定には，従来よりガスクロマトグラフィー（GLC）等を使用し，エステル化などの煩雑な前処理を行ってきた．そのため，日常検査としては大変な困難があり，今回我々は高速液体クロマトグラフィー（HPLC）を用いて，この前処理を必要としない VPA の迅速測定法を確立した．また，この HPLC 法により日常の血中薬物濃度のモニタリングに使用が可能であることを確認することができた．即ち，血漿（血清）200μl にアセトニトリル（AcCN）200μl を加え 1：1 とする．混和後8,000×g，5 分間遠心分離を行い，上清を HPLC に充填する．移動相溶媒は AcCN：水（30：70，%；V/V）で 1 分間に，1.0ml 流速にて行い，これを UV 214nm によって検出する．

その結果 GLC，ELA 法との相関係数もそれぞれ，r=0.913，r=0.955と良好であった．また 7 名のバルプロ酸投与患者の血中 VPA 動態を観察したところ，大部分の投与患者血中濃度が50～120μg/ml の範囲で推移していた．