Research article

A New High-Throughput LC-MS/MS Assay for Therapeutic Level Monitoring of Valproic Acid in Human Plasma

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Abstract

A new high-throughput liquid chromatographic tandem mass spectrometric (LC-MS/MS) assay for the quantification of valproic acid in human plasma was developed and validated. The separation was performed on a Zorbax SB-C18 column under isocratic conditions using a 48:52 (v/v) mixture of acetonitrile and 0.1% (v/v) acetic acid in water at 45 °C with a flow rate of 0.8 mL/min. The detection of valproic acid was performed in SIM mode (m/z 143.1). The human plasma samples (0.2 mL) were deproteinized with methanol and aliquots of 2 μL from supernatants obtained after centrifugation were directly injected into the chromatographic system. The method shows a good linearity (r > 0.9972), precision (CV > 7.8 %) and accuracy (bias > 5.7 %) over the range of 5-200 μg/mL plasma. Lower limit of quantification (LLOQ) was 5 μg/mL and the recovery was between 98-106 %. The method is not expensive, it needs a minimum time for plasma sample preparation and has a run-time of 2.4 min for instrument analysis (retention time of valproic acid was 1.8 min). The developed and validated high-throughput method is very simple, rapid and efficient, with wide applications in clinical level monitoring, pharmacokinetics and bioequivalence studies.

Keywords

Valproic acid • LC-MS/MS • Therapeutic drug monitoring • High-throughput assay

Introduction

Valproic acid (VA; 2-*n*-propylpentanoic acid – Fig. 1) is an anticonvulsant drug, commonly used in the treatment of many forms of epilepsy and many types of seizures, affecting both children and adults. It is also used to treat maniac reactions in bipolar disorder and to prevent migraine headaches. VA and its sodium salt (valproate sodium) are very well absorbed after oral administration and produce peak plasma levels in 1 to 4 hours (15 minutes to 2 hours with the syrup). VA is extensively bound (≥ 90%) to plasma proteins. Over 96% is metabolized mainly in the liver to at least 10 metabolites, who are excreted primarily through the kidneys. Only <3% of VA is excreted unchanged in urine. The therapeutic plasma levels are between 50–120 mg/L and it is recommended to be below 125 mg/L to minimize adverse effects. Therefore, periodic plasma level monitoring is very important both for successful therapy and for evaluating potential drug interactions and adverse effects of VA [1, 2].
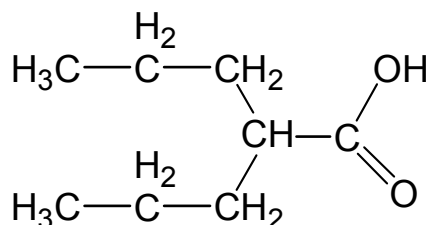
Fig. 1. Chemical structure of valproic acid.

Several methods involving immunoassay [3], gas-chromatography (GC) [4–6] and high-performance liquid-chromatography (HPLC) [6–9] have been reported to determine therapeutic levels of VA in plasma.

VA is a volatile compound. Being an acid, it shows peaks with considerable tails on the GC chromatograms. This inconvenient was usually eliminated by derivatization with different reagents as N-trimethylsilyl-N-methyl-trifluoroacetamide [4], pentafluorobenzyl bromide [5], 2-(2-naphthoxy)ethyl-2-(piperidino)ethane-sulfonate [6].

An alternative at GC assays is HPLC analysis. VA shows a weak optical absorption in ultraviolet range, for this reason only a few HPLC methods used direct analysis of VA [9, 10]. For improving the sensitivity, the most HPLC methods include the conversion of VA into derivatives exhibiting stronger absorption in UV, such as bromophenacyl ester [7], or into fluorescent compounds, such as 4-bromomethyl-6,7-dimethoxycoumarin derivate [8], 6,7-methylendioxy-1-methyl-2-oxo-1,2-dihydro-quinoxaline-3-yl propionohydrazide derivate or N-(7-methoxy-4-methyl-2-oxo-2H-6-chromenyl)-2-bromoacetamide derivate [6]. Because VA is strongly bound to proteins, most methods involve primarily precipitation of proteins, followed by extraction of VA in organic solvents and derivatization. Generally, VA is isolated by liquid-liquid extraction (LLE) [8, 9, 11] or solid-phase extraction (SPE) [12–14] from acidic medium. Both extraction and derivatization are time-consuming steps, increase the cost of assay and can affect the recovery. Moreover, critical loss of VA by volatilization is possible during sample concentration by evaporation of extraction solvent.

