Journal of Pharmaceutical & Biomedical Analysis
Vol. 7, No. 9, pp. 1087–1096, 1989
Printed in Great Britain

0731–7085/89 $3.00 + 0.00

Sample preparation for the HPLC analysis of drugs in biological fluids*

R. D. McDOWALL,†‡ E. DOYLE, G. S. MURKITT and V. S. PICOT

Department of Drug Analysis, Smith Kline and French Research Ltd, The Frythe, Welwyn, Hertfordshire AL6 9AR, UK

Abstract: Sample preparation for the analysis of drugs in biological fluids consists of a number of unit operations that are used for (i) release of the drug from a conjugate or biological matrix; (ii) removal of endogenous compounds that could interfere with the assay; and (iii) techniques for liquid handling. The trends in sample preparation that have occurred over the past 10 years in the authors' laboratory are discussed. In general, there has been a move from the traditional liquid–liquid extraction to methods using bonded-silica which permit rapid throughput and efficient extraction. Automation of sample preparation has seen further gains in productivity; however, the present generation of equipment lack the control and communication systems that are essential for the development of the automated integrated laboratory of the future.

Keywords: *Sample preparation; liquid–liquid extraction; liquid–solid extraction; solid-phase extraction; biological fluids; high-performance liquid chromatographic analysis of drugs; laboratory automation.*

Introduction

Sample preparation is essential when analysing drugs in biological fluids by high-performance liquid chromatography (HPLC) for the following reasons: the necessity to concentrate the analytes to within the detection range of the liquid chromatographic detector; improvement of the specificity of the method by removing or separating as many endogenous interfering compounds as possible; and to free the analyte from those matrix components that are incompatible with the HPLC column.

The objective of the assay method will dictate the detail necessary for the sample preparation scheme. For instance, therapeutic drug monitoring usually requires specificity to distinguish the drug to be monitored from similar compounds, metabolites or co-administered drugs. In contrast, an assay to investigate the pharmacokinetics of a potential drug candidate requires a specific and sensitive analytical method. Therefore, assays are developed and moulded for an individual application.

*Presented at the "International Symposium on Biomedical Applications of Liquid Chromatography", 23–25 March 1988, Bradford, UK.

†To whom correspondence should be addressed.

‡Present address: Analytical and Clinical Pharmacokinetic Services Department, Wellcome Research Laboratories, Langley Park, Beckenham, Kent BR3 3BS, UK.

Unit Operations of Sample Preparation

To aid the analyst, sample preparation should be considered as a number of unit operations, each capable of a specific task. These techniques are the fundamental building blocks for any assay method that can be taken and matched to the analytical challenge at hand. It is important to realise that a particular technique should not exist in isolation, but should be available for use as indicated or required.

A list of some typical unit operations that can be utilized for sample preparation is given in Table 1; these can be classified into three main functions.

(i) *Release of the drug or metabolite from the biological sample*

These techniques are designed to release a drug that has been conjugated (typically with a glucuronide or sulphate moiety) [1] or make available to the assay more drug than otherwise would be the case by the breakdown of the biological matrix by hydrolysis (the hydrolysis can be performed either by enzymes [2, 3] or by an acid or base). Sonication is also a possible means of improving the recovery of a drug from blood [4].

(ii) *Removal of proteins and other endogenous compounds*

The procedures in this group produce the greatest clean-up of all sample preparation unit operations. Ultrafiltration [5] and protein precipitation [6] can be used to remove protein from plasma samples. The membrane filters used for ultrafiltration are efficient, although care must be taken to avoid binding of the drug to the membrane and so

Table 1
Some sample preparation unit operations

(i)	Release of the drug from the biological sample		
	Hydrolysis	Acid	
		Base	
		Enzyme	
			Protease
			Lipase
			β-Glucuronidase
			Aryl sulphatase
	Sonication		
(ii)	Removal of endogenous compounds		
	Liquid–liquid extraction		
	Liquid–solid extraction (Solid-phase extraction)		
	HPLC		
	Precipitation	Organic solvents	
		Inorganic acids and salts	
	Ultrafiltration		
(iii)	Procedures for liquid handling		
	Aspiration		
	Centrifugation		
	Dilution		
	Evaporation		
	Filtering		
	Freezing		
	Mixing		
	Pipetting		
	Salting-out		
	Separation		

producing erroneous results. Precipitation, either using organic solvents such as acetonitrile or methanol [6] or inorganic acids and salts [7] are effective agents for removing proteins. Dependent on the concentration of the analyte and/or the detection mode of the chromatograph the supernatants produced after precipitation can often be injected into the chromatograph without any further treatment.

