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Analytical Survey

Liquid–solid sample preparation in drug analysis

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Abstract: This article compares liquid–solid extraction (LSE) with the more conventional liquid–liquid extraction (LLE) for the preparation of biological samples for assay. The commercially available manual LSE methods, Sep–Pak and Bond Elut, as well as the automated instruments Prep and Analytichem Automated Sample Processor (AASP), are reviewed. Using examples from the literature and the authors' own experiences a practical guide is given to the advantages and disadvantages of LSE.

Keywords: *Sample preparation; biological samples; liquid–liquid extraction; liquid–solid extraction; automated sample preparation; drug analysis; Bond Elut; Sep–Pak; Prep; AASP.*

Introduction

The past two decades have seen many advances in laboratory equipment and instrumentation. During this period high-performance liquid chromatography (HPLC) has matured as an analytical technique, and in twenty years it has risen from relative obscurity to the forefront of analytical techniques, as witnessed by a number of papers in this journal. Advances in HPLC column technology have produced columns able to separate more complex mixtures; the recent introduction of specifically designed micro and narrow bore HPLC systems indicates the trend towards shorter analysis times. During the same period more sensitive, specific and stable detectors have been developed for use with gas–liquid chromatography (GLC), which, with the emergence of reliable fused-silica capillary columns, is once more in a phase of increasing demand.

The development of modern, reliable, microprocessor-controlled analytical instrumentation has enabled analytical work to continue unattended overnight, thereby increasing productivity and freeing staff from mundane tasks. Sophisticated programming and technical innovation have produced systems capable of unattended HPLC method development, thus allowing relatively inexperienced staff to develop mobile phases, whereas previously this was the domain of the intuitive chromatographer. The widespread use of microprocessors and the introduction of low cost integrators have meant that the manual calculation and interpretation of chromatograms has been

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extensively reduced. Laboratory Information Management Systems (LIMS) will have a profound impact on the organization and running of laboratories, as well as on data processing and report generation. Thus, the trend in analytical laboratories has been towards more sophisticated instrumental techniques which, coupled with microprocessor/microcomputer control, allow detection and quantification of analytes at ever lower concentrations. The analyst's responsibility has changed from operating instruments to their supervision; the instrument for the most part has control of its own routine operations.

However, until recently, this had not been matched by similar advances in sample preparation: usually there are still many laborious stages involved in preparing a biological sample for analysis. The accuracy and precision of any assay are dependent upon both the sample preparation and on the instrumental performance, but it is often the former that is the more laborious and less reliable part of the procedure. Advances in sample preparation have been made mainly through the application of the bonded silica chemistry transferred from HPLC column technology to sample preparation. The development of small-particle organic resins from the drug screening programmes of the early 1970s has also been successful. As a result, in many laboratories, these advances are likely to have a major impact on the nature of the work undertaken. Laboratories will also see the benefit of this new technology in quicker sample turn-round times, greater efficiency and better staff motivation, all leading to time and cost savings.

In other areas, where established methods such as immunoassays exist or where the compound is present in relatively high concentrations (e.g. antibiotics), so that little or no sample pretreatment is necessary, then the new technology is likely to have less impact. However, for immunoassays that measure compounds present in very low concentrations, and which require a concentration step, or assays in which the molecule is susceptible to degradation from enzymes, liquid–solid extraction will continue to be useful. Again, the specificity of an assay could be enhanced by selectively removing an interfering metabolite; such an approach would leave the sample in an aqueous medium, thereby preventing the denaturation of antibodies which might otherwise occur in the presence of organic solvents.

During research for this Analytical Survey it became apparent that although biological sample preparation is fundamental for an assay, very few analysts appear to acknowledge it. Searches of the literature were not very productive. It is almost universal that the keywords for a drug assay will inevitably include such topics as: "HPLC", "reversed-phase" or "electrochemical detection" but very few mention the methods of sample preparation employed.

This survey is designed to highlight the recent advances that have been made in the area of sample preparation for the analysis of drugs in biological material. Whilst aimed specifically at a relatively narrow field, there is no reason why the principles outlined here should not be applied to any isolation procedure that requires an organic compound, or a series of compounds, to be purified from a complex matrix. In the authors' opinion this will prove to be a fruitful area for instrument manufacturers to develop and exploit in the coming years.

Objectives of sample preparation

There are various reasons why drugs are quantified in biological fluids; these include the necessity for calculation of pharmacokinetic parameters during drug development,

where a wide range of concentrations will be encountered and as low a limit of reliable quantification as possible will be required. Another example is therapeutic drug monitoring in the hospital environment, where the situation is frequently complicated by concomitant administration of other drugs.

In medico-legal cases (including driving whilst under the influence of drugs and/or alcohol, and instances where the cause of death must be established) the drug must first be identified, then quantified, sometimes in instances where there is a potentially high background due to decomposition of the biological matrix. Assays are required when an investigator needs to know if the medication prescribed was actually taken by the patient; these usually consist of simple qualitative assays, but detection and identification of the analyte of interest, avoiding interference from endogenous compounds or other drugs, is essential for compliance testing.

The main analytical objective, whatever the purpose of the assay, is that specificity is vitally important, since interference by endogenous material, metabolites or other drugs could affect the conclusions drawn by the analyst.

For most drugs the assay of biological materials usually consists of two stages: sample clean-up followed by instrumental analysis of the resulting extract. The reason for the frequent inclusion of an enrichment and purification step is that the drug is present in low concentrations in a medium containing many potential interfering endogenous compounds. The purification is designed to remove as many of these interferences as is practical, while at the same time concentrating the drug and any metabolites so that they fall within the detection limits of the analytical technique employed. Part of the assay specificity derives from the instrumental analytical step, but a substantial contribution is made by the preliminary sample clean-up.

