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Automated protein precipitation by filtration in the 96-well format

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Abstract

The use of automated protein precipitation by filtration in the 96-well format as a rapid sample preparation technique for high throughput bioanalysis using liquid chromatography tandem mass spectrometry is reported. A robotic sample processor is used to aspirate sequentially a plasma sample and acetonitrile separated by air gaps. These are then mixed by being dispensed into individual channels of a 96-well filter block. The resulting supernatant is separated from the precipitated plasma proteins by the application of gentle vacuum using a custom manifold. The filtered supernatants are collected into a deep well microtitre plate, evaporated to dryness using a heated 96-well dry down station and reconstituted in water prior to analysis. The efficiency of the extraction procedure is measured by the Lowry method for determining protein concentration. This method was used to optimise both the volume and the order of reagent addition, and to compare several prototype 96-well filter blocks. Using the optimised procedure a specific, precise and accurate method was developed for the β-agonist salbutamol in rabbit plasma with a calibration range of 1 to 100 ng/ml from 100 μl of sample. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Automated protein precipitation; Filtration; Salbutamol

1. Introduction

Sample preparation prior to chromatographic separation has three principal objectives: the dissolution of the analyte in a suitable solvent, removal of as many interfering compounds as possible and pre-concentration of the analyte. A number of techniques such as liquid–liquid extraction (LLE), protein precipitation, and solid-phase extraction (SPE) are routinely used in bioanalysis to prepare samples prior to chromatographic analysis. Water immiscible solvents such as hexane, diethyl ether and ethyl acetate are used in LLE to separate the analyte by partitioning it between the organic phase and the aqueous phase. While the technique can be very selective, it is prone to emulsion formation and is not well suited to highly polar molecules. Although LLE can be used to efficiently assay large numbers of samples and can be adopted to batch mode, the transfer steps involved make the process labour intensive. In protein precipitation, acids or water miscible organic solvents are used to remove the protein by denaturation and precipitation. Acids, such as trichloroacetic acid (TCA) and perchloric acid, are very efficient at precipitating proteins. Organic solvents, such as methanol and ethanol, although having a relatively low efficiency in removing plasma proteins, have been widely used in bioanalysis because of their compatibility with HPLC mobile phases [1]. In SPE

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the analyte is partitioned between liquid and solid-phases, the latter being packed between two fritted discs in disposable polypropylene cartridges. These extraction cartridges can be mounted on a vacuum manifold that may hold multiple cartridges for batch extraction. The use of bonded silica phases, with their wide range of functionality, coupled with the disposable format have made SPE one of the most popular sample preparation techniques over the last 10 years [2].

The emergence of LC–MS-MS as the method of choice for bioanalysis has increased the pressure on the sample preparation step often making this the rate limiting step. The combination of inherent sensitivity and high specificity of LC–MS-MS enable analysis times to be significantly reduced, run times are typically 1 to 3 min. Both LLE and protein precipitation are difficult to automate compared with SPE because of the liquid transfer steps and the need for centrifugation. The development of SPE in the 96-well format coupled with the use of robotic sample processors (RSP) which offer the advantages of parallel processing, has made SPE the sample preparation technique of choice for LC–MS-MS [3].

Despite the difficulties in automating protein precipitation, its simplicity and wide applicability still make it an important technique particularly in early drug discovery where the generic extraction of 'cassettes' of drug candidates is more important than sensitivity. While the liquid handling involved in conventional protein precipitation can be automated by the use of a robotic sample processor, the tubes or microtitre plate still need to be centrifuged. In our development of automated 96-well format SPE [3,4] the possibility of employing the frit used to secure the sorbent bed to remove precipitated protein by filtration rather than centrifugation was considered. The efficiency of the extraction procedure is measured by using a modification of the Lowry colorimetric method for determining protein concentration [5]. This method, which was automated using a RSP, was used to optimise both the volume and the order of addition of reagents and to compare several prototype 96-well filter blocks. Using the optimised procedure a specific, precise and accurate method was developed for salbutamol in rabbit plasma with a calibration range of 1 to 100 ng/ml from 100 μl of sample.

