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Direct serum injection high-performance liquid chromatographic method for the simultaneous determination of phenobarbital, carbamazepine and phenytoin

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SUMMARY

A method is described for the simultaneous measurement of serum levels of three antiepileptic drugs, phenobarbital, phenytoin and carbamazepine, by direct injection high-performance liquid chromatography on a 25-cm Pinkerton™ internal surface reversed-phase (ISRP) column. Several commonly available compounds were tested and found not to co-chromatograph with the three drugs of interest or the internal standard, 5-(*p*-methylphenyl)-5-phenylhydantoin. Results obtained on patients' samples with this method compared well with those from enzyme-multiplied immunoassay technique (EMIT™).

INTRODUCTION

The value of measurement of anti-epileptic drugs (AEDs) in patient management is widely accepted [1–3]. Modern methods for the measurement of AEDs are based on either chromatographic or immunochemical techniques. A variety of commercially available non-isotopic, homogeneous immunochemical methods (enzyme-multiplied immunoassay technique (EMIT) [4], fluorescence polarization immunoassay (FPIA) [5]) offer fast analysis for individual drugs; however, samples from patients on multiple drug therapy have to be analysed several times. Problems of interference from metabolites with non-isotopic immunochemical techniques has been documented for pheny-

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toin, using both EMIT [6] and FPIA [7] technology particularly in patients with renal failure [6,7]. Chromatographic methods, high-performance liquid chromatography and gas chromatography (GC), exist for analysis of either individual or multiple drugs and have recently been extensively reviewed [8]. An advantage of HPLC over immunochemical analysis is the ability to measure several drugs on one chromatogram. However, the main disadvantage of traditional reversed-phase HPLC methods is the requirement for extraction and/or protein precipitation prior to injection.

Newer HPLC techniques that do not require manual sample pre-treatment have been reported for the measurement of AEDs. Column switching allows analysis on either serum [9–11] or whole blood [12] but generally requires two pumps [10–12] and appropriate valves to prevent proteins entering the analytical column. Addition of an anionic (sodium dodecyl sulphate, SDS) or non-ionic (Brij-35) surfactant to the eluent [13,14] has been reported to permit injection of serum directly onto reversed-phase columns, the proteins being held in solution by micellar formation. Internal surface reversed-phase (ISRP) columns allow direct serum injection without addition of surfactants and have been used to chromatograph spiked serum samples containing AEDs [15] and have been used by us to measure other therapeutic and abused substances in clinical situations [16–18].

Here we present improved eluent conditions using an ISRP column for the rapid separation of three AEDs in human serum and an evaluation of its performance for monitoring therapeutic levels in epileptic patients.

EXPERIMENTAL

Chemicals

Dipotassium hydrogenphosphate, potassium dihydrogenphosphate (AnalaR grade), tetrahydrofuran and acetonitrile (HiPerSolv grade) were obtained from BDH (Poole, U.K.). Carbamazepine was a gift from Ciba Geigy (Horsham, U.K.). Sodium phenobarbital and primidone were gifts from ICI Pharmaceuticals Division (Macclesfield, U.K.). Sodium phenytoin and ethosuximide were gifts from Parke Davies Medical (Eastleigh, U.K.). The internal standard, 5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH) was obtained from Sigma (Poole, U.K.). Other drugs used in the course of this study were obtained either from Sigma or from the hospital pharmacy.

Instrumentation

The HPLC system consisted of LC-XPD pump and LC-UV variable-wavelength detector (Pye-Unicam, Phillips Analytical Department, Cambridge, U.K.) with a light path of 1 cm and a flow cell volume of 8 μl. The signal output was recorded on a SP 4100 computing integrator (Spectra-Physics, Santa Clara, CA, U.S.A.). Sample injection was achieved with an autoinjector, Model SpH

125 Fix (Spark Holland, Emmen, The Netherlands) fitted with a 20-μl injection loop or a manual Rheodyne system with a 20-μl loop.