Liquid–liquid extraction (LLE)

The majority of sample preparation schemes use direct solvent extraction of the biological material. This is frequently a simple partitioning of the drug (and any metabolites) between the parent aqueous phase and an immiscible organic solvent. The choice of solvent will determine the recoveries of the drug, metabolites and any co-extracted endogenous substances.

Liquid–liquid solvent extraction has its origins in the mid-nineteenth century, when work by Stas and Otto on the extraction of drugs from poison victims used tartaric acid as a protein precipitant together with an ethanol extraction. This was followed by acid and alkali extractions of the alcohol residue to isolate the drugs. Since this method of sample preparation was tedious and displayed problems of variable recovery, this led to the development of direct liquid–liquid extraction.

LLE depends upon the greater solubility of the drug (and metabolites) in a suitable organic solvent in comparison to their solubility in the aqueous phase. The pH of the aqueous phase is adjusted so that the drug to be extracted is virtually undissociated, thus facilitating extraction into the solvent. For example, cimetidine, a histamine H2-receptor antagonist, is a monoacidic base with a pK_a value of 7.09; thus in acid solution it is protonated, and can be extracted into an organic solvent from alkaline aqueous solution; re-extraction into dilute acid is utilized to further purify the extract from co-extracted neutral drugs and/or endogenous compounds. The exact procedure can be varied depending on the purity required for the individual analysis.

An advantage of liquid–liquid extraction is its selectivity; depending on the choice of solvent, the drug of interest can be isolated from most of the endogenous components. Moreover, this can be used to great advantage in cases where a non-specific spectrophotometric assay is employed. For example, if a lipophilic drug is extensively metabolized, and the metabolites have the same chromophore as the parent compound, they would then potentially interfere with the assay but the drug can be selectively removed using a lipophilic solvent for extraction, leaving the relatively polar metabolites in the biological fluid. Alternatively, if chromatography is utilized for separation, a hydrophilic solvent can be used to extract both drug and metabolites to allow concomitant determination of each. When considering the use of LLE, any pH can be utilized, depending on the stability of the compound to be isolated and on the extracting solvent employed (cf ethyl acetate). LLE is not, however, suitable for all compounds; for example highly polar molecules cannot usually be extracted by this method. However, the use of a suitable ion-pairing reagent can extend the use of LLE to molecules of this type [1].

The major disadvantage of LLE is emulsion formation; this causes loss of drug by occlusion within the emulsion, leading to lower recoveries. Emulsion formation can be overcome by the use of larger volumes of the extracting solvent, or by using less vigorous mixing methods. If a comparatively large volume of extracting solvent is used relative to sample size, this must be removed from the process at some later stage either by heat, vacuum or by directing a flow of gas upon it. This is frequently the rate limiting step and can present several problems:

(i) There is the safety hazard involved in handling the solvent, as nearly all those used in LLE are toxic or inflammable, so the vapours generated need to be efficiently vented to the atmosphere;

(ii) The conditions used to evaporate the solvent may also cause low recovery of the compound due to degradation by heat, volatilization or adsorption onto glass;

(iii) The process of removing waste solvent from a site is expensive and time-consuming.

LLE can be used to assay large numbers of samples efficiently and can be adapted to batch mode operation; however, the transfer steps involved make the process labour intensive and tedious. Inherent in this technique are drug losses because of the inability to transfer all of the extracting solvent; for example, when deliberate transfer losses are taken into account, the extraction procedure for oxmetidine has a theoretical maximum recovery of 75% [2].

As techniques for analysis become more complex and the limits of quantification fall, the quality of reagents used in the assay can become a critical factor. Although the costs of such chemicals are increasing, it is essential to use the highest purity reagents and solvents available in order to avoid any unpredictable and inconsistent interference to the assay.

The use of some solvents can actually cause a chemical reaction to take place between the solvent and the analyte. Chlorinated hydrocarbon solvents, for example, must be used with care when extracting amines as they can interact and form alkylated derivatives. Where chloroform is used without ethanol as a preservative, carbamate derivatives of tricyclic antidepressants are formed [3]. Artefacts have been reported during chloroform extraction of biological fluids containing norcodeine due to the formation of phosgene and ethylchloroformate during storage [4]. Although ethanol is used to prevent formation of both these compounds, its use will alter the polarity of the solvent, which in turn can affect the selectivity of extraction. Care must therefore be

taken when considering analytical strategies for sample preparation, particularly if the analytical method is expected to be sufficiently robust to be transferred to other parts of the world, where the solvent additives and impurities may differ significantly from those in the country of origin of the assay procedure.

Liquid–solid extraction (LSE): classical methods

An alternative to solvent extraction is the recovery of drugs by adsorption methods. These consist of mixing the biological fluid containing the drug with an adsorbent, separating it and then eluting the drug with an appropriate solvent. The success of this approach depends upon the relative affinity of the drug in a biological matrix for the solid adsorbent and the relative ease of eluting the compound for subsequent analysis. Adsorbents such as carbon (as graphite or charcoal), celite (diatomaceous earth or silica), florisil (activated magnesium silicate) and alumina (aluminium oxide) have been used with varying degrees of success. Charcoal [5] and celite [6] have both been used for the screening of urine for drugs: following adsorption and isolation, the drugs were eluted with organic solvents. This approach, whilst avoiding the formation of emulsions, still requires the eluting solvent to be removed before analysis.

Dole *et al.* [7] introduced ion-exchange resin-loaded papers for the early drug screening programmes. Due to problems in the manufacture of these papers, batch to batch variations were marked, and the effect of the salt content of urine samples precluded quantitative recovery of most drugs: recovery of barbiturates averaged 2% in comparison to 80% for some opiate derivatives. The eluates from these papers were remarkably free of interfering substances. Essentially these methods were utilized for the general screening of many classes of drugs and were not usually optimized for a specific compound. Such methods were useful in cases of overdose, but were generally insufficient when low limits of detection were required.

