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Direct determination of valproic acid in biological fluids by capillary electrophoresis with contactless conductivity detection

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Abstract

Capacitively coupled contactless conductivity detection (C^4D) is a new technique providing high sensitivity in capillary electrophoresis (CE) especially for small ions that can otherwise only be determined with indirect methods. In this work, direct determination and validation of valproic acid (VPA) in biological fluids was achieved using CE with C^4D. VPA is of pharmacological interest because of its use in epilepsy and bipolar disorder. The running electrolyte solution used consisted of 10 mM 2-(*N*-morpholino)ethane sulfonic acid (MES)/DL-histidine (His) and 50 μM hexadecyltrimethylammonium bromide (HTAB) at pH 6.0. Caproic acid (CA) was selected as internal standard (IS). Analyses of VPA in serum, plasma and urine samples were performed in less than 3 min. The interference of the sample matrix was reduced by deproteinization of the sample with acetonitrile (ACN). The effect of the solvent type and ratio on interference was investigated. The limits of detection (LOD) and quantitation (LOQ) of VPA in plasma samples were determined as 24 and 80 ng/ml, respectively. The method is linear between the 2 and 150 μg/ml, covering well the therapeutic range of VPA (50–100 μg/ml).

Keywords: Valproic acid; Contactless conductivity detection; Capillary electrophoresis; Biological fluids; Caproic acid

1. Introduction

Valproic acid (2-propylvaleric acid, VPA) is an anticonvulsant and mood-stabilizing agent widely used in the treatment of epilepsy, bipolar disorder and prophylaxis of migraine headaches [1]. Epilepsy is an episodic illness caused by repeated excessive and abnormal electrical activity of neurons in the brain. There is a fine balance in the brain between factors that initiate electrical activity and factors that restrict it, and there are also systems that limit the spread of electrical activity. During a seizure, these limits break down, and abnormal electrical discharges can occur and spread to whole groups of neighbouring cells. This linkage of electrical discharges creates a storm of electrical activity in the brain. VPA can stop these types of electrical discharges by increasing the concentration of gamma-aminobutyric acid (GABA), which is an inhibitory neurotransmitter in the brain [2,3]. Since VPA shows dose-dependent neurological adverse reactions, the determination of VPA in biological fluids is very important for therapeutic drug monitoring (TDM). In addition to its antiepileptic and antidepressive activities, preclinical results suggest that VPA can influence tumour growth, invasion and differentiation [4].

The analytical methods reported for the determination of VPA include gas chromatography (GC) [5–16], gas–liquid chromatography (GLC) [17–21], high performance liquid chromatography (HPLC) [22–32], chemical ionization mass spectrometry (CI-MS) [33], isotope dilution mass spectrometry (ID-MS) [34] and colorimetric determination [35]. Capillary electrophoresis (CE) is an attractive alternative technique because of its high separation efficiency, short analysis time, relatively low cost, small consumption of samples and reagents. The determination of VPA by capillary electrophoresis has indeed been reported previously [36–39]. However, as VPA lacks a strong chromophore, indirect methods had to be used in order to enable its quantification by UV-absorption [36,38,39] or laser induced fluorescence [37], the standard detection techniques available on commercial capillary electrophoresis instruments. Contactless conductivity detection (for recent reviews see [40,41]) is

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better suited for such analytes than the indirect optical methods because of the poor detection limits of the latter. Indeed, a few studies on the application of this new detection technique to the analysis of blood samples have already been reported. Wan et al. reported the determination of cationic and anionic inorganic blood electrolytes [42]. Petsch et al. described the quantification of fosfomycin in serum, a small, non-UV-absorbing antibiotic substance [43] and very recently the determination of amino acids in human plasma has been reported [44]. The determination of VPA by CE with conductivity detection has also previously been described by Ölvecká et al., who carried out the analysis on a planar electrophoresis chip. The method involved isotachophoretic preconcentration followed by zone electrophoretic separation and subsequent detection by conventional conductivity measurement [45]. Herein, the determination of VPA by standard zone electrophoresis in conventional capillaries and using contactless conductivity detection is described.