Chromatography

For the evaluation of use in the clinical situation the solvent system consisted of an eluent of 0.1 *M* potassium phosphate buffer (pH 7.2) containing acetonitrile and tetrahydrofuran (80:10:10, v/v/v) at a flow-rate of 1.4 ml/min. The chromatographic separation was achieved using a Pinkerton™ ISRP column, 25 cm×4.6 mm I.D., 5 μm particle size) (Orbit Labs. Bangor, U.K.) with a cartridge precolumn of the same material. The chromatography was performed at ambient room temperature. The column and precolumn were stored in acetonitrile when not in use. Prior to use, the columns were washed with distilled water for 5 min, followed by eluent buffer for 10 min. The precolumn was routinely changed after 400 injections and the analytical column has received in excess of 2000 injections without any appreciable increase in back-pressure or decrease in resolution. The direction of the eluent through the analytical column was reversed at regular intervals. A 10-μm filter system was incorporated into the eluent flow lines. Detection of peaks was achieved by monitoring the effluent from the column at 254 nm with an f.s.d. of 0.08 a.u. and an integrator attenuation of 1. Peak-height ratios were used for the calculation of results.

Standards

Serum standards were prepared by the addition of ethanolic solutions of carbamazepine (20 mmol/l), sodium phenobarbital (40 mmol/l) and sodium phenytoin (8 mmol/l) to a single donation of human serum from a female volunteer taking no drug therapy. Five combined serum standards containing carbamazepine (12.5–200 μmol/l), phenobarbital (25–400 μmol/l) and phenytoin (10–160 μmol/l) were used to assess linearity. For routine use, a single standard concentration of 50 μmol/l carbamazepine, 100 μmol/l phenobarbital and 40 μmol/l phenytoin was used to calibrate the assay.

Patient samples

Serum samples were obtained from 100 patients receiving anticonvulsant therapy, 19 of whom were on multiple drug therapy. The samples were stored at 4°C and analysed by routine EMIT analysis for requested analytes within five days of venesection. Samples were then stored at −20°C until HPLC analysis was performed.

Sample preparation

For HPLC analysis the standards, samples and controls were thawed at room temperature and thoroughly mixed. Sera were observed visually for any particulate matter and centrifuged at 2000 *g* prior to analysis if necessary. Stan-

dards, controls and samples (500 μl) were mixed with an equal volume of internal standard (25 μmol/l MPPH, the solid being dissolved in a small volume of methanol before making up to the required concentration in elution buffer) prior to injection onto the ISRP column. No turbidity was observed when serum samples were mixed with the internal standard.

Other procedures

Phenytoin, phenobarbital and carbamazepine were measured by EMIT analysis modified for use on a centrifugal analyser (Multistat MIII, Instrumentation Labs., U.K.).

RESULTS AND DISCUSSION

Optimum chromatographic conditions for the resolution of phenobarbital, carbamazepine, phenytoin and internal standard (MPPH) were acetonitrile–tetrahydrofuran–0.1 mol/l pH 7.2 phosphate buffer (10:10:80, v/v/v) at a flow-rate of 1.4 ml/min, the whole chromatogram taking 12 min to complete (Fig. 1). Since calibration curves for the three drugs were linear over the ranges

