Journal of Pharmaceutical & Biomedical Analysis
Vol. 5, No. 4, pp. 353–360, 1987
Printed in Great Britain

0731–7085/87 $3.00 + 0.00
Pergamon Journals Ltd.

Off-line automated sample preparation — experience in a clinical drug investigation unit*

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Abstract: The authors describe an evaluation of the Du Pont Prep. off-line automatic centrifugal sample processing system, used in the authors' Drug Investigation Unit over the past two years. In comparison to liquid–liquid extraction, the authors found that assay precision has generally been improved, absolute recovery was quantitative, reliability has been good and the use of the system has resulted in notable labour saving. However, the cost per test based on a seven-year amortization of capital cost shows that the system is more expensive.

Assays for urinary opiates, serum tricyclic antidepressants, serum isoxicam, serum valproate, serum metoclopramide and serum pindolol are presented and the merits of the method are compared with those for manual liquid–liquid extraction procedures.

Keywords: *Sample preparation; automation; liquid–solid extraction; urinary opiates; serum–tricyclic antidepressants; isoxicam; valproate; metoclopramide; pindolol.*

Introduction

The majority of methods used for the determination of drugs in biological fluids include a sample preparation stage in order to remove protein, eliminate interferences from metabolites or endogenous small molecules and/or to introduce a concentration step to increase sensitivity.

Traditional sample preparation for drug analysis in biological matrices has used liquid–liquid extraction. Although such procedures offer excellent flexibility they may suffer from problems such as emulsion formation and sample degradation; solvents used may be toxic or flammable and require safe handling and disposal procedures. In addition, where an evaporation stage is included there may be losses of volatile analytes and adsorption of others on to glass surfaces. Extraction efficiencies can be relatively low, necessitating a double or triple extraction procedure, and this, coupled with the time required for evaporation may result in a lengthy procedure which may give rise to loss of precision in the overall method. Liquid–solid extraction (LSE) which avoids many of these difficulties has been less extensively used, although with the increasing

* Presented in part at the 2nd IAEAC Symposium on 'Handling of Environmental and Biological Samples in Chromatography', 24–25 October 1985, Freiburg, FRG.

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availability of commercial columns it is becoming more widespread. High-performance liquid chromatography (HPLC) can allow less complex sample clean-up due to the compatibility of aqueous mobile phases with simple techniques such as protein precipitation.

Automation of sample preparation has the potential for addressing problems such as throughput, precision, reliability and assay complexity. Semi-automated LSE procedures are commercially available such as the AASP system (Analytichem International, Harbor City, USA) which, following off-line sample loading, utilises column switching to load extracts directly on to an LC, and the Prep system (Du Pont de Nemours and Co., Wilmington, USA) in which the sample is loaded on to the column by a centrifugal force followed by pneumatically delivered washing and elution solvents. The use of a bidirectional rotor allows the cartridge eluant to be delivered to either the waste or collection cups. Eluted fractions can be automatically evaporated ready for subsequent analysis by any procedure. For further details on the principles of the operation see ref. 1. The Prep has been used to assay several drugs including anticonvulsants in serum [1, 2], drug screening [3] and tricyclic antidepressants in serum [4].

The instrument has been used in this laboratory for two years in the provision of a routine and research orientated clinical service examining plasma and urine samples, and the authors report their experiences in terms of time, cost and method utility. The analytes examined reflected unit workload patterns and included opiates in urine for addiction screening, serum tricyclic antidepressants for therapeutic drug monitoring of psychiatric patients, valproate used in compliance monitoring and isoxicam, metoclopramide and pindolol during pharmacokinetic studies in selected patient groups.

Materials and Methods

Chemicals

All chemicals used were Analar grade obtained from British Drug Houses (Poole, England).

