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Journal of Pharmaceutical and Biomedical Analysis
25 (2001) 995–1000

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Plasma deproteinization by precipitation and filtration in the 96-well format☆

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Received 16 May 2000; received in revised form 2 January 2001; accepted 8 January 2001

Abstract

The need for fast bioanalytical methods within the pharmaceutical sector is rapidly growing. Sample preparation is often the bottleneck step. A new approach to increasing sample throughput involves precipitated protein removal by filtration in the 96-well format, thereby eliminating the need for centrifugation and manual handling of individual tubes. The potential for such a new technique has been investigated for the determination of an iron chelator, a highly protein-bound compound ($\geq 99.5\%$) in plasma. An analog was used as internal standard. Acetonitrile and plasma were sequentially aspirated, separated by an air gap, using a manual electronic pipettor. They were then dispensed into the channel of an Empore™ filter PPT plate above the filter, and a slight vacuum was applied. The eluate was collected and diluted prior to injection. The compounds were then separated by reversed-phase chromatography and detected by UV at 295 nm. The chromatographic run time was 6 min. The mean recovery following protein precipitation was 78%, which shows that the technique can apply to a highly protein-bound compound. Replicate quality control samples were prepared in drug-free normal human plasma at four different concentrations. The mean accuracy ranged from 87 to 108% with the CV ranging from 3 to 8%. The described procedure is simple, fast and reproducible. It requires minimal equipment. The time required to prepare a plate manually is only about 20 min. The use of 12-channel repeater pipettors reduces the risk of error and improves productivity. Automation should be an aid to further increasing sample throughput when more than one plate a day is to be prepared.

Keywords: Protein precipitation; 96-well format; Filtration

1. Introduction

Bioanalytical methods commonly involve liquid-liquid extraction (LLE), solid-phase extraction (SPE) of the drug and its metabolites from a matrix (blood, plasma, urine, etc), or protein removal, prior to separation by high-pressure liquid chromatography (HPLC). There is a trend today

☆ Presented at the 11th International Symposium on Pharmaceutical and Biomedical Analysis, 14–18 May 2000, Basel, Switzerland

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0731-7085/01/$ - see front matter
PII: S0731-7085(01)00349-1

for SPE to become the most widespread sample preparation technique [1]. Individual columns or cartridges are being replaced by high-throughput 96-well plates in which as many as 96 samples are processed simultaneously. However, the optimization of a SPE procedure using either cartridges or 96-well plates can sometimes be time consuming because it requires the selection of a sorbent and of suitable solvents and pH conditions. The development of a procedure involving protein precipitation is faster due to the simplicity of this technique. Protein precipitation is preferably used in early drug discovery when the rapid availability of a bioanalytical method and the batch extraction of several drug candidates rather than sensitivity are required. This technique involves protein removal from the sample matrix by adding a denaturing agent. In spite of a large choice of denaturing agents, acetonitrile and methanol are widely used because they are compatible with most mobile phases and generally drag an amount of drug down with the precipitate smaller than more efficient precipitants such as trichloroacetic acid or perchloric acid [2].

The application of protein precipitation requires manual handling of individual tubes due to the need for centrifugation. An adaptation of this technique with centrifugation in the 96-well format has recently been reported [3]. Analysts are presently investigating the feasibility to increase the sample throughput of protein precipitation using 96-well filter plates. These plates contain only the frit used to secure the sorbent bed in each well of a SPE plate. This frit is expected to filter the precipitated proteinaceous mass, thereby replacing centrifugation. An increasing number of filter plates are already proposed by various manufacturers [4]. The feasibility for using such plates for protein precipitation has recently been demonstrated by Biddlecombe et al. [5] for the determination of salbutamol in plasma, a relatively polar compound bound to a low level to proteins. The procedure was carried out in conjunction with LC/MS/MS using a robotic sample processor. A deuterated internal standard was used.

The potential of protein removal by filtration in the 96-well format using manual electronic single and 12-channel repeater pipettors [6] was investigated with ICL670, a highly protein-bound iron chelator under development. An analog of ICL670 was used as internal standard (IS). The developed procedure is described herein.