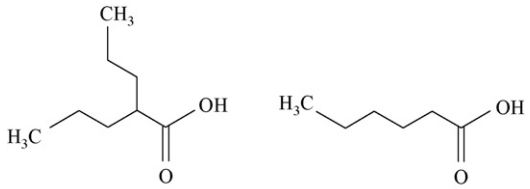
2. Materials and methods

2.1. Chemicals

Hexadecyltrimethylammonium bromide (HTAB), 2-(*N*-morpholino)ethane sulfonic acid monohydrate (MES), DL-histidine (His) and caproic acid (CA) were obtained from Fluka (Buchs, Switzerland). VPA was from Sigma (Buchs, Switzerland). MeOH and acetonitrile (ACN) were obtained from Riedel-de-Haën (Seelze, Germany) and Acros Organics (Geel, Belgium), respectively. VPA test kits which are certified and composed of acetaminophen, carbamazepine, digoxin, gentamicine, lidocaine, *N*-acetylprocainamide, procainamide, tobramycin, valproic acid and vancomycin at different concentrations in bovine serum (Drug Calibrator) were obtained from Dade Behring Limited (Milton Keynes, UK).

2.2. Instrumentation and conditioning

A purpose-made electrophoretic instrument was used for CE analyses. It consists of a perspex box divided into two compartments; the injection and the detection parts. Other components are a dual polarity high voltage power supply with ±30 kV maximum output, function generator, excitation and detection pick-up electronics, and an oscilloscope. The perspex box is fitted with a microswitch that interrupts the power supply on opening the box. The detector electrodes and pick-up amplifier are encased in a small electrically grounded die-cast metal box. An internal metallic shield completely encloses the excitation part of the detector cell, except for a small hole through which the capillary is passed, in order to eliminate any direct coupling between the electrodes. More detailed descriptions are given in the previous papers of one of our research groups [41,46–51]. Analyses were performed in an uncoated fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm I.D. × 360 μm O.D. with a total length of 50 cm and an effective length of 40 cm. For conditioning, a new capillary column was flushed with 1 M NaOH (20 min), water (5 min) and buffer solution (10 min). Ten millimolar MES/His containing 50 μM HTAB at pH 6.0 was used as running electrolyte solution. Ten milliliters of stock solutions of MES (50 mM) and His (50 mM), respectively, and 0.25 ml of HTAB (10 mM) were mixed and completed to 50 ml with deionized water. HTAB (50 μM) was employed to reverse the electroosmotic flow (EOF). Before each injection, the capillary was rinsed with running electrolyte for 2 min. Injections were performed by siphoning at 10 cm height difference for 10 s. The applied voltage was set to −30 kV at the injection end. Data acquisition was performed with a MacLab 4e system (AD Instruments, Hastings, UK).



Valproic acid (VPA) Caproic acid (CA)

Fig. 1. Chemical structures of VPA and CA.

2.3. Standards and sample preparation

Initial stock solutions of VPA and CA (1000 μg/ml) were prepared by dissolving 1 mg of each compound in 1 ml of deionized water. All calibration standards and quality control (QC) samples were prepared from drug-free samples by spiking different concentrations of VPA and internal standard (IS). The structures of both valproic acid and caproic acid are given in Fig. 1. The serum samples were obtained from native human blood after coagulation and centrifugation. The plasma samples were also derived from native blood samples, which had been collected using tubes containing lithium heparinate to prevent coagulation. The urines were samples of spontaneous urine, which were not treated. Plasma and serum samples were stored at −20 °C and urine samples at 4 °C until their analyses. After warming to room temperature the plasma and serum samples were deproteinized with ACN (1:3; v/v) by centrifugation at 6000 × *g* for 15 min before the injection. For this purpose, 250 μl of drug-free plasma or serum sample was taken and mixed with 500 μl of ACN, vortexed and centrifuged. The supernatant liquids of plasma and serum samples were injected directly for the CE analysis. Urine samples were diluted 1:4 (v/v) with deionized water before the injection to reach the expected analyte concentrations. The Drug Calibrators were injected directly after spiking with the known amount of IS (1 μg/ml) for the quantitative study.

