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AUTOMATED SEQUENTIAL PROCESS FOR PREPARING SAMPLES FOR ANALYSIS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new method for the preparation of biological samples prior to high-performance liquid chromatographic analysis is described. The automated technique combines dialysis and trace enrichment. In order to investigate its potential, it has been applied to the estimation of total anticonvulsants and theophylline in serum.

INTRODUCTION

Sample matrices often contain components that influence the quantitation of analytes by high-performance liquid chromatography (HPLC). The matrices of physiological fluids, such as serum, are particularly complex and present numerous sample preparation problems. These can be identified as follows: (a) the analyte concentration/interfering non-analyte ratio is very small; (b) some non-analytes progressively reduce the performance of the analytical column; (c) the non-analyte composition is variable between samples.

Strategies to overcome these problems can be divided into two main groups, namely, direct injection and sample pre-treatment. In the direct injection approach untreated sample is loaded onto the HPLC column[1]. The success of these methods relies initially on the resolving power of the chromatographic system and the specificity of the applied detector. Although this is an apparently simple approach, in practice, the development of suitable chromatographic conditions present many problems. Methods involving sample preparation employ a complementary separation technique to eliminate or reduce the concentration of the non-analytes to a level where they no longer interfere with HPLC. Complementary separation methods that have been used include liquid–liquid extraction[2], protein precipitation[3], ultrafiltration[4], dialysis[5] and the use of solid phase extractions in either pre-column or column switching and backflushing techniques[6,7]. Preliminary investigations[8] have shown that a new sample preparation technique, the automated sequential trace enrichment of dialysates (ASTED) potentially addresses all these problems. By using a combination of dialysis and trace enrichment, ASTED provides a separation process complementary to HPLC, enabling the removal of the interfering non-analytes from the sample matrix prior to HPLC analysis.

This paper describes the performance of two ASTED methods compared with that of two established sample preparation techniques.

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EXPERIMENTAL

Two HPLC separation methods were used to investigate the performance of ASTED for sample preparation. One method separated the anticonvulsants phenobarbitone, phenytoin, carbamazepine and carbamazepine 10,11-epoxide and the other separated theophylline from caffeine. Samples of the same sera were prepared by both ASTED and a reference method (liquid–liquid extraction for the anticonvulsant assay[2] and protein precipitation for the theophylline assay[9]) and quantified by HPLC separation. All the sample preparation techniques were optimised for the assay of total analyte concentrations in serum.

Instrumentation

Unless otherwise stated, all of the equipment in the HPLC and ASTED units was obtained from Gilson Medical Electronics, Villiers-le-Bel, France.

HPLC unit. The HPLC system used consisted of a 303/5SC pump, a Holochrome variable-wavelength UV detector and a Rheodyne 7010 injection valve mounted on a 231 auto-sampling injector (ASI). Chromatographic peak areas were integrated using a 620 Data Master, linked to an Apple IIe microcomputer, which operated a 704 System Manager, Version 2.1.

ASTED unit. A diagram of the unit is shown in Fig. 1. It consisted of a 231 ASI, two 401 dilutors, a polymethyl methacrylate, 150 mm path length flat-plate dialyser, with a donor channel volume of approximately 80 μl, fitted with a cuprophan membrane (10–15 kilodalton cut-off) and a stainless-steel trace enrichment cartridge (TEC), having a packed bed 1 mm high × 4.6 mm I.D., fitted in place of the loop on the injection valve. The 7010 valve was fitted with a 20-μl loop for the injection of samples in the manual preparation methods. The TEC was packed with 15 mg of 10-μm Hypersil ODS (Shandon Southern Products, Runcorn, U.K.). The ASTED process was controlled by the 231 ASI.

