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AUTOMATED SEQUENTIAL TRACE ENRICHMENT OF DIALYSATES AND ROBOTICS

A TECHNIQUE FOR THE PREPARATION OF BIOLOGICAL SAMPLES PRIOR TO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The development of the sample preparation process, the automated sequential trace enrichment of dialysates, in association with a cartesian robotic sampler is described. The system has been applied to the total automation of the preparation of biological samples and high-performance liquid chromatographic analysis. Concepts of the technique are reported together with an examination of its application to free and total analyte estimation. Examples of chromatographic separations obtained from the preparation of a variety of different analytes and sample materials are given.

INTRODUCTION

Total automation of one chromatography cycle may be defined as the preparation of untreated samples and chromatographic analysis without operator intervention. Total automation of chromatography systems is beneficial to capitalise on equipment investment, reduce operator costs and increase the throughput of sample analysis.

Theoretically the automation of sample preparation for high-performance liquid chromatography (HPLC) is generally easier than other separation techniques such as gas chromatography, since liquid samples can be introduced onto the analytical column. However, difficulties arise when analytes of small molecular size need to be quantified in samples with complex matrices such as those of biological origin. Sample preparation is required to remove interfering micro- and macromolecular compounds such as proteins, polymers and low-molecular-weight compounds present at very high concentrations. In particular, the injection of protein onto columns can not only create high column back pressures but will also alter the nature of the stationary phase. The ultimate goal of sample preparation is to protect and prolong the lifetime of the analytical column so producing robust chromatographic

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separations that are free from interferences and will yield the same duration and quality of analytical performance as that obtained when chromatographing pure solutions of analytes. If this goal is achieved the benefits accrued apply both to the experienced and inexperienced HPLC user and the cost effectiveness of HPLC is much improved.

Most methods for sample preparation have involved batch processing, where every sample is treated for one operational step before the next operation is performed. Such techniques include protein precipitation[1], liquid–liquid and solid phase extractions[2–4]. These batch processing techniques do not make efficient use of the HPLC system during sample preparation. Total automation has been achieved using anthropomorphic and cylindrical robots[5,6], to simulate the manually performed procedures such as liquid–liquid extractions but the equipment is expensive. Only partial automation of solid phase extraction has been achieved. This is primarily due to the size of the extraction column employed and manual manipulations for the addition of sample and reagents are required. Of the sequential processing methods, where every operational step is performed on a sample before proceeding to the next sample, direct sample injection[7] and column switching techniques[8] have been totally automated. However operating costs are very high due to rapid deterioration in analytical column and pre-columns employed. Furthermore when analysing proteinaceous samples the techniques impose restrictions on the type of HPLC solvents used and the incorporation of guard columns is essential.

A new, "on-line", technique for sample preparation, the automated sequential trace enrichment of dialysates (ASTED[9]) has been shown to be capable of addressing many of the problems that exist in the total automation of sample preparation[10–13]. Although previous reports have been published on the application and validation of ASTED by reference to the estimation of various analytes in biological fluids, little has been discussed on the theoretical concepts of the process. This paper reports on the development of the commercially available ASTED system in association with a Gilson cartesian robotic sampler, that can be applied at present to the analysis of small organic molecules. It discusses the nature of the process and describes examples of HPLC separations obtained from a variety of samples with complex matrices.

EXPERIMENTAL

Instrumentation

Development of the ASTED component parts and the system process control was made in association with Gilson Medical Electronics, Villiers-le-Bel, France. Unless otherwise stated all other equipment was also obtained from Gilson Medical Electronics.

HPLC unit

The HPLC system consisted of an isocratic and gradient unit. This entailed the use of either one, two or three 303/5SC pumps. Several detectors were used depending upon the application. These included a 116 UV detector, 121 fluorimeter and 141 electrochemical detector. Control of the gradient or isocratic HPLC system and integration of chromatographic peaks was made using a 714 system controller (IBM AT with hard disc, EGA graphic card, mouse, MS DOS 3.1 and Windows software).

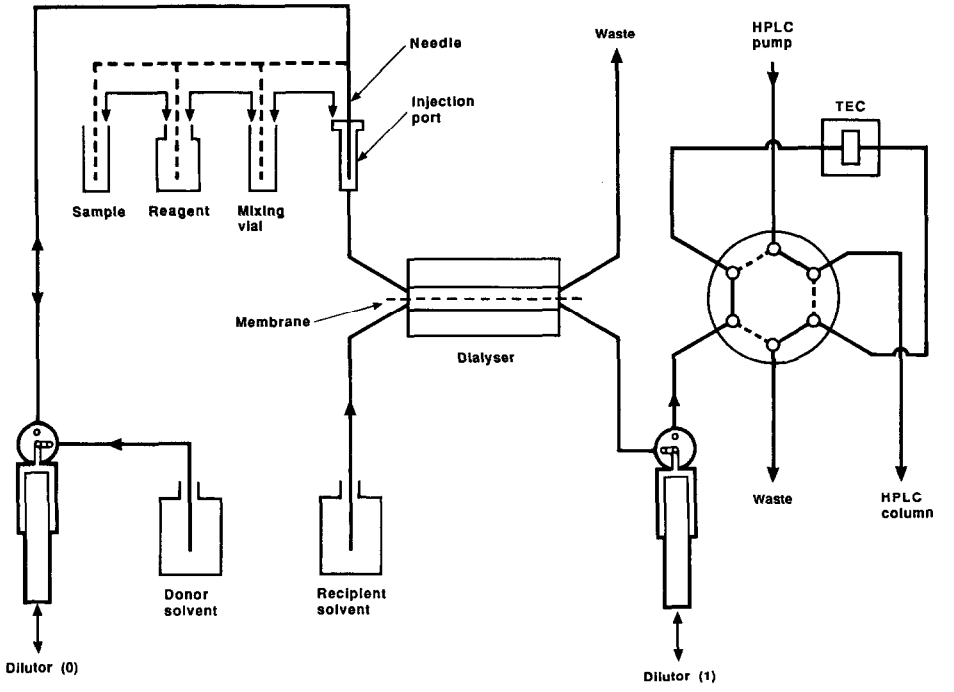

Fig. 1. A schematic diagram of the ASTED system.