Equipment

Liquid chromatography. Liquid chromatography was performed using varied items of equipment pumps: LC–XPS pumps (Pye Unicam, Cambridge, England), Gilson 302 pumps with 5SC heads and 802 manometric module (Gilson, Paris, France) and Varian 8500 pumps (Varian, Walton-on-Thames, England); injection was via either Rheodyne 7125 valves, WISP 710B (Waters, Harrow, England) or Varian 8050 autosamplers; detection was achieved using either a Beckman 160 fixed wavelength detector (Beckman, High Wycombe, England), LC–UV or PU 4020 variable wavelength detectors (Pye Unicam) with CR 6525 (J.J. Instruments, Southampton, England) recorders or Shimadzu CR 3A or CR 2AX integrators (Scotlab Instruments, Bellshill, Scotland).

Columns were packed with a balanced density slurry in the upward mode in the laboratory using a commercial column packer (Shandon, Runcorn, Cheshire, England) with Hypersil packings (Shandon) as specified in the text.

Calibration

Calibration curves were constructed by plotting analyte peak area compared to internal standard peak area, against concentration. Calibrants were prepared in horse

serum (Wellcome, England) or expired transfusion plasma, and extracted as for the samples to cover the following ranges: urinary opiates 25–1000 μg/l; serum: tricyclic antidepressants 5–500 μg/l, isoxicam 0.25–20 mg/l, valproate 2–200 mg/l, metoclopramide 2–1000 μg/l and pindolol 0.05–100 μg/l.

Gas chromatography. Gas chromatography was performed using a 5830A Hewlett–Packard chromatograph (Hewlett–Packard, Winnersh, Berks, England), with a 914 × 6.35 mm i.d. glass column packed with 2.5% CDMS on Chromasorb W using a carrier flow rate of 20 ml/min nitrogen and run isothermally at 140°C with flame ionisation detection. Data was handled using a 18850A terminal (Hewlett–Packard).

Prep conditions

Type W cartridges which contain a hydrophobic styrene–divinylbenzene co-polymer, similar to XAD-2, were used in all assays. Pre-programmed conditions were used. Those specified by programme 15 include 3 min of automatic extraction, 0.5 min wash solvent dispense followed by 2 min elution, a spin reversal of 45 s, dispense of elution solvent (0.5 min), collection (3 min) and evaporation (18 min); total programme time was 30.5 min. Programme 4 is similar but has slightly longer extraction times, slightly shorter elution times and an evaporation period of 15 min giving a total programme time of 28.5 min. *ca* 60 ml each of wash and elution solvent were dispensed on each run.

Scheme for drugs of abuse in urine

Liquid–liquid extraction and TLC. Urine (10 ml) was made alkaline by the addition of 2 g of sodium bicarbonate, shaken for 10 min with 10 ml chloroform–isopropanol (90:10, v/v) then centrifuged and the aqueous layer discarded. The organic layer was filtered through anhydrous sodium sulphate and evaporated to dryness under nitrogen at 60°C.

The residue was dissolved in 100 μl of acetone and 10 μl applied to each of two 10 × 10 HPTLC silica plates with a fluorescent indicator (Merck, Darmstadt, West Germany).

The first plate was run using methanol–ammonia, (100:1.5, v/v) and the second plate ethylacetate–methanol–ammonia, (85:10:5, v/v/v). Lined, saturated, equilibrated mini tanks were used. When the solvent front had run two-thirds the length of the plate (*ca* 20 min), the plates were removed, air-dried, inspected under UV light (254 nm) and dipped in acidified iodopalatinate.

Automated extraction. Type W cartridges were loaded with 2 ml of urine and 2 ml of M ammonium acetate (pH 9.2). Prep programme 4 was used with a water wash and elution performed with chloroform–isopropanol (90:10, v/v) followed by evaporation under a stream of air at 60°C. The TLC procedure was as described above.

