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Note

Measurement of low (sub-therapeutic) phenobarbitone levels in plasma by high-performance liquid chromatography: application to patient compliance studies

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Recently it has been shown that small doses (e.g. 2 mg per day) of phenobarbitone (PB), included in prescribed medication, can be used as a means of assessing patient compliance with drug therapy [1–3]. This has given rise to the need for a simple and reliable method for the determination of low levels of PB in biological fluids. When PB is used to monitor compliance the expected plasma levels will be much lower (< 0.5 mg/l) than its usual therapeutic range (15–40 mg/l). Procedures for therapeutic monitoring of PB include gas chromatography (GC) [4,5], high-performance liquid chromatography (HPLC) [4,6,7] and immunoassays such as enzyme multiplied immunoassay technique (EMIT) [4,8] and fluorescence polarisation immunoassay (Abbott TD_X) [8]. Of these HPLC and immunoassays are preferred because of their relative simplicity and speed.

For patient compliance studies undertaken in this hospital a more sensitive and selective method suitable for the measurement of very low concentrations

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of PB was needed. With this requirement in mind, we have developed an HPLC method for PB which is a modification of the procedure reported by Gill et al. [9]. It utilizes a reversed-phase radial compression column with UV detection at 240 nm. The method is properly validated with respect to very low concentrations of PB and is used on patient samples following administration of low-dose PB as an indicator of compliance.

EXPERIMENTAL

Chemicals and reagents

HPLC grade methanol, hexane, diethyl ether and analytical reagent grade sodium hydroxide and sodium dihydrogen orthophosphate were purchased from BDH (Poole, U.K.). PB sodium and cyclobarbitone (CB) calcium (both BP grade) were purchased from May and Baker (Dagenham, U.K.).

Apparatus

A Radial Pak C_{18} reversed-phase column (10 cm × 0.8 cm I.D., particle size 10 μm) (Waters, Harrow, U.K.) compressed in a radial compression module (Z module) (Waters) was used in conjunction with a C_{18} guard column (Waters), a Model 460 autosampler (Kontron, Watford, U.K.), a Model 1204A variable-wavelength detector (LDC/Milton Roy, Stone, U.K.), a Model III Constametric pump (LDC/Milton Roy), a Model CI-10B integrator (LDC/Milton Roy) and a Model SEK recorder (LDC/Milton Roy). The following HPLC conditions were used: mobile phase (filtered and degassed), 0.1 *M* sodium dihydrogen orthophosphate–methanol (76:24, v/v) adjusted to pH 8.3 with concentrated sodium hydroxide solution; flow-rate, 2 ml/min; wavelength, 240 nm; detector range, 0.05 a.u.f.s.; injection volume, 100 μl; chart speed, 5 mm/min; temperature, ambient.

Procedure

To a screw-cap culture tube (12 ml), a 1.0-ml plasma sample, 0.2 ml of internal standard (5 mg/l CB calcium in water), 0.2 ml of 1 *M* phosphate buffer (pH 7.5) and 5 ml of a mixture of hexane–diethyl ether (1:1) were added. The contents were vortex-mixed for 30 s followed by centrifugation at 750 g for 5 min. The organic phase was transferred to a conical centrifuge tube (10 ml) and evaporated to dryness on a Model SC-3 sample concentrator (Techne, Cambridge, U.K.) at 45°C under a stream of oxygen-free nitrogen. The residue was reconstituted with 250 μl of a mixture of 0.1 *M* sodium dihydrogen orthophosphate–methanol (76:24) and 100 μl were injected onto the chromatograph.

Standard solutions of PB ranging from 0.05 to 0.5 mg/l ($n=5$) were prepared in drug-free plasma. Quality control (QC) samples of 0.1 and 0.4 mg/l were also similarly prepared. A 1-ml volume of each standard was assayed according to the procedure described. The peak-height ratio of drug (PB) to

internal standard (CB) was plotted against concentration of PB (calculated as free acid) and the calibration graph was used for measuring the concentration of PB in the patient samples.

