Determination of Valproic Acid in Human Plasma by High-Performance Liquid Chromatography with Mass Spectrometry Detection

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Background: Free valproic acid is shows no characteristic absorption in the ultraviolet region (above 235 nm), therefore its direct quantification and also the quantification of the corresponding metabolites from human plasma has proven to be challenging. Aim: The aim of our study was to develop and validate an effective LC-MS method for the determination of valproic acid in human plasma without using solid phase extraction as sample preparation, with a short analysis time and high sensitivity.
Materials and methods: Valproic acid was analyzed on a reversed - phase column (Zorbax SB - C18, 100 mm x 3 mm I.D., 3.5 µm) under isocratic conditions using a mobile phase of a 40:60 (v/v) mixture of acetonitrile and 0.1% (v/v) acetic acid in water. The flow rate was 1 mL/min and the column temperature 45 ºC. In these chromatographic conditions, the retention time was 2.3 minute for valproic acid. The detection of the analyte was in single ion monitoring mode using a triple quadrupole mass spectrometer with electrospray negative ionization. The monitored ion was 143.1 m/z derived from 144.2 m/z valproic acid. The sample preparation was very simple and consisted in plasma protein precipitation from 0.2 mL plasma using 0.6 mL methanol.
Results: Calibration curves were generated over the range of 2–200 µg/mL with values for coefficient of determination greater than 0.996 and by using a weighted (1/x) quadratic regression. The values of precision and accuracy for valproic acid at quantification limit were less than 3.3% and 7.2%, for within- and between-run assays, respectively. The mean recovery of the analyte was 104%. Valproic acid samples demonstrated good short-term, post-preparative and freeze-thaw stability.
Conclusion: The method is very simple and allows obtaining a very good recovery of the analyte. The validated LC-MS/MS method could be applied to pharmacokinetics and therapeutic drug monitoring study regarding valproic acid in humans.

Keywords: valproic acid, LC-MS, human plasma, therapeutic drug monitoring, pharmacokinetics

Introduction

Valproic acid is an effective anticonvulsant used in the management of grand mal epilepsy and petit mal epilepsy, often with other adjunctive therapeutic agents. The monitoring of valproic acid serum concentrations is especially required because the pharmacokinetics is highly variable; protein binding depends on concentration following therapeutic dose, and complex interactions between valproic acid and other drugs have been observed. The drug monitoring determination methods need to be rapid, simple and have enough sensibility [1].

Entering the digestive system, valproic acid is liberated from its sodium salt (sodium 2-propylpentanoate), henceforward it dissociates in the circulation to the valproate ion.

Free valproic acid is volatile and liquid, shows no characteristic absorption in the ultraviolet region (above 235 nm), therefore its direct quantification and also the quantification of the corresponding metabolites from human plasma has proven to be challenging. Chemical derivatization methods have been established and used for quantification by gas- or liquid chromatographic determinations of valproic acid in attempts to increase the sensitivity. Several derivatives of phenacyl, including trimethylsilyl, tertbutyldimethylsilyl and 9-aminophenanthrene have been used in the determination of free valproic acid [2,3]. Therefore, analysis using UV or fluorescence detection and gas chromatography requires that valproate should be modified.

Liquid chromatography technique coupled with mass spectrometer detector (LC-MS) provides a more sensitive measurement, without chemical modification of the drug, combining chromatographic separation with a more efficient detection method.

In the analysis of biological samples the purification and preconcentration step plays a very important role, representing the key problem of the analysis and it is the most laborious and lengthy step of the entire analytical procedure. Therefore, it is essential to choose a very simple preprocessing technique for sample preparation.

Several methods were described for quantification of valproic acid in biological environments using LC-MS technique.

Mino et al [3] published in 2001 one of the first LC-MS method for determination of valproic acid in serum, working with atmospheric pressure chemical ionization (APCI), considered to be a soft ionization technique which protects parent ions. The concentration range is quite large (5–1000 µg/mL), the drug being extracted by solid phase extraction (SPE), but using only 20 µL serum sample.

