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Simple procedure for determination of valproic acid in dried blood spots by gas chromatography–mass spectrometry

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ABSTRACT

Valproic acid (VA) is a drug widely used to treat epilepsy and bipolar disorder, at recommended serum concentrations ranging form 50 to 100 μg ml^{-1}. A novel option for therapeutic drug monitoring that has been emerging recently its testing using dried blood spots on paper (DBS), but there are no reports of its application to assaying VA. In this study, a methodology was developed for the determination of VA in 6 mm diameter DBS, equivalent to around 12 μl of blood, using gas chromatography combined with mass spectrometry. DBS were extracted with a mixture of acetonitrile and methanol (1:3, v/v). The method is linear from 5 to 250 μg ml^{-1} with intra-assay and inter-assay precision of 2.67–8.15% and 2.28–3.67%, respectively. Accuracy was 102.84–104.42%. VA was stable in DBS stored at 45 °C for up to 21 days. VA concentrations in DBS correlated with concentrations assayed in serum, with $r = 0.9948$. Mean ratio between VA concentrations in serum and DBS in clinical samples was 1.883. Dried blood spots are a viable option for collection and transport of samples and for assaying VA in the context of therapeutic drug monitoring, especially in Developing Countries.

1. Introduction

Valproic acid (2-propylpentanoic acid) (VA) is a short-chain fatty acid that presents wide-ranging efficacy for control of convulsions, being a first-choice drug for the treatment of mood disorders and epilepsy [1]. Since the drug has a low therapeutic index and there is a wide intraindividual variability in serum VA concentrations, monitoring serum VA concentrations in patients who take this drug is widely recommended in order to achieve the greatest treatment efficacy with a minimum of adverse effects [2,3]. Around 50% of patients exhibit an adequate response to treatment at serum concentrations of 80 μg ml^{-1} [1]. In contrast, just 20% of patients respond adequately to concentrations in the order of 50 μg ml^{-1} [1]. Toxic effects begin to manifest with greater frequency at serum concentrations greater than 200 μg ml^{-1} [1,3]. The consensus therapeutic range for VA is 50–100 μg ml^{-1} in serum [1–3].

A novel option for therapeutic drug monitoring that has been emerging recently is testing in dried blood spots on paper (DBS), collected by finger prick. This type of analytical matrix offers potential advantages such as minimal invasivity, greater patient comfort, greater analyte stability, flexible requirements for time and place of sample collection and the possibility of training patients to take their own samples, since phlebotomy is not necessary [4–6]. This technique has already been used for the measurement of other anticonvulsant drugs as phenobarbital [6] and topiramate [7,8] using liquid chromatography–tandem mass spectrometry (LC–MS/MS), and for phenobarbital, carbamazepine, lamotrigine and phenytoin using high-performance liquid chromatography with UV detection (HPLC–UV) [9]. However, there are no reports in the literature of a method for assaying VA in DBS.

Determination of VA in dried blood spots on paper presents a number of challenges, which are related to the small sample volume and characteristics of the drug itself, since it does not have chromophores that would allow sensitive direct detection by spectrophotometric detectors, and it is highly volatile, requiring the avoidance of solvent evaporation steps in sample preparation [10]. Gas chromatography coupled to mass spectrometry (GC–MS) presents several advantages for the present application due to the characteristics of the VA molecule, especially its high volatility and low molecular weight, associated to the lower acquisition and running costs of GC–MS when compared to LC–MS/MS. Furthermore, for the VA DBS method to be clinically applicable, it is first necessary to determine the correlation between concentrations detected by the standard testing method (in serum samples) and concentrations determined by assaying DBS. In view of the above, the objective of this study was to validate a method for determination

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of VA in DBS using gas chromatography coupled to mass selective detection.

2. Experimental

2.1. Reagents, materials and reference standard samples

Valproic acid (VA) was acquired from the United States Pharmacopeia (Rockville, United States). Cyclohexanecarboxylic acid and butyl acetate were obtained from Sigma–Aldrich (Saint Louis, United States). Glacial acetic acid was obtained from Vetec (Rio de Janeiro, Brazil). Methanol and acetonitrile, HPLC grade, were acquired from Merck (Darmstadt, Germany). Whatman 903 paper was acquired from GE Healthcare (Westborough, United States).

2.2. Preparation of solutions and standards

VA stock solution was prepared in acetonitrile to obtain a concentration of 10 mg ml^{-1}. Valproic acid working solutions were also prepared by dilution of stock solution with acetonitrile, to concentrations of 40, 100, 200, 400, 1000, 1500, 3000 and 5000 μg ml^{-1}. Cyclohexanecarboxylic acid stock solution was prepared in acetonitrile to obtain a concentration of 10 mg ml^{-1}. The stock solution was used to prepare an intermediate solution in acetonitrile at a concentration of 50 μg ml^{-1}. The DBS extraction solvent was a mixture of acetonitrile and methanol (1:3, v/v), containing cyclohexanecarboxylic acid (internal standard, IS) at a concentration of 1 mg ml^{-1}.