2. Experimental

2.1. Solvents and chemicals

ICL670 and IS were provided by Novartis

Table 1
Recovery obtained following protein precipitation[a]

Nominal concentration (μg/ml)	Precipitation in tube			Precipitation in the 96-well format		
	ICL670	IS	Ratio[b]	ICL670	IS	Ratio[b]
	Recovery (%)	Recovery (%)		Recovery (%)	Recovery (%)	
0.8	90	86	1.05	75	71	1.06
	86	88	0.98	82	82	1.00
1	83	86	0.97	58	57	1.02
	84	86	0.98	83	82	1.01
8	90	86	1.05	81	78	1.04
	91	87	1.05	89	87	1.02
Mean	87	87	1.01	78	76	1.03
S.D.	3	1	0.04	11	11	0.02
C.V. (%)	3	1	4	14	14	2

[a] Recovery = (found/nominal concentration) × 100.
[b] Recovery ratio ICL670/IS.

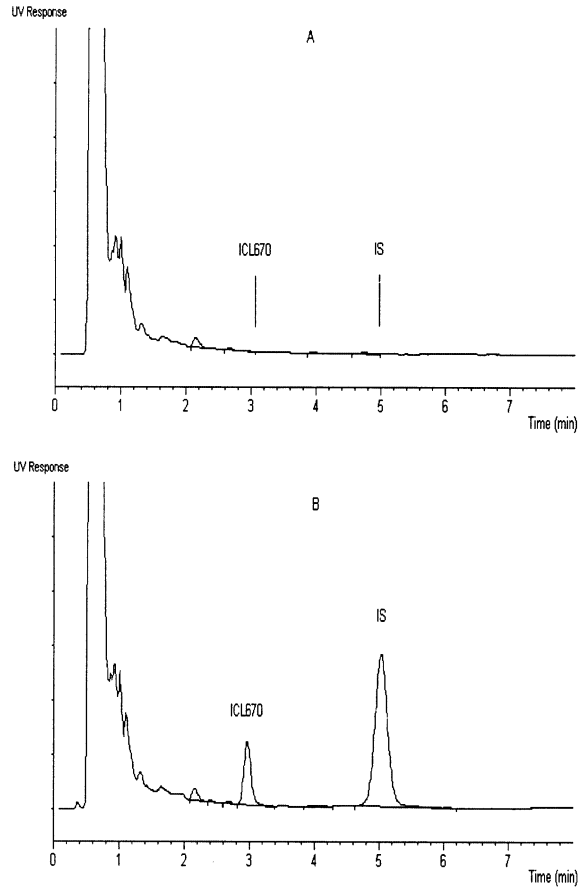

Fig. 1. Representative chromatograms (A) drug-free plasma, (B) plasma spiked with 0.1 μg/ml ICL670 and IS.

Pharma AG, Basle, Switzerland. The IS was an analog of ICL670 with two atoms of fluor instead of hydrogen atoms on phenol groups. Analytical grade methanol and acetonitrile were obtained from Carlo-Erba (Val de Reuil, France). Absolute ethanol was obtained from Prolabo (Fontenay sous Bois, France). *Ortho*-phosphoric acid 85% and Titrisol pH 7 were obtained from Merck (Darmstadt, Germany). Water was deionized, filtered and purified on a Milli-Q Reagent Grade Water System from Millipore (Bedford, MA, USA).

Human plasma was obtained from Etablissement Français du Sang, where blood was collected from volunteers in tubes containing citrate–phosphate–dextrose. After centrifugation, the plasma was transferred and stored at −20°C.

2.2. HPLC apparatus and chromatography

The HPLC system consisted of a Model 305 pump from Gilson (Villiers-le-Bel, France), an autosampler ASPEC from Gilson, a Model UV-975 Jasco detector from Merck monitoring at a wavelength of 295 nm. A chromatography workstation, Model X-Chrom from Thermo LabSystem (Altrincham, Cheshire, UK), was used to perform data aquisition.

Chromatographic separations were performed at 50°C on an Alltima C18 column 50 × 4.6 mm, 3 μm particle size, supplied by Alltech (Deer Field, Illinois, USA). The analytical column was protected with a Lichrospher RP18 precolumn, 5 μm particle size, supplied by Merck.

The mobile phase, 0.005 M *Ortho*-phosphoric acid–ethanol–methanol (40:38:22, v/v/v), was delivered at a flow-rate of 1.3 ml/min. The pressure at the top of the column was about 100 bars.

2.3. Standard solutions

Primary stock solutions of ICL670 and IS were prepared by dissolving 5 mg ICL670 in 10 ml ethanol. Appropriate dilutions of the stock solutions with pH 7 buffer were then made in order to prepare the spiking solutions at concentrations ranging from 1 to 100 μg/ml for ICL670 and 2.5 μg/ml for the IS. Two series of stock solutions were prepared from different weighings to spike the calibration and quality control plasma samples. All the solutions were prepared in glass flasks and stored at 4°C.