The development of non-ionic resins, e.g. Amberlite XAD-2, has found favour in laboratories screening large numbers of urine samples for drugs. This styrene divinylbenzene copolymer does not contract or expand upon hydration and has a macroreticular structure which provides a high surface area-to-volume ratio, giving it the ability to bind relatively lipophilic but water-soluble organic molecules either by van der Waals forces, hydrophobic bonding or dipole–dipole interactions [8]. XAD-2 was first used by Fujimoto and Wang [9] to screen for narcotic analgesics in urine. Small columns were prepared for the sample of urine to pass through and the retained drugs of interest were eluted with methanol.The method was refined by such groups as Hetland *et al.* [10] who were able to screen a single sample for other classes of drugs such as barbiturates, cocaine and strychnine. This method could be used for the simultaneous extraction of acidic, neutral and basic drugs in one step, and saved time and effort in sample preparation.

Recent advances in LSE methods of sample preparation

The development of bonded silica chemistry over the last few years, primarily for HPLC column packings, has provided the impetus for advances in sample preparation using LSE. These are presented in two sections covering manual methods and automatic instruments.

Manual methods of LSE

There are two types of commercially available cartridge capable of containing a variety of sorbents and optimized for sample preparation: these are the Bond Elut and the Sep–Pak cartridges produced, respectively, by Analytichem International and Waters Associates. The Bond Elut cartridge (Fig. 1) consists of a Luer tipped polypropylene reservoir into which 100–500 mg of sorbent is dry packed. This solid phase is held in place at each end by fritted disks. The Sep–Pak cartridge is comprised of radially-compressed sorbent (370–900 mg), sandwiched between two porous frits. This eliminates voids or channels in the sorbent bed. Both types of cartridge are disposable and come in hermetically sealed packages to maximize shelf life, and to avoid contamination or altered surface chemistries.

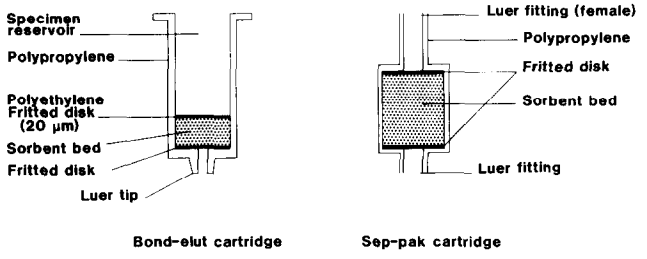


Figure 1
Diagrammatic representation of Bond Elut and Sep–Pak cartridges.

The sample, dissolved in a suitable solvent, is applied to the top of the cartridge, and solvent flow is usually achieved by applying negative pressure to the bottom of the column. The commercial manufacturers have made available vacuum manifolds, which allow batches of eight or ten cartridges to be processed simultaneously. However, solvent flow can also be achieved by the application of positive pressure to the top of the cartridge via a syringe, or alternatively by centrifugation. The eluted analytes are collected in tubes positioned directly beneath each cartridge.

Choice of phase

The usual purpose of sample preparation is to simplify the eventual chromatographic analysis of one or more compounds by removing interferences from a complex sample matrix. A typical sample contains compounds of varying chromatographic polarity, and the analyte could fall anywhere in this polarity spectrum. Thus, the sample preparation should be designed to isolate compounds in a narrow range of polarity, the extremes being either not retained by the solid phase, or not eluted with the compound(s) of interest. LSE separates different solutes by utilizing the principles of modern liquid chromatography. In this process the fluid sample passes over the stationary phase, the analytes being separated according to the degree to which each component is partitioned or adsorbed by the stationary phase. The mobile phase competes with the stationary phase in its affinity for the analytes, so that sample components with a greater affinity for the mobile phase are not retained by the cartridge; whereas compounds with a greater affinity for the stationary phase are retained. In sample preparation using LSE, the extremes of affinity are exploited, i.e. high affinity of the analyte(s) for the solid phase when first applied, and low affinity for subsequent elution.

Although it is outside the scope of this Analytical Survey to provide an in-depth discussion on the selection of a suitable bonded phase, the following guidelines have proved useful to the authors:

(1) Search the literature for chromatographic assay methods published for similar structures;

(2) Consider the physico-chemical properties of the analyte(s), e.g. solubility and ionization;

(3) The physico-chemical properties of the sample matrix should be considered;

(4) Consider the properties of the HPLC mobile phase;

(5) Evaluate the retention of the analyte on various bonded phases if the HPLC separation is reversed-phase. It is important to remember that pH can have a profound effect on retention of a compound;

(6) Compare the recoveries from aqueous and biological matrices. Differences in the behaviour of some drugs in these two matrices have been observed by the authors; for example, oxmetidine has a recovery of >90% from saline, but this falls to 50% in plasma due to competition from endogenous compounds [11];

(7) Elution of the analyte from the cartridge must occur efficiently so that the resulting solution to be assayed is not too dilute;

(8) The sorbent size should be optimized (as is possible with certain commercial systems, e.g. Bond Elut).

To evaluate any sample preparation scheme a radiolabelled form of the drug is used and the amount of radioactivity in the various fractions determined by liquid scintillation counting. This approach will quickly identify any problem areas of the proposed scheme. Generally a stationary phase of similar polarity to the compound of interest is used with the sample dissolved in a solvent of opposite polarity for application to the column. The analyte is eluted by again switching solvent polarity. Thus a relatively lipophilic organic molecule would be applied to a C18 cartridge in a polar solvent, and eluted with a relatively non-polar solvent. At the other end of the theoretical spectrum a polar compound would be applied to a silica phase in a non-polar solvent and eluted with a relatively polar solvent. Table 1 shows typical application and elution schemes for C18 and silica LSE phases.

