Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography B, 830 (2006) 368–371
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

Development and validation of a sensitive assay of valproic acid in human plasma by high-performance liquid chromatography without prior derivatization

Hossein Amini [a,*], Mohammad Javan [b], Abolhassan Ahmadiani [a]

[a] *Department of Pharmacology, Neuroscience Research Center, Shaheed Beheshti University of Medical Sciences, P.O. Box 19835-355, Tehran, Iran*

[b] *Department of Physiology, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran*

Received 11 September 2005; accepted 11 November 2005
Available online 1 December 2005

Abstract

Sensitive and selective determination of valproic acid in plasma by high-performance liquid chromatography (HPLC) is usually achieved with pre-column derivatization. In the present work, the derivatization is omitted due to using a simple but highly selective plasma extraction procedure and an optimized chromatographic condition. Valproic acid and the internal standard octanoic acid were extracted from plasma samples with *n*-hexane under acidic condition followed by back-extraction into diluted triethylamine. Chromatography was performed on a CN column (250 × 4.6 mm, 5 μm) under isocratic elution with acetonitrile–40 mM aqueous sodium dihydrogen phosphate (30:70, v/v), pH 3.5. Detection was made at 210 nm and analyses were run at a flow-rate of 1 ml/min. The method was specific and sensitive with a quantification limit of 1.25 μg/ml and a detection limit of 0.1 μg/ml in plasma. The mean absolute recovery for valproic acid using the present plasma extraction procedure was 75.8%. The intra- and inter-day coefficient of variation and percent error values of the assay method were all in acceptable range. Calibration curves were linear ($r > 0.999$) from 1.25 to 320 μg/ml in plasma.

Keyword: Valproic acid

1. Introduction

Valproic acid (2-propylpentanoic acid) is a simple eight-carbon branched-chain fatty acid with unique anticonvulsant properties against several types of epileptic seizures [1]. Due to the widespread usage of valproic acid as an antiepileptic drug, there is a constant need for simple and specific methods for the determination of this substance in patients' plasma. Since valproic acid is volatile, it is mostly determined by gas chromatography (GC) without derivatization or following alkylation [2]. High-performance liquid chromatography (HPLC) is an attractive alternative to GC for the analysis of most drug, however, its application for the analysis of valproic acid in plasma is limited by the fact that the drug has no suitable chromophore. The very non-specific absorption, where substantial UV absorption occurs, makes it difficult to develop a specific, selective and sensitive HPLC–UV method, particularly when using complex matrices such as biological fluids. Only a few HPLC methods with UV detection at 210 nm are available for valproic acid [3,4] based on deproteination with acetonitrile, which in turn leads to decreased sensitivity and selectivity. It could be suggested that a more specific extraction of valproic acid from plasma would improve HPLC determination of the drug. There are existing HPLC methods that offer sufficient sensitivity and selectivity but require prior derivatization of valproic acid to add either a chromophore or a fluorophore [5–10]. Chromatography of valproic acid without prior derivatization would significantly simplify the method and thus shorten the analysis time.

This paper describes a simple, rapid and efficient (in term of recovery and removal of interferences) liquid–liquid extraction procedure for valproic acid from plasma. The method allows determination of valproic acid at low concentrations without the need for prior derivatization.

* Corresponding author. Fax: +98 21 22403154.
E-mail address: hamini@sbmu.ac.ir (H. Amini).

1570-0232/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2005.11.028

2. Experimental

2.1. Reagents

Valproic acid (purity of 98%) and octanoic acid (99%) were from Aldrich-Chemie (Steinheim, Germany). Analytical grade phosphoric acid, sodium dihydrogen phosphate, triethylamine and HPLC grade methanol were purchased from E. Merck (Darmstadt, Germany). HPLC grade acetonitrile and analytical grade *n*-hexane was obtained from Carlo Erba Reagenti (Rodano, Italy). All other reagents were of analytical grade.

2.2. Instrumentation

The analyses were performed on a Shimadzu chromatographic system (Kyoto, Japan) equipped with an LC-6A solvent delivery pump, SPD-10AVP ultraviolet detector (operated at 210 nm) and C-R8A integrator. The samples were applied by a Rheodyne 7725 loop injector with an effective volume of 100 μl. A Shimpack CLC-CN column (250 × 4.6 mm i.d.; 5 μm particle size) was used for the chromatographic separation. The mobile phase comprised of acetonitrile–40 mM aqueous sodium dihydrogen phosphate (30:70, v/v), pH 3.5. Analyses were run at flow rate of 1 ml/min at ambient temperature.