2. Experimental

2.1. Chemicals and reagents

Both salbutamol and a deuterated internal standard were synthesised at GlaxoWellcome Research Laboratories, Stevenage, UK. HPLC grade water was obtained from Fisher (Loughborough, UK). Analar grade sodium chloride and formic acid were obtained from BDH (Poole, UK). HPLC grade methanol and acetonitrile were obtained from Rathburn Chemicals Ltd. (Walkerburn, UK). The micro protein determination kit was obtained from Sigma (Poole, UK). Control rabbit plasma (New Zealand Whites) was obtained from Charles River Ltd.

2.2. Preparation of standards and controls

Primary stock solutions of salbutamol (1 mg/ml) were prepared by accurately weighing approximately 10 mg into a 10 ml Grade A volumetric flask, dissolving in methanol and making up to volume. Secondary solutions containing 1.0, 0.1 and 0.01 μg/ml were prepared in water by appropriate dilution. These solutions were used to spike control plasma to produce calibration standards at 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 ng/ml of salbutamol. Validation Control (VC) samples were prepared independently (i.e. from a separate weighing) by spiking batches of control plasma at 1.0, 3.0, 20.0, 50.0 and 100.0 ng/ml of salbutamol. A Multi-PROBE II (Packard Sciences, Pangbourne, UK) robotic sample processor (RSP) was used to prepare the plasma spikes from the diluted standard solutions for both the standards and controls.

2.3. Protein precipitation

Three prototype filter blocks were used in this work; the Protein Microlute™ block (Porvair Sciences, Shepperton, UK), the Protein Precipitation Microplate™ (Whatman Ltd., Maidstone, UK) and the Empore™ Filter Plate PPT (3M, St. Paul, USA). Both the Protein Microlute™ and the Protein Precipitation Microplate™ have two filters. The top filter acts as a pre-filter to remove coarse particulate matter. The bottom filter acts as a fine filter removing fine particulate matter when the vacuum is

applied. The low porosity and olephobic nature of the bottom filter in the Whatman Protein Precipitation Microplate™ (0.7 μm) prevents the liquid dripping before the application of the vacuum. Each block was processed using the MultiPROBE II RSP with a custom acrylic vacuum manifold mounted directly onto the deck. A 1 ml square well collection plate (Porvair Sciences) was placed in the base of the vacuum manifold and a protein filtration block was placed on top of the manifold. The RSP aspirates the plasma sample (100 μl), followed by acetonitrile (300 μl) containing internal standard and then dispenses into the channel of a protein filtration block using the default dispense speeds. Once all the samples have been dispensed onto the block a gentle vacuum is applied to the manifold. The supernatants were collected into the square well plate and then evaporated to dryness on a heated 96-well dry down station (Porvair Sciences) using nitrogen heated to 50°C. The supernatants were reconstituted in 100 μl of water, sealed using a heat sealing polypropylene foil (Advanced Biotechnologies Ltd., Epsom, UK) and vortexed before analysis by LC–MS-MS.