Ramakrishna et al [4] in 2005 also used solid phase extraction for processing a quantity of 0.2 mL human plasma

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to obtain a quantification limit of 0.5 μg/mL. Although an analysis time of 4.5 min should be adequate for the analysis of several samples, their preparation is very laborious and time consuming. Furthermore, the upper limit of quantification chosen (60 μg/mL) does not cover the range of therapeutic plasma concentrations of valproic acid (50–100 μg/ml) [1].

Pucci et al [5] have also published a method in 2005, in which they determined valproic acid from plasma of mice with a limit of quantification of 0.15 μg/mL, using a semi-automatic technique for preparation of samples, based on protein precipitation, the most simple and rapid method of pretreatment. Analysis time of 4.5 min is comparable to that from the method mentioned above.

Over two years, Jain et al [1] have developed a fast and effective quantification method of valproic acid (2 min analysis time), using turbo ionspray ionization (TIS) and 0.2 ml of human plasma, with a lower limit of quantification (LLOQ) 2 μg/mL. Although they used a solid phase extraction, the method was applied to a bioequivalence study to analyze approx. 1800 samples in six days.

Cheng et al [2] throughout the same year quantified valproic acid and its major metabolite (4-ene valproic acid) in human plasma by chemical derivatization method with 4-dimethylamino-benzylamine, followed by liquid-liquid extraction, using MS/MS detection with positive electrospray ionization (ESI). The LLOQ was 0.2 μg/mL for valproic acid and 0.02 μg/mL for its metabolite. This method was the first which included the metabolite of valproic acid, with the disadvantage of a long analysis time (20 minutes).

An article published recently by Matsuura et al [6] presents a valid method for the determination of valproic acid, in 0.2 ml human plasma extracted with SPE technique, in the concentration range of 0.5–150 μg/mL and completed in 3 minutes.

A new high-throughput LC-MS/MS assay was developed by Vlase et al [7] using protein precipitation for sample pretreatment. The sensibility was 10 ppb analyte injected to the column.

The aim of our study was to develop and validate an effective LC-MS method for the determination of valproic acid in human plasma without using solid phase extraction as sample preparation, with a short analysis time and high sensitivity.

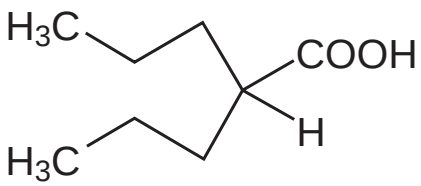

Fig. 1. Chemical structure of Valproic acid

Experimental

Reagents

Valproic acid (European Pharmacopoeia certified reference standard, Figure 1), sodium valproate (Sigma-Aldrich, Germany), acetonitrile, methanol (Merck KgaA, Darmstadt, Germany), acetic acid (Riedel De Haen). Ultrapure, deionised water was produced by a Millipore Direct Q5 (Millipore SA, Molsheim, France) water system. The human blank plasma was supplied from healthy volunteers.

2.2. Standard solutions

The stock solution of sodium valproate was prepared by dissolving the appropriate quantity of reference substance (weighed on an analytical balance from Mettler-Toledo AB54-S) in 10 mL acetonitrile, corresponding to a concentration of 1 mg/mL valproic acid. Eight calibration solutions with concentration between 10–1000 μg/mL valproic acid were then obtained by diluting specific volumes of stock solution with ultrapure water. Then these were used to spike 0.16 mL plasma blank, providing finally eight plasma standards with the concentrations ranged between 2 and 200 μg/mL. Accuracy and precision of the method was verified using plasma standards with concentrations of 6, 60 and 160 μg/mL. Quality control samples (QC) with the same concentrations 6 (QCA), 60 (QCB) and 160 (QCC) μg/mL of analyte were used during clinical samples analysis.

