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Short communication

Comparison of manual protein precipitation (PPT) versus a new small volume PPT 96-well filter plate to decrease sample preparation time

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1. Introduction

The advent of large combinatorial libraries and high-throughput screening has greatly increased the number of compounds flowing through the drug discovery pipeline [1]. To support the fast pace of drug research, liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) has become the bioanalytical technique of choice due to its sensitivity, selectivity and speed. Sample preparation in turn is often viewed as the rate-limiting step in bioanalysis. Sample preparation approaches to address this issue include automated solid-phase extraction in a 96-well plate format [2,3] and direct injection of plasma onto a small diameter, large particle size LC column followed by the removal of plasma constituents using a largely aqueous mobile phase under turbulent flow conditions [4,5].

Recently, a 96-well small volume protein precipitation (PPT) filter plate (3M Empore™) was introduced to remove precipitated proteins using a simple three-step process, thereby eliminating the time consuming centrifugation steps associated with manual protein precipitation. To evaluate the utility of the PPT filter plate, several structurally unrelated compounds (natural product, alcohol, acid and amines) were examined using the PPT filter plate and traditional centrifugation following protein precipitation. The results from this investigation are presented below.

2. Experimental

2.1. Reagents and chemicals

A variety of proprietary substances from the Novartis Institute for Biomedical Research in-

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cluding two amines, an acid, an alcohol and a natural product were investigated. HPLC grade acetonitrile and methanol were purchased from Sigma–Aldrich (St. Louis, MO/Milwaukee, WI), ethanol (190 proof) was purchased from Quantum Chemical Corporation (Tuscola, IL) and acetic acid (HPLC grade) and formic acid (88%, certified A.C.S.) were both purchased from Fisher Scientific (Fair Lawn, NJ). In-house deionized water was further purified with a Milli-Q water purifying system purchased from Millipore Corporation (Bedford, MA). Ultra high purity nitrogen (99.99%) and ultra high purity argon were purchased from AGL Welding Supply Co., Inc. (Clifton, NJ). Control, drug free, heparinized rat plasma from fasted animals was obtain in-house or purchased from Harlen Biological Research (Indianapolis, IN).

2.2. Equipment

LC/MS/MS analyses were performed using a Hewlett Packard 1100 system (Agilent Technologies, Paramus, NJ) coupled to a Micromass Quattro LCZ (Micromass UK Limited, Manchester, UK). The mass spectrometer was operated using an electrospray atmospheric pressure ionization source in positive ion mode (ESI^+) with multi reaction monitoring (MRM). An ODS-AM, 100×2.0 mm, 5 μm analytical HPLC column from YMC Inc. (Wilmington, NC) was incorporated as the stationary phase. For the filtration technique, a 3M small volume PPT 96-well filter plate, which removes 98% of all particles ≥ 10 μm and 50% of all particles between 2 and 10 μm, was used (3M Empore, St. Paul, MN). For the centrifugation process, Eppendorf 1.5 ml micro centrifuge tubes were purchased through Fisher Scientific and centrifuged using a Biofuge A from Baxter Scientific Products (Heraeus Instruments, Germany). A Branson 3200 sonicator from Branson Ultrasonics, Corp. (Danbury, CT) was used.

2.3. Standard and quality control (QC) preparation

Stock and spiking solutions of all compounds were prepared in methanol. Calibration standards and QC's were prepared from the same set of stock and spiking solutions. An internal standard was prepared in the same manner as the spiking solutions. All solutions were stored at approximately -80°C when not in use.

The calibration curves for all compounds consisted of seven concentrations and were prepared in duplicate on a daily basis. The calibration standards were prepared by adding 10 μl of each respective spiking solution to 100 μl of control blank rat plasma. The standard curve had a dynamic range of 1.0–100 ng/ml for both centrifugation and filtration techniques. QC's were also prepared by spiking control blank rat plasma at three different concentrations (2, 10 and 50 ng/ml) and distributing them evenly throughout the analytical run.

