The analysis of drug levels in biological fluids is an important part of pre-clinical and clinical trials. Sample preparation by protein precipitation is routinely used for the removal of proteins contained in biological fluids, usually plasma, prior to bioanalysis by LCMS/MS.

Protein precipitation is a generic technique applicable to a wide variety of drug types and is the method of choice when other methods e.g. Solid-Phase Extraction, seem too complex or time consuming to develop or perform. The development of mass spectrometry has enabled this technology to become a standard technique.

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PROTEIN PRECIPITATION

Traditional methods of protein precipitation are carried out in tubes or microplates. A precipitating agent, typically acetonitrile, is added to the vessel or plate, followed by the plasma or serum sample in a ratio of 3:1 respectively. After mixing by shaking or vortexing, the mixture is centrifuged and the supernatant, containing the drug, removed for analysis. This is a laborious method and when high sample numbers are processed, it is very time consuming and can be susceptible to human error. High throughput protein precipitation methods have recently focused on vacuum filtration methods. However, these have experienced various difficulties, the most important being the uncertainty of total filtration for every sample. Many users find this method needs constant supervision to ensure each well empties. There can be issues with wells blocking with plasma, and recovery has been reported to vary across a plate. In addition, the filtration plates used for protein precipitation are expensive and can range from £30 to £50 per plate.

With advances in liquid handling, centrifuges have been incorporated into the deck of such instruments enabling full automation of protein precipitation by the traditional centrifugation method. Not only is this method more reliable, but it can be automated using conventional plates, which are much less expensive.

This article describes one such fully automated system – The Flexus DMPK. This article compares the on-board Flexus centrifuge with a long-established, manual bench centrifuge. The data presented will show superior results with the Flexus centrifuge even with shorter spin times and at a slower speed.

The Flexus, with built-in centrifuge *(Figure 1)* is one model in the Anachem Flexus Automated Liquid Handling range. The flexible, modular and upgradable design of the Flexus units makes them the ideal choice for a wide range of applications.

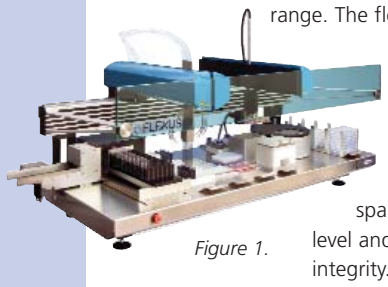

Figure 1.

The Flexus DMPK comes complete with 8 washable or disposable flexi-span probes enabled with both liquid level and clot detection to ensure assay integrity. A plate gripper on the second arm allows plates to be moved in and out of the centrifuge. An optional bar code reader is available for both sample tubes and plates to allow for sample tracking. A choice of bed size i.e. 1.0, 1.5 or 2.0 meter accommodates low to medium throughput processes. The high-speed centrifuge can pull up to 2000g and can be mounted within the deck or at the side of the unit *(Figure 2)*.

Pre-configured software routines allow users to adjust the sample number, sample volume and precipitant volume at the beginning of each run. Hundreds of samples can be processed per cycle.

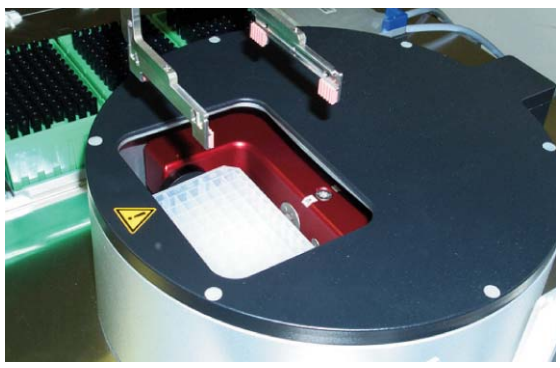

Figure 2.

MATERIALS AND METHODS

Samples were taken from either tubes or plates and prepared in deep well plates by adding 150 µl of acetonitrile, followed by 50 µl of plasma. The sample and precipitant were mixed and the plates then placed in the centrifuge for a defined period of time. 10 µl aliquots of the supernatant were injected onto an Applied Biosystems API 3000 MS/MS and a Waters UPLC system for analysis.

The Flexus centrifuge was compared to a bench-top plate centrifuge at various times at 3200g. Several compounds were investigated in this study comprising a mixture of Neutral, Polar and Non Polar. The Flexus centrifuge was run at three different time periods i.e. 5 minutes, 10 minutes and 15 minutes, compared to a single time point of 10 minutes for the bench centrifuge. Aspects investigated included: average peak height; average background height; and average signal to noise ratio.

RESULTS AND DISCUSSION

Table 1 shows a comparison of each criterion for compounds 1 and 2. For compound 1, a 5-minute spin in the Flexus centrifuge was not sufficient to achieve an average peak height as obtained with a 10-minute spin in the bench centrifuge. However, a 10-minute and greater spin produced a much superior average peak height in the Flexus centrifuge. For compound 2, superior results were found with only a 5-minute centrifugation time in Flexus.

Table 1.

Compound 1				
Centrifuge	Current	Ixion		
Length of spin time (mins)	10	5	10	15
Average peak height	3375	3166	3450	3500
Average background height	700	500	400	350
Average signal to noise ratio	4.8	6.3	8.6	10.0
Compound 2				
Centrifuge	Current	Ixion		
Length of spin time (mins)	10	5	10	15
Average peak height	1783	2220	2533	3483
Average background height	47	40	40	38
Average signal to noise ratio	42.8	55.5	63.3	90.9

The average background noise and average signal-to-noise ratios for both compounds, were found to be improved with only a 5-minute Flexus centrifuge over the 10-minute bench top centrifugation.

Protein precipitation is known to be a quantitative technique to determine drug levels in plasma. This has been demonstrated by Covance, a Contract Research Organisation in England, using Glibenclamide, a drug used in the treatment of diabetes. Figure 3 shows the standard curve, generated on the Flexus DMPK, measuring levels of Glibenclamide from 1 - 400ng/ml of human plasma. Samples processed by the Flexus DMPK showed low background matrix levels enabling drug detection at these low levels.

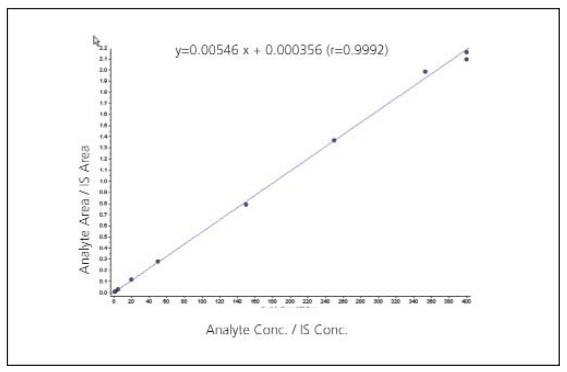

Figure 3.

CONCLUSION

As the numbers of samples requiring precipitation of proteins increase and the technique grows in popularity, the method ideally should be automated. The problems inherent in the vacuum method for protein precipitation can be overcome using the traditional centrifugation method. The Flexus with its on-board centrifuge is the ideal solution, not only to automatically process large numbers of samples, but also to provide cleaner samples. Having cleaner samples for LCMS/ MS analysis will give clearer results and assist in prolonging column lifetime. A longer column life and the elimination of an expensive vacuum plate also create valuable cost savings.

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