2.4. LC–MS-MS method

Concentrations of salbutamol in protein precipitation supernatants were determined by a modification of a recently described LC–MS-MS method [6]. Supernatants were analysed by LC–MS-MS using Perkin-Elmer series 200 micropumps, a Perkin-Elmer series 200 Autosampler, a Jasco CO 965 column oven, and a PE-Sciex API-365 triple quadrupole mass spectrometer (Ontario, Canada). A 25-μl aliquot of the sample supernatant was injected onto a 100×2.1 mm I.D. Inertsil ODS-3 HPLC column (Capitol HPLC Ltd, Braburn, UK) operated at 40°C using a fast gradient (100% A to 100% B over 2 min, where A=0.1% formic acid and B=95:4.9:0.1 acetonitrile:water:0.1% formic acid) at a flow-rate of 0.8 ml/min. The total cycle time including re-equilibration was 4 min. The flow from the column was split using a T-piece such that only 200 μl/min was directed to a TurboIonspray (TISP) interface operating at 450°C in positive ion mode, using nitrogen as both the nebuliser and auxiliary gas. The analytes were detected by tandem mass spectrometry using selected reaction monitoring (SRM) of the transitions m/z 240 to 148 (400 ms dwell) and m/z 243 to 151 (100 ms dwell) for salbutamol and its $^2H_3$ internal standard, respectively. The instrument was operated with a TISP needle voltage of 5000 V, unit mass resolution on both quadrupoles and nitrogen as the collision gas (indicated target thickness of two) with a collision energy of 26 eV (laboratory frame). All LC–MS-MS SRM peaks were integrated by the PE-Sciex processing software MacQuan™. Calibration curves were constructed by plotting peak area ratios of analyte to internal standard against concentration, using a weighted $(1/X)$ linear regression model in all instances. Concentrations of the VC samples were subsequently interpolated from these curves. A one-way analysis of variance (ANOVA) of the validation data was performed by an Excel macro (Microsoft Corp.).

2.5. Protein determination

A primary stock solution of bovine serum albumin (1 mg/ml) was prepared by pipetting 0.5 ml of the kit protein standard into a 50 ml Grade A volumetric flask, and making up to volume with 0.85% sodium chloride. Calibration standards at 250, 500, 750 and 1000 ng/ml of protein were prepared by the RSP for each assay by diluting the primary stock with 0.85% sodium chloride. The protein precipitation supernatants were dried down and reconstituted in 0.85% sodium chloride before analysis for protein concentration. The protein determination assay was also automated by the RSP. Using a multiple liquid transfer procedure, 220 μl of the Biuret reagent is aspirated followed by 20 μl of the standard or unknown and dispensed into a standard microtitre plate (Beckman, High Wycombe, UK). The RSP mixed the solutions by twice aspirating and dispensing 50% of the dispensed volume. The samples were then allowed to incubate at room temperature for 10 min, the RSP software allows a timed delay to be included in the method. The Folin Ciocalteu's phenol reagent (10 μl) was then added to each well using a reagent addition procedure and the contents of each well were mixed by twice aspirating and dispensing 100 μl of the well contents before incubating for a further 30 min at room temperature. The microtitre plate was then read at 650 nm using a Thermo_{max} microplate reader (Molecular Devices, Crawley, UK)

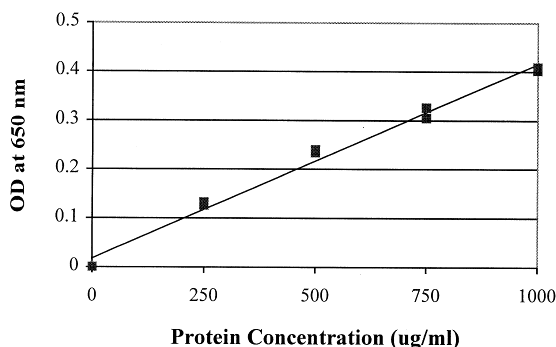

Fig. 1. Typical standard curve for automated Lowry method. Duplicate measurements of the OD at 650 nm are made at each standard concentration.

and the unknown protein concentrations were interpolated from the calibration curve. A typical calibration curve is shown in Fig. 1.

3. Results and discussion

3.1. RSP procedure

All the liquid handling for both the protein determination assay and the protein precipitation procedure was automated using a MultiPROBE II robotic sample processor (RSP). The software of this RSP with its pre-defined library makes it very easy to set up the deck, the selected labware are simply dragged and dropped into position on the screen. The procedure templates allow easy set up for standard pipetting operations such as single or multiple liquid transfers, reagent additions, pre-dilutions, or serial dilutions. This RSP allows disposable tips and fixed tips to be used in the same procedure, thereby eliminating carry over when dispensing from the standard solutions. In addition, the use of file based sample lists make complex dilution schedules easy to set up. The software also allows complete control of the vacuum for the protein precipitation assay.