2.3. Chromatographic and mass spectrometry system and conditions

The HPLC system was an 1100 series model (Agilent Technologies) consisting in a quaternary pump, an in-line degasser, an autosampler, a column thermostat, and a G6410A model Triple Quad mass spectrometer detector (Agilent Technologies). Chromatograms were processed using MassHunter software.

The detection of the analyte was in the single ion monitoring mode (SIM) using an electrospray negative ionization (ESI negative). The monitored ion was 143.1 m/z derived from 144.2 m/z valproic acid (Figure 2). Other detector parameters: dry temperature 350°C, nebulizer 50 psi, voltage 4000 V, dry gas – nitrogen at 10 L/min.

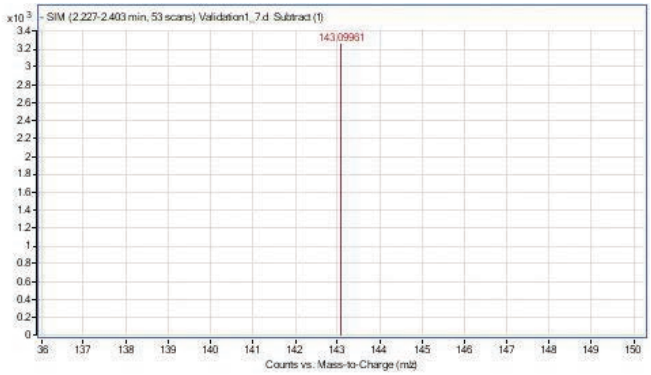

Fig. 2. MS spectra of Valproic acid (MS2 SIM mode) 143.1 m/z

Table I. Within-run accuracy and precision results

Level	LLOQ		QCA		QCB		QCC	
Replicate	2 µg/mL	%nom.	6 µg/mL	%nom.	60 µg/mL	%nom.	160 µg/mL	%nom.
1	1.85	92	7.3	123	69	114	154	96
2	1.75	88	6.5	108	66	109	163	102
3	1.79	89	6.7	111	64	107	171	107
4	1.69	85	6.4	106	65	108	164	103
5	1.74	87	6.5	108	65	108	162	101
Mean	1.76		6.7		66		163	
SD		0.05		0.4		2		6
Accuracy	88		111		110		102	
% CV		3.3		5.7		2.6		3.7

LLOQ = lower limit of quantification; QCA, QCB, QCC = quality control samples at three levels of concentration

Table II. Between-run accuracy and precision results

Level	LLOQ		QCA		QCB		QCC	
Replicate	2 µg/mL	%nom.	6 µg/mL	%nom.	60 µg/mL	%nom.	160 µg/mL	%nom.
1	1.76	88	6.7	111	66	110	163	102
2	1.79	89	6.4	107	64	107	151	94
3	1.70	85	6.0	100	62	104	149	93
4	1.98	99	6.3	104	61	102	148	93
5	1.99	99	6.7	112	63	105	146	91
Mean	1.84		6.4		63		151	
SD		0.13		0.3		2		7
Accuracy	92		107		106		95	
% CV		7.2		4.7		2.9		4.3

LLOQ = lower limit of quantification; QCA, QCB, QCC = quality control samples at three levels of concentration

Chromatographic separation was performed at 45°C on a Zorbax SB-C18 100 × 3 mm, 3.5 µm column (Agilent Technologies), protected by an in-line filter.

2.4. Mobile phase

The mobile phase consisted of a mixture of water containing 0.1% acetic acid and acetonitrile (60:40 v/v), each component being degassed, before elution, for 10 minutes in a Clifton 64426 ultrasonic bath. The pump delivered the mobile phase at 1 mL/min.

2.5. Sample preparation

Standard and test plasma samples were prepared just before being analyzed. In an Eppendorf tube 0.2 mL plasma was shaken for 30 seconds with 0.6 mL methanol, then centrifuged for 10 minutes at 10000 rpm. The supernatant was transferred in an autosampler vial and 2 µL were injected into the LC-MS system.