Table 1
General procedures for using C18 and silica solid phases for sample preparation

	C18	Silica
Packing polarity	Low	High
Polarity of solvent in which analyte applied to cartridge	High, e.g. aqueous	Low, e.g. hexane, tetrahydrofuran
Solvent polarity to wash column	High	Low
Solvent change required to elute analytes	Decrease solvent strength, e.g. methanol, acetonitrile	Increase solvent strength, e.g. water, buffer
Sample elution order	Most polar first	Least polar first

General procedure for the use of bonded phases

A general procedure for sample preparation is shown in Fig. 2. The example given is for a C18 bonded phase but the same principles apply to almost all the non-polar bonded phases shown in Table 2, the diol, cyanopropyl and amino-propyl phases can be used according to the above scheme, but can also be utilized in the normal-phase mode. The procedure is as follows:

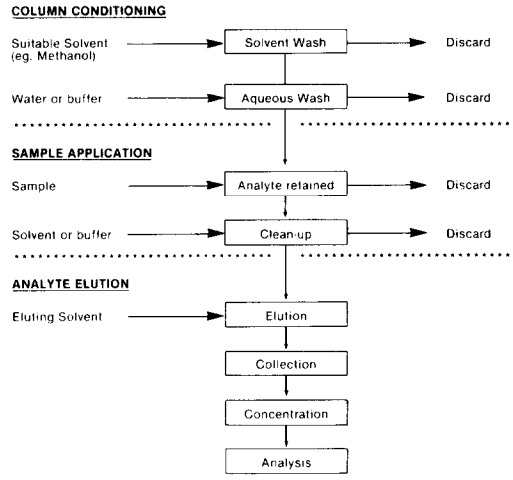


Figure 2
Liquid–solid extraction scheme for the preparation of aqueous samples.

STEP 1: Pre-wet the column with an organic solvent, e.g. methanol. This serves two purposes: (i) to open up the hydrocarbon chains and hence increase the surface area available for interaction with the analyte; (ii) to remove residues from the packing material that might interfere with the analysis. In the authors' experience, failure to carry out this stage will result in poor recoveries of drug due to reduced retention on the column and may lead to interference peaks in the chromatogram which are unrelated to the original sample.

STEP 2: Wash the sorbent bed with HPLC-grade water or a suitable buffer. This will remove excess methanol and prepare the surface for the sample. However, if excessive washing takes place, the column will no longer be sufficiently 'wetted' and a reduction in recovery will be observed. It has been suggested that to overcome this problem, an organic solvent, e.g. methanol (1–3%) should be added to the sample prior to processing. This will help to maintain the equilibrium between the solid and liquid phases.

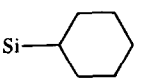
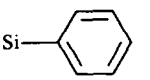
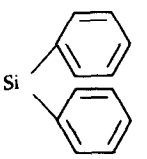
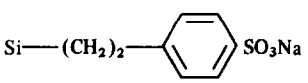
STEP 3: Apply the sample, allow it to flow through the sorbent bed and discard to waste.

STEP 4: Wash the column with water or a suitable solvent to selectively remove from the sample matrix those endogenous compounds, which might interfere with the subsequent chromatography.

STEP 5: Elute the sample with a suitable solvent and collect the eluent for immediate analysis or further work-up.

After the use of a simple, solid-phase extraction clean up step, there may still be in the eluate compounds from the sample matrix, which will interfere in the chromatogram. One solution may be to vary the nature of the washing solvent to selectively elute the unwanted compounds. If this is unsuccessful it may be necessary to use a second

Table 2
Packings available for Bond Elut cartridges

Phase	Bonded moiety
Non-polar phases	
Methyl (C-1)	$Si—CH_3$
Ethyl (C-2)	$Si—CH_2—CH_3$
Butyl (C-4)	$Si—(CH_2)_3—CH_3$
Hexyl (C-6)	$Si—(CH_2)_5—CH_3$
Octyl (C-8)	$Si—(CH_2)_7—CH_3$
Cyclohexyl (CH)	
Phenyl (PH)	
Diphenyl (2PH)	
Octadecyl (C18)	$Si—(CH_2)_{17}—CH_3$
Polar and weak ion exchange phases	
Cyanopropyl (CN)	$Si—(CH_2)_3—CN$
Diol (2OH)	$Si—(CH_2)_3—O—CH_2—CH(OH)—CH_2(OH)$
Aminopropyl (NH_2)	$Si—(CH_2)_3—NH_2$
Primary/secondary amino (PSA)	$Si—(CH_2)_3—N(H)—(CH_2)_2—NH_2$
Propyl carboxylic acid (CBA)	$Si—(CH_2)_3—COOH$
Strong ion exchange phases	
Propyl sulphonic acid (SCX-P)	$Si—(CH_2)_3—SO_3Na$
Benzene sulphonic acid (SCX-B)	
Quaternary amino (SAX)	$Si—(CH_2)_3—N(CH_3)_3\ ^+Cl^-$

extraction column to provide a more efficient clean-up. Excellent guidelines are offered on the use of Bond Elut cartridges by the manufacturers in what is referred to as 'Chromatographic Mode Sequencing'. An example is shown in Fig. 3 for the analysis of tricyclic antidepressants in human plasma [12]. The drug is retained on a C2 cartridge and interfering material is washed to waste with dilute sodium hydroxide. Chloroform is used to elute the drug from the C2 cartridge onto a cyanopropyl column, effecting a further purification; the analytes are then eluted with HPLC mobile-phase and aliquots taken for analysis.

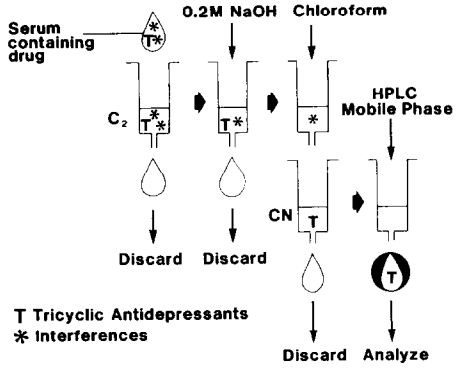


Figure 3
Chromatographic Mode Sequencing (CMS) using Bond Elut cartridges.