The HPLC columns were either 100 mm or 150 mm × 4.6 mm I.D. containing Spherisorb 5 μm ODS 2 (Phase Separations, Queensferry, U.K.). No guard columns were used.

Sample preparation (ASTED) unit

This consisted of a 231 sample injector module incorporating a 7010 Rheodyne injection valve, two 401 dilutors (identified as 401 dilutor 0 and 1) with 1.0-ml syringes for liquid transfer, a polymethyl methacrylate flat plate dialyser fitted with a Cuprophan membrane, and a stainless-steel trace enrichment cartridge fitted in place of the loop on the injection valve. A schematic diagram of the ASTED system is shown in Fig. 1.

Development of the ASTED system

Dialyser unit

A flat plate dialyser was adopted since, with this design replacement of the membrane was simple and had good filling and purge characteristics. For mechanical strength and ease of dismantling the donor and recipient halves, a rectangular shape was chosen, clamped together with bolts. Two sizes of units were developed with donor volumes of 100 μl and 370 μl having path lengths of 186 and 677 mm respectively. By connecting combinations of units in series a range of donor volumes can be achieved.

The channel through which the donor and recipient liquids flowed was a "U" design allowing adjacent inlet and outlet connections. The width and depth of the channels were optimised with respect to a 20-μm thick (wet) Cuprophan membrane. This membrane has a tensile strength of 1050/220 (longitudinal/transversal) cN/15 mm. The membrane has a molecular weight cut-off of approximately 15 kilodalton (kD). The donor channel was 2 mm wide and 0.2 mm in depth, whilst the recipient channel was 0.75 mm in depth. This permitted some flexing of the membrane, problem-free filling and purging of the dialyser.

Trace enrichment cartridge (TEC)

The internal dimensions of the TEC were 1.6 mm (height) × 4.6 mm (diameter). This retained 20 mg of 10 μm reversed-phase spherical silica between stainless-steel meshes held in place by Kel-F end seals. It was necessary to use material with 10 μm (or less) in diameter and materials were slurry packed under vacuum. With these quantities rapid regeneration of the TEC retaining conditions were obtained with small volumes of solvent. The TEC holder was constructed in two threaded halves that could be screwed together and finger-tightened to withstand pressure drops of 48 MPa. This enables rapid interchange of TEC's whilst the holder remains in place connected to the Rheodyne injection valve. The holder and associated 1/16 in. attachment tubing to the Rheodyne injection valve was 316L stainless steel.

Process control

Software on ROM within the 231 controls preparation and clean-up of samples using ASTED, communications with the HPLC system and selects the ASTED process option required.

Preparation and sample clean-up. The ASTED process consists of pre-column sample treatment of the sample by the 231 (up to 5 different reagents may be added, mixed and incubated with the sample, if required). The sample (or sample–reagent mixture) is loaded into the donor channel of the dialyser via the loop filler port of the 231 using the 401 dilutor 0. Larger sample volumes can be dialysed by "pulsing" the sample through the dialyser at pre-determined times. The low-molecular-weight analytes diffuse across the membrane and are continously swept to the TEC in the recipient solvent using the 401 dilutor 1. The retained analytes on the TEC are eluted onto the HPLC column by the HPLC solvent after switching the Rheodyne injection valve to the inject position. After purging the system of excess sample and dialysate, the injection valve is returned to the load position and the TEC retaining conditions regenerated with a small volume of recipient solvent.

Communications with the HPLC unit. After the first sample has been processed the system operates with the HPLC in a constant cycle. Samples can be analysed in a sequential mode, *i.e.* one sample is treated and the analytes separated before the next sample is prepared, or in a concurrent sequential mode[14] when a sample is prepared while the previous one is being chromatographed. When derivatised analytes are unstable, samples must be analysed in a sequential mode.

Selection of process option. ASTED uses two, combined processes to effect sample clean-up. Together these create three different options, dialysis and trace enrichment (process 1), dialysis alone (process 2) and trace enrichment alone (process 3). Fig. 2 shows which option should be selected depending upon the sample complexity and analyte dilution.

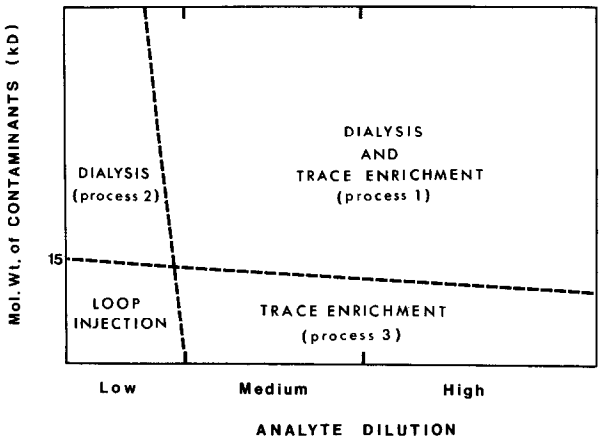

Fig. 2. The areas of application of three alternative ASTED procedures in terms of analyte dilution and molecular weight of contaminants.