3.2. Optimisation of procedure

In conventional protein precipitation using centrifugation it has been shown that at least 2 volumes of acetonitrile are required to give a greater than 99% efficiency of protein precipitation [1]. The optimal volume of acetonitrile for efficient protein precipitation for each protein filtration block was determined using a modified Lowry method for protein determination. The original Lowry method is limited by poor stability of the combined reagents, non-reproducibility of colour especially at low protein concentration and a non-linear chromogenic response with protein concentration [5]. In the modified method the sample is mixed first with a diluted Biuret reagent and later with an undiluted phenol reagent for colour development [7]. This method, which was automated to support these studies, shows good linearity over the calibration range as shown in Fig. 1. For each filtration block the RSP was used to sequentially aspirate 100 μl of rabbit plasma followed by an air gap and then varying volumes of acetonitrile (100, 200, 300 or 400 μl). The total volume was dispensed into a protein filtration block and a gentle vacuum applied to separate the supernatant from the precipitated plasma proteins. The extracts were evaporated to dryness using a 96-well heated dry down station and reconstituted in 100 μl of 0.85% sodium chloride prior to protein determination. By measuring a 100-fold dilution of the pooled rabbit plasma, the total protein content was determined and the respective percentage efficiencies for 2, 3 and 4 volumes of acetonitrile were calculated. The results are presented in Fig. 2 and Table 1 and show that for all three blocks at least 3 volumes of acetonitrile to 1 volume of plasma are required to obtain efficient protein precipitation by filtration. No reliable estimates of the protein concentrations could be obtained when using 1 volume of acetonitrile because the amount of protein breakthrough made it impossible to reconstitute the dried down supernatants. The filtration block data compare favourably with the conventional tube data, see Fig. 2. The data in Fig. 2 also show that although using 4 volumes of acetonitrile improves the efficiency of the extraction compared to 3 volumes, the difference is not significant. The smaller, 3 volumes, was used in all further experiments in order to reduce the time taken for liquid handling and the dry down time. The low efficiency of extraction shown by the Porvair block when using 2 volumes of acetonitrile was due to protein breakthrough in a few wells. The 3 and 4

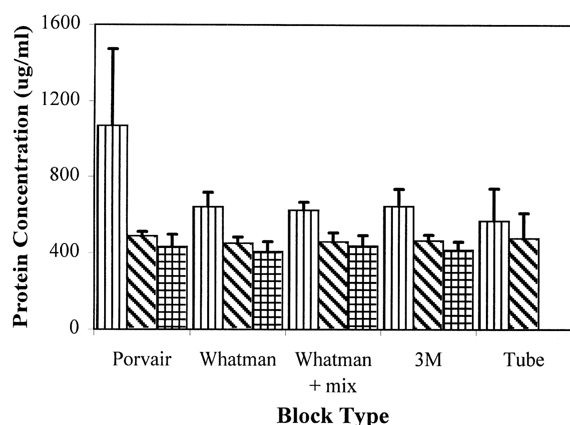

Fig. 2. Optimisation of acetonitrile volume. Maintaining the sample size at 0.1 ml the effect of varying the volume of acetonitrile, from 0.2 ml, 0.3 ml, and 0.4 ml, on the protein concentration (μg/ml) of the extracts is shown. The error bar represents the standard deviation (n=8).

volume data for the Porvair block show no evidence of any gross protein breakthrough, both sets of data are very precise and compare well with the data obtained using the other two blocks.