Recently, the LC-MS/MS assay offers considerable advantages by its powerful performances: speed, selectivity, sensitivity and robustness. Sample preparation is more simple and rapid and often includes precipitation of proteins (PP) [15] and/or extraction [11–14] before chromatographic analysis.

The aim of this work was to develop and validate a new simple and efficiently high throughput LC-MS/MS assay for quantification of VA in human plasma in clinical level monitoring.

Results and Discussion

We propose a very simple and rapid pretreatment of plasma samples including only PP by methanol and direct injection into chromatographic system from supernatant obtained after centrifugation. Pucci et al. [15] have also determined VA by LC-MS/MS after PP from mouse plasma and have obtained a good sensitivity (lower limit of quantification – LLOQ was 150 ng/mL). However, as the therapeutic plasma levels of VA are generally between 50–120 µg/mL [1, 2], the LLOQ of 5 µg/mL established in our method can be accepted in routine purposes for therapeutic drug monitoring of VA in human plasma.

Several researchers prefer to include in plasma sample preparation an extraction step to eliminate the impurities and to increase the sensitivity. But this operation increases the time of analysis, increases the costs and can affect the recovery. In our method, the sample preparation is very simple and rapid and offers a shorter time of analyses and a lower cost in comparison with the other longer methods which used extraction prior to LC assays. Therefore, Matsuura et al. [14] have determined VA from plasma by LC-MS/MS after SPE and they obtained an LLOQ of 0.5 µg/mL and a recovery of only 84%. Jain et al. [13] have also reported an LC-MS/MS method using an SPE of VA prior to LC analysis with an LLOQ of 2 µg/mL and a good recovery of 99.73%. Cheng et al [11] have obtained a similar sensitivity of 2 µg/mL for VA by HPLC-MS/MS after LLE, but they have performed a pre-column derivatization of VA using 4-dimethylaminobenzylamine dihydrochloride (at 37°C for 1 h) to increase the sensitivity of method, the time of plasma sample preparation being longer.

LC-MS/MS assay

The chromatographic conditions, especially the composition of mobile phase, were optimized in several trials to achieve a good MS signal, a short retention time of VA and consequently a high-throughput analysis. The best results were obtained with the mixture of acetonitrile and 0.1% (v/v) acetic acid in water (48:52, v/v) under isocratic conditions. In the selected chromatographic conditions the retention time of VA was 1.8 min and the analytical run-time was 2.4 min. Matsuura et al. [14] have also obtained a good run-time of 3 min with a retention time of VA of 1.2 min, but using a non-porous silica column and after SPE of VA from plasma matrix. The method reported by Jain et al. [13] is a high-throughput method too, with a run-time of 1.8 min and a retention time of VA of 1.41 min. They also have included a SPE step prior LC-MS/MS assay.

The electrospray source ionization (ESI) of VA produced the [M-H]$^-$ ion at 143.1 (m/z) under negative ion mode. No marked fragment ions were observed in the MS/MS spectra

while variations in the collision energy. Therefore, the detection of VA was performed in selected ion monitoring (SIM) mode.

Assay validation

The method was validated in accordance with international regulations [16, 17]. Representative chromatograms of drug-free plasma and plasma spiked with VA at LLOQ are shown in Fig. 2. No interfering peaks from the endogenous plasma components were observed in the retention time of VA.