2.3. Standard solutions

The octanoic acid (internal standard) was dissolved in methanol to produce a final concentration of 1 mg/ml. Standard stock solution of valproic acid was prepared in methanol by dissolving 800 mg of free acid in 50 ml of methanol and stored at −5 °C. Working standard solutions for valproic acid were prepared daily in methanol producing 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 and 0.0625 mg/ml.

2.4. Calibration curves and quantitation

Plasma standards for calibration curves were prepared by spiking different samples of 1 ml drug-free plasma each with 20 μl of one of the above mentioned valproic acid working standards to produce 320, 160, 80, 40, 20, 10, 5, 2.5 and 1.25 μg/ml. They were shaked for 2 min, and then stored at least 15 min at room temperature before use. The prepared calibration standards and quality control standards (250 μl) were pipetted into 4 ml polypropylene tubes and stored at −20 °C pending analysis. In each run, a plasma blank sample was also analyzed, but results for blank samples were not used as part of the calibration curves.

Calibration curves were constructed by plotting peak height ratio (y) of valproic acid to the internal standard versus valproic acid concentrations (x). A linear regression was used for quantitation.

2.5. Extraction procedure

A 250 μl volume of plasma was transferred to a 4 ml polypropylene tube (10 mm × 70 mm). The internal standard (25 μl, equal to 25 μg of octanoic acid in methanol) was added and vortex-mixed for 10 s. Extraction was performed by adding 50 μl of 1M H_3PO_4 and 3.5 ml of *n*-hexane and vortex-mixing for 2 min. After centrifugation at 12,000 × *g* for 3 min, the whole organic layer was separated and transferred into another tube. Then, 100 μl of 0.5% triethylamine in water was added. The mixture was vortex-mixed for 2 min. After waiting for 30 s, some of the upper organic phase was discarded and the remaining mixture (about 1 ml) was transferred into a 1.5 ml microcentrifuge tube. After centrifugation at 11,300 × *g* for 2 min, the upper organic phase was discarded completely. Finally, a volume of 50 μl of aqueous phase was injected into the chromatograph.

2.6. Assay validation

The specificity of the method was evaluated by comparing the chromatograms obtained from the samples containing valproic acid and internal standards with those obtained from blank samples. Additionally, plasma sample spiked with various other drugs were prepared and investigated for their possible interferences. Besides calibration standards, additional standards were prepared for the determination of intra-day ($n = 5$) and inter-day ($n = 5$) of the assay accuracy and precision. The absolute recoveries ($n = 5$) was calculated by comparing peak heights obtained from prepared sample extracts with those found by direct injection of drug solution made in 0.5% triethylamine in water at the same concentration.

The limit of quantification (LOQ) was estimated by analyzing valproic acid at low concentrations of the calibration curves. The LOQ was defined as a concentration level where accuracy and precision were still better than 10%. To determine the limit of detection (LOD), lower plasma concentrations than the lower end of the calibration curves were used. The LOD was then defined as the concentration, which caused a signal three times the noise ($S/N = 3/1$).

3. Results and discussion

3.1. Sample preparation

Valproic acid could be extracted from plasma into various organic solvents under acidic condition. However, *n*-hexane, which is a weak solvent for most drugs was preferred to prevent co-extraction of other drugs and endogenous interferences from plasma. Both phosphoric and hydrochloric acid were suitable for plasma acidification. Using these acids, valproic acid could be extracted from plasma into *n*-hexane even at pH 5 with absolute recovery of 50%, but very poor recovery was obtained with acetic acid at the same or lower pH. Therefore, the choice of proper acid is also important.

One major problem associated with valproic acid is the critical loss of sample during sample concentration steps. Possible unequal losses of valproic acid during evaporation of extraction solvent can cause large variations in the measurements. To circumvent concentration by evaporation, several methods [5,11,12] use a small volume of extraction solvent and inject it directly. However, in reversed-phase chromatography direct

injection of water immiscible solvent can cause serious problems. Back-extraction into a small volume aqueous solution is the best way to concentrate valproic acid while it can also offer more selective extraction and cleaner chromatogram. Although, back-extraction into 0.5 M sodium hydroxide has been proposed [13,14], it must be noticed that direct injection of sodium hydroxide solution with such a high concentration can destroy injector and column. We first tried to back-extract valproic acid from *n*-hexane into 0.01–0.05 M sodium hydroxide solutions, but it was not successful. We evaluated several alternatives to sodium hydroxide solution and observed that diluted triethylamine solution can effectively extract valproic acid from *n*-hexane. Interestingly, although 0.5% of triethylamine in water had a pH of about 8, it was decreased to the pH of 6 after back-extraction of plasma extract. This observation may be explained that most of triethylamine goes to *n*-hexane phase because of better solubility in it, but the complex of valproic acid with triethylamine prefers to be in aqueous phase.