It was thought that the observed differences in the relative efficiencies between filtration in the 96-well format and centrifugation in tubes could be explained by differences in the efficiency of mixing. In conventional protein precipitation the tubes are vortex mixed prior to centrifugation, while in the filtration blocks the only mixing occurs when the liquids are dispensed into a 96-well block. In order to test this, the experiment needed to be repeated with the sample and acetonitrile being vortex mixed in the block prior to the vacuum step. This is only possible with the Whatman block which is unique in that the porosity of the bottom filter retains the content of the wells without any dripping. As can be seen in Fig. 2 there is no significant difference between the mixed and unmixed data, indicating that efficient mixing is occurring during the dispense step.

Table 1
Efficiency of protein precipitation for each prototype filtration block

Plate	2 vols	3 vols	4 vols
Porvair	97.8	99.0	99.1
Whatman	98.7	99.1	99.2
Whatman (mixed)	98.7	99.1	99.1
3M	98.7	99.0	99.1

The order of the addition of reagents was similarly investigated by determining the efficiency of protein precipitation. All three blocks were evaluated using three different methods of addition. In the first method, 'P then A', the sample is transferred to the block and the acetonitrile added as a second step. In the second method, 'P+A', the sample and the acetonitrile were sequentially aspirated separated by an air gap and dispensed together into the block. In the third method, 'A+P', the acetonitrile is aspirated before the sample, again separated by an air gap and dispensed together into the block. The results for all three blocks are presented in Fig. 3.

The data show that for both the Porvair and 3M filtration blocks, that the 'A+P' was the best order for reagent addition. No data were obtained for the Whatman filtration block using the 'A+P' order of reagent addition as all the wells blocked. Indeed it was very difficult to pull the supernatant through the block regardless of the order of the addition of the reagents. As discussed earlier, the bottom frit of this block has the lowest porosity of all three prototypes and it might be beneficial to load the samples with

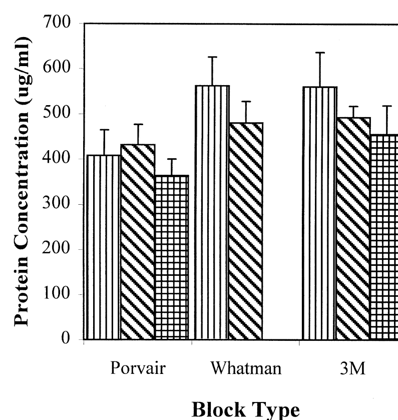

Fig. 3. Optimisation of reagent addition order. The effect of different methods of reagent addition on the protein concentration (μg/ml) of the extracts is shown. The error bar represents the standard deviation (n=8). The sample is transferred to the block and the acetonitrile is added as a separate step. The sample and then the acetonitrile are sequentially aspirated separated by an air gap and dispensed together into the block. The acetonitrile and then the plasma sample are sequentially aspirated separated by an air gap and dispensed together into the block.

the vacuum continuously on. The data show that, for the 3M block, both sequential methods for reagent addition were significantly better than the two step method. For the Porvair block, 'A+P' was the best order for reagent addition, although statistically there was no difference from the two step method.

3.3. Validation of procedure

In order to evaluate the performance of these prototype filtration blocks in more detail salbutamol was used as a test compound. Salbutamol, [2-(*tert*.-butylamino)-1-(4-hydroxy-3-hydroxymethylphenyl) ethanol], also known as albuterol, is an effective β_2-adrenoreceptor agonist which is used for the relief of bronchospasm associated with reversible obstructive airways disease. This is a relatively polar basic compound with a calculated log *P* value of 0.11 and a low level of protein binding [8], the chemical structure is included in Fig. 4. The blocks were used to extract rabbit plasma standards and controls using the optimised volumes and order of reagent addition. The extracts were analysed by a modified LC–MS-MS method for the determination of salbutamol in human plasma [6]. The intra-assay precision and accuracy of each of the blocks were assessed by analysing 100 μl validation control (VC) samples in replicates of six at five different concentrations. The results obtained are shown in Table 2. While all three blocks show reasonable accuracy and precision, only the Porvair block would meet our acceptance criteria of less than 15% at all VC concentrations. Both the Whatman and the 3M blocks show unacceptable precision at the lowest VC of 1 ng/ml. Table 2 also shows the intra-assay accuracy and precision data obtained for a standard method of protein precipitation for salbutamol using tubes and centrifugation. The data obtained is very similar to that obtained using the Porvair block. The SRM chromatograms for a blank and a 1 ng/ml salbutamol standard are presented in Fig. 4, and show that the method is specific and sensitive.