Methods such as liquid–liquid extraction and liquid–solid (solid-phase) extraction [8] are used in many sample preparation schemes, these can produce clean extracts for analysis very efficiently. The chromatograph itself can also be used as a means of sample preparation, the HPLC column is a very efficient method of separating components and very simple yet elegant assays can be developed [9].

(iii) *Procedures for liquid handling*

Procedures in this section are involved in the addition, mixing, separation or removal of liquids, and provide the links between the unit operations in the two previous sections.

Whatever unit operations are used, the final aim of the sample preparation scheme must be to isolate and purify the compound of interest and present it to the liquid chromatograph in a manner that is compatible with the mobile phase and does not perturb the LC detector. As mentioned above, the liquid chromatograph is also capable of separation and clean-up, so that it can enhance any preparation scheme already undertaken, or perform the extraction and quantification stages. It has been argued, according to Frei and Zech [10], that elaborate purification procedures make redundant the separating power of a chromatographic column, but insufficient purification can lead to deterioration of the column efficiency. There is a balance to be struck between an external clean-up and that due to the chromatographic column. If there is a strong element of selectivity and trace enrichment in the sample preparation step, fewer demands will be placed on the quality of the chromatography. On the other hand, the choice of a selective detection mode may simplify the preparation stage to a point where it is relatively easy to automate [10].

Sample Preparation Methods: Trends 1978–1988

To illustrate the advances and trends in sample preparation, Table 2 summarizes the methods used in the authors' laboratories over the past decade. Methods developed in the period from 1978 to 1983, relied on traditional liquid–liquid extraction as the means of sample preparation, this produced clean extracts but suffered from the waste of analyte through sample transfer and emulsion formation, as well as being labour

Table 2
Liquid chromatographic assay methods for drugs in biological fluids [Department of Drug Analysis, SK&F Research (1978–1988)]

Sample preparation method	Number of assay methods
Liquid–liquid extraction	7
Off-line liquid–solid extraction	3
AASP liquid–solid extraction	6
Semi-automated (robot arm) liquid–solid extraction	1
Fully automated (Gilson-AASP) liquid–solid extraction	6
HPLC column switching	4

intensive with a poor potential for automation to cope with large sample throughput. The assay methods consisted of two separate stages: off-line extraction followed by HPLC analysis.

During the early 1980s attention was focussed on disposable extraction cartridges, packed with chemically bonded-silica as a means of overcoming some of the practical disadvantages of liquid–liquid extraction [8]. Sample throughput with these columns was higher than the comparable liquid–liquid extraction [11] but the extraction and analysis stages were still separate processes. Lack of experience with method development, hampered effective use of these columns; this was offset by the use of radioactively labelled drugs to monitor and modify the extraction scheme [8].

Relatively few analytical methods were developed using these disposable cartridges because 1983 saw the introduction of a semi-automated sample preparation system (advanced automated sample processor, AASP, Varian Associates), which enabled the elution of the analyte from the solid phase. Methods still involved off-line extraction of a cassette of 10 solid-phase cartridges which was then transferred to an autosampler where it became a pre-column of the chromatograph with the mobile phase being used to elute the analytes onto the analytical column [8, 12]. Several methods were developed using this approach, all of which provided little analyte loss with fast throughput. However, the preparation and analysis were still two separate processes.

The AASP was automated further in two ways: the first used a robotic arm and the second an autosampler with a probe. The robotic arm was essentially automating the manual sample preparation stage, which remained off-line as the AASP cartridges had to be transferred manually to the chromatograph [13, 14]. The second approach, using the autosampler connected to the purge connection of the AASP valve, allowed on-line activation of the solid-phase, application of the sample and washing of the cartridge before automated elution onto the analytical column [15, 16]. This configuration is currently operating in our laboratories on several applications. The full potential of the unit, however, has to be fully explored in pre-column derivatization either within the working envelope of the autosampler or on the extraction cartridge itself.