2.4. Sample preparation

About 100 μl of thawed plasma were transferred in a 96-deep well format plate and 10 μl of the appropriate ICL670 spiking solution were added. An aliquot of 10 μl of the internal standard solution was then added using a 12-channel repeater pipettor, and the plate was vortexed for a few seconds. A 3M Empore™ filter plate PPT was placed on top of a 3M vacuum manifold. About 200 μl of water were dispensed above each filter of the filter plate using a 12-channel repeater pipettor, and a slight vacuum was applied. A collection

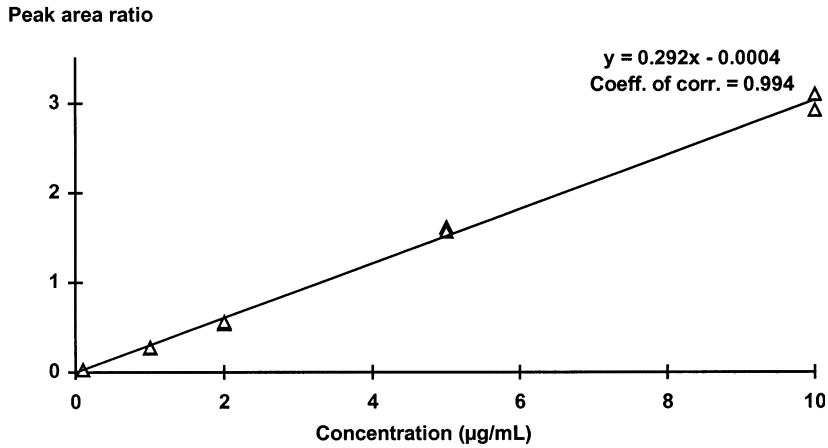

Fig. 2. Representative calibration curve.

plate with 96 polypropylene tubes fit in the standard 9 mm centre-to-centre spacing was placed in the bottom of the manifold. Then, 300 µl acetonitrile and 100 µl spiked plasma were subsequently withdrawn, separated with an air gap, using an electronic single-channel pipettor working in the dilution mode. The liquids were then dispensed together above each filter, to the inner side of the well. Once all wells were filled, a gentle vacuum was applied until the collection tubes were filled and each well was dried. Then, the collection tubes were placed on the autosampler rack refrigerated at 4°C. Prior to injection of each filtrate, 200 µl of 1 M *ortho*-phosphoric acid were automatically added into the collection tube by the ASPEC system, and 200 µl were injected.

3. Results and discussion

Attempts to precipitate the plasma proteins in the 96-well format were first done by adding the liquids in a conventional manner in two steps. The plasma was aspirated using a manual electronic pipette and dispensed in a well above the filter. Then, in a second step, acetonitrile was aspirated and dispensed above the plasma. The proteins precipitated, but the filtrate contained particles in suspension regardless of the order of addition of plasma and acetonitrile. A saturated solution of NaCl was then added above the filter prior to plasma addition in order to avoid dripping of plasma within the filter before the vacuum is applied. But this also failed to produce a clear filtrate. Hence, acetonitrile and plasma were sequentially aspirated using an electronic pipette in the dilution mode, separated with an air gap, and subsequently dispensed together into a channel of the filter plate. This permitted a more efficient mixing of acetonitrile and plasma. The dripping of the liquids prior to protein precipitation might have been suppressed, thereby allowing for a higher effectiveness in precipitating proteins. A clear filtrate was obtained, but the reproducibility was poor. Prior wetting of the filtrate with water enhanced reproducibility.

Perchloric acid, an agent more efficient in precipitating proteins than acetonitrile [2], was tested. Several filters became blocked, and the volume of the filtrate largely differed from one well to an-

Table 2
Within-day reproducibility

Nominal concentration (µg/ml)	Mean accuracy (%)[a]	Precision CV (%)
0.1	87	8
0.8	102	6
1	99	3
8	108	4

[a] Accuracy = (found/nominal concentration) × 100. Five replicates were prepared at each concentration.