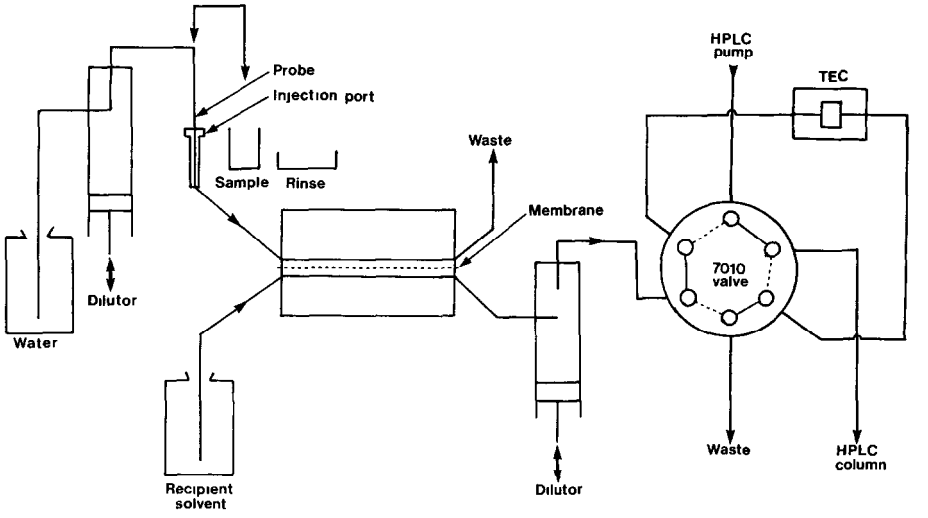

Fig. 1. Schematic diagram of the ASTED system.

Operation of the ASTED unit. The coordination of the operations of the ASTED and HPLC system has been described previously[10] and permitted concurrent operation of the two processes. In this way, a sample can be pretreated during chromatographic separation of the previous sample. The only rate-limiting step is the chromatographic analysis time.

An ASTED cycle can be divided into four stages:

(A) Loading the sample. Internal standard solution is added to an aliquot of the sample, and the mixture (bracketed by air segments to minimise dispersion of the sample mixture) injected into the donor channel of the dialyser by the 231 ASI. The sample mixture in the donor channel is then held static.

(B) Dialysis and trace enrichment. The dialysate containing diffusible solutes is then moved continously from the recipient channel of the dialyser by the 401 dilutor and loaded onto the TEC, with the injection valve held in the LOAD position.

(C) Injection and purging. The injection valve is turned to the INJECT position, The analytes retained on the TEC are eluted into the analytical column, and both the donor and recipient channels of the dialyser are purged.

(D) Regeneration. The injection valve is returned to the LOAD position, and the TEC is re-equilibrated with dialyser recipient solvent (see below) to regenerate the initial conditions.

Reagents

Unless otherwise stated, all chemicals were of analytical grade, obtained from BDH Chemicals., Poole, U.K. The water used for all reagent preparations was purified by passing through activated carbon and an ion-exchange resin (Spectrum C system, Elga, High Wycombe, U.K.).

Combined stock standards. All drugs were obtained from Sigma (Poole, U.K.) unless otherwise stated. The stock standard contained: 1.5 g/l phenobarbitone, 1.5 g/l phenytoin, 0.5 g/l carbamazepine, 0.125 g/l carbamazepine 10,11-epoxide (supplied by Geigy Pharmaceuticals, Macclesfield, U.K.), 1.0 g/l theophylline and 1.0 g/l caffeine. All standards were dissolved in methanol.

Working standard. The stock standard was diluted 1:50 with drug-free human serum to give analyte concentrations of 30 mg/l phenobarbitone, 30 mg/l phenytoin, 10 mg/l carbamazepine, 2.5 mg/l carbamazepine 10,11-epoxide, 20 mg/l theophylline and 20 mg/l caffeine.

Anticonvulsant internal standard. A 200-mg amount of hexobarbitone was dissolved in 1 l of 1.0 *M* ammonium phosphate buffer containing 250 m*M* trichloroacetic acid (pH 7.0).