3. Results and discussion

3.1. Deproteinization

The use of MeOH and ACN was investigated for the deproteinization of plasma and serum samples at different volume ratios. At a plasma:reagent volume ratio of 1:3, the VPA peaks for these two different precipitation reagents remain clearly separated from the other plasma constituents, with almost identical

electrophoretic patterns. 1:1, 1:2, 1:3 and 1:4 volume ratios of plasma to organic solvent were investigated to reach the optimum conditions. A 1:3 (v/v) ratio gave the best results, since a 1:4 ratio caused a deterioration of the resolution between VPA and the other plasma components. On the other hand, lower ratios are not adequate for deproteinization of the sample and cause higher peak areas for the extraneous plasma peaks. Since the peak shapes were better and the analysis time was shorter than for MeOH, ACN was selected as deproteinization solvent for the serum and plasma samples in this work.

3.2. Separation voltage and buffer optimization

The effect of the applied separation voltage was investigated in the range of 15–30 kV (at the injection end) and −30 kV was found to be the optimum. Under the highest possible voltage, complete baseline separation of VPA from the endogenous plasma components could be achieved within 3 min and, except for the extended time, no significant differences were observed for the electropherograms obtained with lower applied voltages. For optimization of the buffer system, PeakMaster 5.1, which is a simulation programme predicting the behaviour of background electrolytes and analytes in zone electrophoresis with different detection techniques, was used [52]. High ionic strength and buffer concentrations generate high currents and Joule heating, which may cause fluctuations in the electrophoretic conditions throughout the whole system, mostly lead to dispersion and peak broadening. On the other hand, the pH-value of the running electrolyte solution is an important factor for optimizing the electrophoretic conditions, especially for the ionizable analytes. Since the pK_a value of VPA is 4.6, it is completely ionized under the physiological pH conditions and migrates as the negatively charged valproate anion with a high electrophoretic mobility to the anodic side.

Because of its good separation efficiency and low specific conductivity value, MES/His buffers have been widely used as running electrolyte solution, especially for the applications using conductivity detection [51,53]. Furthermore, its pH-value is ideal for the complete ionization of VPA. Therefore, 10 mM MES/His buffer at pH 6.0 was selected for direct conductivity detection of VPA. Because of the migration to the anodic side, the analysis of anionic species in CE requires the reversal of the direction of EOF in the capillary. In order to reverse the surface charge of the capillary, different methods like polymerization [54] or dynamic coating procedures are widely used [55,56]. In this work, the addition of 50 μM HTAB to the MES/His buffer was found sufficient to reverse the EOF direction and the generated current was measured as 44 μA for the final buffer composition in a 50 cm long capillary with 50 μm ID.

3.3. Method validation and quantification

Validation of the proposed method was performed with the evaluation of linearity, precision, recovery, limit of detection (LOD) and limit of quantitation (LOQ). Caproic acid (hexanoic acid) was selected as IS to verify the absolute recovery, linearity, precision and accuracy of the method developed due

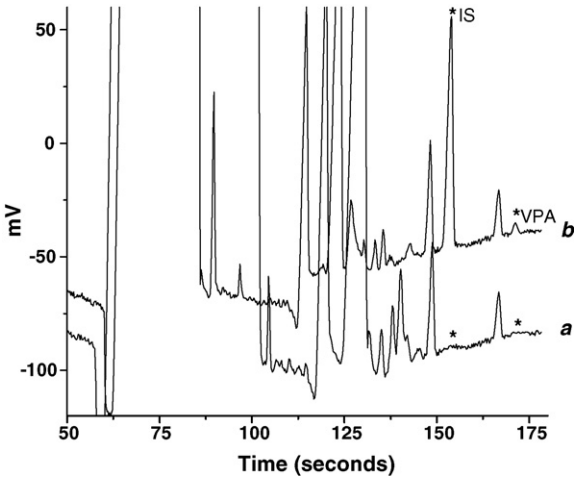


Fig. 2. Electropherograms of human plasma samples. (a) Blank plasma sample; (b) plasma sample spiked with 2.0 μg/ml IS and 100 ng/ml VPA, respectively. CE conditions: 10 mM MES/His +50 μM HTAB pH 6.0, applied voltage: −30 kV.

