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BRIEF COMMUNICATION

A new rapid micromethod for the assay of phenobarbital from dried blood spots by LC-tandem mass spectrometry

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Summary

Advantages of dried blood spot include low invasiveness, ease and low cost of sample collection, transport, and storage. We used tandem mass spectrometry (LC-MS/MS) to determine phenobarbital levels on dried blood spot specimens and compared this methodology to commercially available particle enhanced turbidimetric inhibition immunoassay (PETINIA) in plasma/serum samples. The calibration curve in matrix using D_5-phenobarbital as internal standard was linear in the phenobarbital concentration range of 1–100 mg/L (correlation coefficient 0.9996). The coefficients of variation in blood spots ranged 2.29–6.71% and the accuracy ranged 96.54–103.87%. There were no significant differences between the concentrations measured using PETINA and LC-MS/MS (both had similar precision and accuracy) however, LC-MS/MS allows at least 1.5 times higher throughput of phenobarbital analysis and additionally offers ease of sample collection which is particularly important for newborns or small infants.

KEY WORDS: Phenobarbital, Liquid chromatography-tandem mass spectrometry, Epilepsy, Anticonvulsant, Antiepileptic, Particle enhanced turbidimetric inhibition immunoassay.

Phenobarbital (PB) is widely used in the treatment of partial and generalized tonic–clonic seizures in all age groups (Schmidt et al., 1986) and is considered a first line drug for treating seizures and status epilepticus in the newborn. Due to the narrow therapeutic index of the drug and a wide interindividual variability in the rate of metabolism and clearance, therapeutic monitoring of PB concentrations has been recommended for optimizing treatment. In the clinical setting, therapeutic PB blood concentrations usually range from 10 to 40 mg/L (Patsalos et al., 2008).

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Phenobarbital has been assayed by several analytical techniques (Queiroz et al., 2008; Saka et al., 2008; Subramanian et al., 2008) but routine determinations are usually performed by particle enhanced turbidimetric inhibition immunoassay (Tietz, 1994) or other immunoassay techniques (e.g., fluorescence polarisation immunoassays, homogeneous enzyme immunoassays). However, analytical methods imply time consuming chromatographic runs and blood sample volumes for immunoassay methods range 0.5–2 ml, which may be a limitation in newborns or small infants. In addition, some chromatographic methods have used unspecific wavelengths for the detection of PB. Analysis on dried blood spot (DBS) implies lower costs in that samples can easily be sent to the lab via mail, avoiding the costs for traveling and personnel in charge of venipuncture; an ease of sampling represents a major practical advantage especially in small infants.

Many clinical applications for the measurement of biological markers on DBS have been reported (Mei et al., 2001), including the assay of the antiepileptic drug topiramate (la Marca et al., 2008), the immunosuppressive tacrolimus (Hoogtanders et al., 2007), some antiretroviral drugs (Koal et al., 2005) and methylene blue (Burhenne et al., 2008).

We evaluated the use of tandem mass spectrometry (MS/MS) to assay of PB using DBS. We demonstrated that minimal sample preparation, high sensitivity/specificity and high throughput make this method a good candidate for large-scale routine use.

Experimental

Materials

Phenobarbital (purity > 98%) was purchased from Sigma-Aldrich (Steinheim, Germany); the internal standard, Phenobarbital-D_5 (99.8% D purity) was from Cerilliant (Round Rock, TX, U.S.A). Stock solutions (corresponding to 2,000 mg/L) of both were made in methanol. Working solution of Phenobarbital-D_5 was 0.08 mg/L. All chemicals and solvents were of the commercially highest purity available.

DBS preparation

The venous blood in heparin tube was shipped on cold packs from hospital blood collection unit to the laboratory. Within 6 h of collection, DBS were prepared by spotting 20 μl whole blood onto Whatman 903 specimen collection paper (903, Whatman GmbH, Dassel, Germany). Blood spots were dried for 2–3 h at room temperature. The DBS were stored in sealed plastic bags containing desiccant (silica pellets) and daily sent to the mass spectrometry laboratory. The remaining whole blood was centrifuged at 620 g for 5 min to obtain serum. Sera (400 μl) were transferred to the Dimension Vista integrated system (Dade Behring Inc., Newark, DE, U.S.A) sample cups and analyzed with low (10.0 mg/L), medium, (30.0 mg/L), and high (60 mg/L) control serum samples.