Reagents

All HPLC solvents were of analytical grade, obtained from BDH, Poole, U.K. Unless otherwise stated all other chemicals were obtained from Sigma (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.). Unless otherwise stated the TEC contained 10-μm Hypersil ODS material (Shandon Southern Products, Runcorn, U.K.).

Methods

Most investigations of the ASTED system (using process 1) were carried out on dilute solutions of some anticonvulsant and xanthine drugs. The HPLC conditions for the separation of these analytes have been described previously[12]. Unless otherwise stated, all the methods used a dialyser block with a donor volume of 100 μl.

The performance of dialysis and trace enrichment

(i) The effect of varying flow-rates on dialysis rates was investigated by attaching the dialyser recipient outlet to the UV detector and aspirating recipient solvent continuously through the flow cell using a 401 dilutor (incorporating a 5-ml syringe), located after the detector. The detector response (wavelength 220 nm) was monitored for the dialysis of a static aqueous phenobarbitone solution (800 mg/l) in the donor channel.

(ii) The system was compared with and without incorporating trace enrichment. To do this the TEC was replaced by a 50-μl injection loop and the donor channel was filled with an aqueous phenytoin solution (20 mg/l). After dispensing recipient solvent continuously (1.1 ml/min) through the loop, a 50-μl volume of the dialysate was injected onto the column, at various timed intervals. The same phenytoin solution was processed, replacing the loop with a TEC, and the procedure repeated. The performance of the TEC, when operated in conjunction with dialysis, was also assessed by analysing aqueous solutions (20 mg/l) of theophylline, phenobarbitone and

phenytoin. Various recipient volumes were dispensed through the TEC by altering the flow-rate using the 401 dilutor 1 to maintain a constant 3-min dialysis time. At each flow-rate the amount of analyte loaded onto the HPLC column was measured.

Application of the ASTED system

Free analyte estimation. The ASTED system was examined for its application to the estimation of free analyte concentrations. The drugs phenobarbitone, phenytoin and phenylbutazone were used as models. Protein binding isotherms were determined by adding 15 mg/l of each drug to a range of human serum albumin (HSA) concentrations (0–35 g/l). The HSA was dissolved in sodium phosphate buffered saline (PBS). A volume of 100 μl of each solution was loaded into the donor channel of the dialyser and 2000 μl of PBS aspirated through the recipient channel in a 2-min period. Also a series of different serum samples (free of phenytoin) were obtained from patients with a variety of pathological disorders known to affect protein binding. Each sample was supplemented with 15 mg/l of phenytoin and analysed with the same ASTED conditions as those used for the protein binding isotherms. The isocratic HPLC solvent conditions for the separation of phenylbutazone used a solvent of acetonitrile–20 m*M* ammonium phosphate buffer, pH 5.0 (40:60). The phenylbutazone peak was detected at a wavelength of 263 nm.

Total analyte estimation. For total analyte estimates in serum samples containing protein binders, the effects of competing or releasing reagents on protein-bound theophylline and phenytoin were investigated. Firstly the effect of pH by the addition of varying concentrations of monochloracetic acid (MCA) to serum on the release of theophylline from protein and secondly by the addition of buffered trichloroacetic acid (TCA), pH 7.0, on the release of the anticonvulsant drug phenytoin from serum protein. Amounts of 15 mg/l of each drug were added to pooled human sera. For estimating the efficiency of a releasing agent to minimise matrix effects on the recovery of analytes, 30 different serum samples (free of phenytoin) were obtained from patients with chronic renal failure. Each sample was supplemented with 15 mg/l of phenytoin and the imprecision of the method determined with and without the addition of buffered TCA. For these investigations, 100 μl of sample–reagent mixture (4:1, v/v) was loaded into the donor channel of the dialyser and 2000 μl of a 20 m*M* ammonium phosphate buffer, pH 7.0, was aspirated through the recipient channel of the dialyser for 3 min.

Practical examples. To validate ASTED for the preparation of different types of sample materials and analytes, a range of various materials, were prepared by ASTED, using the various process options described (Fig. 2). Samples were loaded, untreated, directly onto the rack of the 231 and after sample preparation, chromatographed with the HPLC conditions of the publications cited. For ASTED process 1: UV detection of serum total glucocorticoids[13], postmortem blood warfarin[15] and a yeast extract food product for water soluble vitamins[16]. For glucocorticoids, a dialyser block with 370-μl donor channel volume was used. For ASTED process 2: fluorescence detection of amino acids using pre-column derivatisation with *o*-phthalaldehyde–2-mercaptoethanol (OPA–MCE)[17]. Sample types examined were homogenised cerebral cortex brain tissue and red cell lysates. For ASTED process 3: electrochemical detection of urinary free catecholamines[18]. The TEC was packed with hydroxyethyl methacrylate (HEMA) sulphobutyl (Anachem, Luton, U.K.).

RESULTS AND DISCUSSION

Prototype systems implementing the ASTED process have been described[14,19]. However, to automate totally the analytical system it was necessary to incorporate some form of robotics, which have the requisite flexibility[20,21] for the development of total automation applied to sample preparation and HPLC analysis. The ASTED system permits association with any type of HPLC/integrator units, and possible use for other types of analysis. ASTED is a combination of two separate processes, and, on occasion dialysis or trace enrichment alone, can be sufficient to prepare samples depending upon the sample composition. Fig. 2 shows the areas of application for the three alternatives that can be pre-selected in the system. Dialysis combined with trace enrichment has the widest application.