One technique that has been available during the period under discussion is column switching, where the HPLC is used for sample preparation and analysis. The chromatograph consists of a pre-column and an analytical column through which the flow of mobile phase can be switched using HPLC switching valves. The pre-column is dry packed and meshes rather than frits are used to avoid blockage. The column is "primed" two or three times with the control sample before use, to block active sites on the silica surface, and the samples are injected directly onto the pre-column to extract the analytes before eluting them onto the analytical column. With such a system, the pre-column can be used many times (approximately 150 injections of 100 μl of plasma). If gradient elution is required for the assay then the pre-column must be flushed separately from the analytical column with a suitable solvent during the equilibrium time between assays. The reader is referred to the paper by Brinkman for more detailed coverage of this topic [17].

Sample Preparation Applications

One application using each of the sample preparation techniques described in the last section will be outlined here, along with the unit operations that are required, which are listed in Table 3.

Table 3
Comparison of the unit operations required for some sample preparation schemes

Liquid–liquid extraction [20]	Off-line liquid–solid [14]	AASP [14]	Gilson-AASP [18]
Aliquot sample	Centrifuge sample	Centrifuge sample	Centrifuge sample
Add IS	Aliquot sample	Aliquot sample	Aliquot sample
Mix	Add IS	Add IS	
Add octanol	Mix	Mix	
Mix	Activate phase 1	Activate phase 1	
Centrifuge	Activate phase 2	Activate phase 2	
Transfer organic	Apply sample	Apply sample	
Add acid	Wash cartridge	Wash cartridge	
Mix	Elute analytes	HPLC analysis	
Centrifuge	Transfer to vials		
Aspirate organic	HPLC analysis		
Transfer aqueous			
Add acetonitrile			
Mix			
Add solid Na_2CO_3			
Mix			
Centrifuge			
Aspirate acetonitrile			
Transfer to vials			
HPLC analysis			

Table derived from Huber and Zech [9].

Liquid–liquid extraction

Cimetidine [18] and subsequent H_2-receptor antagonists [19–22] have been determined in the authors' laboratories using liquid–liquid extraction prior to HPLC analysis. An outline of the basic scheme is given in Table 3, it can be seen that the method involves several extraction stages coupled with liquid transfer stages. The technique required a degree of manual dexterity, especially when isolating the final acetonitrile layer that had been salted out from an aqueous solution. The original method was used in the analysis of many thousands of blood, plasma and urine samples from clinical and pre-clinical studies.

Manual liquid–solid extraction

Manual liquid–solid extraction prior to HPLC analysis has been used for the assay of SK&F 93574, a histamine H_2-receptor antagonist, [23]. This technique was used in preference to liquid–liquid extraction, as it utilized different mechanisms for extraction of the drug from the biological matrix, and because its retention on the sorbent was pH dependent, which greatly increased the specificity of the assay. Although a base, SK&F 93574 is extracted from plasma onto C18 sorbent at an acidic pH (4.5). Once absorbed onto the phase, the sample is washed with sodium carbonate without removal of the drug, now retained by reversed-phase mechanisms. In this way both acidic and basic endogenous material in the matrix are removed, without significant loss of drug. Traces of carbonate are then removed from the phase by water washes, and a final sample clean-up is achieved through an aqueous–organic wash. Since this wash contains a lower percentage of organic solvent than the final elution solvent, this removes material which would normally run close to the solvent front on the HPLC system, but not the analyte.

Extraction of samples by this method is much less laborious, and considerably faster, than a liquid–liquid extraction. By linking three vacuum assemblies together, up to 30

samples can be extracted simultaneously, with a total extraction time of approximately 30 min. Precision, accuracy and day to day variation of the assay are all well within acceptable limits (±15%).

Semi-automated sample preparation

The AASP has been used for a number of applications; in one such application it was used to determine SK&F 94120, a cardiovascular drug, and its four metabolites (one of which was an intact glucuronide conjugate) from a single plasma sample. The existing assay [12] involved the extraction of the drug and the unconjugated metabolites on a C18 cartridge, washing with water and eluting the analytes in the AASP LC module with a mobile phase of acetonitrile and 0.01 M, pH 4.5–5 ammonium acetate (20:80 v/v). The availability of a pure reference standard of the glucuronide metabolite allowed the development of an analytical method capable of measuring all five analytes. Experiments revealed that the glucuronide was quantitatively extracted from plasma but was lost during the wash stage. Retention of the glucuronide on the extraction cartridge was achieved by using an acidic wash to suppress the ionization of the carboxylic acid moiety of the glucuronide molecule.