Sample preparation

A pooled mixture of blood samples was spiked with PB and 20 μl were put on filter paper from six adult healthy volunteers after they had signed an informed consent.

Two 3.2 mm DBS (containing about 3 μl of blood each) were punched (DBS-Puncher, 1296-071; Wallac, Turku, Finland) and extracted in a Polypropylene 96-well plate v-shape (Tomtec Plastics, Budapest, Hungary) with 200 μl 20/80 of water/methanol solution containing 0.08 mg/L of D_5-PB. Samples were put in an orbital shaker and kept at 37°C for 20 min. Fifty DBS from healthy controls and 50 spots from patients with confirmed epilepsy whose PB levels had also been monitored with the PETINIA method were tested. For all subjects, hematocrit levels were determined using an automated Coulter counter (Beckman-Coulter ACt.8, High Wycombe, United Kingdom).The procedure was approved by the review board of the Meyer Children's University-Hospital. Age of patients and controls ranged 48 h of life-81 years (controls: median 49 years, SD 15.1 years; patients: median 53 years, SD 16.8 years). DBS were stored at room temperature until analysis which was on the same day of their receipt in the laboratory. The stability of 5 and 100 mg/L of PB on DBS were measured in triplicates during a 33 days period at room temperature, +4 and −20°C, with no significant differences being apparent. (see supplemental Table S1). The median storage time between the drawing of the blood sample and the analysis was 40 h (range 2–60 h).

Methods

Particle enhanced turbidimetric inhibition immunoassay (PETINIA)

The phenobarbital assay method is based on a particle enhanced turbidimetric inhibition immunoassay technique that uses a latex particle-phenobarbital reagent (PR) and phenobarbital-specific monoclonal antibody (Ab).

Heparinized plasma/serum samples (50 healthy controls and 50 patients) were analyzed by Petinia by using the commercially available Dimension PHNO/Flex reagent cartridge on an Dimension Vista integrated system (Dade Behring Inc.). Company-provided calibrators at concentrations 0.0, 16.0, 32.0, 48.0, 64.0, and 80.0 mg/L were used to establish the calibration. The analytic procedure was conducted according to the manufacturer's instructions.

Mass spectrometry

An Applied Biosystems-Sciex (Toronto, Canada) API 4000 Triple-Quad Mass Spectrometer equipped with the TurboIonSpray source operating under negative ion mode at a voltage of −4,500 V was used. Declustering Potential (DP), Collision Exit Potential (CXP) and Collision Energy (CE) were automatically optimized for PB and D_5-PB. The resulting DP was −48 V, CE and CXP were found at −15 and −9 V. Standard solutions of 1 μg/L of PB and D_5-PB in an aqueous solution of 80% methanol were infused at 5 μL/min by connecting the infusion pump directly to the source.

Quantitation was achieved by use of a Series 1100 Agilent Technologies (Waldbronn, Germany) CapPump and Micro ALS autosampler. Liquid chromatography was performed using a Phenomenex Synergi 4u Fusion-RP 80A 4 μm, 2 × 150 mm chromatographic column (Phenomenex Italia, Anzola Emilia, Italy). Column flow was 0.2 ml/min using an aqueous solution of 80% methanol. Column eluent was directed to the TurboIonSpray probe without split ratio.

Two μL of the extracted sample were injected for the LC-MS/MS experiments. Phenobarbital concentrations were measured by, and compared to the PETINIA technique.

Statistical procedures

The two methods were compared by use of the Bland–Altman plot (Bland & Altman, 1986) and by linear correlation.

Results

Results are summarized in Fig. 1 and in Table 1. Supplemental Fig. S1 shows the MS/MS spectrum obtained by fragmenting the precursor ion [231.2 Th] of PB and D_5-PB [236.2 Th] and a blank DBS, PB standard in water and a typical patient sample. The ion-pair transition for the quantitative experiment (SRM) is 231.2 > 188.1 for PB and 236.2 > 193.1 for D_5-PB. Supplemental Fig. S2 shows an extract ion chromatogram from DBS of a patient with 12.1 mg/L of PB versus a PB standard solution in water at the concentration of 12 mg/L (IS 0.08 mg/L) and a control. No interferences or quenching of the intensity of the signal were revealed, indicating that the assay performances are independent from the sample matrix. Additionally, the combined effects of the components of the sample on the measurement of the analyte, do not seem to affect the MS response, owing to the sample dilution rate (30 times) and the isotopic dilution strategy. The choice of a water/methanol 20:80 solution for extraction purposes from DBS was made considering the chromatographic column conditions. The chromatographic conditions were set up in order to speed-up the running time (4 min) since specificity is provided by the MS/MS measurement. Sample dilution rate (3–3.4 μL diluted to 200 μL) and injection volume (2 μL) were selected in order to avoid overloading the chromatographic column. No deterioration in column efficiency was observed after the analysis of 200 DBS samples.