The calibration curves were linear over the concentration range of 5.1 – 204.0 µg/mL in human plasma, with a correlation coefficient greater than 0.9972. The LLOQ was 5.1 µg/mL. The values obtained for intra-day and inter-day precision and accuracy during the validation for plasma are shown in Tab. 1 and Tab. 2, respectively. All values for accuracy and precision were within recommended limits. The recovery values were between 98-106 %, which means no loss of analyte during sample preparation due to its volatility or adsorption on precipitated proteins. VA showed good stability in plasma at room temperature for 4 h (CV of 6.4% at lower level and -2.1% at higher level, respectively) and a good post-preparative stability in autosampler for at least 24 hours at 25°C before the chromatographic assay (CV < 15%).
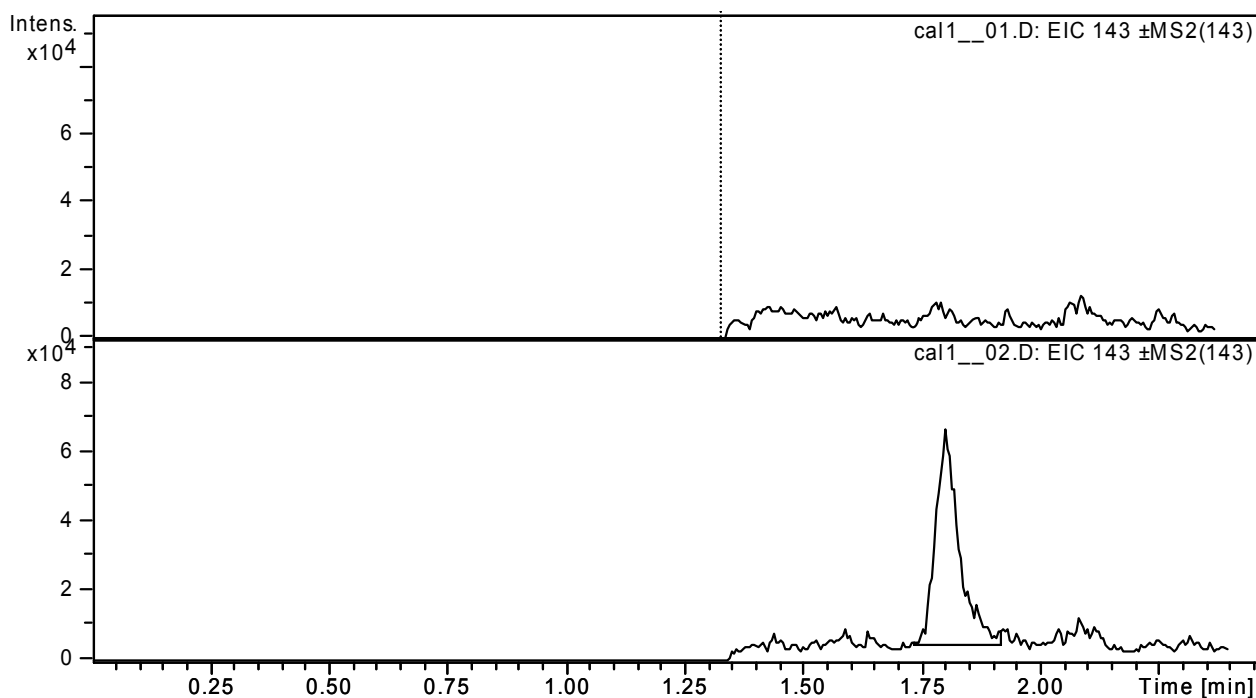
Fig. 2. Representative chromatograms of (up) blank plasma and (down) plasma spiked with valproic acid at lower limit of quantification (5.1 µg/mL).

Tab. 1. The intra-day precision (CV %) and accuracy (bias %) and recovery data for the measurement of valproic acid in human plasma (n = 5):

Nominal concentration (µg/mL)	Found concentration mean µg/mL	± SD	CV (%)	Bias (%)	Recovery (%)	± SD
5.100	5.128	0.550	10.7	0.5	106.4	12.2
12.750	13.158	0.412	3.1	3.2	102.1	3.3
81.600	80.717	3.859	4.8	−1.1	101.1	4.9
163.200	155.082	7.022	4.5	−5.0	102.3	4.6

Tab. 2. The inter-day precision (CV %) and accuracy (bias %) and recovery data for the measurement of valproic acid in human plasma (n = 5):

Nominal concentration (µg/mL)	Found concentration mean µg/mL	± SD	CV (%)	Bias (%)	Recovery (%)	± SD
5.100	5.210	0.754	14.5	2.1	103.4	6.9
12.750	13.472	1.047	7.8	5.7	98.5	5.5
81.600	79.068	3.465	4.4	−3.1	102.3	4.5
163.200	154.973	3.874	2.5	−5.0	99.0	7.4

Our developed LC-MS/MS assay is simple, rapid, accurate and not expensive. In comparison with other published LC-MS/MS assays [11–14] for VA level monitoring in human plasma our method performs better in terms of speed (both sample preparation and chromatographic run-time) and costs, which are essential attributes for methods used in routine analysis. The method was validated over the concentration range of 5.1–204 µg/mL which cover both therapeutic (50–120 µg/mL) and toxic (>125 µg/mL) plasma levels of VA. This high-throughput method can be successfully applied in clinical level monitoring of VA, but it can have wide applications in pharmacokinetics and bioequivalence studies too.