Elution of the glucuronide into the existing mobile phase resulted in the compound eluting with the solvent front, which was unsuitable for quantification. The elution was modified to include two sequential elutions from the same AASP cassette. The first elution, with a mobile phase containing 10% acetonitrile, selectively removed the glucuronide metabolite. The use of the valve reset facility, which controls the volume of mobile phase flowing through the cartridge, is crucial for the success of this stage. The cassette is then transferred to an identical chromatograph where a second elution with a mobile phase containing 20% acetonitrile is used to determine the parent drug and the three remaining metabolites [24].

Summary of manual extraction procedures

The unit operations of the three manual examples are summarized in Table 3, it can be seen that the many stages required for the extraction of cimetidine are reduced by the use of liquid–solid extraction and reduced further when the AASP is used. This results in less labour involvement and faster throughput of the assay. The main area where speed and time is gained is in the separation of extraction and wash solvents; the solid phase and a liquid are relatively easy to separate, compared with two immiscible liquids. The use of liquid–solid extraction is effective in dealing with analytes with a range of analyte polarities; this would not always be practical with liquid–liquid extraction.

The use of robotics in routine analysis and method development

Originally, a fully customized robotic system was purchased to automate the manual off-line sample preparation for the AASP [14]; this was followed by human transfer of the extracted cassettes to the AASP LC module. The robot was very successful in its task, the results produced by the manual and robot methods were essentially the same [13]. When the HPLC assay was not sufficiently sensitive and a radioimmunoassay was used in further development of the compound, the opportunity was taken to re-engineer the robot to undertake a different rôle.

This alternative task was to screen the different bonded-silica phases to aid the selection of the most appropriate sorbent for an assay method. The configuration of the arm and the peripherals is shown in Fig. 1. The robot will service up to 50 solid-phase

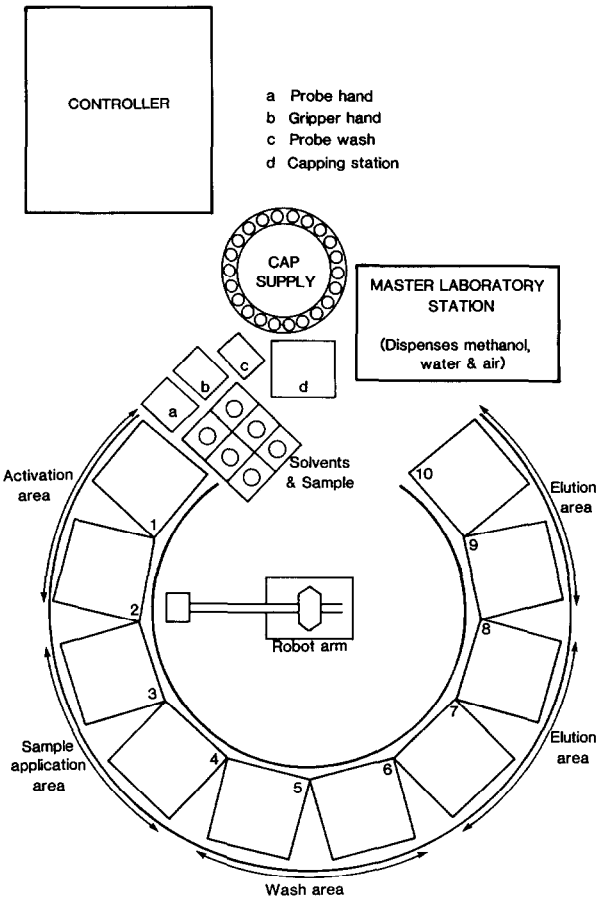

Figure 1
Configuration of a robotic system to screen solid phase cartridges as an aid to method development.

cartridges (most commonly, 10 different phases, each with five replicates) which are held on a gantry, driven to and fro around a track by the robotic arm. The track rails are positioned over eight scintillation vial racks each holding 25 vials in a five by five matrix. The robot arm, with a dispenser probe attached will condition the column, introduce either a plasma sample or an aqueous buffer spiked with radiolabelled drug, and pressurize the column. The fluid emerging from the cartridge after the application of the sample, wash or elution solvents is collected in separate vials which are automatically filled with scintillation fluid and capped before manual transfer to a liquid scintillation counter.

