A phenytoin assay using dried blood spot samples suitable for domiciliary therapeutic drug monitoring

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SUMMARY. A commercially available substrate-labelled fluorescent immunoassay procedure has been modified to create a simple rapid method for the determination of capillary phenytoin concentration in dried filter paper blood spots of patients on phenytoin therapy. The specimens are collected and despatched to the laboratory by the patient, thus domiciliary monitoring of phenytoin therapy can take place. The technique was validated by comparing the phenytoin results of simultaneously collected capillary dried blood spots and conventional plasma samples. The technique offers a convenient method for overcoming many of the practical problems of monitoring phenytoin therapy in epilepsy.

Monitoring of anti-epileptic drug levels is important in the management of patients with seizure disorders. Repeated visits by patients for venesection to obtain plasma samples may be traumatic, inconvenient, and time consuming. This is particularly true for children and the elderly, in those whose employment is precarious, and when their epilepsy is complicated by physical or mental handicap. For the general practitioner the difficulty of obtaining a sample for laboratory analysis may result in over-cautious prescribing (leading to sub-therapeutic levels, poor control, and multiple drug therapy), or a failure to recognise drug toxicity. A more acceptable method of obtaining samples which are suitable for analysis by standard laboratory methods is therefore required. As dried blood spots (DBS) collected at home and posted to the laboratory for glucose assay were well accepted by diabetic patients,¹ we have established a DBS-phenytoin assay by adapting the Ames (Miles Laboratories, Stoke Poges, Slough) TDA Fluorostat method to assay the concentration of phenytoin in discs punched from DBS collected on to filter paper cards by patients using an Autolet at home. A gas chromatographic phenytoin assay for DBS has also been described,² but it requires more complex equipment and sample preparation.

METHODS

SUBJECTS

Fifty-six adults (25 male, 28 female) and three paediatric (male) unselected patients with epilepsy receiving conventional single or combination anticonvulsant therapy were studied. Ten members of the laboratory staff known not to be taking anticonvulsant therapy were also studied.

BLOOD SAMPLES

Plasma samples

Venous blood was obtained from an antecubital vein at the same time as the capillary specimen, and the plasma was stored at −20°C until assay.

Capillary blood samples

Filter paper (Whatman Ltd, grade 160, Catalogue No. 2160917) was cut into 15 × 10 cm cards and stamped with a series of 1 cm diameter circles. This paper has recently been discontinued by the manufacturer and replaced by PKU–31 paper. The blood volume contained in a DBS disc depends on the characteristics of the paper punch and paper batch. Each labora-


tory should therefore establish their own relationship between capillary DBS and venous plasma phenytoin samples using their own assay materials.

Capillary blood was obtained from the palmar surface of a warmed terminal digital pulp space using an Autolet® (Owen Mumford Ltd, Woodstock Oxon) and applied to the centre of the circle so that the circle was filled on both sides of the paper. The spot was labelled with the date and time of collection, allowed to air dry at room temperature, and stored at 4°C in a sealed plastic container until assayed.

**Haematocrit experiment samples**
Aliquots of venous blood, collected from five patients on phenytoin therapy, were placed in tubes, centrifuged and the plasma supernatants adjusted to produce a range of haematocrit values. Each aliquot was then mixed, the haematocrit measured by standard techniques, and spotted on to filter paper cards. The phenytoin concentrations of these samples was then determined using the DBS phenytoin assay.

**ASSAY PROCEDURE**

**Plasma phenytoin assay**
The procedure described by the manufacturer in the TDA booklet was used. A dedicated dilutor was used to perform a four-stage procedure: stage (i) 50 µL of test sample were diluted with 2·5 mL assay buffer (bicine buffer); stage (ii) 50 µL of the initial dilution were placed with 0·5 mL of assay buffer in a reaction cuvette; stage (iii) 50 µL of antibody/ enzyme reagent (rabbit or goat antiserum to phenytoin/galactosidase) and 0·5 mL assay buffer was added to the reaction cuvette; stage (iv) 50 µL fluorogenic drug reagent (phenytoin labelled with umbelliferyl-β-D-galactoside/formate buffer) and 0·5 mL assay buffer were added to the reaction cuvette. After 20 min incubation at room temperature, fluorescence (proportional to the phenytoin concentration of the test sample) was measured in the Ames Fluorostat (using 405 nm narrow band pass excitation and 450 nm emission filters) whose microprocessor converts the results into concentration units (mg/L or µmol/L) using a standard curve constructed from supplied calibrants.

**Capillary dried blood spot phenytoin assay**
A 6 mm diameter disc was punched from each DBS and placed in a round-bottomed polyurethane tube (Sarstedt type 500, catalogue number 55.484, 55 x 12 mm). The elution reagent (490 µL aqueous 5'-sulphosalicylic acid, 50 g/L, British Drug Houses Ltd, Poole, Dorset) was added, the tubes were capped and phenytoin eluted from the DBS by agitation on a shaking platform for 4 h. The tubes were then centrifuged (5 min at 1000 g). Fifty microlitres of eluate was combined with 0·5 mL assay buffer. As the DBS represented the plasma and cell water of approximately 9·8 µL of whole blood, this gave a dilution of the test sample equivalent to stage (i) of the plasma assay. The assay was completed by performing stages (ii) to (iv) of the plasma assay. For the clinical assay service, duplicate discs from a single capillary sample were assayed and the mean result reported in mg/L.

**Statistical methods**
The analysis of Cornbleet and Gochman was used with the computer program of Dr P Wood, Department of Chemical Pathology, Southampton General Hospital, Southampton SO9 4XY.