Dialysis

Dialysis is a separation process that depends on the differential transport of solutes of different sizes across a semi-permeable barrier separating two liquids and operates according to Fick's law of diffusion:

$$\frac{dm}{dt} = -DA\frac{dc}{dx}$$

where D = diffusion coefficient, A = membrane surface area, dm/dt = mass flow diffusion rate, and dc/dx = concentration gradient. Whilst dialysis is a highly efficient molecular filter it is inefficient in terms of solute diffusion rates. To increase the diffusion rate the dialyser was designed with a maximum ratio of membrane surface

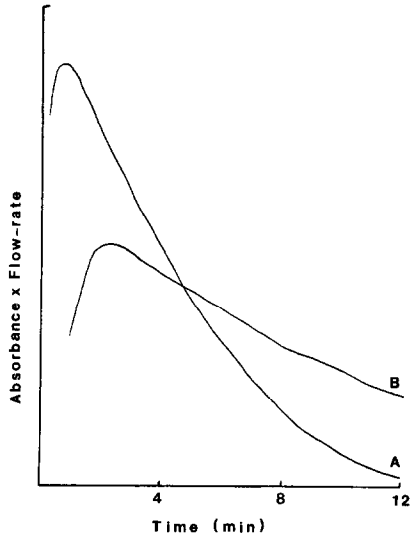

Fig. 3. Effect of flow-rate on dialysis rate. Rate of dialysis of a 800 mg/l phenobarbitone solution exhaustively dialysed at (A) 1.7 ml/min and (B) 0.3 ml/min. Absorbance × flow-rate corrects for the dilution of phenobarbitone.

area/sample volume. To minimise the volume of sample required it was held static in the donor channel of the dialyser whilst continually aspirating solvent through the recipient channel. This form of exhaustive dialysis serves to produce a high solute concentration gradient across the membrane at all times, increasing the rate of diffusion. This is shown in Fig. 3, where a greater diffusion rate for the analyte, phenobarbitone, is obtained at the higher recipient flow-rate. The amount of analyte passing into the recipient decreases exponentially with the volume of recipient passed through the dialyser (Fig. 3). This is due to the falling concentration of analyte in the donor channel of the dialyser resulting in a decrease in the dialysis rate in accordance with Fick's law. For this reason, complete removal of the analyte from the donor channel will require an infinite volume of recipient solvent to be aspirated through the dialyser. The curves in Fig. 3 have been corrected for the dilutions incurred at the two different flow-rates and approximately 68% of the analyte in the donor channel was recovered in 12 min at a recipient flow-rate of 1.7 ml/min. The amount of analyte removed from the sample in the donor channel per unit time will vary with the flow rate of the recipient solvent. The proportion of the analyte transfered into the recipient solvent during a fixed time period is independent of the solute concentration in the donor channel and the time required to obtain a fixed proportion of the analyte transfered is inversely related to the flow-rate of recipient solvent aspirated through the dialyser. Quantitative diffusion rates were always obtained due to the precise aspiration rates obtained using the 401 dilutor.

The body of the dialyser was polymethyl methacrylate so that visual monitoring of the liquid flow in the donor channel could be made. However, if it is necessary to use high concentrations of organic solvents for the donor or recipient solvents the body of the dialyser can be constructed from more solvent resistant polymers. Organic solvents do not appear to adversely affect the performance of the Cuprophan membrane. The choice of membrane with a molecular weight cut-off of 15 kD was made on the basis that ASTED was initially designed to analyse small molecules with molecular weights less than 2 kD. Membranes with higher-molecular-weight cut-off's are available that could extend the analytical range of ASTED. Dialysis, using Cuprophan, has been applied successfully for many years and problems that may be recognised, such as protein binding to the surface of the membrane, can be overcome by increasing the variable purge volume available in the system and occasionally washing the donor channel with weak alkali solution. The sample–sample interaction with ASTED applications has been shown to be less than 1%[10–13].

Trace enrichment

Trace enrichment is a procedure for concentrating solutes from dilute solutions and generally cannot be used for the treatment of samples with complex matrices. Many different types of material can be used for trace enrichment which can provide a range of selectivities.

For the on-line process of ASTED, the height of the trace enrichment bed was kept relatively small so that, packing was simplified and regeneration of retaining conditions could be achieved rapidly. An important consideration was the particle size of the sorbent used in the TEC. In agreement with a previous report[22], sorbents of 10 μm (or less) in diameter were used to suppress band broadening and enable good loading characteristics when analytes are eluted from the TEC onto the HPLC column.

Rigid packing materials must be used with on-line trace enrichment techniques such as ASTED. For analytical techniques, it is unlikely that, using the TEC dimensions and material mass described, the TEC loading capacity[23], *i.e.* the maximum capacity of the stationary phase for the solute when the amount of solute retained is proportional to the mass of stationary phase, will be exceeded. In practice, it is necessary to consider only the TEC working capacity. The TEC working capacity is defined as the amount of solute retained on the TEC when the solute retention rate equals the elution rate. This state exists when the volume of mobile phase passed through the TEC is greater than the solute breakthrough volume and the concentration of solute in the mobile phase is not so high that the loading capacity is exceeded. For a constant mobile composition and mass of stationary phase, the working capacity is proportional to the solute concentration in the mobile phase. Solute breakthrough volume has been clearly defined in an earlier report and a method for its determination described[23]. Solute breakthrough volume is dependent on the nature of the mobile phase and nature and mass of the stationary phase. It is independent of solute concentration in the mobile phase and is the volume of mobile phase passed through the TEC at which elution of solute commences. For quantitative analysis the TEC can be operated at its working capacity provided that the breakthrough volume of the analyte(s) under investigation remains constant.