The calibrators, containing the internal standard at 0.08 mg/L, were at concentrations of 0, 1, 2, 5, 10, 20, 50 and 100 mg/L. For spiking studies, we evaluated linearity by analyzing supplemented 3.2 mm DBS prepared at 0, 1,

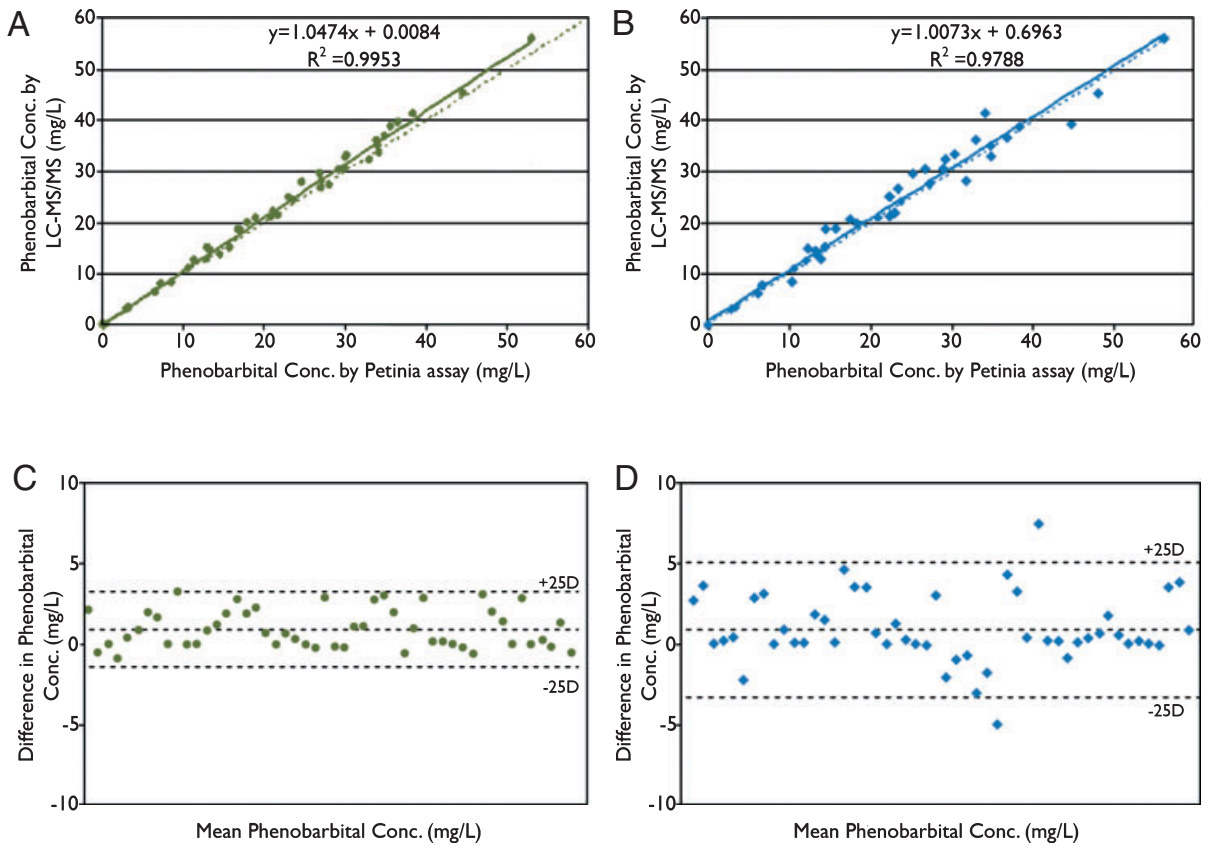

Figure 1.
LC-MS/MS versus Petinia: (**A**) and (**C**) adjusted for measured haematocrit. (**B**) and (**D**) adjusted for theoretical haematocrit (40% in adults; 55% in newborns). The dashed line is the line of identity, and the solid line is the regression line.