After counting the vials, the results from each phase can be expressed as four columns, as shown in Fig. 2. The first column shows the efficiency of the extraction by the various sorbent phases, the low recovery values from all phases show that extraction is efficient and breakthrough is low in all instances. The wash stage is depicted in the second column which shows the effect of the wash solvent on removing the analyte from the column. The final two columns show the effect of eluting solvents, which can be two aliquots of the same or single volumes of two different solutions. The results are assessed and are

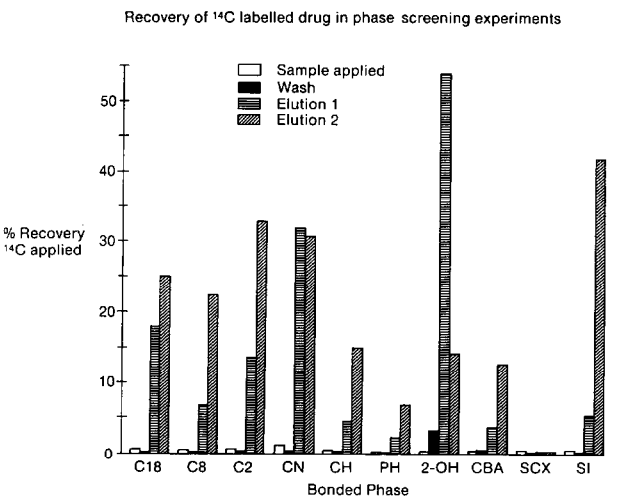

Figure 2
Results from a typical robot screening experiment.

then used as a basis for choosing either an appropriate bonded-phase for further work or different elution conditions for the same phases.

The use of dedicated automation in routine analysis

Total automation of the sample preparation and analysis has been achieved in the authors' laboratories by linking solid-phase extraction with HPLC separation and UV detection. This has been accomplished by using a Gilson 222 autosampler with 401 Dilutor for sample manipulation, conducting the liquid–solid extraction in an AASP LC module, and then at the appropriate time, the HPLC mobile phase is switched through the cartridge allowing elution of the compounds of interest directly onto the analytical column [16]. The Gilson autosampler assumes responsibility as the master controller of the system configuration and communicates with the AASP via a 12-V interface relay box. In this way the Gilson can "remote-start" the AASP and advance the cassette at a predetermined time. In addition, switching of the Valco valve is also effected by the controller. In the only manual part of the procedure, plasma samples for assay were first thawed at ambient temperatures and microfuged at 10,000*g* for 1 min; to remove the fibrous material that might otherwise block the extraction cartridges. Plasma aliquots are transferred to 1.5-ml polypropylene tubes and placed in the Gilson 222 sample rack.

Future Developments in Automation

The present automated systems lack three factors; these are integration, control and communication. These are not limiting factors at the moment but will become so.

Regarding integration, instruments such as the Gilson-AASP are modular, developed from two separate instruments, but the user is paying for two development costs. To overcome this problem, a manufacturer could develop a totally integrated unit with one set of overheads and a smaller footprint on the bench.

Concerning control of the instrument, at present as there is no overall control, when a malfunction or blockage occurs valuable sample will be lost. Thus, developers should consider feedback control mechanisms to monitor the operation of a complex instrument which would be sampling, preparing and analysing simultaneously. If an error in this operation were detected and if the fault could not be corrected, the unit would shut down preventing the further loss of any samples. The development of these workstations, able to make decisions, may involve chemometrics, where there will be feedback between the analytical measurement and the experimental design.

Communication with LIMS and other data handling computers is essential to produce an integrated analytical laboratory. Communications must be incorporated so that the workstation should be able to receive instructions such as what method to run, the analysis conditions to employ as well as the identification of the samples under analysis. If this were possible then the administration time and transcription errors associated with each analysis would be reduced.

