GASP and FASP are Complementary for LC–MS/MS Proteomic Analysis of Drug-Metabolizing Enzymes and Transporters in Pig Liver

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Sample preparation is a critical step in the proteomic workflow. Numerous different approaches are used, tailored to the type of sample, the aims of the experiment, analytical method, and to an extent, user preference. This has resulted in large variation in reported protein abundances. In this study, the complementarity of two different sample preparation techniques is demonstrated for the study of absorption, distribution, metabolism, and excretion (ADME) related proteins from pig liver tissue. Filter-aided sample preparation (FASP) is a well-established and widely used method, while gel-aided sample preparation (GASP) is a relatively new method optimized and simplified from previous gel-associated digestion techniques. To investigate each method, the number of peptides and proteins characterized, reproducibility of results, and their real-time application are examined. While both methods have their merits and limitations, for example, FASP is the less technical of the two methods, while GASP is time efficient, ultimately the two methods show significant differences in the peptides identified and therefore, the use of both methods should be considered when examining and quantifying ADME related proteins. Data are available via ProteomeXchange with identifier PXD011324.

1. Introduction

Shotgun proteomics is set to transform clinical practice, notably through drug dosage adjustments for populations deviating from the ‘average’ patient model commonly used by the pharmaceutical industry.[1] Advances in LC–MS/MS hardware and accompanying software have led to low abundance proteins in complex mixtures,[2] including drug-metabolizing enzymes and transporters within tissue samples, being detectable and quantifiable,[3,4] providing a basis for in silico pharmacokinetic modeling and simulation in under-represented populations.[1] However, these models are data hungry and, despite numerous proteomic studies, there is no consensus as to the proteomic workflow[3] resulting in large variations in the reported protein abundances.[5] Several independent groups have highlighted the need for consensus methodology.[6–8]

Here, we compare filter-aided sample preparation (FASP)[9] and gel-aided sample preparation (GASP)[10] in LC–MS/MS-based analysis of drug absorption, distribution, metabolism, and excretion (ADME) proteins from pig liver microsomes. The main classes of proteins under study were membrane-associated cytochrome P450 (CYP) enzymes, and membrane-embedded UDP-glucuronosyltransferase (UGT) enzymes and transporters. Their quantification is especially susceptible to variations in sample preparation but is essential for building models to translate observations in animals and in vitro systems into clinical situations. Uncertainties in these models are related to existing gaps in system parameters, such as protein abundance, and the translatability (scaling) of their use.[5]

FASP is popular because of its relative simplicity and ability to characterize more of the proteome when compared to its in-solution predecessors.[11–13] It has been adapted for different purposes by varying concentrations and types of solubilizing agents, centrifugation times, and filter sizes.[14] Unfortunately, protein loss on the filter and occasional filter failure can still affect reproducibility of results.[15] The GASP method, optimized from previous in-gel and tube-gel digestion techniques,[16] was demonstrated by Fischer and Kessler[10] in a preliminary study to be similarly effective. We now aimed to determine whether the GASP method was a serious rival to FASP for the quantification of mammalian ADME proteins. Of interest were i) number of peptides and proteins characterized by each method, ii) reproducibility of the methods, iii) technical difficulty and
2. Methods

Figure 1 describes FASP and GASP method workflows. Materials were purchased from Sigma–Aldrich (Poole, UK) unless otherwise indicated. Our in-house FASP method utilizes sodium deoxycholate (SDC) in place of SDS to reduce interference with downstream LC–MS analysis.\[11\] To ensure a direct comparison between the two methods, this step was kept the same for GASP. Two biological replicates, Pig Liver 1 (PL1) and Pig Liver 2 (PL2), were processed in triplicate, making a total of six samples in each arm of the study. Microsomal fractions were prepared as previously described\[17\] with amendments to the initial homogenisation step. One gram of liver tissue from two pigs (Axons Butchers, Manchester, UK) was homogenized using a digital handheld homogenizer TT-30K (Cambio Ltd., Cambridge, UK) in homogenization buffer (1 mM EDTA, 10 mM HEPES, 150 mM KCl, 1 mM DTT, 0.2 mM Pefabloc, pH 7.4), at 10 mL buffer per gram of tissue, containing 1 cOmplete mini protease inhibitor cocktail tablet (Roche Applied Sciences, Mannheim, Germany). The microsomal fraction was extracted using differential centrifugation and stored in storage buffer (0.25 M potassium phosphate, pH 7.25, 30% v/v glycerol) at −80 °C. Microsomal protein concentration was determined by BCA assay in triplicate.

Sample preparation was carried out in triplicate for each method (technical replicates) using 50 µg of microsomal protein. Solubilization and reduction of proteins was achieved using 5% SDC and 0.1 M DTT, followed by incubation for 30 min at 56 °C. The protocols for GASP and FASP are described in Figure 1. Sequential enzymatic digestion using Lys-C (Wako, Japan) and Trypsin (Promega, Mannheim, Germany) has been shown to improve digestion performance.\[18,19\]

Extracted peptides were cleaned using Pierce C18 spin columns (Thermo Fischer Scientific, Rockford, IL) as per the manufacturer’s guidelines. Eluted peptides were analyzed by LC–MS/MS using an UltiMate 3000 Rapid Separation LC (Dionex Corporation, Sunnyvale, CA) coupled to an Orbitrap Elite (Thermo Fisher Scientific, Waltham, MA) mass spectrometer. Peptide mixtures were separated using a multistep gradient from 95% Buffer A (0.1% formic acid in water) and 5% Buffer B (0.1% formic acid in acetonitrile) to 7% B at 1 min, 22% B at 58 min, 30% B at 73 min and 60% B at 75 min at 300 nL min⁻¹, using a 75 mm x 250 µm id 1.7 µm CSH C18 analytical column (Waters). Peptides were selected for fragmentation automatically by data-dependant acquisition (see Supporting Information Method).