Table 1. Intra- and interday imprecision. Considering all the examined samples, discrepancies between expected and measured values were within 6.8%. A pooled DBS sample was processed 10 times, resulting an intraday repeatability below 3.9% for all values. Values better than 3.5% were obtained in an interday repeatability test

Expected concentration (mg/L) (n = 10)	Mean (mg/L)	Standard deviation	% CV	% Accuracy
Intraday				
0	0	0		
1	0.99	0.07	6.69	99.05
2	2.08	0.13	6.24	103.87
5	5.07	0.16	3.13	101.47
10	9.86	0.34	3.48	98.62
20	20.43	0.60	2.92	102.15
50	50.12	1.26	2.52	100.24
100	99.86	2.29	2.29	99.86
Interday (10 days)				
0	0	0		
1	0.97	0.04	4.20	97.34
2	2.03	0.09	4.63	101.31
5	5.03	0.23	4.53	100.61
10	9.65	0.32	3.33	96.54
20	19.42	1.30	6.71	97.12
50	51.54	2.53	4.91	103.08
100	99.38	4.57	4.60	99.38

2, 5, 10, 20, 50 and 100 mg/L. Intra- and inter-day imprecision data are reported in Table 1.

With the proposed parameters, the estimated limit of detection (signal to noise ratio >5) in DBS was 17 μg/L (0.5 mg/L, 30-times diluted), the limit of quantitation (signal to noise ratio >10) was 34 μg/L (1 mg/L, 30-times diluted).

Mean hematocrit levels for adult patients (43/50) (age: mean and median 53 years; SD: 16.82 years) were 39.6% (median 40%; SD: 4%); for adult controls (43/50) (age: mean 49 years; median 50 years; SD: 12.12 years) were 38.3% (median 39.1%; SD: 4.6%). Mean hematocrit levels for newborns (7/50) (age: <1 month) were 53.8% (median: 53.8%; SD: 1.7%); for newborn controls (7/50) (age: <1 month of life) were 55.6% (median: 55.4%; SD: 2.5%).

Discussion

We observed a significant inverse correlation between levels of PB in plasma and in whole blood levels, owing to the influence of hematocrit on plasma/whole-blood distribution of PB. Considering this, all samples' value obtained from DBS were multiplied by the factor 100/(100-HCT value) and compared to values obtained from the PETINIA method. Linear correlation analysis and Bland/Altman tests (summarized in Fig. 1A and 1C) showed an optimal agreement between the two methods. Correlation coefficient was >0.995 and the mean difference (PETINIA minus LC-MS/MS) was +0.89 mg/L (error 4.66%) with 95% limits of agreement of Bland and Altman −1.45 mg/L + 3.23 mg/L. All differences lie between the mean difference ±2 SD.

An objective of this new micromethod is to demonstrate its suitability for paediatric applications and potentially for domiciliary therapeutic drug monitoring. Therefore, assuming an hematocrit range between 50–60% (mean 55%) in newborns and 35–50% (mean 40%) in adults we also converted the PB measurements from DBS to plasma concentration multiplying by the factor 100/(100–55) and by factor 100/(100–40) for newborns (up to 1 month of life) and adults. Results showed an acceptable correlation (R = 0.979) and a good agreement (Fig. 1B and 1D). Considering the mean difference ±2 SD, only two values exceeded limits of agreement. They corresponded to PB values obtained from two patients (two 58 years old males) exhibiting hematocrit levels of 27% (this patient had undergone hemodialysis in the last 24 h) and 49%. The large statistical difference of these values in comparison with the applied theoretical value (40%, see above) might explain these data. Results in these two patients demonstrate that assay from remotely collected DBS has limitations if hematoctrit levels are far removed from normal. Ignoring this limitation could lead to serious error in drug level determinations compared with plasma.

Therapeutic monitoring of PB is essential for optimizing patient management. Monitoring of the drug from a simple DBS that can be obtained even at home is greatly advantageous compared with venipuncture especially in small children.