Packings available

Presented in Table 2 are the various bonded silica phases available for Bond Elut cartridges, grouped according to functionality, e.g. polar, non-polar etc. There are four packings for the Sep–Pak system: C18, silica, florisil and alumina. The latter packing is available washed with acidic (pH 4–5), neutral (pH 7–8), or basic (pH 9–10) buffers and comes fully activated and ready for use. All remaining Sep–Pak and Bond Elut phases are dry packed and must be activated before use (except the cyanopropyl, aminopropyl and diol packings when used in normal-phase mode).

Applications of manual LSE methods

Tricyclic antidepressant drugs have been prepared for analysis using Sep–Pak [13] and Bond Elut [12] cartridges. Narasimhachari [13] reported extracts from this method as clean as those obtained by LLE, but with more consistent recoveries. However, the best feature was the saving in time. A novel application of LSE is represented by a colorimetric screening method for methamphetamine [14]; here the LSE cartridge was used to isolate and retain the analyte from urine and was then utilized as a vessel for the reaction to determine the presence of the drug. Modification of the surface chemistry, with reference to HPLC, has been used to determine paraquat and diquat in urine [15].

The C18 phase was pretreated with cetrimide to block any free silanol groups that might interfere with the extraction. This was followed by sodium heptane sulphonate to convert the packing to a cation exchanger for isolation of the analytes. The method was very rapid, avoided emulsion formation and the recoveries of the two compounds were in excess of 90%.

Automated LSE methods

At present there are two companies manufacturing first-generation automated sample processors that utilize the principle of LSE. These are the devices of Du Pont (Prep) and of Analytichem International (AASP).

The Du Pont 'Prep' system

This microprocessor-controlled automatic sample preparation instrument, developed by Du Pont, is based on a bidirectional centrifuge that combines the preparation and concentration steps in the same machine. Up to 12 samples per run are prepared using extraction cartridges. Each cartridge consists of a cap, an extraction column, plus effluent and recovery cups (Fig. 4). These four parts are stacked together, so that the cap and the effluent cup seal the extraction column to prevent the sorbent bed from drying out. All units, apart from the recovery cup, are made from polyethylene, the recovery cup being made from aluminium to facilitate the evaporation of eluting solvents. The cap can be used to fit either the recovery or effluent cups. The maximum volume of solvent that can be dispensed from each of the two solvent reservoirs is approximately 40 ml, which allows up to 3 ml to be dispensed either as a wash or eluting solvent into each cartridge. Care must be taken not to over fill the effluent cup, as a maximum of approximately 4 ml of sample and buffer can be processed initially.

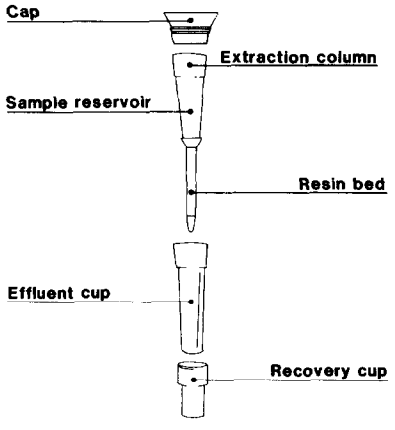


Figure 4
Extraction cartridges for the Prep sample processor.

The unique rotor consists of an inner and an outer concentric ring of swinging buckets mounted on the same drive shaft. The inner ring is fixed, but the outer ring can move relative to it. When each cartridge is separated and loaded into the rotor the extraction column is placed in the inner ring, while the effluent and recovery cups are placed into the outer ring. When the rotor swings clockwise the tip of the extraction column lines up with the effluent cup (Fig. 5). Centrifugal force pushes the sample through the column, which extracts the compounds of interest, unretained material being collected as waste in the effluent cup.

A wash solvent is then dispensed from a reservoir through the rotor head to the top of each extraction column to flush any residual sample and to remove more potentially interfering components. A high speed spin then removes excess solvent from the resin bed before the elution cycle begins. The direction of the rotor is then reversed and the tip

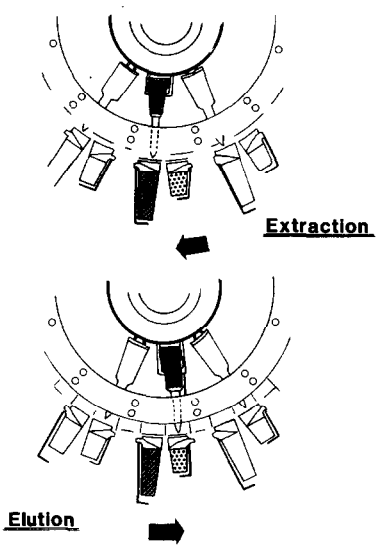


Figure 5
Prep rotor assembly showing alignment during extraction and elution.

of the extraction column lines up with the recovery cup (Fig. 5); the eluting solvent is pumped onto and centrifuged through the column to wash the drug into the cup. There is an optional drying cycle which allows compressed air (heated up to 60°C) to be blown over the extract, after which the cups are cooled before manually reconstituting the extracts for analysis.

Fifteen microprocessor-controlled programs make the machine versatile, although the extraction scheme has to be adapted to the programs available, rather than vice versa. There is the facility for manual intervention during method development; however, this is limited to the possibility to cause advancement of the cycle steps used at the time. The time taken for the complete cycle varies from 8 to 30 min depending on the program chosen.

The machine needs to be fully loaded for each run in order to avoid the spillage of wash and eluting solvents into the centrifuge bowl; or effluent cups can be placed in the inner ring of the rotor, this being sufficient to collect the two aliquots of solvent dispensed by the machine. It should be pointed out that the cups must be balanced so that rotor stability is not affected in any way.

Wash and elution solvents reach the cartridge via the centre of the aluminium rotor. It is recommended that corrosive solvents are not used, particularly halide acids, and that the instrument is washed thoroughly each day to protect the rotor head.