Theophylline internal standard. A 160-mg amount of β-hydroxyethyltheophylline was dissolved in 1 l of 500 m*M* monochloroacetic acid.

Dialyser recipient solution. This was a diammonium hydrogen orthophosphate buffer, 20 m*M*, pH 5.0.

Chromatographic conditions

A 100 × 4.6 mm I.D. column, packed with 5-μm Spherisorb ODS 2 (Phase Separations, Queensferry, U.K.) was used for all HPLC separations. For the anticonvulsant assay, the eluent used was: acetonitrile–tetrahydrofuran (THF)–1.0 *M* ammonium phosphate buffer (pH 5.0)–water (25:4.25:2:69, v/v/v/v) at a flow-rate of

2.5 ml/min. The detector was set at 220 nm and 0.2 absorbance units full scale (a.u.f.s.). The eluent for the theophylline assay was THF–1.0 *M* ammonium phosphate buffer (pH 5.0)–water (1.5:2:96.5, v/v/v) at a flow-rate of 2.5 ml/min with the detector set at 273 nm and 0.1 a.u.f.s.

Methods

Sample preparation. (1) Anticonvulsant liquid–liquid extraction method. An 800-μl aliquot of either test or standard solution was mixed with 200 μl anticonvulsant internal standard reagent and 8.0 ml diethyl ether. After extracting for 10 min on a rotary mixer, the organic phase was removed and evaporated in another tube. The residue was dissolved in 100 μl anticonvulsant eluent, and 20 μl was injected into the HPLC system via the 231 ASI Rheodyne 7010 valve, fitted with a 20-μl loop.

(2) Anticonvulsant ASTED method. A 100-μl aliquot of either test or standard solution was mixed with 25 μl anticonvulsant internal standard reagent, and 100 μl of this mixture was loaded into the dialyser. Dialysate (2 ml) was passed through the TEC during a time interval of 3 min. The analytes retained by the TEC were then loaded into the HPLC.

(3) Theophylline protein precipitation method. A 200-μl aliquot of either test or standard solution was mixed with 50 μl theophylline internal standard. The proteins were precipitated by the addition of 300 μl of 10% (w/v) trichloroacetic acid, and 20 μl of the supernatant was injected into the HPLC system.

(4) Theophylline ASTED method. A 100-μl aliquot of either test or standard solution was mixed with 25 μl theophylline internal standard and 100 μl of this mixture was loaded into the dialyser. After passing 700 μl dialysate through the TEC during a time interval of 3.5 min, the analytes retained by the TEC were loaded into the HPLC.

All ASTED operations were performed by the 231 ASI in both the anticonvulsant and theophylline methods.

Quantitation. Analyte concentrations were calculated by proportion, using the ratio of the peak area to the internal standard, the peaks being identified by their retention time.

RESULTS

Chromatographic performance

The effect of the serum matrix on chromatographic performance was assessed in the anticonvulsant HPLC method by repeatedly injecting into the analytical column 20 μl aliquots of the working standard (Fig. 2a). There was a progressive loss of column performance, and the first analyte eluted was undetectable, due to the large concentration of poorly retained components in the serum. When the same sample was prepared by ASTED, no loss of column performance was observed, and all the analytes were quantifiable (Fig. 2b).