3.2. Chromatographic condition

3.2.1. Chromatographic column and mobile phase

We first tried C8 and C18 columns with acetonitrile–phosphate buffer as mobile phase. There were several plasma interferences close to the retention times of valproic acid and the internal standard. We evaluated different mobile phases and changed buffer concentration, pH values and organic to aqueous phase ratio, but all these efforts did not give satisfactory results. We finally overcame this problem by using a CN column, which showed superior separation results than other tested columns. A simple buffered acetonitrile mobile phase under isocratic elution on CN column was found appropriate for separation of valproic acid and internal standard from plasma interferences.

3.2.2. Injection solvent

The sharp and narrow peak for valproic acid and internal standard was obtained when they were injected in very dilute triethylamine solutions, but peak broadening was observed when injecting the analytes dissolved in methanol. The peak broadening could be due to the fact that methanol has a higher solvent strength than the mobile phase, leading to defocusing of the peaks at the start of the chromatographic process.

3.3. Method validation

Representative chromatograms of drug-free plasma, plasma spiked with valproic acid at LOQ and a high concentration, and a patient sample are shown in Fig. 1. The retention times for valproic acid and internal standard were 8.5 and 9.9 min, respectively. No interfering peaks from the endogenous plasma components were observed in the retention time of valproic acid or internal standard. All analyzed patients samples showed an additional peak at 7.4 min, which could be one of the metabolites of valproic acid. A number of drugs were added to blank plasma at sufficient concentrations and these samples were analyzed according the present method. They include: mefenamic acid; indomethacin; sodium diclofenac; naproxen; sodium benzoate; sodium salicylate; phenytoin; primidone; ethosuximide; carbamazepine; theophylline; ciprofloxacin; ofloxacin; acetaminophen; codeine; dextromethorphan; ranitidine; cime-