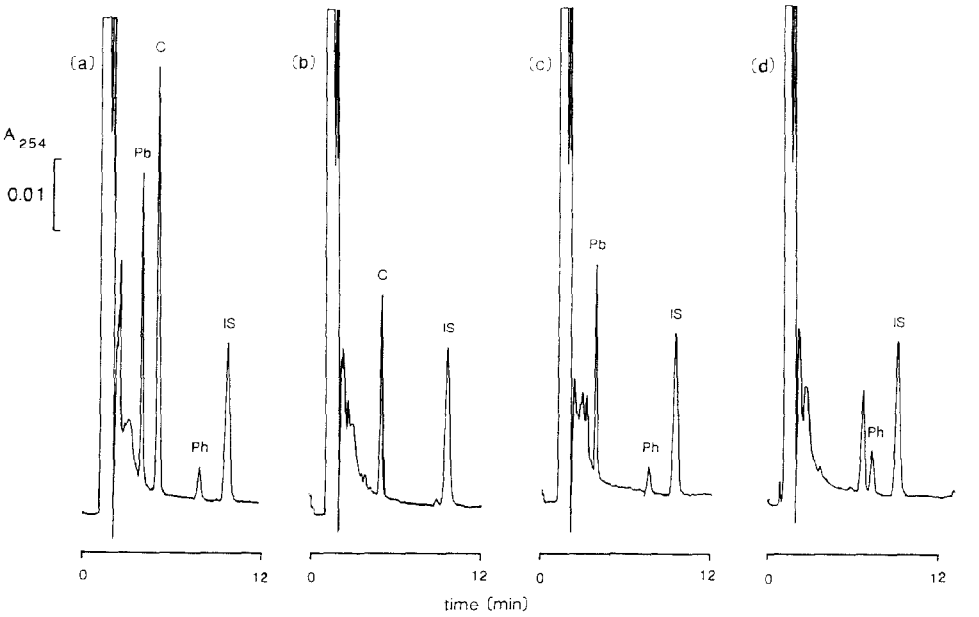

Fig. 1. Chromatograms of (a) serum standard containing 100 μmol/l phenobarbital (Pb), 50 μmol/l carbamazepine (C), 40 μmol/l phenytoin (Ph) and internal standard (MPPH) (IS), and sera from patients on (b) carbamazepine therapy (25 μmol/l), (c) phenobarbital and phenytoin therapy (75 and 32 μmol/l, respectively) and (d) phenytoin and clobazam therapy (51 μmol/l phenytoin).

TABLE I

RETENTION INDICES RELATIVE TO INTERNAL STANDARD OF ANTI-EPILEPTIC DRUGS AND OTHER DRUGS AND METABOLITES

Compound	Retention index
Internal standard[a]	1.00
Phenobarbital	0.45
Carbamazepine	0.60
Phenytoin	0.83
Ethosuximide	0.30
Caffeine	0.31
Theophylline	0.31
Salicylate	0.31
Paracetamol	0.34
Primidone	0.37
Carbamazepine 10,11 epoxide	0.41
DF118	0.53
Morphine	0.54
Codeine	0.55
5-(*p*-Hydroxyphenyl)-5-phenylhydantoin	0.65
Clobazam	0.69
Methadone	1.39

[a]Retention time = 9.4 min.

TABLE II

INTER-ASSAY COEFFICIENTS OF VARIATION FOR DIRECT INJECTION HPLC ($n = 7$)

Compound	Concentration (μmol/l)	Coefficient of variation (%)
Carbamazepine	23.6	3.2
	97.8	3.8
Phenobarbital	51.1	9.5
	195.0	2.3
Phenytoin	20.3	5.2
	81.0	4.3

0–400 μmol/l for phenobarbital, 0–200 μmol/l for carbamazepine and 0–160 μmol/l for phenytoin, a single-point calibration was used routinely (Fig. 1a). Sera from two volunteers taking no drug therapy showed no interfering endogenous peaks.

Retention indices (RIs) were measured for a number of commonly available drugs (Table I). None of the drugs tested had an RI close enough to those of the AEDs of interest to present a problem. The carbamazepine metabolite,