Analytical performance

The imprecision of the different methods are summarised in Table I. The within-run imprecision was estimated by assaying two pools of sera, supplemented to two different levels (low and high therapeutic) for all the analytes, 20 times in the same

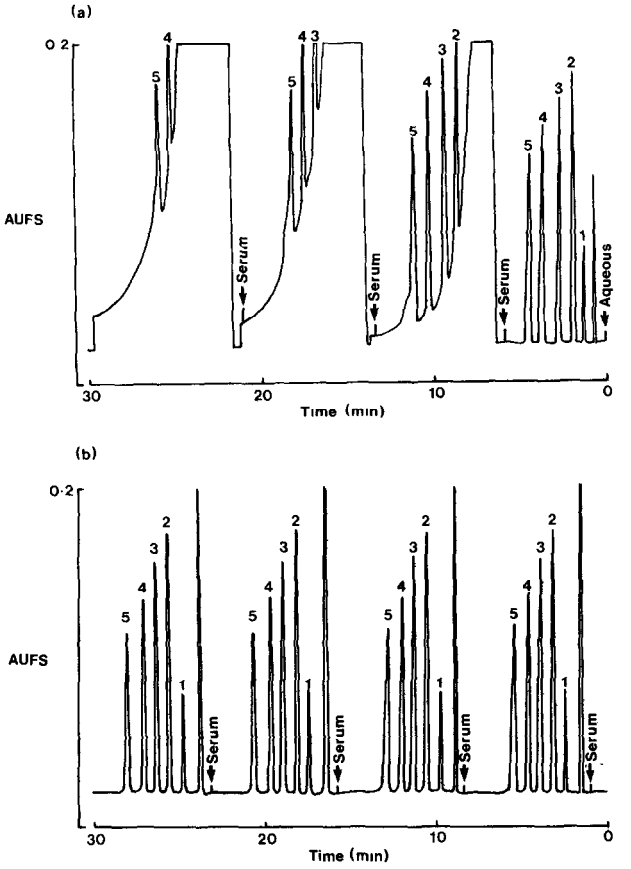

Fig. 2. Chromatograms of anticonvulsants using (a) direct 20 μl injection, and (b) ASTED. The arrows indicate the times of injection. The HPLC conditions were identical, and the serum samples were taken from the same serum pool. Peak identification: 1 = carbamazepine epoxide, 2 = phenobarbitone, 3 = carbamazepine, 4 = hexobarbitone and 5 = phenytoin.

run. The between-run imprecision was estimated by assaying the same two serum pools on 20 different days. For all the methods the peak areas varied linearly with concentration for all analytes up to at least three times the top of their respective therapeutic ranges. No carry-over between samples was observed, when both the donor and recipient channels of the dialyser were purged with 2 ml water and recipient solution, respectively.

The analytical recoveries of all the methods ranged from 93–106%. The absolute recovery of analytes using the ASTED method was estimated from the peak areas obtained by assaying an aqueous solution of analytes by the ASTED method and those obtained by injecting 20 μl of the same aqueous analyte solution into the HPLC column. For a dialyser donor channel volume of 80 μl, it was estimated that approximately 50% of each analyte originally in the dialysed aqueous analyte solution was loaded into the HPLC, using the ASTED method.

The sensitivities of the anticonvulsant and theophylline ASTED methods were

TABLE I

IMPRECISION (COEFFICIENTS OF VARIATION, C.V., %) OF THE METHODS

n = 30. Ref = reference method. Level 1 = low therapeutic level, level 2 = high therapeutic level.

Compound	*Within-run C.V. (%)*				*Between-run C.V. (%)*			
	Level 1		*Level 2*		*Level 1*		*Level 2*	
	Ref	*ASTED*	*Ref*	*ASTED*	*Ref*	*ASTED*	*Ref*	*ASTED*
Phenobarbitone	3.3	1.8	1.7	1.4	5.1*	2.7*	2.5	2.0
Phenytoin	5.0*	2.5*	2.8	2.4	7.6	4.5	4.3	3.8
Carbamazepine	2.8	2.3	2.7	1.9	4.0	3.6	4.1	3.2
Carbamazepine epoxide	4.6	2.9	3.0	2.5	6.6	4.7	4.4	4.0
Theophylline	5.0*	2.6*	3.5	2.1	7.2	4.4	5.2	3.6
Caffeine	3.5	2.6	2.9	1.8	5.0	3.9	4.3	3.5

* Variances are significantly different (p = 0.05).

established by analysing 30 drug free sera after standardising the procedures with the calibration serum. The detection limit for each drug was calculated as the mean concentration (±2 S.D.) of the zero standard peaks. For the detector settings used the detection limits, expressed as mg of analyte per litre of serum sample were phenobarbitone 0.08, phenytoin 0.17, carbamazepine 0.07, carbamazepine 10,11-epoxide 0.05, theophylline 0.06 and caffeine 0.08.