Determination of tricyclic antidepressants in serum

Liquid–liquid extraction and LC. Glass tubes (MF 24/1/5 Q and Q, Corning Ltd., Staffs, England) were silanized (to minimise adsorption of analyte) in a fume cupboard with 2% dimethyldichlorosilane in 1,1,1 trichloroethane, rinsed with methanol and allowed to dry. Into each tube were pipetted 2 ml of serum, 100 μl of concentrated ammonia and 50 μl of a 40 mg/l appropriate internal standard in aqueous solution. The internal standards, in parentheses, were as follows: amitriptyline–nortriptyline (desmethylimipramine); imipramine–desmethylimipramine (nortriptyline); doxepine–desmethyldoxepin (amitriptyline); chlorpromazine and metabolite (amitriptyline). The

tubes were shaken for 5 min after addition of 2 ml of dichloromethane, centrifuged, and the aqueous layer discarded; 2 ml of ethanol were then added, followed again by mixing and centrifugation. The supernatant was decanted into a second freshly silanized tube and evaporated to dryness at 60°C under nitrogen.

The residue was dissolved in 50 μl of mobile phase and 25 μl injected into the LC. A normal phase system was used with a 10 cm × 5 mm i.d. 5μ silica Hypersil column (Shandon, Runcorn, Cheshire, England) with a dichloromethane–isopropanol–ammonia (100:1.4:0.2, v/v/v) eluant pumped at 1.5 ml/min using detection at 254 nm (0.01 AUFS). This is similar to previously published procedures from this laboratory [5, 6].

For the purposes of evaluation, the assay of amitriptyline–nortriptyline with desmethylimipramine as internal standard was examined.

Automated extraction. To a type W cartridge was added 1 ml of serum, 2 ml of ammonium acetate buffer (pH 9.2) and 25 μl of a 40 mg/l internal standard solution (see above). Programme 15 was used with a water wash and elution performed with chloroform–isopropanol (90:10, v/v) with automatic evaporation at 60°C under a stream of air. LC analysis was as described above.

Measurement of isoxicam and piroxicam in serum

Liquid–liquid extraction. 1 ml of serum acidified with 500 μl of 2 M citric acid was extracted for 15 min using a lateral shaker (Gallenkamp, London) with 3 ml of chloroform containing 1.25 mg/l of appropriate internal standard. Piroxicam was used in the isoxicam assay and isoxicam for piroxicam. The organic layer was transferred to a second tube and evaporated at 60°C under vacuum.

The residue was dissolved in 50 μl of LC mobile phase and 25 μl injected on to a 10 cm × 5 mm i.d. 3 μ ODS Hypersil column. The eluant was acetonitrile–0.75% acetic acid containing 0.1% octane sulphonic acid (40:60, v/v) with a flow rate of 1.5 ml/min; detection was performed at 320 nm (313 nm for fixed wavelength detector with filter).

Automated extraction. Type W cartridges were loaded with 200 μl of serum, 2 ml of 0.1 M citric acid and 50 μl of a 50 mg/l aqueous internal standard solution. Programme 15 was used with a water wash and chloroform–isopropanol (90:10, v/v) elution followed by automatic evaporation at 60°C under air. Liquid chromatographic analysis was performed as described above.

Measurement of valproate in serum

Liquid–liquid extraction. Serum (200 μl) and 200 μl of 150 mg/l *n*-hexanoic acid (internal standard) in 2 M sulphuric acid were extracted with 9 ml of diethyl ether for two min, then centrifuged. The supernatant was transferred to a second tube, 20 μl of 0.5 M ethanolic potassium hydroxide added, and evaporated at 45°C under air. To regenerate the free acid 50 μl of 98% formic acid and 200 μl of acetone were added to the extract and 5 μl of the free acid solution injected into the GC.

Automated extraction. Serum (200 μl) with 200 μl of 150 mg/l *n*-hexanoic acid in 2 M sulphuric acid and 2 ml of 0.1 M citric acid were added to a Type W cartridge. Programme 15 was used with a water wash and the chloroform–isopropanol (90:10, v/v) eluant contained 0.005 M potassium hydroxide. Extracts were evaporated at 25°C under air then treated as described above.