2.6. Validation

As a first step of method validation [8–10], specificity was verified using five different plasma blanks (normal, hyperlipemic and hemolyzed) obtained from healthy human volunteers who had not previously taken any medication.

The concentration of analyte was determined automatically by the instrument data system using the external standard method. Calibration was performed using singlicate calibration standards on five different occasions. The calibration curve model was determined by the least squares analysis. The applied calibration model was a quadratic one: $y = ax^2 + bx + c$, weight 1/x, where y – peak area and x – concentration. Distribution of the residuals (% difference of the back-calculated concentration from the nominal concentration) was investigated. The calibration model was accepted, if the residuals were within ±20% at the lower limit of quantification (LLOQ) and within ±15% at all other calibration levels and at least 2/3 of the standards met this criterion, including highest and lowest calibration levels.

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

The within- and between-run precision (expressed as coefficient of variation, CV%) and accuracy (expressed as relative difference between obtained and theoretical concentration, Bias%) of the assay procedure were determined by analysis on the same day of five different samples at each of the lower (6 µg/mL), medium (60 µg/mL), and higher (160 µg/mL) levels of the considered concentration range and one different sample of each on five different occasions, respectively.

The relative recoveries at each of the previously three levels of concentration and limit of quantification were measured by comparing the response of the treated plasma standards with the response of standards in solution with the same concentration of analyte as the prepared plasma sample.

Table III. Recovery results

Nominal	6 µg/mL	60 µg/mL	160 µg/mL
Replicate	Rec A	Rec B	Rec C
Area Rec	1.80 10^5	1.82 10^6	3.93 10^6
Area QC	1.86 10^5	1.87 10^6	4.17 10^6
% REC	103	103	106

Table IV. Results of the stability studies (n=3)

$c_{nominal}$, µg/mL Valproic acid	RTS		PPS		FTS	
	30	160	30	160	30	160
Bias%	-12	1.2	-5.4	2.9	-6.7	0.17

RTS = room temperature stability (24°C, four hours); PPS = post-preparative stability (15°C, 24 hours); FTS = freeze-thaw stability (3 freeze-thaw cycles)

The stability of the analyte in human plasma was investigated in different ways, in order to characterize each operation during the process of therapeutic drug monitoring studies: room-temperature stability (RTS), post-preparative stability (PPS) in the autosampler, freeze-thaw stability (FTS). For all stability studies, plasma standards at each of the lower (30 µg/mL) and higher (160 µg/mL) levels were used. Four plasma standards at each of the levels were prepared and let at room temperature four hours before processing (RTS study). Other four pairs were prepared, immediately processed and stored in the HPLC thermostated autosampler (15°C) (PPS study). The samples were injected after 24 hours, the expected longest storage times of the samples in autosampler before injection. For the freeze-thaw stability (FTS), aliquots at the same low and high concentrations were prepared. These samples were subjected to three cycles of freeze-thaw operations in three consecutive days. After the third cycle the samples were analyzed against calibration curve of the day. The mean concentration calculated for the samples subjected to the cycles and the nominal ones were compared. The requirement for stable analyte was that the difference between mean concentrations of the tested samples in various conditions and nominal concentrations had to be in ±15% range.

The ability to dilute samples with concentrations above the upper limit of quantification was also investigated. Plasma standards (n=5) with 480 µg/mL valproic acid were six times diluted with plasma then processed and analyzed. The mean found concentration was compared with the nominal value. The accuracy and precision had to be within ±15% range.

Results and Discussion

No significant interference at the retention time of valproic acid (2.3 min) was observed in different plasma blank chromatograms due to the specificity of selected signals (Figure 3).

The analyte carryover was verified using a blank injection made right after an injection of the most elevated level of concentration from calibration curve. No interference at retention time of analyte due to carryover was observed.