A full validation consisting of four runs was then carried out using the Porvair block. The inter-assay precision and accuracy data are presented in Table 3. Both the intra- and inter-accuracy and precision would meet our acceptance criteria of less than 15% for all VC concentrations. Although not as sensitive as the SPE method which has an LLOQ of 10 pg/ml based on 500 μl of human plasma [6], this protein precipitation method would be more than adequate to support early discovery work. The recovery was estimated by comparing the peak areas obtained for six replicates of the 50 ng/ml VC sample with six replicates of blank plasma that were spiked after protein precipitation at an equivalent of 50 ng/ml. The recovery was calculated to be 80% which compares favourably with the 80–90% obtained by SPE [6].

An important consideration in the choice of the sample preparation technique for LC–MS-MS is the amount of ion suppression [9]. The supernatants from blank plasma processed using each of the protein precipitation blocks and by standard method of protein precipitation were spiked with salbutamol, dried down and reconstituted in mobile phase (20:80 acetonitrile:0.1% formic acid) prior to injection onto the LC–MS-MS system running isocratically. The responses for the spiked blanks were compared with an equivalent spiked aqueous solution to show the relative level of ion suppression. The response obtained for duplicate injections for each protein filter block and the standard tube method is shown in Fig. 5. The data shows that there are no differences in the level of ion suppression obtained using either the filter blocks or the standard protein precipitation method.

3.4. Further automation

The filtration procedure is relatively simple and fast, it takes just over 20 min to process a complete block of 96 samples on the RSP and a further 20–30 min to evaporate the supernatants to dryness using a heated 96-well dry down station. The potential to process two to three blocks an hour makes protein precipitation by filtration a very attractive sample preparation technique for high throughput bioanalysis. As with SPE in the 96-well format this potential is only fully realised if the process can be fully automated and we have previously reported a custom built Zymark system for automated 96-well SPE [4]. These systems consist of a Zymate XP robot, a cooled storage carousel that acts as both a warehouse for all the labware (SPE blocks and collection blocks) and as a refrigerated storage for