Once the experimental parameters have been defined, then, apart from loading the extraction cartridges into the instrument, the process is fully automatic. This approach is very reproducible and avoids some of the operator errors associated with conventional sample preparation. The limitation of the instrument is that only twelve samples can be prepared at one time; furthermore, there is a relatively long delay if the instrument is used to evaporate the sample extracts. This could be rate-limiting if an assay with a rapid through-put were employed. However, the authors have found that if the extracts are transferred and subjected to off-line solvent removal, then an excellent rate of sample through-put can be maintained.

Packing materials for Prep. Four packing materials available for the Prep are the XAD-2 type resin (Type W), a C18-bonded silica (Type OD), and strong anion and cation exchange resins (Types AS and CS respectively).

The cation and anion exchange resins comprise sulphonic acid or quaternary ammonium moieties attached to an organic resin; they are therefore more stable to strong acids or bases than are silica bonded phases. Both resins are compatible with either aqueous or organic solvents; this means that methanol or other organic solvents can be used to wash the column, after which the same solvent containing a volatile acid or base can then be used to elute the drug of interest. The C18 cartridges, as for any other bonded silica phases, need activation with aliquots of methanol and water before use. This is a potential disadvantage, but since the instrument already has the capability to utilize four solvents, consideration should be given to fitting the two extra solvent reservoirs. Additional solvent capability would also allow more sophisticated washing or differential elution cycles for the other packing materials.

A well written pamphlet is produced by the manufacturer to aid the selection of phases, taking into account the physico-chemical properties of the molecule(s) to be isolated. A method development guide then outlines general strategies to follow for each cartridge in order to achieve the desired extraction.

Applications of Prep. Prep has been used in the determination of tricyclic antidepressants [16], ibuprofen and flurbiprofen [17]. The assays were reported as being quicker, more precise and more sensitive compared with the manual LLE methods. Barbiturates in tissue homogenates and post-mortem blood have been extracted by this method; considering the viscosity and fibrous content of the samples, this approach was shown to be very successful and compared well with the conventional LLE technique [18]. Warfarin, a highly protein-bound drug, has been successfully extracted (97% recovery) from biological samples using Prep [19].

The 'Analytichem Automated Sample Processor' (AASP) system

The AASP Liquid Chromatography Module is a microprocessor-controlled instrument specifically designed to integrate extraction on bonded-phase columns, with automated on-line syringe-free injection of the eluent into a high-performance liquid chromatograph. Samples are prepared at the bench using a cassette of ten miniature extraction columns (Fig. 6). These are similar in design to Bond Elut columns but fit into a modified Vac-Elut manifold, in which positive pressure (nitrogen or air) is applied to the top of each column in order to drive the sample through the sorbent bed. The extraction procedure follows the general rules for the use of bonded-phases. The packings listed in Table 2 are available in AASP cassettes. After the samples have been applied, the columns may be washed to remove undesirable compounds. Elution, however, is not carried out in the normal manner. The prepared cassettes containing the analytes of interest are then loaded into the Auto-Injector. Up to ten cassettes (i.e. 100 samples) can be loaded at any one time.

The AASP Auto-Injector consists of a high-pressure sealing chamber (Fig. 7) which encapsulates each cartridge in turn. Elution is effected by switching the solvent flow from the HPLC pump through this chamber and onto the column. The fluid pathway is determined by a pneumatically activated Valco ten-port injection valve controlled by the AASP microprocessor (Fig. 8). This valve can also be re-set after a pre-determined time period. Thus, the analytes of interest are selectively eluted from the AASP cartridge

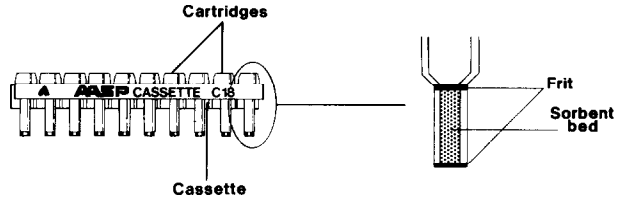


Figure 6
Analytichem-automated sample preparation (AASP) cassette.

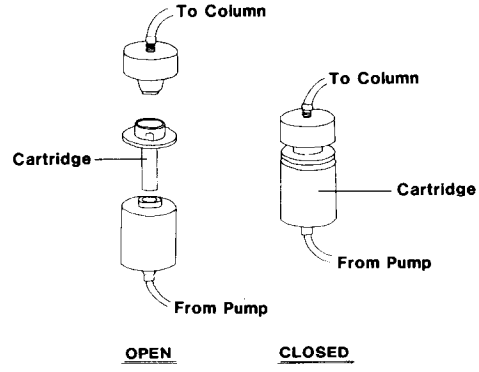


Figure 7
Schematic of AASP compression chamber in open and closed positions.

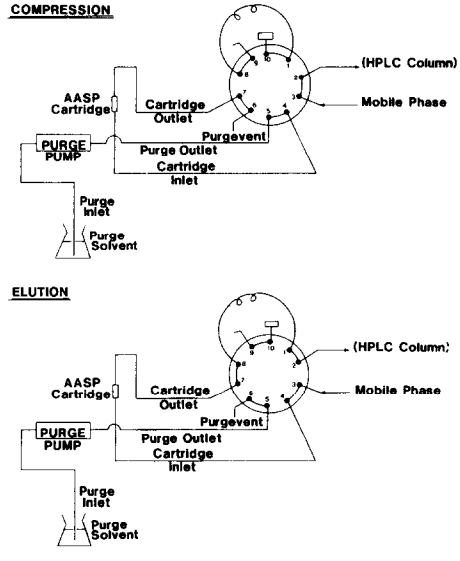


Figure 8
AASP fluid pathways during compression and elution cycles.

leaving unwanted endogenous compounds behind. These latter compounds, if eluted onto the analytical column, would greatly increase the chromatographic run-time and hence reduce sample throughput.