Dialysis and trace enrichment combined

Trace enrichment combined with dialysis enables dilute solutes in the recipient solvent to be enriched resulting in high analyte recoveries that could not be obtained using dialysis alone. This is demonstrated in Fig. 4 which compares the recovery (detector response) of phenytoin in the dialysate obtained with and without using trace

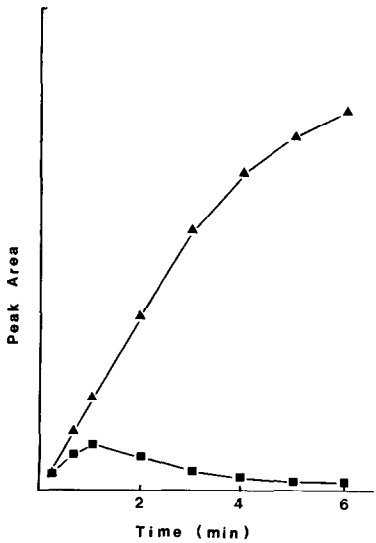

Fig. 4. Recovery of analyte from the dialysate with and without trace enrichment. The peak area (recovery) of phenobarbitone from a 20 mg/l solution exhaustively dialysed for various times and then loaded onto the HPLC using a 50-μl loop (■), and a TEC (▲). The dialysate flow-rate was 1.1 ml/min.

enrichment. When a loop was substituted for the TEC on the injection valve the dialysis kinetics observed (lower curve on Fig. 4) were identical to those observed when the analyte concentration was directly monitored in the dialysate (Fig. 3). To obtain sufficient analyte recovery for detection and quantification it is necessary to enrich the dilute solute(s) in the recipient solvent (Fig. 4). At the constant recipient flow-rate used (1.1 ml/min) approximately 58% of the analyte in the aqueous donor solution was recovered after 5 min dialysis time. The decrease in rate of analyte recovery in time, observed on the upper curve shown in Fig. 4, is a function of the exponential fall of dialysis rate of the analyte, knowing that the breakthrough volume for the analyte under investigation (phenytoin) has not been exceeded. Under these conditions it is inefficient to increase the dialysis time beyond a point where this does not produce a directly proportional increase in the amount of analyte retained on the TEC. When, due to poor assay sensitivity, it is necessary to load larger amounts of analyte onto the HPLC, this should be done by introducing more sample into the dialyser and the enrichment repeated.

Classically, trace enrichment has been used for concentrating minute quantities of hydrophobic compounds such as polynuclear aromatic hydrocarbons in water analysis[22,23]. These are compounds with very large capacity factors and breakthrough volumes. For analytical purposes ASTED must also be capable of dealing with molecules having low capacity factor, and consequently low breakthrough volumes even when the TEC bed height is relatively small. Fig. 5 shows the relationship between the volume of dialysate enriched, and the amount of solute retained by the TEC for three drugs with widely differing polarities. While maintaining a constant dialysis time,

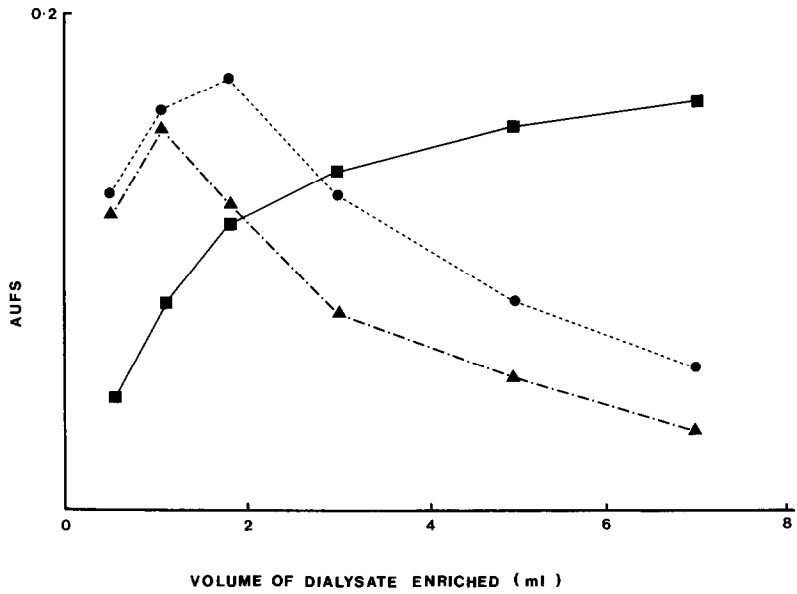

Fig. 5. Recovery of analytes on the TEC with various enrichment volumes. The peak absorbance by three drugs when different volumes of dialysates were enriched and then loaded onto the HPLC column is given. A constant 3-min dialysis time was used. Key: (▲) theophylline, (●) phenobarbitone, (■) phenytoin.

the volume of dialysate enriched was varied by altering the recipient flow rates. As dialysis of the sample in the donor channel proceeds the solute concentration falls causing a concomitant reduction in solute concentration in the recipient solvent. When the breakthrough volume is exceeded the recipient solvent, containing a now much reduced concentration of solute causes solute to be eluted off the TEC. This can be seen by comparing the traces for theophylline (having a low breakthrough volume of approximately 1 ml) and phenytoin (having a high breakthrough volume not identifiable) in Fig. 5. By measuring analyte recovery at different recipient flow-rates and constant dialysis times approximately breakthrough volumes can be established for those analytes having low capacity factor.

