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Dried blood spot assay for the quantification of phenytoin using Liquid Chromatography-Mass Spectrometry

Fabio Villanelli [a], Elisa Giocaliere [a], Sabrina Malvagia [a], Anna Rosati [c], Giulia Forni [a], Silvia Funghini [a], Engy Shokry [d], Daniela Ombrone [a], Maria Luisa Della Bona [a], Renzo Guerrini [a,b,c], Giancarlo la Marca [a,b,*]

[a] Newborn Screening, Biochemistry and Pharmacology Laboratories, Department of Neurosciences, Meyer University Children's Hospital, Florence, Italy
[b] Department of Neuroscience, Pharmacology and Child Health, University of Florence, Italy
[c] Pediatric Neurology Unit, Meyer University Children's Hospital, Florence, Italy
[d] Pharmaceutical Chemistry Department Faculty of Pharmaceutical Sciences and Pharmaceutical Industries, Future University in Egypt, Cairo, Egypt

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ABSTRACT

Phenytoin (PHT) is one of the most commonly used anticonvulsant drugs for the treatment of epilepsy and bipolar disorders.
The large amount of plasma required by conventional methods for drug quantification makes mass spectrometry combined with dried blood spot (DBS) sampling crucial for pediatric patients where therapeutic drug monitoring or pharmacokinetic studies may be difficult to realize. DBS represents a new convenient sampling support requiring minimally invasive blood drawing and providing long-term stability of samples and less expensive shipment and storage.
The aim of this study was to develop a LC-MS/MS method for the quantification of PHT on DBS.
This analytical method was validated and gave good linearity ($r^2 = 0.999$) in the range of 0–100 mg/l. LOQ and LOD were 1.0 mg/l and 0.3 mg/l, respectively.
The drug extraction from paper was performed in a few minutes using a mixture composed of organic solvent for 80%. The recovery ranged from 85 to 90%; PHT in DBS showed to be stable at different storage temperatures for one month. A good correlation was also obtained between PHT plasma and DBS concentrations.
This method is both precise and accurate and appears to be particularly suitable to monitor treatment with a simple and convenient sample collection procedure.

1. Introduction

Phenytoin (PHT) is an anticonvulsant drug widely used in the treatment of acute seizures and epilepsy, and less commonly for other conditions such as bipolar disorder [1].

PHT levels in the blood must be maintained within a narrow therapeutic range. Low levels may cause the patient to suffer seizures or precipitate into status epilepticus; high levels may result in toxicity with adverse effects [2,3].

Maintaining a therapeutic level of PHT in the blood can be a challenge for several reasons. Hepatic enzymes metabolize PHT with different speed among people, under the influence of age, genetic factors, coexisting disorders and organ function. Some patients are very sensitive to small changes in bioavailability and even small increases in dose can cause large increases in blood concentrations, enhancing the severity of side effects and causing PHT toxicity [4].

PHT in blood is approximately 90% bound to proteins and 10% (the active portion) free [5,6]; thus, a low amount of protein in the blood causes an excess of active drug. Furthermore, PHT often interacts with other drugs, affecting the safety and effectiveness of other medications [7].

Therapeutic drug monitoring (TDM) in pediatric samples is necessary because of the very different responses in terms of drug availability [8,9].

Over the last years, a number of validated methods for PHT measurement have been reported, usually requiring a large amount of plasma sample [6,10–13]. In pediatric patients the available amount of sample

Abbreviations: PHT, phenytoin; TDM, therapeutic drug monitoring; DBS, dried blood spot; LC-MS/MS, liquid chromatography-tandem mass spectrometry; Hct, hematocrit; ICH, International Conference on Harmonisation; LOD, limit of detection; LOQ, limit of quantitation.

* Corresponding author at: Newborn Screening, Biochemistry and Pharmacology Laboratories, Pediatric Neurology Unit, Meyer University Children's Hospital, Florence, Italy. Tel.: +39 0 55 5662988; fax: +39 0 55 5662489.
E-mail addresses: f.villanelli@meyer.it (F. Villanelli), e.giocaliere@meyer.it (E. Giocaliere), s.malvagia@meyer.it (S. Malvagia), a.rosati@meyer.it (A. Rosati), giuliaforni1984@libero.it (G. Forni), s.funghini@meyer.it (S. Funghini), engyshoukry@fue.edu.eg (E. Shokry), daniela.ombrone@libero.it (D. Ombrone), m.dellabona@meyer.it (M.L. Della Bona), r.guerrini@meyer.it (R. Guerrini), g.lamarca@meyer.it, giancarlo.lamarca@unifi.it (G. la Marca).

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is usually low and blood drawings that are required for pharmacokinetic studies are highly impractical and hampered by ethical issues.

In this scenario, dried blood spot (DBS) sampling represents a new convenient method of sample collection owing to minimally invasive blood drawing, plasma molecules stability ensured by filter paper, in addition to feasible shipment and storage especially from outermost geographic areas [14].

In 1984, Coombes et al. introduced a substrate-labeled fluorescent immunoassay procedure to quantify phenytoin on DBS to monitor therapy in epilepsy [15]. However, antibody-based approach can be expensive with multistage sample handling. In addition, quantitative data obtained by antibody-based assays can be strongly affected by the potential cross reactivity, mainly in a complex biological matrix at lower concentrations [16].