The applied calibration curve model proved to be accurate over the concentration range 2–200 µg/mL for valproic acid, with a determination coefficient greater than 0.996. The mean calibration curve, y = a (±SD) x^2 + b (±SD) x + c (±SD) with SD standard deviation, were y = -2.52 (±0.76).10^7 x^2 + 2.95 (±0.14).10^7 x – 8 (±6).10^3, N = 8 calibration points, n=5 determinations for each calibration point. The residuals had no tendency of variation with concentration.

The method had within- and between-run accuracy and precision (Tables I and II), in agreement to international regulations regarding bioanalytical methods validation [8–10]. The lower limit of quantification was established at 2 µg/mL valproic acid, with accuracy and precision less than 20% (Tables I and II).

The recovery was consistent and reproducible (Table III).

The analyte proved its stability under various conditions (Table IV), the Bias% of found concentration being less than 15%, the maximum accepted value for method's accuracy.

The sample dilution could be made with accuracy within ±3% range and precision less than 8.5%, both for within- and between-assay (Table V).

The use of a triple quadrupole mass spectrometer permitted to achieve a very good sensitivity (LLOQ of 2 µg/mL – 4 ppb injected to column) and to improve other important parameters regarding bioanalytical methods used in busy laboratories, with better or equal results than in the previously published LC-MS methods (Table VI).

Table V. Sample dilution results

Dilution	DS intra-day	DS between-day
1	83	82
2	84	80
3	82	82
4	79	92
5	56	73
Mean	82	82
SD	2.2	6.7
Accuracy	103	102
% CV	2.6	8.2

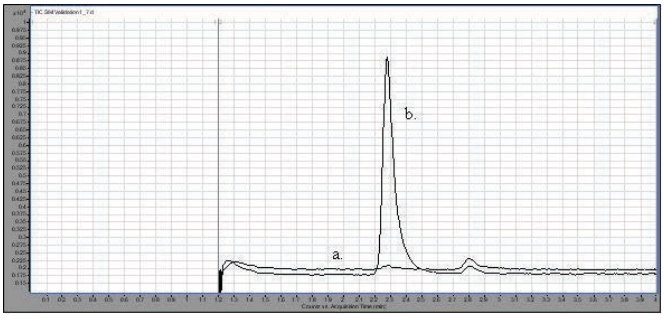

Fig. 3. Chromatograms of a blank (a) and an LLOQ sample (b)

Table VI. Comparison between the proposed and already published methods

No.	Sample treatment	Range (µg/mL)	Ionization	Analysis time (min)	LLOQ (µg/mL)	Injected volume (µL)	ppb injected	Reference
1	plasma, SPE	2–200	TIS Neg	2	2	5	10	[1]
2	plasma, deriv, LL	0.2–5	ESI Pos	20	0.2	40	8	[2]
3	serum, SPE	5–1000	APCI Neg	6	5	50	250	[3]
4	plasma, SPE	0.5–60	ESI	4.5	0.5	?	?	[4]
5	plasma mice, PP semiautomated	0.15–100	ESI Neg	4.5	0.15	?	?	[5]
6	plasma, SPE	0.5–150	ESI Neg	3	0.5	?	?	[6]
7	plasma, PP	5.1–204	ESI Neg	2.4	5.1	2	10.2	[7]
8	plasma, PP	2–200	ESI Neg	4	2	2	4	current method

Conclusions

The proposed method provides accuracy and precision for quantitative determination of valproic acid in human plasma.

The sample preparation by protein precipitation, besides its simplicity, uses less organic solvent with small amounts of sample plasma volume and allows obtaining a good recovery of the analyte without using the derivatization step.

The relatively short run time and the selected signals for monitoring allowed a specific and efficient analysis of plasma samples, making the method more productive and thus more cost effective.

The method can be applied to accurately measure valproic acid concentration on a large number of plasma samples from a clinical-bioanalytical study.

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