**Results**

**ASSAY REPRODUCIBILITY**
Venous whole blood DBS from two patients on phenytoin with plasma concentrations of 4·6 and 13·9 mg/L gave within-assay coefficients of variation (n = 10) of 3·95% and 4·05% respectively, and between-assay coefficients of variation (n = 12) of 8·7% and 6·1% respectively. These studies were performed without changing the filter paper or TDA reagent batch.

**Stability**
Venous whole blood DBS from three patients with plasma phenytoin concentrations of 6·7, 8·8 and 13·9 mg/L were stored at 4°C, room temperature, and 37°C, and assayed after 1, 12, 19 and 35 days. No significant change in phenytoin concentrations was detected, indicating phenytoin is stable in a DBS matrix for at least 1 month. Storage in unsealed containers (as might happen in the home) had no effect on phenytoin stability.

**Haematocrit**
Over a typical normal venous haematocrit range (40–54%) DBS phenytoin concentration showed a fall of up to 2·0 mg/L in three out of the five samples (Fig. 2). At haematocrit values of less than 40% two samples demonstrated a fall in DBS phenytoin concentrations of up to 1·0 mg/L.
The capillary DBS samples from subjects not taking anticonvulsants gave a mean ± SD phenytoin concentration of 0.4 ± 0.14 mg/L (n = 10). Blank filter paper discs and elution reagent only were also assayed using the capillary DBS assay procedure. These gave mean ± SD phenytoin concentrations of 0.09 ± 0.10 (n = 10) and 0.03 ± 0.07 (n = 10) mg/L respectively. These values were sufficiently low for a blank correction to be unnecessary.

**RELATIONSHIP BETWEEN VENOUS PLASMA PHENYTOIN AND CAPILLARY DBS PHENYTOIN CONCENTRATION**

As shown in Fig. 1, this was highly significant (n = 56, r = 0.9889, y = 0.0818 + 0.6743x, standard error of regression = 3.4383), validating the capillary-DBS assay procedure. This relationship was obtained over 13 separate assay batches, indicating that the punching and eluting steps were adequately reproducible. The typical serum phenytoin therapeutic range of 10 to 20 mg/L gave a DBS-phenytoin range of 6.8 to 13.6 mg/L.

To detect changes in this relationship with time, simultaneously collected capillary DBS and venous plasma samples should be regularly compared as part of the quality assurance of the assay. The mean ± SD capillary DBS to plasma phenytoin concentration ratio was 0.70 ± 0.13.

**Discussion**

Phenytoin is frequently prescribed because it can be administered in a single daily dose and is inexpensive. Routine monitoring of phenytoin therapy is necessary as the oral dose cannot predict the circulating drug concentration because of the wide variability in the drug's half-life. This variability is caused by the saturation of the drug's hepatic degradative...
enzyme system that occurs within the drug's therapeutic range. In general practice drug levels have been reported to influence patient management decisions in 70% of epileptics, thus the availability of such estimations would be of great value.

A DBS-phenytoin assay was possible because substrate-labelled fluoroimmunoassays require only very small sample volumes in their reaction cuvettes, and aqueous sulphosalicylic acid was found to elute the drug from a blood spot/paper matrix leaving non-specific fluors with the DBS disc. This technique has already been applied to the monitoring of theophylline therapy. The stability of phenytoin in this matrix and the significant correlation between the DBS assay and conventional plasma measurements in patient samples meant the assay was suitable for clinical application. A tendency for the DBS-phenytoin concentration to fall with increasing haematocrit was demonstrated (Fig. 2); however, capillary haematocrit is significantly less than that of venous blood, and this effect was less at low haematocrit values. These results contrast with those of Albani et al. probably because of the differences in the elution techniques used, and the compromise in our technique between eluting the drug (which is highly protein-bound) without producing assay interference by also eluting non-specific fluors from the sample. The relationship between venous plasma and DBS samples in the patients (Fig. 1) suggests a haematocrit effect is of little practical importance. Approximately 90% of total circulating phenytoin is bound by albumin. Intra-erythrocyte phenytoin may represent the free (or active) drug concentration in plasma water, which may correlate better with clinical toxicity than total drug concentrations. A DBS sample (representing whole blood) combined with haematocrit and plasma concentration results therefore has the interesting potential for providing an index of the intra-erythrocyte phenytoin concentration, of possible benefit in patients with hypoalbuminaemia, e.g. ureaemic subjects. The mean whole blood to plasma phenytoin ratio of 0.70 in our subjects was similar to the mean of 0.73 (n = 9) reported by Borondy et al. The cost penalty of these commercially prepared reagents and dedicated instruments is offset by the convenience of the collection system to the patient, and the postal despatch which allows centralisation of the service to a single (perhaps regional) laboratory. The technique requires basic laboratory skills only, and the range of reagents available gives it potential for monitoring other drugs. The favourable comments of the patients who took part in this study suggests this technique would be well accepted in practice. Clearly, it depends on the quality of the sample provided and patient selection and instruction would be vital, but experience with diabetics suggests that most patients would cope satisfactorily.

Patients' compliance should improve as they become more aware of the value of the sample they are providing. DBS assays are particularly suitable for patients in whom venesection may be difficult, e.g. children, the physically and mentally handicapped, and the elderly.

A DBS phenytoin assay service therefore provides a convenient method for tailoring therapy to individual patient needs and satisfies the demand for making drug assays more readily available to general practitioners with less inconvenience to their patients.

References

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