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) offers many advantages compared with the immunoassay or conventional HPLC methods [17,18]. This technology allows achieving high specificity, simple and inexpensive sample-handling process and fast acquisition time. These advantages combined with DBS sampling make LC-MS/MS technology the most effective tool for the purpose of pediatric pharmacokinetic studies.

The aim of this study was to develop a simple, precise, accurate, sensitive and reliable method for determination of PHT in DBS by LC-MS/MS.

2. Material and methods

2.1. Patients and sample collection

Both plasma and whole blood specimens were collected from 17 pediatric patients. Parental informed consent was obtained for each patient. The correlation between PHT concentrations in matched plasma and blood spot samples were evaluated.

Plasma and DBS samples were stored at −20 °C until the analysis.

2.2. Standards

Chemical standard for PHT (5,5-Diphenylhydantoin) was supplied by Sigma-Aldrich (Steinheim, Germany).

Stock solution and successive dilutions were prepared in methanol. All solvents were of highest purity commercially available and were used without any purification (Panreac, Barcelona, Spain).

2.3. Sample preparation

A 3.2 mm diameter disk from DBS (~3 μl blood) was punched directly into a 96-well plate and extracted with 330 μl of methanol/water (80/20) + formic acid (0.1%).

The 96-well plate was closed with a polypropylene plate cover and then enveloped with aluminum foil; the extraction was performed in an orbital shaker for 25 min at 37 °C. The extract was transferred in a new 96-well plate and sealed with plastic film.

For plasma samples, 10 μl was diluted with 490 μl of methanol/water (80/20) + formic acid (0.1%), vortexed and centrifuged to precipitate proteins.

2.4. Validation procedure

The DBS analytical method was validated in accordance with the International Conference on Harmonisation (ICH) guideline [19].

2.4.1. Preparation of calibration standards

Validation procedures were assessed on calibration points prepared by spiking with known amounts of PHT into blank blood samples from healthy donors. The spiked blood tubes were gently mixed every 10 min and incubated at room temperature for different times (15, 30, 60, 120, and 360 min). 20 μl of each spiked blood was spotted on filter paper (Whatman, 903, Gmbh, Dassel Germany) and left to dry.

In order to identify the best solvents to extract PHT from DBS, different mixtures of acetonitrile, methanol, water and formic acid, for different extraction times and different temperatures were tested.

2.4.2. Limit of detection and limit of quantitation

The limit of detection (LOD) and the limit of quantitation (LOQ) were determined preparing a specific calibration curve in blood in the range of 0–5 mg/l; the residual standard deviation of the regression line and its slope were used to calculate LOD and LOQ as reported in ICH guideline [19].

2.4.3. Linearity

Quantification analysis was performed using an external standard calibration curve, covering a concentration range from 0 to 100 mg/l (0–0.1– 0.5–1–5–10–20–50–75–100). This range, larger than the therapeutic range, was chosen also to quantify the samples in case of intoxication (two or three times the upper limit of the therapeutic range).

2.4.4. Accuracy and precision

The accuracy and precision of the developed method were determined by the analysis of DBS samples (calibrators) spiked with PHT at four concentration representing LOQ (1 mg/l), a concentration within the therapeutic range (10 mg/l), medium and high (50 and 100 mg/l) levels of the standard curve. Ten punches from each calibrator were analyzed in the same batch for intraday test and in three replicates for ten different days for inter-day test.

2.4.5. Recovery and matrix effect

The efficiency of the extraction procedure was also determined by analysis of DBS samples spiked at two different concentration levels, 5 and 20 mg/l. The analyte response obtained from extracted spiked samples was compared to a spiked blank matrix sample and the recovery was calculated.

As reported by van Eeckhaut et al. [20] there are different methods available to evaluate the matrix effect. In the present method matrix effect was calculated by comparing the signal obtained by PHT at 5 and 20 mg/l concentrations, injected directly in mobile phase and the signal obtained from the same amount of compound added to an extracted drug-free matrix sample [21,22].

2.4.6. Spot homogeneity

The spot homogeneity was also verified by punching at different locations, including left, right, center, top and bottom edge of three different spots at three different concentrations.

2.4.7. Stability and storage

The drug short term stability on DBS was evaluated up to four weeks under the following storage conditions: −20 °C, +4 °C, room temperature and +37 °C to ensure that patient samples are stable during shipping and storage prior to analysis. The stability of samples during storage was determined by analyzing DBS samples at LOQ, medium and high concentrations ($n = 3$ replicates) weekly over one month, after storage at the previously mentioned conditions.

2.5. Mass spectrometry

The data were obtained using an API 3200 triple quadrupole mass spectrometer (AB SCIEX, Toronto, Canada) equipped with Turbo Ion Spray source and operating in MRM positive ion mode. The ion spray voltage was set to 5500 V, the gas 1 and gas 2 were set both at 40 (arbitrary unit) and the temperature was 500 °C.

The transitions were optimized by infusing a standard solution (1 mg/l) and the following MS/MS conditions were monitored: the transition m/z 253.2 > 182.2 was used as quantifier, with 24 V as