The instrument has three operational modes: 'manual', for processing individual cartridges; 'remote', for automatic analysis controlled from an external device; and 'auto' for automatic analysis controlled by the AASP itself. In addition to the off-line column wash procedure a purge facility is available. Prior to injection the cartridge can be flushed with a pre-determined volume of solvent from a separate reservoir (Fig. 8). The eluent from the purge process may be either switched to waste or directed into a holding loop and injected onto the HPLC column. This latter technique can, in theory, be used to overcome one of the major drawbacks of the AASP system, viz. the HPLC solvent may not be of sufficient strength to elute the analytes from the cartridge. The use of a stronger solvent in the purge reservoir may overcome this problem, but the limiting factor remains the volume that can be injected, without disturbing the chromatography on the analytical column.

Although the instrument is relatively new the authors have, with relative ease, successfully transferred a manual Bond Elut method to run on the AASP. In this instance the machine offers higher recoveries, increased sample throughput, greater precision, faster sample preparation and smaller sample volumes compared to the regular Bond Elut assay.

Discussion of liquid–solid extraction methods

Advantages of LSE

The modern LSE methods show the following advantages. There is minimal introduction of impurities into the assay by modern LSE methods; the use of high-purity polypropylene vessels to contain the packings means that leaching of plasticizers is virtually eliminated. There is total elimination of emulsion formation, which, as noted above, is the major disadvantage of LLE. As a result of the high extraction efficiencies, smaller biological sample volumes can be contemplated than would otherwise be the case. Plasma samples of 50–100 μl can now be assayed where previously it would have been 10-fold higher. The columns are disposable and only require removal from the laboratory as biohazard waste. The solvents used in the final elution are for the most part water soluble and constitute a reduced safety hazard.

The great advantage of these methods of sample preparation is speed, enabling more samples to be processed per day; the time saved can be spent performing other tasks. This method of sample preparation is especially suitable for molecules that are volatile or labile, as all operations are carried out at room temperature. Because the majority of packing materials are bonded silicas, it might be expected that they would only be stable over a small pH range. This is not the case, as the packing is only in contact with the liquid for a relatively short time; thus, a wider range of solution pH can be used in practice.

Disadvantages of LSE

The authors have found that frozen and thawed plasma often contains precipitated fibrins that will prevent free flow through LSE columns. Thus their removal is essential for uninterrupted analysis; this material is removed by routinely centrifuging every plasma sample. Glass beads and glass wool have also been used to overcome this

problem with Prep cartridges [18]. Batch-to-batch variation has been reported for Prep cartridges: the normal recovery of ibuprofen was 95%, but with one batch of the cartridges this fell to 60%; the assay reproducibility remained constant, but the limit of detection was not as low as previously reported [17].

It is essential for analysts using manual LSE methods to avoid any untimely disturbance, as this may affect up to 10 samples that are being processed simultaneously; in particular, the addition of the eluting solvent at the wrong time would result in loss of the analytes.

The robustness of solid-phase extraction methods

In developing an analytical method it is of prime importance that the accuracy and precision obtained are not affected by variations outside the control of the analyst. In the case of bonded-phase extraction columns the robustness of the assay may be affected by variations in the sample matrix, the quality and homogeneity of the sorbent and, in particular, by the choice of bonded phase.

In general, the authors have found that good reproducibility can be obtained if the recovery of the analytes is high (>90%). However, in some instances where this is not achieved, the poor recovery is associated with an increase in the relative standard deviation for the assay. Ideally, good recovery would be the aim in all methods, but this cannot always be achieved given the time restraints imposed by an industrial pharmaceutical environment.

Comparison with column-switching in HPLC

One aspect of solid-phase sample preparation which is not covered in this analytical survey is that of column-switching. This is a technique in which analytes are concentrated on a pre-column which forms an integral part of the liquid chromatograph. The pre-column is automatically back-flushed with the HPLC mobile phase, which transfers the analyte onto the analytical column. The cycle starts again and one pre-column may be used to process hundreds of samples. The technique has been recently reviewed [20]. However, it is worth looking at the advantages and disadvantages of on-line and off-line sample pre-treatment.

The major disadvantage of on-line pre-concentration is that some material may become irreversibly bound to the bonded-phase packing material, hence decreasing its capacity to retain further samples. Alternatively, endogenous material may move very slowly through the pre-column and be eluted in a subsequent analysis giving rise to spurious peaks on the chromatogram. Column-switching has been evaluated in the authors' laboratories for the analysis of cardiovascular drugs in urine; in practice these problems have not been experienced. However, this may depend on the sample, and it can be envisaged that blood, plasma and especially bile may be more difficult to handle by this technique.

With off-line sample pre-treatment it is recommended that each column be used once only and hence 'memory effects' are not observed. Yee *et al.* [21] reported reusing Bond Elut columns in the analysis of cyclosporin in serum without deleterious effects on the recovery or on the chromatography. Although this is an obvious way to cut costs if funding is limited, the authors would not recommend it if, as is often the case, the overall cost of the study itself far exceeds that of the analytical materials.

A major disadvantage of on-line sample preparation is its lack of flexibility. Unless very complex switching systems are employed, one is limited to a simple wash step followed by elution with HPLC mobile phase. In contrast, with off-line extraction

columns analytes can be sequentially eluted with a variety of solvents, used either directly for analysis, or for transfer to a second column for further clean-up. This latter approach, although elegant, increases the sample preparation time and the cost per sample. It is on these criteria that column switching has the advantage. The authors have used commercially available pre-columns which, even if disposed of daily, are considerably cheaper than individual extraction columns. Column-switching also has the advantage of being fully automated, although some might argue that with the unreliability of some HPLC equipment this is not an advisable direction in which to be heading. One useful feature of off-line sample preparation is that sequential solvents do not have to be miscible, as the sorbent bed can be dried between applications. It is also possible to use extremes of pH, as the transit time of solvent through the column is insufficient for degradation of the bonded phase or silica backbone to occur. Neither condition is true with on-line preparation, as all solvents must be miscible with each other and compatible with the solid-phase.