Assay of metoclopramide in serum

Liquid–liquid extraction. This procedure is based on that of Meyer *et al.* [7]. 2 ml of serum, 100 μl of sodium hydroxide (5 M) and 25 μl of *N*-propionylprocainamide (16 mg/l internal standard) were extracted with 6 ml of chloroform–isopropanol (96:4, v/v). The organic layer was evaporated to dryness at 70°C under a stream of nitrogen. 50 μl of methanol was added to the residue and 10 μl injected into the LC. A 25 cm × 5 mm i.d. 5μ ODS Hypersil column was used with an eluant consisting of acetonitrile–0.1 M sodium acetate (pH 5.0)–1-hexanesulphonic acid (75:25:0.2, v/v/w) with detection at 280 nm (0.02 AUFS).

Automated extraction. Serum (2 ml), 25 μl of 16 mg/l *N*-propianylprocainamide and 100μ of concentrated ammonia were added to Type W cartridges. Programme 15 was used with a water wash and chloroform–isopropanol (90:10, v/v) elution followed by evaporation at 60°C under a stream of air. LC assay was performed as described above.

Assay of pindolol in serum

Liquid–liquid extraction. This assay is based on that of Bangah *et al.* [8]. Two ml of serum were made alkaline with 0.5 ml of sodium hydroxide (1 M) and extracted with diethyl ether (8 ml) for 2 min. After centrifugation, six ml of ether were transferred to a second tube containing 200 μl of sodium dihydrogen phosphate (0.1 M, pH 3.0) and vortexed for 20 s. The aqueous layer was frozen by immersing the tubes in a dry ice–ethanol mixture and the ether decanted, the aqueous phase was thawed then washed with 2 ml of *n*-heptane by vortexing for 20 s. The aqueous phase was frozen again, the *n*-heptane discarded and 50 μl of the aqueous phase injected into the LC.

Chromatography was performed using a 25 cm × 5 mm column containing 5μ SAS Hypersil, with an eluant of 0.01% aqueous perchloric acid–acetonitrile (80:20, v/v) at a flow rate of 1.5 ml/min. UV detection was at 233 nm.

Automated extraction. Serum (2 ml) was buffered to pH 10.5 using 2 ml of ammonium acetate buffer, and 50 μl of 50 mg/l propanolol were added as internal standard on a Type W Cartridge. Programme 15 was used with a water wash and a chloroform eluant, followed by evaporation at 50°C under a stream of air. LC assay was performed as described above.

Results and Discussion

The performance characteristics of all assays were examined in both sample preparation procedures and compared as to total sample preparation time, labour time, absolute and relative recovery, between-batch precision, sensitivity, sample volume and apparent overall reliability (Table 1). A batch of 12 (i.e. a full Prep rotor) was considered for the calculation of the time to completion.

Less time was required for the automated assay and it was less labour-intensive; however, if batches greater than 12 are to be run, then due to the serial nature of Prep use, the time component becomes an arithmetic progression which is not necessarily the case for manual methods. Since a full critical path analysis is beyond the scope of this presentation it is not feasible to define the rate-limiting step in each manual procedure. A reasonable limitation in most manual assays would be 50 analyses per batch. For serum pindolol we estimate that manual and automated methods would be equally time

Table 1
Characteristics of assay methods using automated extraction

Criteria Analyte (conc.)		Time (h) Total	 Labour	Recovery (%) Absolute	 Relative	Precision (%)* (Between-batch CV)	Limit of detection (3 × S/N) (ng on column)	Sample volume (ml)	Reliability
Urinary opiates	M	3.5	2.25	80	—	—	250	10	Good
(qualitative)	P	0.75	0.25	90	—	—	50	2	Excellent
Serum amitriptyline	M	2	1.5		94 ± 2	11.6	10	2	Poor
(45 μg/l)	P	0.75	0.25		105 ± 2.5	8.0	5	1	Excellent
Serum isoxicam	M	3	2	72 ± 8	98 ± 48	5.3	500	1	Fair
(10 mg/l)	P	0.75	0.25	99 ± 3	99 ± 3	3.2	50	0.2	Excellent
Serum valproate	M	1.5	1.0	60 ± 8	98 ± 4	3.5	600	0.2	Very good
(140 mg/l)	P	0.75	0.25	97 ± 5	99 ± 5	3.2	300	0.2	Very good
Serum metoclopramide	M	0.75	0.5	61 ± 10	94 ± 10	10.0	250	2	Good
(500 μg/l)	P	0.75	0.25	100 ± 2	100 ± 2	4.2	6	2	Excellent
Serum pindolol	M	1.25	1.0	96 ± 3	99 ± 2	4.0	4	2	Good
(50 μg/l)	P	0.75	0.25	96 ± 3	99 ± 2	4.2	0.1	2	Excellent