Experimental

Reagents

Valproic acid (VA) was reference standard from SynFine Research (Ontario, Canada). Acetonitrile (ACN) of HPLC-grade and methanol and acetic acid of analytical-reagent grade were purchased from Merck KGaA (Darmstadt, Germany). Bidistilled, deionised water pro injections was purchased from Infusion Solution Laboratory of University of Medicine and Pharmacy Cluj-Napoca (Romania). The human blank plasma was supplied by the Bleeding Centre Cluj-Napoca (Romania) from the healthy volunteers, men and women.

Apparatus

The following apparatus were used: 204 Sigma Centrifuge (Osterode am Harz, Germany);

Analytical Plus and Precision Standard Balance (Mettler-Toledo, Switzerland); Vortex Genie 2 mixer (Scientific Industries, New York, USA); Ultrasonic bath Elma Transsonic 700/H (Singen, Germany). The HPLC system used was an 1100 series Agilent Technologies model (Darmstadt, Germany) consisting of a G1312A binary pump, an in-line G1379A degasser, an G1329A autosampler, a G1316A column thermostat and an Agilent Ion Trap Detector 1100 VL.

Chromatographic and spectrometric conditions

Chromatographic separation was performed on a Zorbax SB-C18 (100 mm x 3.0 mm i.d., 3.5 μm) column (Agilent Technologies) under isocratic conditions using a mobile phase of a 48:52 (v/v) mixture of acetonitrile and 0.1% (v/v) acetic acid in water at 45°C and a flow rate of 0.8 mL/min. The detection of VA was in the selected ion monitoring (SIM) mode (monitored ion: m/z 143.1) using an ion trap mass spectrometer equipped with an atmospheric pressure electrospray ionisation ion source (capillary 4000 V, nebulizer 70 psi (nitrogen), dry gas nitrogen at 12 L/min, dry gas temperature 350°C).

Standard solutions

A stock solution of VA (15 mg/mL) was prepared by dissolving an appropriate quantity of VA in acetonitrile. A working solution (0.510 mg/mL) was prepared by appropriate dilution in drug-free human plasma. This solution was used to prepare plasma standards with the concentrations of 5.1, 10.2, 20.4, 30.6, 45.9, 91.8, 153.0 and 204.0 μg/mL. Quality control (QC) samples of 12.8 μg/mL (lower), 81.6 μg/mL (medium) and 163.2 μg/mL (higher) were prepared by adding the appropriate volumes of working solution to drug-free human plasma. The resultant plasma standards and quality control standards were pipetted into 15 mL polypropylene tubes and stored -20°C until analysis.

Sample preparation

Standards and plasma samples (0.2 mL) were deproteinized with methanol (0.6 mL). After vortex-mixture (10 s) and centrifugation (6 min at 5000 rpm), the supernatants (0.15 mL) were transferred in autosampler vials and 2 μL were injected into the HPLC system.

Method validation

The specificity of the method was evaluated by comparing the chromatograms obtained from the plasma samples containing VA with those obtained from different plasma blank samples (n=6).

The concentration of VA was determined automatically by the instrument data system using pick areas and the external standard method. The calibration curve model was determined by the least squares analysis: $y = b + ax$, weighted $(1/y)$ linear regression, where y - peak area and x - analyte concentration.

The intra-day precision (expressed as coefficient of variation, CV %) and accuracy (expressed as relative difference between obtained and theoretical concentration, bias %) were determined by the analysis on the same day of five different samples (n = 5) from each QC standards (at lower, medium and higher levels). The inter-day precision and accuracy were determined by the analysis on five different days (n = 5) of one sample from each QC standards (at lower, medium and higher levels).

The lower limit of quantification (LLOQ) was established as the lowest calibration standard with an accuracy and precision less than 20%.

The absolute recoveries were measured by comparing the response of VA from spiked plasma samples with the response of VA from a standard solution with the same analyte concentration, prepared in mobile phase and processed in the same manner with plasma sample. The absolute recovery was evaluated for quantification limit and also for each QC level.

The VA stability in plasma at lower and higher levels (n=5) was investigated. For the room-temperature stability (RTS) study, the samples were prepared and kept for 4 h at room temperature, then they were processed and analyzed by HPLC. For the post-preparative stability (PPS) study, the samples were prepared, processed and kept for 24 h in the autosampler of the HPLC system at 25°C before the chromatographic analysis. The requirement for the stability of drug is that the difference between mean concentrations of the tested samples in various conditions and nominal concentrations was in ±15% range.

Authors' Statement

Competing Interests

The authors declare no conflict of interest.

Acknowledgements

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