RESULTS AND DISCUSSION

Chromatograms of drug-free plasma and plasma samples of PB are shown in Fig. 1. The retention times of PB and CB are 5 and 10 min, respectively. The calibration curve was linear within the concentration range studied. The regression equation for the calibration graph was $y=0.0021x+0.0085$, $r=1.000$. Good reproducibility of the method was indicated when replicate determinations of 0.05, 0.25 and 0.5 mg/l samples of PB gave within-day coefficients of variation (C.V.) of 3.9, 2.0 and 1.0% and day-to-day C.V. of 4.8, 2.5 and 1.3%, respectively ($n=10$ in each case). The limit of detection (signal-to-noise ratio of 3) was found to be 0.02 mg/l. Percentage recovery (extraction efficiency) of PB was calculated by comparing the peak height of the plasma sample (after extraction) with that of an aqueous solution containing the appropriate concentration of this drug. Three concentrations within the calibration range, i.e. 0.05, 0.25 and 0.5 mg/l ($n=5$ in each case) were investigated for recovery studies. The addition of buffer (pH 7.5) to the sample and the use of hexane–diethyl ether mixture as an extractant yielded samples with a mean ($\pm$S.D.) recovery of $87\pm2\%$.

Whenever a batch of samples was analysed, calibration standards were also included in the run to allow a standard curve to be constructed. In addition,

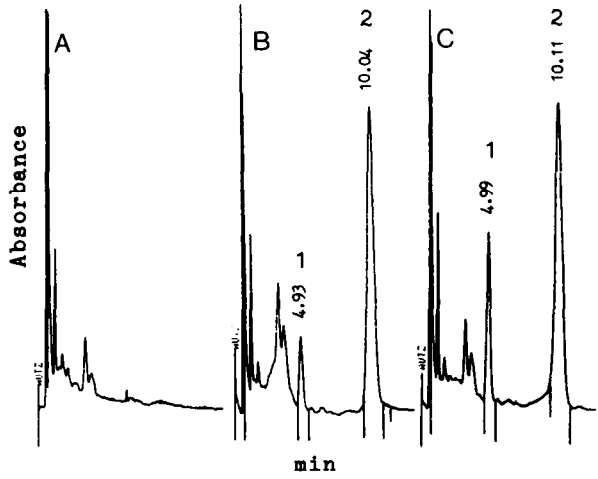

Fig. 1. Chromatograms of blank plasma (A), patient plasma containing 109 ng/ml phenobarbitone (B) and 254 ng/ml phenobarbitone (C). Peaks: 1 = phenobarbitone; 2 = cyclobarbitone (internal standard).

two QC samples at 0.1 and 0.4 mg/l were run along with each batch of samples. For each batch the results of QC samples were within 10% of known values. No peaks were observed at the retention times of PB or CB from the blank plasma nor from commonly used drugs such as phenytoin, carbamazepine, atenolol, propranolol, isoniazid/rifampicin, glibenclamide, metoprolol, lisinopril and amitriptyline with which PB might be used as a compliance indicator. Warfarin and nifedipine gave peaks, but they were eluted after CB at 15 and 30 min, respectively. When stored at $-20\,^{\circ}C$ the plasma standards were stable for at least six weeks.

During the preliminary work on selection of a suitable technique for compliance studies we explored the possibility of using immunoassay (EMIT) because of its simplicity and speed, but using this technique we were unable to obtain the necessary accuracy and precision at very low levels of PB (<0.5 mg/l). The criteria of accuracy, precision and selectivity were easily met by HPLC, and therefore we decided to use HPLC in our studies. In one study the steady state concentrations of PB measured over a year in a compliant patient (who received 2 mg PB daily, incorporated with lisinopril) ranged from 321 to 371 ng/ml with a mean value of 342 ng/ml ($n=9$). Table I shows the plasma levels in a small group of volunteers following four weeks 'treatment' with 2 mg PB per day when they were fully compliant and when they simulated poor (incomplete) compliance with the same dose over a similar (four weeks) period. Poor compliance was simulated by missing one day (dose) every three days. Several clinical studies have demonstrated that 7.5 mg PB daily (or less) is non-sedative [3] and non-enzyme-inducing [10] and may safely be used as an indicator of compliance. The method is currently being used in further compliance studies. The results reported here demonstrate the usefulness of the

TABLE I

PLASMA LEVELS IN VOLUNTEERS FOLLOWING FOUR WEEKS 'TREATMENT' WITH 2 mg PB PER DAY

(A) The volunteers were fully compliant. (B) The volunteers simulated poor (incomplete) compliance with the same dose over a similar (four weeks) period. (Poor compliance was simulated by missing one day (dose) every three days.)

Volunteer	PB concentration (ng/ml)	
	A	B
1	412	348
2	389	254
3	411	251
4	361	226
5	353	260
6	255	140

present method for the determination of low levels of PB in plasma samples, during its use as a potential compliance indicator.

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