to its similar molecular structure and therefore similar physicochemical properties to that of the analyte (Fig. 1). Plasma samples from four different drug-free persons were analysed in order to assess inter-individual variability, and suitability of the method. Electropherograms of blank plasma (Fig. 2a), and plasma spiked with IS (2.0 μg/ml) and VPA (100 ng/ml, Fig. 2b) were compared to test the applicability of the proposed method. The optimized buffer consisting of 10 mM MES/His +50 μM HTAB at pH 6.0 was employed. As can be seen from Fig. 2a and b, the blank plasma sample did not show any interference at the migration times of VPA and the IS. All other major plasma components were observed before the VPA-peak and the electrophoretic patterns of different plasma samples were almost identical. Serum and urine samples were also investigated (Fig. 3). As shown in the figure, the electropherograms of serum (Fig. 3A) and urine samples (Fig. 3B), show similarities to those obtained for plasma. Since the type and the concentration of endogenous components in human serum, plasma and urine samples are different; some variabilities in their electrophoretic patterns could be expected. While the migration time of VPA and the IS showed slight variations depending on the matrix, they were well separated from the major constituents for all applications. The linearity was tested by preparing blank plasma samples spiked with IS at a constant concentration (20 μg/ml) and VPA at 12 different concentration levels ranging from 1 to 250 μg/ml. The calibration curve was linear in the concentration range from 2 to 150 μg/ml for duplicate injections of the spiked plasma sample at each concentration. The therapeutic plasma concentration range (50–100 μg/ml) of VPA is found in this linear range. The parameters for the calibration curve in plasma were $y = 0.1051x - 0.0503$, $R^2 = 0.9993$. In this equation, y shows the normalized peak area, (the ratio of the migration time corrected peak area of the analyte to the internal standard), x the theoretical concentration of the spiked standards in micrograms per milliliters and R^2 the correlation coefficient. The precision of the method was determined by measuring the repeatability and intermediate precision as relative standard

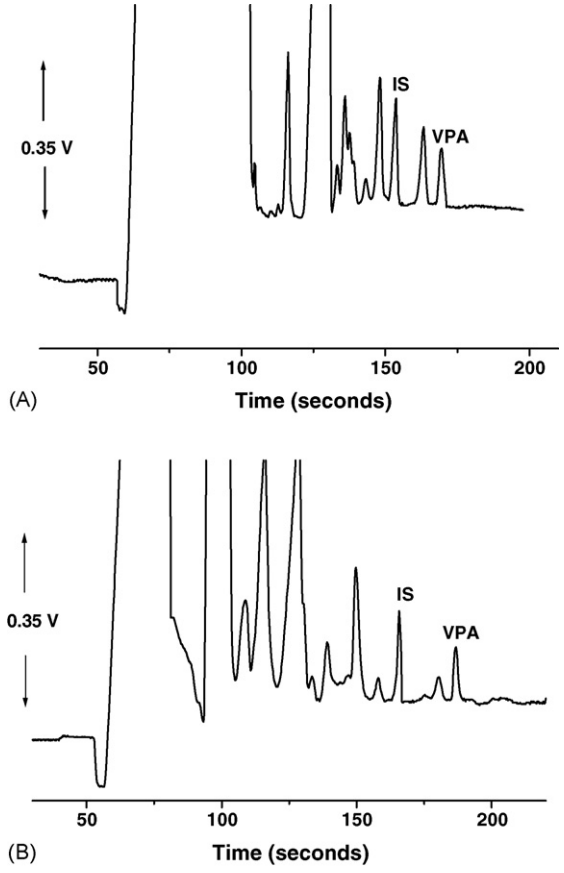


Fig. 3. Electropherograms of (A) human serum and (B) human urine sample spiked with 2.0 μg/ml IS and 5.0 μg/ml VPA. Other conditions are the same as for Fig. 2.