2.3. Chromatography equipment and conditions

Analyses were conducted using a Focus gas chromatograph coupled to a Thermo ISQ single-quadrupole gas chromatograph (GC/MS) system, from Thermo Scientific (San Jose, USA). Splitless injection mode was used, with injector temperature of 250 °C. Chromatographic separation was performed in a 30 m CP-WAX column (Varian, The Netherlands) with 0.25 mm internal diameter and 0.25 μm thick internal coating, at a constant helium flow rate of 1 ml min^{-1}. The column temperature was held at 80 °C for 2 min and then increased at a rate of 40 °C min^{-1} until reaching 250 °C, at which temperature it was maintained for a further 2 min. The total chromatographic analysis run time was 8.25 min. The transfer line temperature was 280 °C and the ion source temperature was 300 °C. The ions monitored for VA were 102 (quantifying ion), 73 and 115 (qualifying ions). The ions monitored for the IS were 126 (quantifying ion), 81 and 108 (qualifying ions).

2.4. Sample preparation

Quality control and calibration samples were obtained by pipetting 50 μl aliquots of VA-spiked blood onto Whatman 903 paper and leaving them to dry at room temperature for 3 h before processing. DBS discs were obtained by perforation, using a 6 mm punch cutter, using one disc per extraction. Discs were transferred to polypropylene microtubes and 200 μl of extraction solution was added. After 1 h in an ultrasonic bath, 100 μl of extract was transferred to an autosampler vial and a 1 μl aliquot was injected into the GC.

2.5. Selectivity

Blank DBS samples obtained from 10 different human sources were prepared as described above to check for the presence of chromatographic peaks that might interfere with detection of VA or IS.

2.6. Benchtop stability

For estimation of stability of processed samples under the conditions of analysis, two DBS control samples containing VA at 10 and 250 μg ml^{-1} (n = 8 each) were extracted as described above. The extracts obtained at each concentration were pooled. Aliquots of these pooled extracts at each concentration were transferred to autosampler vials and injected under the conditions of a regular analytical run at time intervals of 1 h, during 12 h. Stability of analytes was tested by regression analysis plotting absolute peak areas corresponding to each compound at each concentration vs. injection time. Using the obtained linear regression, the concentration after 12 h was calculated. A decrease or increase of up to 10% in the measured peak areas was considered as acceptable.

2.7. Linearity

Aliquots of 950 μl blank blood (% Hct = 40) were enriched with 50 μl of the corresponding VA working solutions and applied to paper to obtain DBS calibration samples at the concentrations of 5, 10, 20, 50, 75, 150 and 250 μg ml^{-1}, that were analyzed within 24 h. Replicates (n = 6) at each concentration were analyzed as described above. Calibration curves were calculated relating the area ratios from VA peak to the IS peak and with the nominal concentrations of the calibration samples. Homoscedasticity of calibration data was evaluated with F-test at the confidence level of 95%. Curves were fitted using least-squares linear regression using several weighting factors ($1/x$, $1/x^2$, $1/y$, $1/y^2$). Calibration models were assessed using coefficients of correlation (r) and cumulative percentage relative error ($\sum$%RE) [11]. Daily calibration curves using the same concentrations (single measurement at each concentration level) were analysed with each batch of validation and clinical samples.

2.8. Precision and accuracy

Aliquots of blank blood were enriched with the working solutions and applied to paper to obtain quality control DBS samples containing VA at concentrations of 25 (quality control low, QCL), 125 (quality control medium, QCM) and 200 μg ml^{-1} (quality control high, QCH), that were analyzed within 24 h. The quality control samples were analyzed as described above in triplicate on each of 5 days. Within-assay precision and between-day precision were calculated by one-way ANOVA with the grouping variable “day” and were expressed as CV %. Accuracy was defined as the percentage of the nominal concentration represented by the concentration estimated with the calibration curve. The acceptance criteria for accuracy were mean values within ±15% of the theoretical value and for precision a maximum CV of 15% [12].

2.9. Lower limit of quantification

An independent DBS quality control sample at the lowest point of the calibration curve, 5.0 μg ml^{-1}, was included in the accuracy and precision experiments (quality control at the limit of quantitation, QCLOQ) and was tested in triplicate on three different days. The acceptance criteria established for the limit of quantification was accuracy within 100 ± 20% of the nominal value and a maximum CV of 20% [12].

2.10. Extraction yield

Aliquots of 12 μl of blood (% Hct 35 and 45) containing VA at concentrations of 25 (quality control low, QCL) and 200 μg ml^{-1} (quality control high, QCH), and non-spiked blood were added to 6 mm punches of Whatman 903 paper and allowed to dry at room temperature for 3 hours. Non-spiked extracts were added with VA