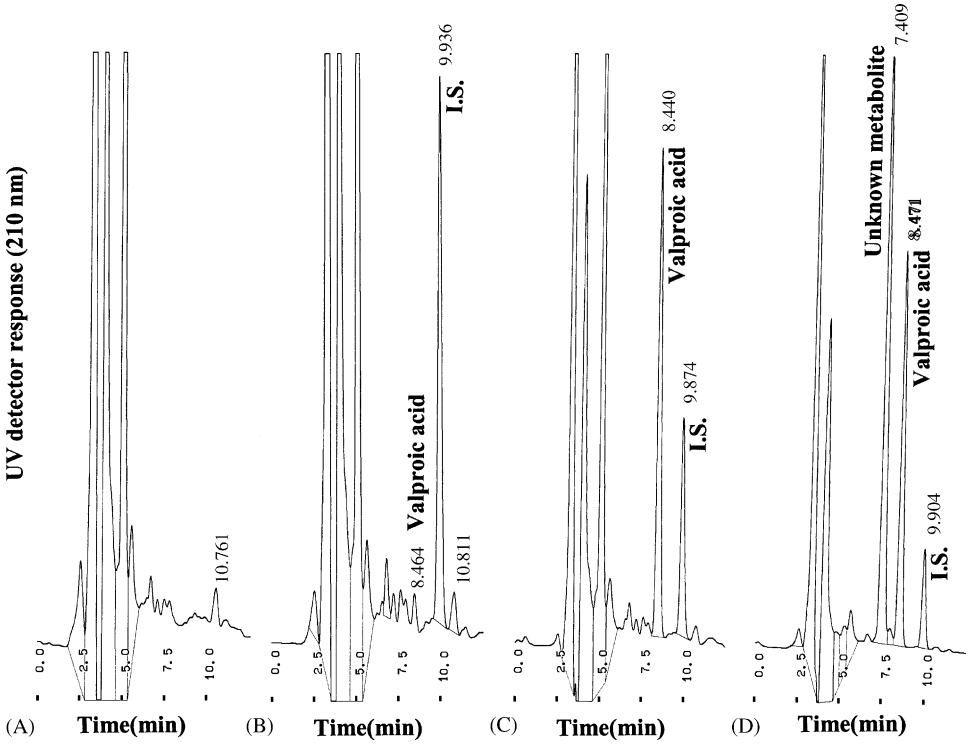

Fig. 1. Representative chromatogram of: (A) a blank plasma, attenuation = 2; (B) plasma spiked with 1.25 μg/ml valproic acid, attenuation = 2; (C) plasma spiked with 80 μg/ml valproic acid, attenuation = 3 and (D) a sample from an epileptic patient containing 166.7 μg/ml valproic acid, attenuation = 4.

Table 1
The intra- and inter-day precision and accuracy, and recovery data for the measurement of valproic acid in human plasma ($n = 5$)

Nominal concentration (μg/ml)	Recovery (%)	Intra-day			Inter-day		
		Mean ± S.D.	Precision (%)	Accuracy (%)	Mean ± S.D.	Precision (%)	Accuracy (%)
1.25	78.9 ± 6.4	1.13 ± 0.05	4.4	−9.6	1.27 ± 0.11	8.7	1.6
5	74.2 ± 3.8	5.1 ± 0.15	2.9	2	4.9 ± 0.38	7.8	−2
80	73.4 ± 5.1	78.4 ± 1.55	2	−2	77.3 ± 2.35	3	−3.4
320	76.5 ± 4.7	318.6 ± 8.16	2.6	−0.4	327.5 ± 6.72	2.1	2.3

tidine; famotidine; diltiazem; verapamil; caffeine; amlodipine; cyclosporine; metformin; ketoconazole; clonazepam; diazepam; acyclovir; loratadine; moclobemide and losartan. None of them interfered with the valproic acid assay.

The calibration curves were linear over the concentration range of 1.25–320 μg/ml in human plasma, with a correlation coefficient greater than 0.999. The LOQ was 1.25 μg/ml and the LOD was about 0.1 μg/ml. The values obtained for intra-day and inter-day precision and accuracy during the 5-day validation for plasma are shown in Table 1. All values for accuracy and precision were within recommended limits. The mean absolute recoveries for valproic acid and internal standard using the present extraction procedure were 75.8 and 55.2%, respectively.

Acknowledgements

We wish to thank Dr. Abbas Kebriaeezadeh (Department of Pharmacology and Toxicology, Faculty of Pharmacy and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran, Iran) for kindly donating octanoic acid to us.

References

[1] W. Loscher, Prog. Neurobiol. 58 (1999) 31.
[2] J.T. Burke, J.P. Thénot, J. Chromatogr. 340 (1985) 199.
[3] K. Kushida, T. Ishizaki, J. Chromatogr. 338 (1985) 131.
[4] L.J. Lovett, G.A. Nygard, G.R. Erdmann, C.Z. Burley, S.K.W. Kalil, J. Liq. Chromatogr. 10 (1987) 687.
[5] M.C. Lin, H.S. Kou, C.C. Chen, S.M. Wu, H.L. Wu, J. Chromatogr. B 810 (2004) 169.
[6] S. Rompotis, M. Parissi-Poulou, E. Gikas, M. Kazanis, A. Vavayannis, I. Panderi, J. Liq. Chromatogr. Rel. Technol. 25 (2002) 2833.
[7] R. Alric, M. Cociglio, J.P. Blayac, R. Puech, J. Chromatogr. 224 (1981) 289.
[8] S. Hara, M. Kamura, K. Inoue, M. Fukuzawa, N. Ono, T. Kuroda, Biol. Pharm. Bull. 22 (1999) 975.
[9] H. Liu, L.J. Montoya, L.J. Forman, C.M. Eggers, C.F. Barham, M. Delgado, Ther. Drug Monit. 14 (1992) 513.
[10] C. Lucarelli, P. Villa, E. Lombaradi, P. Prandini, A. Berga, Chromatographia 33 (1992) 37.
[11] H.J. Leis, W. Windischhofer, G.N. Rechberger, G. Fauler, J. Chromatogr. B 784 (2003) 69.
[12] D.J. Hoffman, W.R. Porter, J. Chromatogr. 276 (1983) 301.
[13] L.J. Dusci, L.P. Hackett, J. Chromatogr. 137 (1977) 145.
[14] C.J. Jensen, R. Gugler, J. Chromatogr. 137 (1977) 188.