For quantitative analysis using ASTED it is important to operate below the breakthrough volume of the analyte under investigation, since the breakthrough volume can be affected by changes in the material bed due to ageing or compression after many regeneration cycles. The heterogeneity of the mobile phase being enriched may also have some effect on solute breakthrough volume. This is especially the case when using process 3 for samples, which, by virtue of their complexity, may contain molecules that exhibit competitions for the TEC material with the analytes under investigation. Modifications made to the recipient solvent can alter the avidity of the analyte for the sorbent in the TEC and therefore the analyte breakthrough volume. Provided that the breakthrough volume of the analyte(s) under investigation is not exceeded then the final recovery of the analyte(s) on the TEC is proportional to the time of dialysis and the volume of recipient solution passed through the TEC. These parameters are controlled by the volume and flow-rate of the recipient solvent dispensed by the 401 dilutor 1. Therefore under specified ASTED conditions where membrane permeability, sample volume, area of membrane in contact with the sample, dialysis time and volume of recipient solvent passed through the TEC are all constant, the dialysis diffusion rate is proportional to the concentration of analyte in the donor channel of the dialyser (Fick's law of diffusion), and quantitative analyte recoveries on the TEC are obtained. Thus the amount of analyte retained on the TEC and loaded onto the HPLC is directly proportional to the concentration of the analyte in the original sample. The use of high recipient flow-rates maintains high concentration gradients and enables shorter sample preparation times with increased analyte recoveries.

Dialysis combined with trace enrichment are two separate processes that complement and overcome the limitations of each other. The low-molecular-weight cut-off of the Cuprophan membrane protects the TEC from compounds that would otherwise reduce its performance. Up to 1000 samples can be handled by a single TEC. Since only molecules with masses of less than 15 kD pass onto the HPLC column the service life of the analytical column is greatly increased.

ASTED applications

Free analytes. Frequently analytes in biological liquids are present in two states: in combination (or bound) with another molecules and as the unbound (or free) compound. In the case of analytes, such as drugs, which bind to tissue and blood proteins it is often necessary to estimate the free analyte concentration since this is the physiologically active fraction[24]. Binding of a drug with a protein is usually a reversible reaction obeying the law of mass action and the affinity between a drug and

its binding site is expressed as the concentration ratio of the drug in the bound form to the product of the unbound drug and protein:

$$P + D \underset{K_2}{\overset{K_1}{\rightleftharpoons}} PD$$

with

$$\frac{[PD]}{[P] \cdot [D]} = \frac{K_1}{K_2} = K_a$$

where [P] is the free protein concentration, [D] is the free drug concentration, [PD] is the drug–protein complex concentration, K_1 and K_2 are the rate constants for the forward and reverse reactions, and K_a is the association constant expressed in mol/l. The greater the affinity between drug and protein the larger the K_a. Its reciprocal value is the dissociation constant K_d. The processes of association and dissociation of drug and protein have half-times of a few milliseconds. Many factors can affect the equilibrium *e.g.* pH, protein concentration, competition of other molecules for the binding sites and temperature. Previous methods that have been used to estimate free analyte concentrations include equilibrium dialysis[25], ultrafiltration[26] and antibody extraction[27]. In spite of the fact that equilibrium dialysis has been recognised as the definitive method, criticisms of the technique have been published[28]. Automation of such procedures at present are impracticable and long dialysis times required to achieve steady state. With the ASTED technique, in common with all procedures incorporating membrane technology, diffusion of only free drug from the donor channel into recipient solvent occurs. As this transfer proceeds, some of the drug–protein complex will dissociate to maintain the equilibrium. It has been shown that the percentage binding of highly-protein-bound drugs in human serum is fairly constant within the therapeutic range of the analytes[29]. Since the rates of diffusion are governed by Fick's law and are proportional to the diffusible solute concentration gradient across the membrane, it should be theoretically possible to estimate a measure of free drug concentrations using ASTED even when the concentration gradients are changing due to the continual movement of the recipient solvent. This would be accomplished by comparing the rates of diffusion (measured as the amount of analyte retained on the TEC) of a drug in protein solution with that obtained with a solution containing 100% free drug (*i.e.* an aqueous solution). As the diffusible free drug passes into the recipient solvent, bound drug dissociates to maintain the equilibrium. This maintains the diffusible drug concentration in the sample at a higher level than the aqueous solution, in proportion to its degree of binding. Therefore to obtain an index of free drug concentration using ASTED the dialysis time must be limited so that deviations in the initial dialysis rates of diffusible drug between the aqueous and protein solutions are negligible.

Some evidence to support these arguments are shown in the protein binding isotherms (Fig. 6a) obtained using ASTED for the three drugs, phenylbutazone, phenytoin and phenobarbitone. These drugs were chosen, as models, since their affinities for albumin differ markedly. The results in Fig. 6a are expressed as

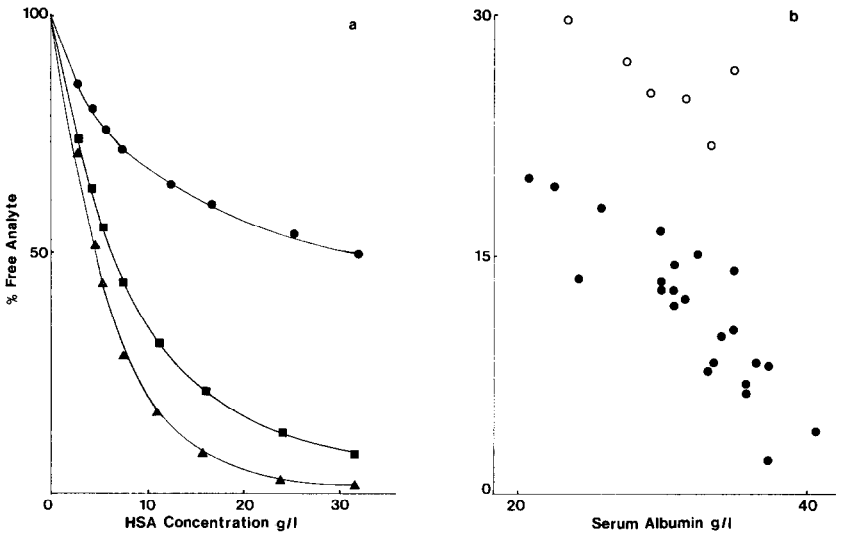

Fig. 6. (a) The binding isotherms for phenobarbitone (●), phenytoin (■), and phenylbutazone (▲) to human serum albumin (HSA). The % free analyte is the amount of solute recovered compared with that from an aqueous solution. (b) The % free phenytoin of various patients' sera each supplemented with 15 mg/l phenytoin compared with their albumin concentration. (○) Patients suffering from chronic renal failure, (●) patients suffering from diseases other than chronic renal failure.