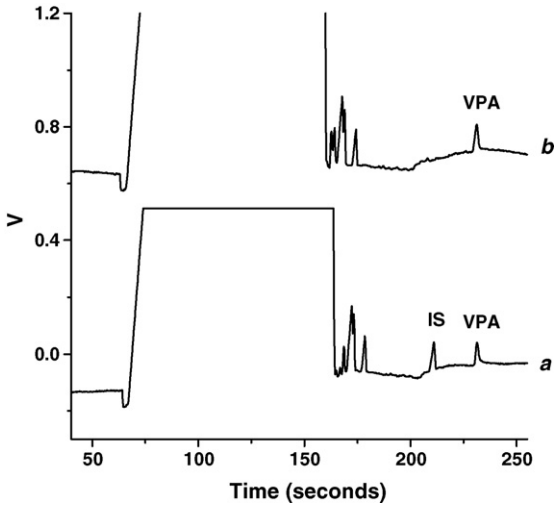


Fig. 4. Electropherogram of the Drug Calibration Standard 1. Dilution factor: 1/4. (a) With 1.0 μg/ml, (b) without IS spiking. Other conditions are the same as for Fig. 2.

deviation (R.S.D.%) of the same homogeneous sample spiked with three different concentrations of VPA; 5.0 μg/ml for lower limit, 50.0 μg/ml for middle point and 100.0 μg/ml for upper limit. R.S.D. values for the peak areas and migration times of a plasma sample spiked with VPA (5.0, 50.0 and 100.0 μg/ml) and IS (2.0 μg/ml) are shown in Table 1. LOD and LOQ values of VPA in plasma samples were determined as 24 ng/ml (signal/noise = 3) and 80 ng/ml (signal/noise = 10), respectively. The mean recovery in a drug-free plasma sample spiked with 5.0 μg/ml VPA was found as 98% ($n = 5$). The quantitative analyses were performed by using Drug Calibrator Standard solutions ($n = 5$, Fig. 4). Because of the bovine serum content of these standard solutions, some differences were observed in the electrophoretic pattern compared to human serum. Results of the quantitative analyses of these standards by the method developed with CE–capacitively coupled contactless conductivity detection (C^4D) are shown in Table 2. The data shows that our method is both accurate and precise at different concentrations of VPA and that there is no interference from the other drugs contained in this mixture (namely acetaminophen, carbamazepine, digoxin, gentamicine, lidocaine, *N*-acetylprocainamide, procainamide, tobramycin and vancomycin) which may also be used concurrently by the patients.

Table 1
Precision results for VPA spiked plasma sample

[VPA] (μg/ml)	Repeatibility (R.S.D.%, $n = 10$)		Intermediate precision (R.S.D.%, $n = 10$)	
	Retention time (t)	Peak area (A)	Retention time (t)	Peak area (A)
5.0	0.25	2.5	0.36	3.1
50.0	0.25	2.7	0.38	3.2
100.0	0.26	2.9	0.36	3.5

Table 2
Quantitative results for Drug Calibration Standards

Samples	Dilution factor	Concentration of VPA found (μg/ml, $n = 5$)	Manufacturer's values (μg/ml, $n = 5$)
Drug Calibration Standard 1	1:4	19.9 ± 0.8	20.6
Drug Calibration Standard 2	1:4	39.6 ± 0.5	39.8
Drug Calibration Standard 3	1:4	78.0 ± 1.0	77.7
Drug Calibration Standard 4	1:4	154.3 ± 2.5	152.0

4. Conclusion

C^4D is a powerful and competitive detection technique especially for the species which do not absorb UV-radiation. The main advantages of this detection technique are high sensitivity, simple electronic circuitry, good suitability for conventional and microchip capillary electrophoresis and also relatively low cost. Since VPA is a non-UV-absorbing drug, C^4D is a desirable detection method for its direct analyses. In this work, we demonstrated the applicability of this technique to biological samples. As mentioned in Section 1, Ölvecká et al. [45] determined valproate only in serum by capillary electrophoresis with isotachophoretic preconcentration. The obtained detection limits in the range from 23 to 50 ng/ml are comparable with the results presented in this study achieved without the preconcentration method (24 ng/ml) but the separation time (roughly 10 min) is longer than that obtained in this work (roughly 3 min). The analysis time reported by Pucci et al. [38], for their method with indirect UV determination in plasma is comparable (roughly 3 min) but the detection limit obtained (150 ng/ml) is markedly higher than ours. The simple sample preparation procedure, short analysis time, R.S.D. and LOD values obtained from the optimized electrophoretic conditions make our method suitable for routine analyses of VPA in biological fluids like plasma, serum and urine.

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