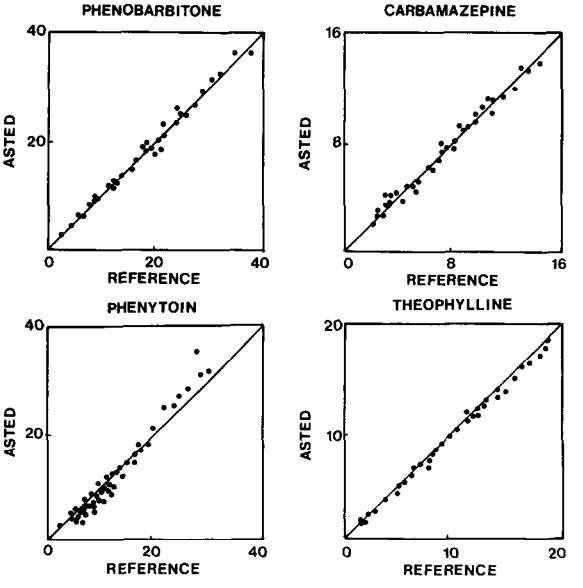

Fig. 3. Comparison of ASTED and reference methods for phenobarbitone, carbamazepine, phenytoin and theophylline. Phenobarbitone: $y = 0.2 + 0.98x$, $r = 0.989$, $n = 34$; carbamazepine: $y = 0.14 + 0.98x$, $r = 0.989$, $n = 36$; phenytoin: $y = -0.25 + 1.15x$, $r = 0.993$, $n = 62$; theophylline: $y = 0.25 + 0.97x$, $r = 0.996$, $n = 36$.

The results from the HPLC assay of a series of serum samples by the different sample preparation methods were compared using an orthogonal regression[11] and are shown in Fig. 3.

Serum samples from the Heathcontrol external quality assesdsment scheme (Department of Pharmacology and Therapeutics, University of Wales, Cardiff, U.K.) were assayed by both ASTED methods, and the results for four analytes were compared with the mean of the results obtained by laboratories using reversed-phase HPLC. The deviation of the ASTED method results from the concensus mean, over a period of eight months, is shown in Fig. 4.

DISCUSSION

The ability of ASTED to eliminate gross effects of the serum matrix on the chromatographic performance can be seen in Fig. 2. The HPLC conditions for the ASTED and reference methods were identical and, therefore, any differences between their analytical performances would be due to the different sample preparation procedures used.

ASTED was a significantly less imprecise sample preparation technique than the reference methods for the assay of phenobarbitone, phenytoin and theophylline (Table I). This is probably due to total automation of the ASTED method. Similar estimates of linearity and analytical recovery were obtained for the assay of the various analytes by both the ASTED and reference methods.

