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ORIGINAL ARTICLE

Comparison of different serum sample extraction methods and their suitability for mass spectrometry analysis

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Abstract Mass spectrometry has been widely used, particularly in pharmacokinetic investigations and for therapeutic drug monitoring purposes. Like any other analytical method some difficulties exist in employing mass spectrometry, mainly when it is used to test biological samples, such as to detect drug candidates in mammalian serum, which is rich in proteins, lipids and other contents that may interfere with the investigational drug. The complexity of the serum proteome presents challenges for efficient sample preparation and adequate sensitivity for mass spectrometry analysis of drugs. Enrichment procedures prior to the drug analysis are often needed and as a result, the study of serum or plasma components usually demands either methods of purification or depletion of one or more. Selection of the best combination of sample introduction method is a crucial determinant of the sensitivity and accuracy of mass spectrometry. The aim of this study was to determine the highest serum protein precipitation activity of five commonly used sample preparation methods and test their suitability for mass spectrometry. We spiked three small molecules into rabbit serum

Abbreviations: PP, protein precipitation; MS, mass spectrometry; LC, liquid chromatography

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and applied different protein precipitation methods to determine their precipitation activity and applicability as a mass spectrometry introductory tool.

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1. Introduction

Plasma is frequently used as a biological matrix as it is easy to collect (Olsen et al., 2004; Sjoholm et al., 1979). Typically, it is widely used in studies of analytical method development and validation, just prior to the animal trials. Indeed, appropriate sample preparation is essential for obtaining reliable and meaningful results. Consequently, sample preparation is still an area of high importance when a liquid chromatography and mass spectrometry (LC/MS/MS) method is developed to assay biological samples (Xu et al., 2005). It is predominantly used in the '*optimisation*' of a sample for analysis with mass spectrometry (MS) techniques. The importance of sample preparation is to ensure that the analytical method maintains certain essential elements of robustness and consistency that are expected in any bioanalytical assay (Xu et al., 2005).

Generally, the two main sample preparation methods used for the MS analysis of blood, serum plasma and urine samples are liquid–liquid extraction or solid-phase extraction (SPE) (Bouzas et al., 2009). However, for drug discovery and pharmacokinetics, protein precipitation (PP)/extraction is the most common sample preparation procedure, which is the simplest approach that requires minimal method development and removes the majority of the protein from the sample (Xu et al., 2005). PP with miscible organic solvents (usually acetonitrile or methanol) is the most commonly used sample preparation method because of its low cost and minimal method development requirements (Ma et al., 2008). While, there are many PP solvents that are widely used including organic and inorganic solvents (Bouzas et al., 2009; Lawson, 1989), the selection predominantly depends on the investigational compound used. Usually, the use of methanol is especially valuable for support of preclinical pharmacokinetic studies conducted during the lead optimisation stages of drug discovery, where rapid development of assays for new compounds is essential (Henry et al., 2013; Ma et al., 2008). In an attempt to investigate the suitability of each of the solvents used for MS analysis of small molecules in pharmacokinetics studies, we performed PP using five different solvent systems and compared their ability to precipitate serum proteins and extract potential drug molecules for MS analysis.

2. Materials and methods

2.1. Serum samples

Blood was collected from a healthy rabbit housed at the Small Animal Facility of the CSIRO Australian Animal Health Laboratory. Serum was obtained by allowing the blood to clot at room temperature for 2 h. The clotted blood was then centrifuged for 10 min at 12,000*g*. Serum was then collected and stored at −20 °C.

2.2. Confirmation of compounds identity and purity using MS

Three potential antiviral compounds of small molecular weight (pending patent) were selected for this study and given different codes (AAHL 13, AAHL 18 and AAHL 42). The compounds were initially dissolved in methanol at a concentration of 0.5 mg/ml, then diluted in 50% methanol/0.2% formic acid to a final concentration of 10 μg/ml. Diluted samples were analysed by direct infusion at a rate of 10 μl/min into the electrospray ionisation source of an LCQ ion-trap mass spectrometer (Thermo, San Jose, CA, USA). Spectra were acquired and averaged over 50 consecutive scans. Full scans were acquired over the mass range m/z 50–500 to give an indication of sample purity. High resolution zoom scans were also performed that allowed determination of the mass/charge state of the selected ion and hence an accurate mass measurement of the selected ion.

2.3. Detection of compounds in rabbit serum

Rabbit serum was spiked with three investigational compounds (AAHL 13, AAHL 18 or AAHL 42) at a concentration of 0.5 mg/ml. The spiked serum then underwent protein precipitation using the described methods. The supernatants from each treatment were collected and diluted 1:1 with 0.4% v/v formic acid to give a final solvent composition of 50% methanol/0.2% formic acid and analysed by MS.

2.4. Methanol extraction method

Briefly, 100 μl of serum was mixed with 900 μl of HPLC-grade methanol. Following centrifugation, aliquots of 100 μl of the supernatants were dried and then resuspended in electrophoresis sample buffer (MES) and analysed by electrophoresis, or aliquots were diluted in 50% methanol/0.2% formic acid for MS analysis.

2.5. Folch extraction method

A mixture of chloroform–methanol in the ratio of 2:1 by volume was prepared and 400 μl of this mixture was added to a 100 μl of serum. The upper phase of each sample was used for analysis, because the proteins were precipitated in the middle and lower phases.

2.6. Acetone extraction method

Briefly, 900 μl of acetone was added to 100 μl of serum. The supernatant only was used for analysis. For electrophoresis, samples were dried and then resuspended in sample buffer (MES) and for MS analysis samples were diluted in 50% methanol/0.2% formic acid.

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