Raw data were processed using MaxQuant version 1.6.2.3 default parameters except where specified in Table 1, Supplementary Information. The resulting peptide files were examined in Excel and peptides with PEP scores < 0.01 were removed. To rationalize protein nomenclature, porcine enzymes and transporters were putatively identified using iterative Needleman–Wunsch time taken for each method, and iv) applicability to ADME proteins.
A) Total number of nonrepeating peptides identified for the pig proteome using FASP and GASP performed in triplicate. GASP consistently identified more peptides than FASP. This is primarily due to the increased number of miss cleaved peptides (lighter bars) that were introduced by design to increase protein sequence coverage. Minimal variation was witnessed between technical replicates, highlighted by the low SD of each data set (PL1 FASP 37, PL1 GASP 69, and PL2 GASP 45). However, one replicate of PL2 FASP characterised 600 less peptides, which resulted in a larger SD of 342.

B) Percentage identical peptide (PIP) highlights the similarity of peptide sequences between technical, biological, and methodological replicates. Three distinct analyses were carried out: GASP versus GASP, FASP versus FASP, and GASP versus FASP, reflecting two distinct groups. C) Principal coordinate analysis (PCoA) confirmed the existence of two clusters with the main source of variability being methodological (FASP vs GASP), followed by technical variability almost exclusively in FASP replicates, with little biological variability. Comparison of samples within the same method showed higher PIP when comparing peptide sequences between GASP and FASP. Therefore each method appears to characterize different types of peptides as illustrated by (D).

3. Results & Discussion

On average between 1750 and 1930 proteins were identified in each of the 12 samples with FASP identifying 60–180 more proteins than GASP. This performance was not mirrored when examining the total number of peptides for each method (Figure 2A). FASP identified an average of 7517 (PL1) and 6758 (PL2), with GASP identifying up to 11% more, 8120 (PL1) and 7612 (PL2). The GASP method produced a greater number of missed cleaved peptides (20–23%) compared to FASP (15–17%). This can be attributed to the nature of the GASP method; the use of acrylamide introduces an additional modification on lysine residues preventing the cleavage by trypsin at this site and characterizes a greater number of peptides unique to each protein thereby improving protein identification. Allowing for an additional missed cleavage (2) in GASP samples to account for the modification of lysine further increases the number of peptides characterized by at least 600 and should be considered for future analysis. Furthermore, over 50% of the miss cleaved peptides containing a modified lysine were identical across samples and more than 90% within technical replicates (Figure 1, Supporting Information) which, may at this stage, indicate further candidate peptides for protein quantification.

Figure 2A also highlights that one FASP replicate gave fewer peptides. A number of factors can influence the recovery of peptides and proteins during the proteomic workflow. In our laboratory, school children have successfully completed FASP and therefore in experienced hands the outlier is unlikely to be
Mineral variability was seen across technical replicates for all protein groups in each method. However, GASP identified more peptides for CYP, UGT, and transporters despite identifying similar numbers of proteins. B) Percentage identical peptide (PIP) analysis revealed similar results to the global proteome analysis; variability in identified peptides was mainly methodological, followed by technical variability in FASP replicates. The higher number of peptides per protein and high PIPs are promising for improving protein characterization and quantification, as this increases the protein sequence coverage and the opportunity to quantify different samples using the same peptides.

In terms of speed and technical difficulty, the FASP method is very straightforward but took 4 h to complete mainly because of long centrifugation steps. The GASP method includes several tricky aspiration steps, which are made easier with the use of gel loading pipette tips, though there is still a risk of losing gel pieces as well as the use of potentially hazardous acrylamide solutions. This method was completed in 2 h for the same number of samples.

4. Conclusion

In summary, our findings are consistent with the original study[10] in that similar numbers of proteins were identified by both methods and GASP identified more peptides overall. We add an unbiased assessment of the applicability of these peptides than FASP, although this did not translate into additional proteins, with both methods identifying an average of 11–12 CYPs, 4–5 UGTs, and 27–31 transporters. PIPs for CYPs, UGTs, and transporters (Figure 3B) reflected the global results. The higher number of peptides per protein and high PIPs are promising for improving protein characterization and quantification, as this increases the protein sequence coverage and the opportunity to quantify different samples using the same peptides.
methods to analysing clinically relevant proteins. The two methods tended to identify different peptides and their complementary use should be considered for the quantification of ADME proteins.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[25] partner repository with the dataset identifier PXD011324.

Conflict of Interest
The authors declare no conflict of interest.

Keywords
drug metabolism, FASP, GASP, global proteomics, pig liver

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