Techniques based on diffusion or dialysis are often very slow. It may take

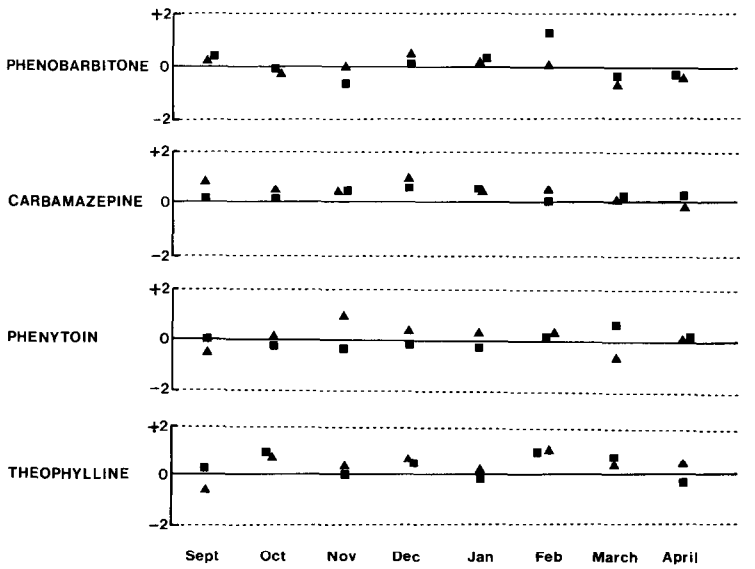

Fig. 4. Performance of the ASTED method compared with the reversed-phase method group mean on the Heathcontrol external quality assessment scheme over a period of eight months. Units are standard deviations of the method group mean. The different symbols represents results from two different quality assessment samples.

many hours of dialysis before the concentrations of diffusible solutes in the two chambers of an equilibrium dialysis cell reach equivalence. If the chambers have the same volumes, the maximum absolute recovery achievable is only 50%. However, the absolute recovery of analytes by ASTED was estimated to be approximately 50% after dialysis for only 3 min. This relatively high recovery is achieved rapidly by the ASTED process, since a high diffusion gradient across the membrane is maintained during the dialysis stage, the recipent/sample volume ratio is large (2000:60 for the anticonvulsant method and 700:60 for the theophylline method), and the design of the dialyser provides a large area of membrane in contact with the sample. Moreover, in the reference methods, the absolute recoveries of analytes from the sample were significantly less than 50%, due to losses during phase separation, and liquid transfer. The relatively high recoveries obtained in the ASTED methods can enable quantitation of sub-therapeutic levels of the drugs assayed in the ASTED methods described.

Many analytes bind to serum proteins. If these serum proteins are injected into the HPLC column, they can bind the analytes and adversely affect the chromatographic separation[1]. Dialysis techniques have been used successfully to measure the degree of binding of diffusible ligands to non-diffusible binders[12]. Using these techniques, it has been shown that phenytoin is 90%, phenobarbitone 50%, carbamazepine 50% and theophylline 40% bound to serum proteins[13]. While this indicates that dialysis should efficiently separate serum proteins from these analytes, any variation in the concentration of binding proteins or factors affecting the binding, would cause variable recovery of the analytes in the dialysate. The correlation of the ASTED results with the reference methods (Fig. 3) suggests that the effect of any binding proteins is the same for both methods. Furthermore, the results from the Heathcontrol external quality assessment scheme (Fig. 4), derive from the same samples being assayed by methods employing approximately the same HPLC conditions but a variety of sample preparation techniques. The absence of any consistent bias of the ASTED results from the consensus mean suggests that there is no systematic difference between the performance of ASTED and the other sample preparation methods, indicating that matrix effects due to protein binding are avoided. The influence of protein binding on the ASTED assays was eliminated by: (a) using a serum based calibrant, (b) reducing the degree of binding by the addition of buffered trichloroacetic acid to samples for the anticonvulsant assay and the addition of monochloroacetic acid to samples for the theophylline assay and (c) providing a large surface area of membrane in contact with the sample. The ASTED process is applicable to any total analyte assay in physiological fluids, provided a suitable releasing agent is used to reduce the variance, due to protein binding, to an analytically acceptable level. Furthermore, it is probable that free analyte concentrations can be determined automatically using the ASTED process.

ASTED has been shown to be a reliable procedure for the automatic preparation of serum samples prior to HPLC for the analysis of some anticonvulsants, theophylline and caffeine in serum. The technique has the potential for the preparation of any type of homogeneous liquid sample prior to HPLC analysis.

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