a percentage of free drug calculated by the percentage ratio of the peak area of the drug in protein/peak area of the drug in zero protein solution. At the most concentrated albumin solution the percentage free analyte values were 97.5, 88, and 56 for phenylbutazone, phenytoin and phenobarbitone respectively. These values were in general agreement with the established equilibrium dialysis values of 99, 93, 50% for phenylbutazone, phenytoin and phenobarbitone respectively[30]. A further example using ASTED to measure an estimate of free analyte concentrations is shown in Fig. 6b. Here a range of different patient sera supplemented with the same amount of phenytoin were assayed. The concentration of free phenytoin was found to be inversely proportional to albumin concentration. However, two distinct populations were apparent, the sera from patients suffering from chronic renal failure, all having higher free phenytoin levels than other patients with comparable albumin concentrations. This is due to the disturbed protein binding that occurs in disorders of this type, which results from accumulated serum compounds competing for the protein binding sites[31]. The percentage phenytoin binding ranges observed using ASTED for renal and non-renal patients sera agrees with previous published material[29].

The accurate measurement of free analyte concentrations is recognised as a difficult problem due to the many limitations of methods used. Rate dialysis methods for estimating free analyte concentrations have been proposed[32] but suffered from analyte detection due to the dilute solute in the dialysate. The incorporation of trace enrichment in ASTED has to some extent overcome this limitation although for highly protein bound analytes where solute concentrations are extremely low detection will still be a problem. The automation and speed of analysis using ASTED for free analyte

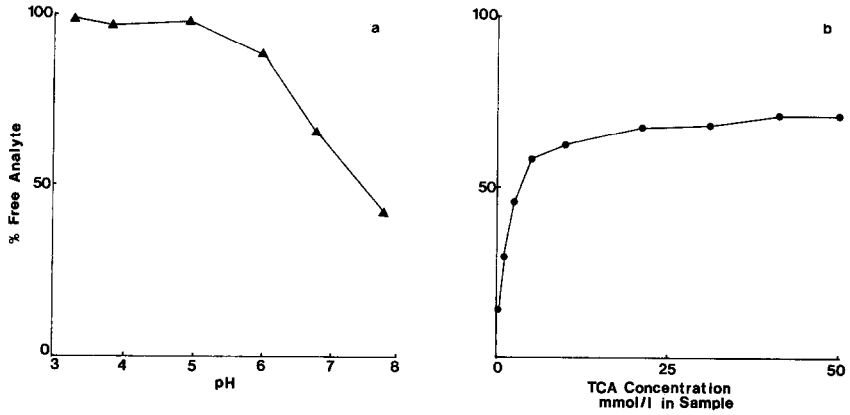

Fig. 7. (a) The effect of sample pH on the release of theophylline from serum proteins expressed as % free analyte. (b) The effect of TCA, at pH 7.0, on the release of phenytoin from serum proteins expressed as % free analyte.

examination is an obvious advantage compared with other methods using membrane separation. However it would only be realistic at this time to suggest that ASTED may be capable of measuring an estimate of free analyte concentrations and much work remains to be completed on this assay potential.

Total analytes. Sample matrix interferences are common in a wide variety of analytical techniques and ASTED is no exception. For the assay of total analytes, it is necessary to overcome any molecular binding effects. Theoretically this can be achieved in a number of ways: either by diluting the binder (*e.g.* protein) in the sample, altering the structure of the binder, adding competing reagents for the binding sites, using calibrants containing an identical binder, or by altering the chemical nature of the analyte.

If the sample dilution is high then analyte detection becomes difficult. Altering the structure of binders such as proteins can be successfully accomplished simply by changing the pH as shown with theophylline where most of the bound drug could be released from serum protein by the addition of MCA (Fig. 7a). MCA was used since a small volume of concentrated reagent generates a lowering of pH without excessive dilution of the sample or risk of precipitating serum proteins. The binding of other xanthines *e.g.* caffeine, and the glucocorticoid cortisol have also been shown to behave in a similar manner[12,13]. The competing effect of reagents for binding sites was demonstrated by the release of phenytoin from serum proteins (Fig. 7b) using buffered TCA[12]. Although phenytoin is not completely released, the competing effects of TCA are sufficient to reduce the between-sample imprecision from 25.2 to 5.1% (C.V.). Altering the chemical nature of the analyte has recently been described to eliminate protein binding of the amino acid cystine prior to its analysis[33].