other. Methanol was also tested as precipitant, but no clear filtrate could be obtained. Three different filter plates were tested. A 3M Empore™ filter plate PPT with each well containing a filter secure in place with a sealing ring was used. The filter is composed of polypropylene microfibers of graded densities, the coarsest one on top, and the finest at the bottom. The microfibers are oriented so that a tortuous path of liquid flow is created. The filter has been experimentally determined to retain 98% of all particles larger than 10 μm in size and 50% of particles as small as 2 μm. A Porvair Protein Microlute™ filter plate, equipped with a combination of Polyolefin (Vyon™) frits was also used. Clear filtrates were obtained with the two plates, but reproducibility tests were performed with the Empore™ plate only. A Whatman Protein Precipitation Microplate™ plate equipped with a dual membrane consisting of two distinct layers was also tested. The top layer acts as a prefilter removing coarsest particulates. The bottom layer has an oleophobic nature for retaining the well contents without dripping and acts as a fine filter for removing fine particulates (0.7 μm). However, probably due to the low porosity of the bottom filter, some wells became blocked with this filter plate.

The extraction recovery was assessed following conventional precipitation in tubes and in a 3M Empore™ filter plate. The precipitation in tubes was applied with the volumes of liquid described in Section 2.4. After addition of plasma and then acetonitrile, the tubes were shaken on a vortex mixer for a few seconds and then centrifuged for 5 min at 4000 rpm. The supernatant was diluted before injection as described in Section 2.4. The recovery was calculated from the comparison of the peak areas obtained from spiked extracted plasma samples and blank plasma extracts spiked with ICL670 before injection (Table 1). The mean ICL670 recovery obtained following application of the conventional precipitation in tube (87%) was slightly higher than that obtained following filtration (78%). With both methods, the bulk of the proteins precipitated spontaneously upon addition of plasma and acetonitrile. The mixture was shaken for further mixing with the conventional protein precipitation in tubes only. Since ICL670 is highly bound to plasma proteins (protein bound fraction $\geq 99.5\%$), this suggests that some residual binding of ICL670 to proteins might have been disrupted when the tube was shaken, resulting in removal of ICL670 from the precipitated proteinaceous mass. However, a recovery of 78% is by far above the acceptance criteria ($\geq 50\%$), and this shows that protein precipitation in the 96-well format is a viable technique, even for highly protein-bound compounds. The reproducibility with the conventional precipitation in tube was better than that obtained with the filtration procedure as indicated by the coefficients of variation (C.V.), 3 versus 14% (Section 1), respectively. However, the variability observed with the filtration procedure was balanced with the internal standard. The coefficient of variation on the ratio of the recoveries ICL670/IS was even lower with the filtration procedure (2%) than with the precipitation in tubes (4%).

Representative chromatograms are exhibited in Fig. 1. Calibration samples were prepared at five different concentrations in duplicate in the range 0.1–10 μg/ml. The calibration curves, represented by the plots of the peak area ratio ICL670/IS versus ICL670 concentration in the calibration sample, were generated using weighted ($1/x^2$) linear regression. A representative calibration curve is shown in Fig. 2. Five replicate quality control samples were prepared in drug-free normal human plasma at four different concentrations. The mean accuracy ranged from 87 to 108% with the C.V. ranging from 3 to 8% (Table 2). The acceptance criteria, mean accuracy within 85–115% and C.V. $\leq 15\%$, were met.

The time required to process the 96 samples of a plate manually was about 20 min, as earlier reported when using a robotic sample processor [5]. This time may be further decreased by using an electronic 12-channel repeater pipettor to aspirate and dispense the plasma and acetonitrile in 12 wells simultaneously. The samples were diluted prior to injection so that no band broadening occurred, and they were injected without further treatment. This avoided a time-consuming evaporation and reconstitution step. The compounds were detected at a wavelength of 295 nm, where a few endogenous plasma compounds absorb. The

chromatographic run time was 6 min, when using a short column packed with 3 μm particles.

4. Conclusion

The described procedure involving protein precipitation and filtration in the 96-well format is fast, simple and reproducible. It can apply to a highly protein-bound compound. The use of a plate eliminates the need for centrifugation and manual handling of individual tubes. The procedure requires minimal equipment. The time required to prepare a plate manually is about 20 min. The use of 12-channel repeater pipettors reduces the risk of error and improves productivity by decreasing the number of fills and dispenses. Automation should be an aid to further increasing sample throughput when more than one plate a day is to be prepared. Then, the plasma transfer from the clinical tubes to the plate (manually performed within about 15 min) should also be automated to take full advantage of the automation. With the emergence of LC/MS/MS and its inherent specificity, this sample preparation technique should find a broad applicability in bioanalysis.

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