Failure to incorporate these facilities will mean that advances in laboratory automation will proceed at a slow pace and the piecemeal approach used up to now will put pressure on existing parts of the system. Betteridge [25] has noted that automation in analytical chemistry provides a solution to today's problem but this sows the seeds for tomorrow's problem. To illustrate this point, a sample processing system such as the Gilson-AASP with very high throughputs, will put strain on the data handling components that were not designed for these large numbers of samples. This is a problem that the authors are currently experiencing in their own laboratories. What is required is a quantum leap to the next generation of equipment where all components can cope with the anticipated throughput.

References

[1] A. C. Metha, *Talanta* **33**, 67–73 (1986).
[2] L. K. Turner, *J. Forensic Med.* **11**, 24–32 (1964).
[3] M. D. Osselton, M. D. Hammond and P. J. Twitchett, *J. Pharm. Pharmac.* **29**, 460–462 (1977).
[4] N. Haagsma, H. G. J. M. Pluijmakers, M. M. L. Aerts and W. M. J. Beek, *Biomed. Chromatogr.* **2**, 41–45 (1987).
[5] G. R. Granneman and L. T. Sennello, *J. Chromatogr.* **229**, 149–157 (1982).
[6] J. Blanchard, *J. Chromatogr.* **226**, 455–460 (1981).
[7] R. P. Maickel, in *Drug Determination in Therapeutic and Forensic Contexts* (E. Reid and I. D. Wilson, Eds), pp. 3–16. Plenum Press, New York (1984).
[8] R. D. McDowall, J. C. Pearce and G. S. Murkitt, *J. Pharm. Biomed. Anal.* **4**, 3–21 (1986).
[9] R. Huber and K. Zech, in *Selective Sample Handling and Detection in High Performance Liquid Chromatography, Part A* (R. W. Frei and K. Zech, Eds), pp. 81–143. Elsevier, Amsterdam (1988).
[10] R. W. Frei and K. Zech, in *Selective Sample Handling and Detection in High Performance Liquid Chromatography, Part A* (R. W. Frei and K. Zech, Eds), pp. 1–3. Elsevier, Amsterdam (1988).
[11] R. D. McDowall, G. S. Murkitt and J. A. Walford, *J. Chromatogr.* **317**, 475–481 (1984).
[12] J. C. Pearce, J. A. Jelly, K. A. Fernandes, W. J. Leavens and R. D. McDowall, *J. Chromatogr.* **353**, 371–378 (1986).
[13] J. C. Pearce, M. P. Allen and R. D. McDowall, *Bioactive Analytes, Including CNS Drugs, Peptides and Enantiomers* (E. Reid, B. Scales and I. D. Wilson, Eds), pp. 293–296. Plenum Press, New York (1986).
[14] J. C. Pearce, M. P. Allen, S. A. O'Connor and R. D. McDowall, *Chemometrics Intell. Lab. Systems* **3**, 315–321 (1988).
[15] R. D. McDowall and J. C. Pearce, *Developments in Analytical Methods in Pharmaceutical, Biomedical and Forensic Sciences* (G. Piemonte, F. Tagliaro, M. Marigo and A. Frigerio, Eds), pp. 217–225. Plenum Press, New York (1987).
[16] G. S. Murkitt, J. C. Pearce and R. D. McDowall, *Chromatographia* **24**, 411–415 (1987).
[17] U. A. Th. Brinkman, *J. Pharm. Biomed. Anal.* In press.
[18] R. M. Lee and P. M. Osborne, *J. Chromatogr.* **146**, 354–360 (1978).

[19] R. M. Lee and R. D. McDowall, *J. Chromatogr.* **273**, 335–345 (1983).
[20] R. D. McDowall, G. S. Murkitt and R. M. Lee, *J. Chromatogr.* **305**, 214–221 (1984).
[21] R. D. McDowall and R. M. Lee, *J. Chromatogr.* **275**, 377–385 (1983).
[22] D. A. Barrett, A. J. Dean, E. Doyle and R. M. Lee, *J. Pharm. Biomed. Anal.* **3**, 385–390 (1985).
[23] V. S. Picot, L. Read and E. Doyle, *Chromatographia* **24**, 282–286 (1987).
[24] J. C. Pearce, W. J. Leavens, J. A. Jelly, K. A. Fernandes and R. D. McDowall, *J. Pharm. Biomed. Anal.* **6**, 75–86 (1988).
[25] D. J. Betteridge, *Analyt. Proc.* **24**, 106–108 (1987).

[*Received for review 24 November 1988*]