Whatever technique is employed on-line or off-line, it is evident that semi or fully automated solid-phase sample preparation will offer an answer to many of the analytical problems encountered in the pharmaceutical industry. More recently the Zymark Corporation have applied robotics to the automation of solid-phase sample preparation. At present the authors are evaluating the possibility of combining the best features of on- and off-line sample preparation in a fully automated configuration.

Cost comparisons

To compare the advantages of one method with another, one must inevitably make comparative costings; in trying to do this the authors can only look at the effects that the newer methods have had in their own laboratories. The factors that have been taken into account are:

(a) the cost of materials for both methods;
(b) the time taken either to do a set task or to process a given number of samples;
(c) the capital cost of equipment (automated LSE equipment only);
(d) staff motivation.

The cost of chemicals and materials for the LLE of oxmetidine [2] was found to be approximately 15% more than that for the corresponding extraction using Bond Elut [11]. The time taken to process 60 plasma samples for chromatographic analysis was 1.5 h by LSE, compared to 5 h by the LLE method [11], a saving of 3.5 h that could be used for further sample processing or for alternative tasks. The capital cost of automated instrumentation is usually justified on the grounds of increased sample throughput and hence productivity. However, equally as important are the scientific criteria of better precision and the ability to utilize smaller sample volumes.

Staff motivation is an aspect that is not always covered in analytical reviews, but unless the staff are thoroughly briefed on the potential impact of alternative working practices and their co-operation obtained, then any moves in this direction are doomed to failure. Equally as important, there are also the positive advantages for laboratory personnel, viz. release from what is quite often monotonous work would be expected to improve the working atmosphere.

Future perspectives

The trends of liquid–solid sample preparation will probably proceed along two lines. The first will be the development of more specific phases for the separation of distinct

classes of compound, phases may even be specific to one molecule. Such silica-bonded phases are already becoming available commercially. The phenylboronic acid phase (Analytichem International) is specific for the isolation of coplanar vicinal hydroxyl molecules; one application for this phase will be the isolation of catecholamines. After activation by an alkaline solution, the phase will covalently bind catecholamines as shown in Fig. 9. This type of binding will allow harsher washing conditions to remove interfering compounds. The elution of the catecholamines is accomplished by using an acidic solution, since the covalent bond cleaves at pH values below 8.0.

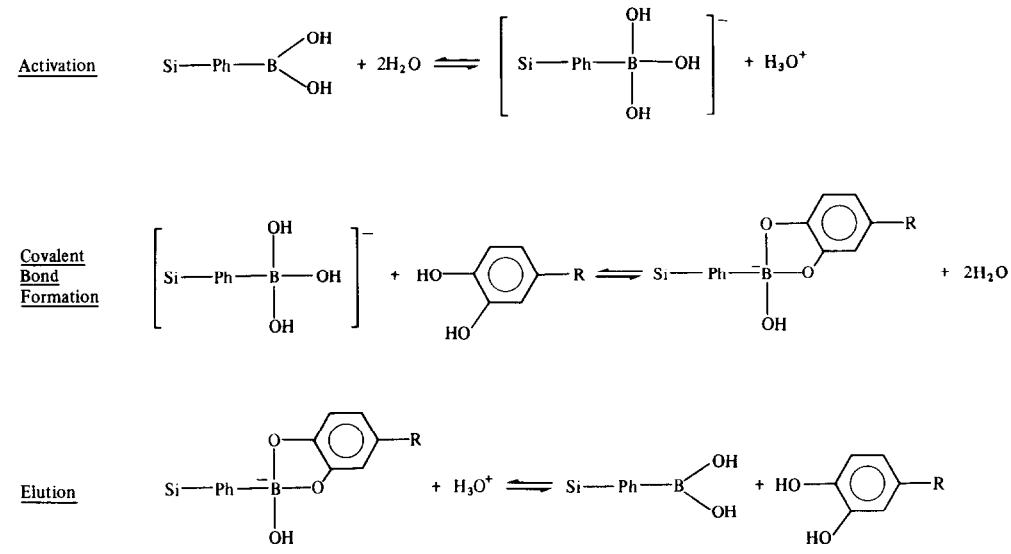


Figure 9
Covalent binding of catecholamines to phenylboronic acid phase.

An interesting application of the XAD-2 resin recently published shows the potential usefulness of LSE methods [22]. The authors impregnated the resin with pentafluorobenzyl bromide and used it for the simultaneous extraction and derivatization of tetrahydrocannabinol carboxylic acid and phenobarbitone. The reaction time was 1 h at ambient temperature and the analytes were eluted from the resin with diethyl ether; after the solvent was evaporated, the residue was taken up in acetonitrile and an aliquot analysed by GLC. The great advantage of this method is that the derivatizing agent is rapidly isolated from the analytes and the isolation and derivatization steps are combined. Although this approach is only in its infancy, it may point the way to the future. Automation of this approach should be relatively easy given the present state of the art.

A second line of development in liquid–solid extraction methods will concern the instrumentation, as machines to exploit fully the advantages of LSE have still not been marketed. The areas for exploitation will be the full automation of sample preparation; AASP and Prep are the first generation of such instruments. The final goal will be the total integration of sample preparation with the instrumental measurement step (most probably HPLC), so that the analyst will, in principle, only apply the biological sample to the instrument and then obtain the final assay result automatically. Sample preparation,

chromatographic analysis, integration and report generation will be programmed by the analyst. It is the opinion of the authors that prototype instruments should soon be available for simple tasks. Further advances in this area are to be anticipated during the remainder of this decade and beyond.

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Addendum: Since this article was written Varian Associates have been marketing the AASP under the acronym Advanced Automated Sample Processor.