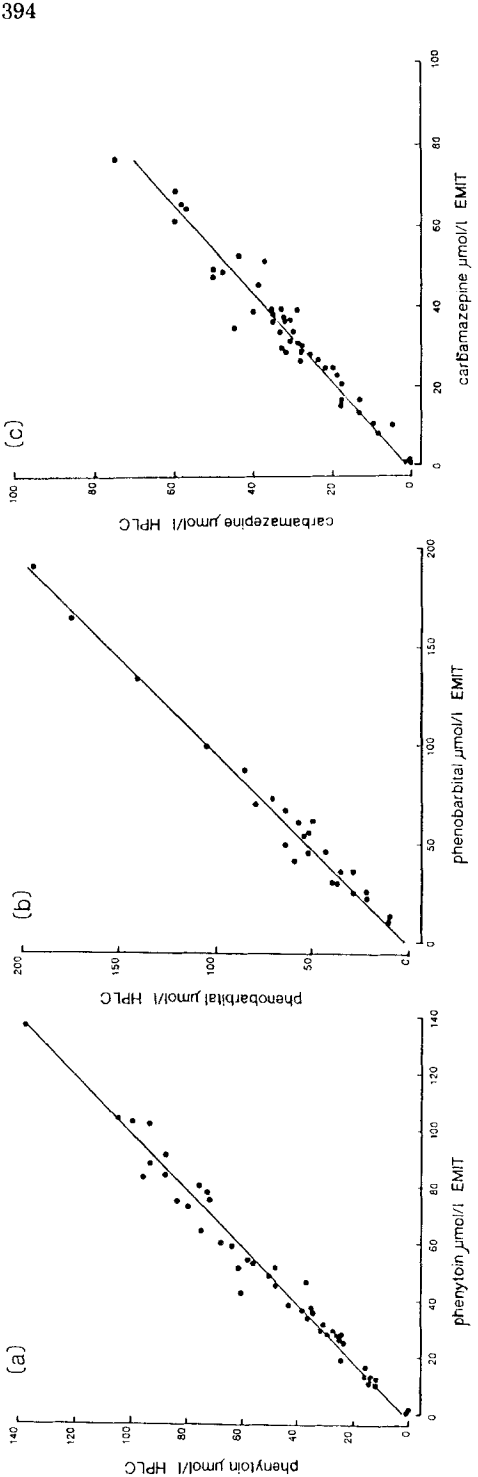

Fig. 2. Comparison of results obtained by direct injection HPLC and EMIT analysis for (a) phenytoin ($y = 0.997x + 2$; $r = 0.99$; $p < 0.001$; $n = 46$), (b) phenobarbital ($y = 1.047x + 1$; $r = 0.99$; $p < 0.001$; $n = 25$) and (c) carbamazepine ($y = 0.914x + 1.3$; $r = 0.98$; $p < 0.001$; $n = 46$).

carbamazepine 10,11 epoxide, and phenytoin metabolite, 5-(*p*-hydroxyphenyl)-5-phenyl hydantoin, were resolved from the parent compounds. One hundred patient samples were analysed during the course of this study and the only significant peak seen, other than the AEDs for measurement, was N-desmethylclobazam, the bioactive metabolite of clobazam (Fig. 1d).

Imprecision data are shown in Table II. This is similar to that of EMIT analyses and at least as good as published extraction HPLC methods. There is good correlation ($r=0.98$, $p<0.001$) between the results obtained by EMIT analysis and this new non-extraction HPLC method for all three drugs measured (Fig. 2). Carbamazepine levels were noted to be lower ($y=0.914x+1.3$) when measured by HPLC than by EMIT. A similar finding has been reported with extraction HPLC methods [8].

A large number of HPLC methods have been reported for measurement of AEDs using reversed-phase columns with preliminary extraction/protein precipitation of serum [8]. More recently, investigators have perceived the need to eliminate the manual pre-analytical stage using column-switching techniques, both manual [9] and automated [10–12] which claim to be "direct serum injection" methods. However, these techniques are essentially automated column extraction procedures and the analytical column receives only the pre-concentrated analytes. Moreover, column switching is relatively complex and expensive, generally requiring two pumps and multiport injection systems.

Direct sample injection has been achieved by the inclusion of anionic and non-ionic surfactants in the eluent, allowing the serum proteins to directly pass through the analytical column [13,14]. While these techniques have been successfully used for the quantitation of phenobarbital, carbamazepine and phenytoin in serum standards and controls, no results have been reported for patients' samples. The development of ISRP columns allows proteins to be excluded from the internal pores and pass around the hydrophilic exterior of the biphasic packing material while analytes of less than 2000 daltons react with the hydrophobic internal surfaces. We have previously shown ISRP columns to be suitable for the measurement of paracetamol and salicylate in overdoses [17,18] and of theophylline and caffeine in neonatal samples [16].

Here, it is shown that use of a Pinkerton™ ISRP column also offers a fast direct serum injection HPLC method for the simultaneous measurement of phenobarbital, carbamazepine and phenytoin.

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