2.4. Sample preparation

2.4.1. Protein precipitation and centrifugation

A 100-μl aliquot of control blank rat plasma was transferred to a 1.5 ml Eppendorf microcentrifuge tube for each calibration standard and QC. A 10-μl aliquot of the appropriate spiking solution and IS (internal standard) were then added to each sample followed by vortexing for approximately 5 s. Then, 500 μl of a precipitation mixture (90:10/v:v, acetonitrile:ethanol + 0.1% acetic acid) was added to each plasma sample and sonicated for 10 min. Each sample was then centrifuged at 12 000 RPM for 15 min. The supernatant was transferred to a 13×100 mm polypropylene tube and evaporated under nitrogen at 45°C. All samples were then reconstituted in 100 μl of mobile phase (5:95/v:v acetonitrile:water containing 0.1% formic acid). The reconstituted samples were then transferred to 0.6 ml Eppendorf microcentrifuge tubes and again centrifuged at 12 000 RPM for approximately 15 min to remove any reconstituted particulate matter. The samples were transferred to 300 μl polypropylene autosampler vials and 10 μl was injected on the HPLC column. The total sample preparation time for this technique was approximately 4–5 h for 96 samples.

2.4.2. Protein precipitation and filtration

A 100-μl aliquot of control blank rat plasma was transferred to a 96-deep well 2-ml plate for each calibration standard and QC. A 10-μl aliquot of the appropriate spiking solution and IS were added to each sample followed by vortexing for approximately 5 s. Then, 500 μl of a precipitation mixture (90:10/v:v, acetonitrile:ethanol + 0.1% acetic acid) was added to each plasma sample and sonicated for 10 min. The samples were transferred manually to a 96-well small volume PPT filter plate and pulled through using a vacuum manifold into a 96-well collection plate and evaporated under nitrogen at 45°C. All samples were reconstituted in 100 μl of mobile phase (5:95/v:v acetonitrile:water containing 0.1% formic acid) and 10 μl was injected on column. The total sample preparation time for this technique was approximately 1–1.5 h for a 96-well plate.

2.5. Chromatographic conditions

Separations were achieved using gradient elution. The mobile phase consisted of solvent A: water containing 0.1% formic acid (pH ≈ 2.7), and solvent B: acetonitrile containing 0.1% formic acid. A 3 min gradient from 5 to 95% B followed by a hold at 95% B for 2 min was executed at a flow rate of 0.3 ml/min. All compounds were eluted from the LC column in less than 5 min.

2.6. Mass spectrometer conditions

The parent $[M + H]^+$ to daughter ion transition was monitored with an inter channel delay of 0.03 s. The source capillary voltage was set at 3.5 kV, extractor was 5 V, RF lens was 0.35 V and the source block and desolvation temperatures were 150 and 350°C, respectively. The nebulizer and drying gas (both nitrogen) were set at ≈ 70 and ≈ 750 l/h, respectively. The analyzers were set accordingly; for quad 1, the low mass and high mass resolutions were both set at 14.0 with an ion energy of 0.5 V. The collision cell had the entrance lens set at 25 V and the exit lens set at 0.0 V. For quad 2, the low mass and high mass resolutions were both set at 12.5 with an ion energy of 1.0 V. The analytes tested in this study were low molecular weight compounds ranging from 304.3 to 620.7 amu.

Calibration curves ($y = mx + b$), represented by the plots of the peak area ratios (y) of the compounds to IS versus the concentrations (x) of the calibration samples, were generated using weighted ($1/x$) linear least-squares regression as the mathematical model. Specificity was demonstrated by the lack of interfering peaks in control rat plasma at the retention times of all compounds and the IS for both procedures. The limit of quantification was 1 ng/ml for each compound.

3. Results and discussion

Innovative techniques that reduce sample preparation time, while maintaining data quality, are essential to effective drug research. To assess the utility of the 96-well small volume PPT filter plate technology in combination with protein precipitation, a systematic comparison versus centrifugation was undertaken. Initially a 3-day validation was performed using an acidic compound. Reproducibility assessments following centrifugation or filtration indicated good accuracy (mean back-calculated accuracy 88.7–108 and 93.2–103%, respectively) and precision (%CV of 5.68–13.4 and 3.87–10.1, respectively) for both methods over a concentration range of 1–100 ng/ml (Table 1). Linearity of the calibration curves, as measured by r^2, was comparable for both sample preparation techniques. The precision of the calibration curve parameters were acceptable for both methods (slope %CV = 18.9 for centrifugation and 4.03 for filtration). The filtration results, however, appeared to be more reproducible as represented by a lower %CV. The relative recovery of QC's (Table 2) ranged from 88.4 to 104% for centrifugation and 101–108% for the filter plate. The inter-day precision of the QC's, measured as %CV, were acceptable and spanned 12.6–16.0% for centrifugation and 6.46–8.75% for the filtration method. Specificity was demonstrated for both methods by the absence of interfering peaks at the retention time for all compounds. The total ion current (TIC) chromatograms for blank and 2 ng/ml QC samples