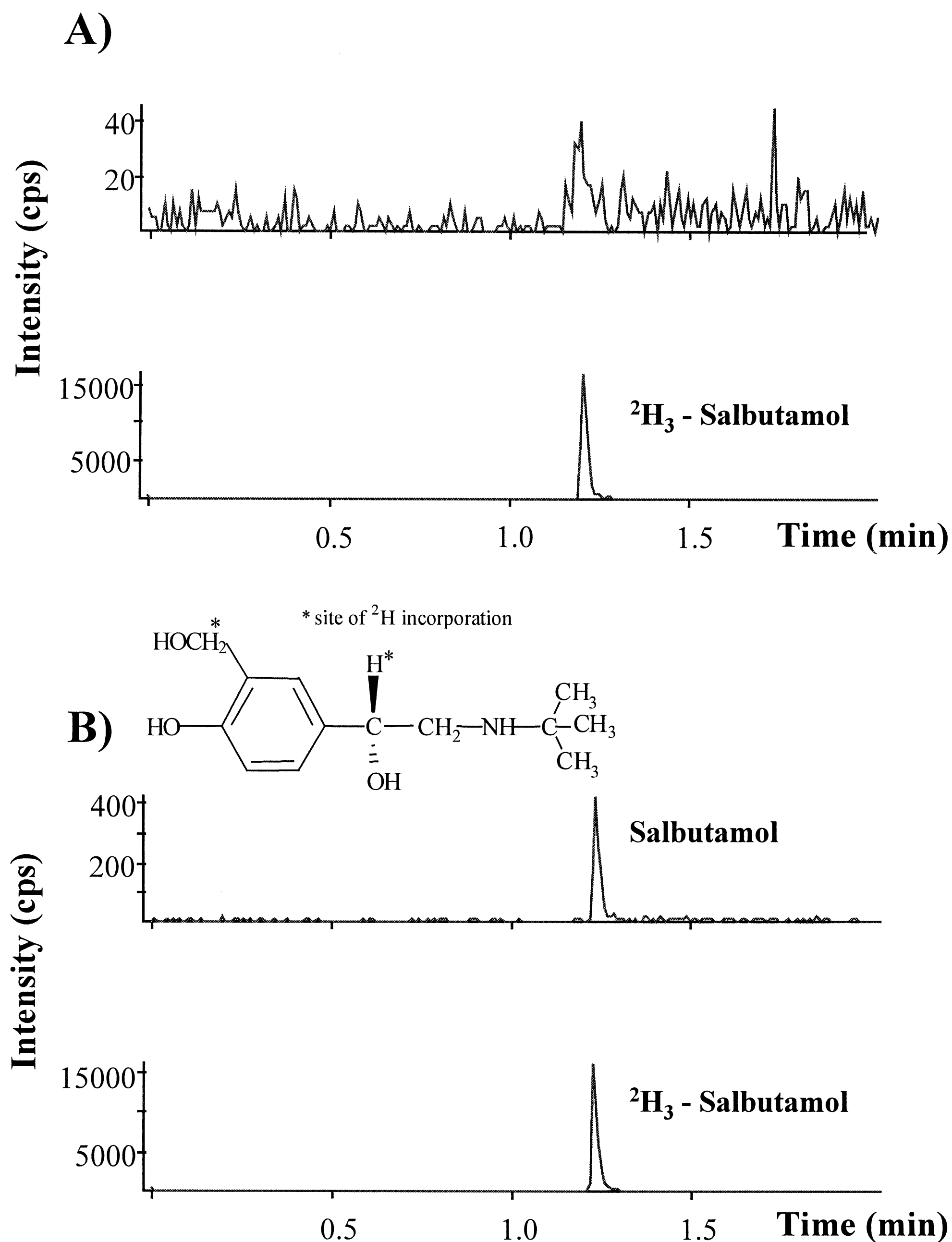

Fig. 4. LC–MS-MS SRM chromatograms for (A) blank and (B) 1 ng/ml calibration standard.

the sample extracts, an SPE station, and an RSP. The SPE station is a modified reagent addition station which incorporates a two stage vacuum manifold. Reagents are added a row at a time and a switching valve allows up to 12 different reagents to be used on the system. The SPE station performs all the vacuum, conditioning, washing and eluting steps and the RSP is used to dilute samples, add internal

Table 2
Intra-assay accuracy and precision data for salbutamol for each prototype filtration block and classical centrifugation ($n=6$)

Validation control	VC1	VC2	VC3	VC4	VC5
Nominal concentration (ng/ml)	100	50	20	3	1
Porvair					
Accuracy (% bias)	−2.1	0.1	−1.0	−9.4	1.4
Intra-assay precision (% C.V.)	5.5	4.0	3.7	13.4	8.4
Whatman					
Accuracy (% bias)	−2.0	4.3	0.7	−8.8	−4.9
Intra-assay precision (% C.V.)	8.3	6.8	9.5	9.9	22.0
3M					
Accuracy (% bias)	−10.0	−9.6	−12.2	−10.1	18.3
Intra-assay precision (% C.V.)	8.6	12.0	11.9	10.8	22.1
Tube					
Accuracy (% bias)	6.1	−0.1	0.6	−3.6	−11.0
Intra-assay precision (% C.V.)	6.8	4.1	4.4	11.6	11.1

standard and transfer samples from tubes to the SPE block. These systems are capable of processing a complete block of 96 samples in around 30–40 min, depending on the reagent volumes and number of steps, and of processing up to 384 samples from tubes in a single batch. More recently, a heated 96-well dry down station, a plate vortex and a plate sealer have been added to these systems [10].