The analytical performance of ASTED for a number of analytes in different biological samples has been reported previously and in accordance with Fick's law of diffusion, recovery of analyte on the TEC has been shown to vary linearly with solute concentration in the donor channel[10–13]. For total analyte estimations the analytical recovery has been shown to be approximately 100% when using protein based

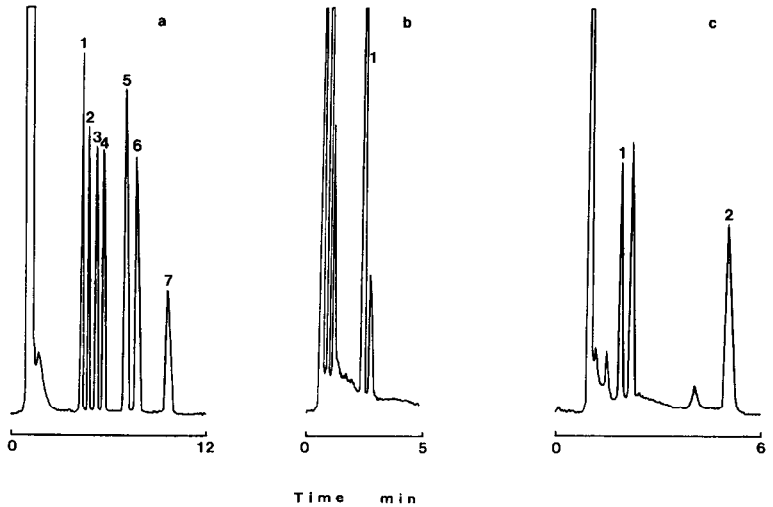

Fig. 8. Chromatograms showing: (a) Serum total glucocorticoids in human serum supplemented with glucocorticoids. Peaks: 1 = prednisone; 2 = cortisone; 3 = prednisolone; 4 = cortisol; 5 = fludrocortisone internal standard (I.S.); 6 = corticosterone; 7 = 11-deoxycortisol. (b) Grossly elevated warfarin level in a sample of *post mortem* blood. Peak 1 = warfarin. (c) Water-soluble vitamins in a food yeast product. Peaks: 1 = thiamine; 2 = riboflavin.

calibrants and compounds to minimise protein matrix effects. The absolute recovery achieved using the ASTED procedure (*i.e.* the amount of analyte recovered from an aqueous solution in the donor channel) is about 50% within a 3-min time interval[12]. Future developments in membrane technology and dialyser design could improve this analytical recovery.

ASTED advantages:

ASTED has been applied to the preparation of a range of different complex homogeneous liquid materials[10–13,34,35] (Figs. 8 and 9) using one of the three different processes. The peaks in the chromatograms represent analyte concentrations found under normal conditions of therapy and metabolism or, in the case of vitamins in the yeast product, concentrations recommended by the manufacturer. The examples used in this report to identify the potential of ASTED have been limited to analytes frequently examined in the routine clinical environment and the theoretical considerations given to these analytes should apply to other molecules of a different chemical structure. Furthermore, the efficiency of the sample clean-up described reduces cost in view of the fact that the TEC can be regenerated many times, no guard column is required and robust chromatographic separations are produced that sustain very long analytical column life times. No restrictions are imposed on the types of HPLC columns, detectors, and HPLC solvent conditions required. Moreover, high sample throughput is achieved using concurrent sequential operations of the sample preparation and HPLC analysis. In general the rate limiting stage is the chromatographic separation time. In conclusion, ASTED in association with robotics offers total automation of sample preparation and HPLC analysis, reducing labour,

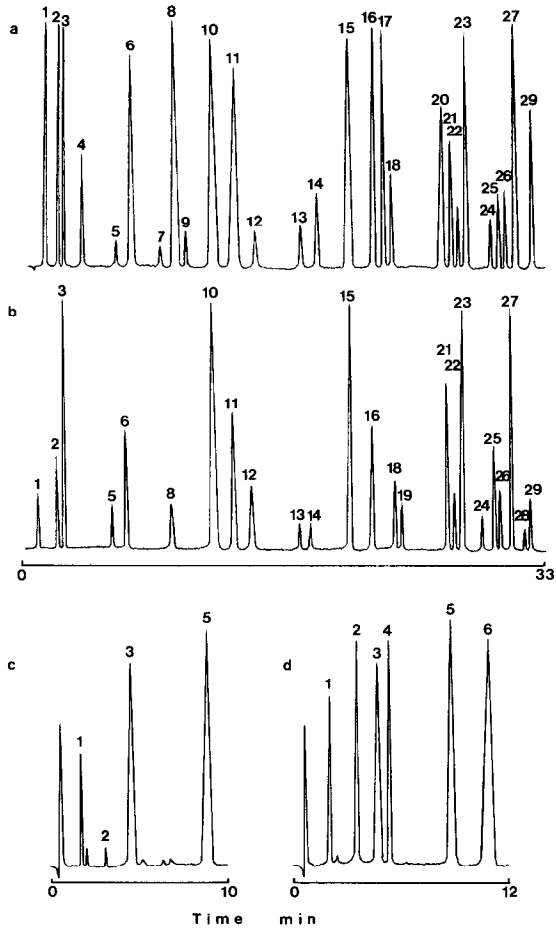

Fig. 9. Chromatograms showing: (a) Amino acids in homogenised brain tissue; (b) in red cell lysate. Peaks: 1 = aspartate; 2 = glutamate; 3 = homocysteic acid (I.S. 1); 4 = cystine; 5 = asparagine; 6 = serine; 7 = histidine; 8 = glutamine; 9 = phosphoethanolamine; 10 = homoserine (I.S. 2); 11 = glycine; 12 = threonine; 13 = citrulline; 14 = arginine; 15 = alanine; 16 = taurine; 17 = γ-aminobutyric acid; 18 = tyrosine; 19 = α-aminobutyric acid; 20 = ethanolamine; 21 = valine; 22 = methionine; 23 = norvaline (I.S. 3); 24 = tryptophan; 25 = phenylalanine; 26 = isoleucine; 27 = leucine; 28 = ornithine; 29 = lysine. (c) Catecholamines in urine from a healthy adult; (d) from a patient with a phaechromocytoma. Peaks: 1 = noradrenaline; 2 = adrenaline; 3 = dihydroxybenzylamine (I.S.); 4 = normetadrenaline; 5 = dopamine; 6 = metadrenaline.

consummable costs and capitalising on equipment investment in a simple but flexible manner.

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