Table 1
Reproducibility, linearity and precision of three calibration curves for an acidic compound in rat plasma using centrifugation or a 96-well PPT small volume filter plate

	Procedure	Nominal concentration (ng/ml)							Calibration curve parameters**	
		100	50	20	10	4	2	1	Slope	r^2
Mean ± S.D.*	Centrifugation	99.2 ± 11.6	50.6 ± 3.21	19.4 ± 1.10	10.7 ± 1.26	3.95 ± 0.39	1.77 ± 0.24	1.08 ± 0.09	0.0352 ± 0.0067	0.9850 ± 0.0183
% CV		11.7	6.35	5.68	11.8	9.85	13.4	8.41	18.9	1.86
Mean back-calculated accuracy %		99.2	101	96.9	107	98.7	88.7	108		
Mean ± S.D.*	Filtration	99.2 ± 3.84	50.4 ± 3.32	19.9 ± 1.20	10.3 ± 1.04	4.10 ± 0.33	2.04 ± 0.12	0.93 ± 0.06	0.0344 ± 0.0014	0.9967 ± 0.0044
% CV		3.87	4.59	6.03	10.1	7.97	5.79	6.42	4.03	0.44
Mean back-calculated accuracy %		99.2	101	99.5	103	103	102	93.2		

* $n = 6$.
** $n = 3$.

were comparable for both the PPT filter plate and centrifugation procedures as illustrated in Fig. 1.

To test the general utility of the filter plate method, four additional structurally unrelated compounds were examined. All compounds had similar results compared to the acidic compound (Table 3). The overall mean back-calculated accuracy was 73.8–114% for centrifugation and 91.0–113% for filtration. The centrifugation and filtration calibration curve slopes were similar for each compound while the r^2 values were marginally better for filtration. The relative recovery of QC's (Table 4) ranged from 75.8 to 107% and from 93.8 to 121% for centrifugation and filtration, respectively. There was lower recovery of the 2 ng/ml QC sample across all compounds following centrifugation. This finding was statistically significant (t-test, $P < 0.05$).

Table 2
Percent recovery of daily quality control samples for an acidic compound using centrifugation or a 96-well PPT small volume filter plate

	Procedure	Nominal concentration (ng/ml)		
		50	10	2
Mean ± S.D.* % recovery	Centrifugation	104 ± 13.1	96.8 ± 12.5	88.4 ± 14.1
% CV		12.6	12.9	16.0
Mean ± S.D.* % recovery	Filtration	101 ± 6.52	101 ± 7.37	108 ± 9.45
% CV		6.46	7.29	8.75

* $n = 6$.