The present LC-M-MS assay and PETINIA are both sufficiently precise and accurate for routine TDM of PB. Moreover, there was a good agreement between DBS concentration and PETINIA plasma concentration when corrected for haematocrit (both actual and theoretical). The greatest advantage of the new micromethod is an ease sampling and transport, as well as easy sample preparation. The potential for reduction of costs should also be considered. It is conceivable that a prick of a finger for DBS can be performed at home. We observed that PB is stable at room temperature for at least 1 month. The DBS sample can easily be sent to the lab via mail avoiding the costs for personnel in charge of venipuncture transportation and for traveling to the venipuncture site. The actual, direct and indirect savings are very difficult to calculate in that they vary in different health systems. Instrumental analytical time is <4 min. For PETINIA assay, the instrument requires 6 min to test one sample. The improvement

achieved in terms of throughput is at least 1.5 times if the new LC-MS/MS method is used.

The LC-MS/MS assay from DBS is well suited for pediatric applications and, potentially, for domiciliary therapeutic drug monitoring.

Acknowledgments

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Disclosure: None of authors has any conflict of interest to disclose.

References

Bland JM, Altman DG. (1986) Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1:307–310.

Burhenne J, Riedel KD, Rengelshausen J, Meissner P, Müller O, Mikus G, Haefeli WE, Walter-Sack I. (2008) Quantification of cationic antimalaria agent methylene blue in different human biological matrices using cation exchange chromatography coupled to tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 863:273–282.

Hoogtanders K, van der Heijden J, Christiaans M, Edelbroek P, van Hooff JP, Stolk LM. (2007) Therapeutic drug monitoring of tacrolimus with the dried blood spot method. *J Pharm Biomed Anal* 44:658–664.

Koal T, Burhenne H, Römling R, Svoboda M, Resch K, Kaever V. (2005) Quantification of antiretroviral drugs in dried blood spot samples by means of liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* 19:2995–3001.

la Marca G, Malvagia S, Filippi L, Fiorini P, Innocenti M, Luceri F, Pieraccini G, Moneti G, Francese S, Dani FR, Guerrini R. (2008) Rapid assay of topiramate in dried blood spots by a new liquid chromatography-tandem mass spectrometric method. *J Pharm Biomed Anal* 48:1392–1396.

Mei JV, Alexander JR, Adam BW, Hannon WH. (2001) Use of filter paper for the collection and analysis of human whole blood specimens. *J Nutr* 131:1631S–1636S.

Patsalos PN, Berry DJ, Bourgeois BF, Cloyd JC, Glauser TA, Johannessen SI, Leppik IE, Tomson T, Perucca E. (2008) Antiepileptic drugs–best practice guidelines for therapeutic drug monitoring: a position paper by the subcommission on therapeutic drug monitoring, ILAE Commission on Therapeutic Strategies. *Epilepsia* 49:1239–1276.

Queiroz RH, Bertucci C, Malfará WR, Dreossi SA, Chaves AR, Valério DA, Queiroz ME. (2008) Quantification of carbamazepine, carbamazepine-10,11-epoxide, phenytoin and phenobarbital in plasma samples by stir bar-sorptive extraction and liquid chromatography. *J Pharm Biomed Anal* 48:428–434.

Saka K, Uemura K, Shintani-Ishida K, Yoshida K. (2008) Determination of amobarbital and phenobarbital in serum by gas chromatography-mass spectrometry with addition of formic acid to the solvent. *J Chromatogr B Analyt Technol Biomed Life Sci* 869:9–15.

Schmidt D, Einicke I, Haenel F. (1986) The influence of seizure type on the efficacy of plasma concentrations of phenytoin, phenobarbital and carbamazepine. *Arch Neurol* 43:263–265.

Subramanian M, Birnbaum AK, Remmel RP. (2008) High-speed simultaneous determination of nine antiepileptic drugs using liquid chromatography-mass spectrometry. *Ther Drug Monit* 30:347–356.

Tietz NW. (1994) Anti-epileptic drugs. In Tietz NW (Ed) *Textbook of clinical chemistry*, W.B. Saunders Co. Philadelphia, PA, pp. 1116–1117.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The MS/MS spectrum obtained by fragmenting the precursor ion (231.2 Th) of PB and D_5-PB (236.2 Th).

Figure S2. Extract ion chromatograms from a PB standard solution in water at the concentration of 12 mg/L (IS 0.08 mg/L) (panel **A**), a DBS of a patient with 12.1 mg/L of PB (panel **B**), and a DBS of a control (panel **C**).

Table S1. Stability of PB in dried blood spot at room temperature, +4°C, −20°C

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