M = manual procedure.
P = Prep procedure.
* = $n \geq 15$ samples in each case.

consuming with a nominal total time of 3 h, however the labour intensity is always less for the automated procedure.

Most procedures use a good internal standard so absolute recovery is not important provided precision is acceptable. Improved absolute sensitivity limits with decreased levels of interferences and improvements in absolute recovery are reflected in reduced sample volumes required for the automated technique. This may be of particular importance in kinetic and paediatric work and some notable improvement was found for certain analytes, e.g. isoxicam, urinary opiates, etc.

For some assays such as the tricyclic antidepressants automated extraction showed considerable improvement in between-batch precision; the liquid–liquid extraction of these drugs is well-known to suffer losses due to adsorption, necessitating the silanisation of glassware [5, 9]. In our experience unsatisfactory assays occurred too frequently but no problems have been encountered since introducing the automated liquid–solid technique.

A disadvantage of the current Prep configuration is the use of aluminium receiver cups; although these allow good heat transfer they can catalyse degradation reactions causing analyte losses, a problem we have experienced with methotrexate. This may be avoided by using glass liners.

Although cartridge types other than type W (XAD-2 type) are available they are not necessary for this type of work and all assays can be modified to suit type W cartridges. A major advantage of the system is that relatively inexperienced staff have found it possible to establish the main assay requirements and readily define performance criteria.

Occasionally problems of cartridge blockage were encountered but centrifugation of samples prior to loading avoided this difficulty.

Cost is an important factor. In the past year, not including calibration and quality control the following approximate number of Prep analyses were performed: in urine — 500 opiate measurements; in serum — 200 tricyclic antidepressants, 600 valproates, 300

Table 2
Comparative costs for 250 extractions*

Item	Manual	£	Automatic (Prep) £
Equipment amortization	H_2O bath	100	2000
	shaker	50	
	vortexer	20	
Consumables	pipettes	100	1800
	test tubes	500	(cartridges and pipette tips)
Reagents	solvents	250	50
	other reagents	220	100
Labour (including reagent preparation)†		1850	300
Total		3090	4250
Approximate cost per test		1.25	1.70

*Breakdown as shown in text.
†Labour costs calculated for an analyst paid £11,400 p.a. working a 40-h week, i.e. hourly rate = £5.50.

metoclopramides, 400 pindolols and 500 isoxicams. The cost of the Prep and manual analyses are compared in Table 2. The following assumptions have been made: (i) The initial purchase price of the Prep instrument of £14,000 was amoritized over 7 years. Other costs were derived from annual requirements costed at current prices; (ii) Analyses were performed by an analyst paid at £5.50/h; (iii) There were no assay failures for either type of sample preparation procedure.

The cost per test is nearly 40% greater using the Prep. Thus, the decision to utilise this instrument cannot be justified on cost savings but rather on the basis of a more efficient use of the labour force. However, this is a single justification on the basis of cost to replace expensive commercial non-isotopic immunoassay procedures by the use of the Prep and chromatography.

In conclusion the Prep has been found to be reliable and simple to use. Its application has improved the laboratory work pattern although this has been realised at an increased cost.

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[*First received for review 25 October 1985; revised manuscript received 12 August 1986; final version received 24 October 1986*]