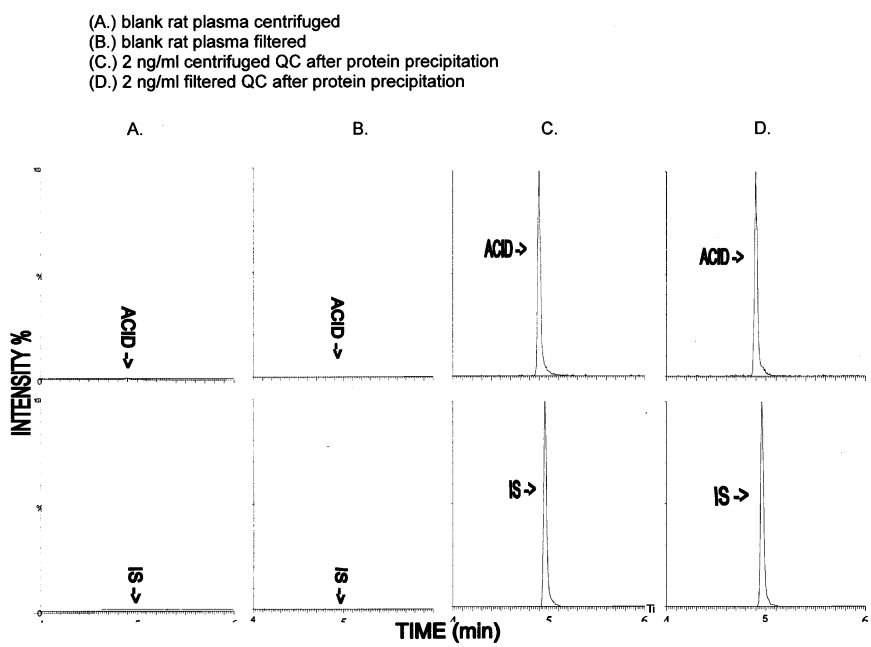

Fig. 1. Typical TIC chromatograms of a low molecular weight acid and internal standard in rat plasma extracts: (A) blank rat plasma centrifuged; (B) blank rat plasma filtered; (C) 2 ng/ml centrifuged QC after protein precipitation; (D) 2 ng/ml filtered QC after protein precipitation.

Table 3
Back-calculated mean* and calibration curve parameters for four structurally unrelated compounds in rat plasma using centrifugation or a 96-well PPT small volume filter plate

Compound	Procedure	Nominal concentration (ng/ml)							Slope	r^2
		100	50	20	10	4	2	1		
Natural product	Centrifugation	88.6	56.1	20.75	10.5	4.27	2.13	0.77	0.0053	0.9855
	Filtration	94.7	50.7	20.8	11.5	4.01	1.88	0.89	0.0044	0.9961
Amine	Centrifugation	85.4	53.7	20.5	12.4	3.56	1.41	ND[a]	0.0215	0.9873
	Filtration	86.6	50.5	20.9	11.7	3.93	1.66	0.98	0.0226	0.9954
Alcohol	Centrifugation	93.7	56.5	22.5	11.1	3.91	1.90	0.70	0.0023	0.9890
	Filtration	97.3	51.1	20.0	10.5	3.89	1.85	1.09	0.0022	0.9987
Amine	Centrifugation	88.6	51.4	23.5	11.7	4.48	1.73	0.73	0.0037	0.9787
	Filtration	95.7	49.8	21.0	11.7	3.76	2.04	0.70	0.0028	0.9958
Overall mean back-calculated accuracy %	Centrifugation	89.1	109	109	114	101	92.1	73.8	0.0082	0.9851
	Filtration	92.2	101	103	113	97.4	92.8	91.0	0.0080	0.9965

[a] ND, not determined, back calculated concentration not within $\pm 30\%$ criteria.
* $n = 2$.

Table 4
Percent recovery of daily quality control samples for four structurally unrelated compounds using centrifugation or a 96-well PPT small volume filter plate

Compound	Procedure	Mean % recovery*		
		50 ng/ml	10 ng/ml	2 ng/ml
Natural product	Centrifugation	88.4	90.6	87.5
	Filtration	97.8	113	108
Amine	Centrifugation	101	107	ND[a]
	Filtration	93.8	110	98.0
Alcohol	Centrifugation	100	104	75.8
	Filtration	100	98.7	96.3
Amine	Centrifugation	96.3	95.4	76.0
	Filtration	101	103	121

[a] ND, not determined, back calculated concentration not within $\pm 30\%$ criteria.
* $n = 2$.

4. Conclusions

Both centrifugation and filtration after protein precipitation provided comparable accuracy, precision, reproducibility and linearity results for several structurally unrelated compounds. Overall, the mean data appear to be more reproducible for the filtration method. This is most likely due to less human intervention when using the filter plates. The general utility and robustness of the 96-well PPT small volume filter plate method has been demonstrated. Most significantly, the sample preparation time (≈ 96 samples) was reduced approximately 4-fold (from 4–5 to 1–1.5 h) us

ing the filter plate compared to centrifugation. The 1–1.5 h sample processing time reported here for the manual 96-well PPT small volume filter plate procedure is comparable to the sample processing time reported for direct plasma injection followed by turbulent flow chromatography [4,5]. The sample processing time for the filter plate procedure may be further reduced as the procedure is adaptable to an SPE workstation.

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