It was hoped that the Zymark SPE system could be used to fully automate the protein precipitation procedure. Unfortunately, the preferred option to add the sample and acetonitrile simultaneously prevents the use of the SPE station for reagent addition and vacuum. However, by modifying the top section of the acrylic vacuum manifold so that it can be lifted by the robot hand, it is possible for the XP robot to build the manifold on the deck of the RSP. This makes it possible for both the collection and the filtration block to be changed automatically offering the potential of multiple unattended runs. These modifications allow our robot systems to offer both protein precipitation and SPE as fully automated procedures.

4. Conclusions

Protein precipitation by filtration in the 96-well format has been shown to be a viable alternative to conventional centrifugation. A 3:1 ratio of acetonitrile to plasma, sequential aspiration of the sample followed by acetonitrile and simultaneous dispensing have been found to be optimal when automating the protein precipitation procedure using a robotic sample processor. The use of 96-well filtration blocks eliminates the need for centrifugation and the attendant manual handling of individual tubes and vials. This substantially reduces the time taken to extract

Table 3
Inter- and intra-assay accuracy and precision data for salbutamol for the protein microlute filtration block

Validation control	VC1	VC2	VC3	VC4	VC5
Nominal conc. (ng/ml)	100	50	20	3	1
Number of determinations	24	24	23	23	24
Accuracy (% bias)	3.4	2.0	0.7	−6.2	−5.6
Intra-assay precision (% C.V.)	8.5	9.9	9.5	8.9	12.2
Inter-assay precision (% C.V.)	2.0	2.0	1.7	Negligible	5.1
Overall precision (% C.V.)	8.7	10.1	9.6	8.9	13.3

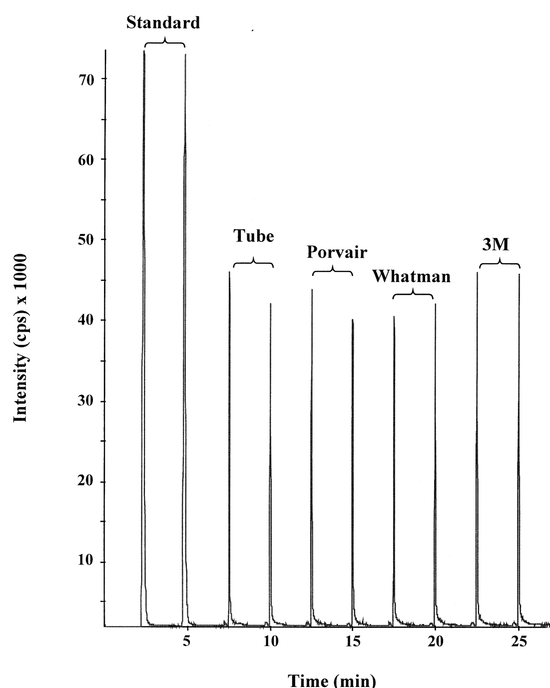

Fig. 5. Ion suppression data comparing the protein precipitation blocks with classical protein precipitation.

plasma samples by protein precipitation with a block of 96 samples taking just 20 min. As has been found with solid-phase extraction there is also an advantage in consumable costs in moving to the 96-well format. A method for the determination of salbutamol in rabbit plasma using protein precipitation by filtration has been developed and was used to compare three prototype filtration blocks. By making a simple modification to the acrylic vacuum manifold it has been possible to fully automate the protein precipitation procedure using the Zymark SPE robotics system in our laboratory. This will enable these systems to provide both sample preparation techniques and extend their applicability, for example to support early drug discovery. Work is on-going to evaluate the technique for a wide variety of drug molecules, including highly protein bound drugs, with a more detailed comparison with conventional protein precipitation using centrifugation.

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