



Metabolic Profiling of Human Blood Plasma by Combined Ultra Performance Liquid Chromatography / Mass Spectrometry (UPLC/MS)



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Introduction

Metabolic profiling (metabolomics / metabonomics) is concerned with the analysis of low molecular weight compounds present in complex samples such as human bio-fluids^[1,2]. Measuring such compounds could provide deeper insights into mechanisms of disease and novel markers for diagnosis. Recent developments in analytical techniques such as UPLC/MS are proving to be powerful tools for metabolic profiling on complex samples such as plasma. Within the field of metabolic profiling, traditionally NMR spectroscopy has been the technique of choice, however, the sensitivity of NMR spectroscopy is relatively poor compared to MS methods, and potential biomarkers within samples may be at such low concentrations that they are simply undetectable.

Much of the biochemical analysis performed on plasma using LC/MS has focused on looking specifically at individual or specific groups of compounds^[3], or at drug metabolites from induced doses. Advances in LC/MS instrumentation has meant that some researchers have started to perform metabolic profiling type analysis using LC/MS on bio-fluids^[4]. However, so far there has been a relatively small amount of work published on sample treatment / preparation, compound identification, data handling, robustness and reproducibility of results. When trying to measure and model large numbers of metabolites in large numbers of complex samples it is vital to make sure the data obtained is accurate and consistent over the whole experimental run.

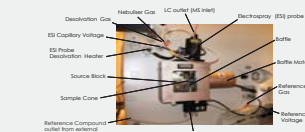
Instrumentation

1. Waters Acquity UPLC System



The UPLC system uses smaller column particle sizes compared to conventional HPLC (1.7 µm), and can handle back pressures up to 15,000 psi. These column particles and large back-pressures help achieve faster, more efficient separations at lower flow rates.

2. Waters - Micromass LCT Premier time-of-flight (ToF) Electrospray Mass Spectrometer



The LCT Premier system comprises of an electrospray source unit with accurate mass lock spray capability, enabling the system to correct for changes in m/z during an experimental run by continuous correction via a reference compound (leucine enkephalin).

Experimental

Fresh EDTA anti-coagulated blood was collected from 20 healthy volunteers (10 male and 10 female). Blood plasma was prepared by centrifuging at 14000 rpm for 10 minutes at 4 °C. Individual samples and sample pools were prepared and stored in 100 µl aliquots at -80 °C.

Frozen plasma's were thawed on ice. 400 µl of extraction mix (methanol solutions) were added to each plasma and vigorously extracted at a frequency of 30 Hz for 2 minutes. The samples were left for 2 hours on ice, after which they were centrifuged at 14000 rpm for 10 minutes at 4 °C. The supernatant (400 µl) was removed and placed in a 0.22 µm ultrafiltration tube to remove any undissolved particles. The tubes were centrifuged (10000 rpm for 2 minutes), and the resulting supernatant evaporated to dryness.

The samples were reconstituted in 50 µl of the corresponding solution of methanol to that of the original extraction mix (with the assumption that plasma = 100% aqueous). The resulting extracted plasma samples were then transferred to vials for UPLC/MS analysis. All plasma samples were injected in triplicate, in three successive batches, each batch having a different order of injection. Blank samples and a standard mix sample were also injected throughout the run.

The data was processed using peak detection and alignment followed by data reduction and compression methods previously reported using in-house software^[5]. The data reduction step was achieved by dividing the chromatographic dimension into a number of narrow, equally sized time windows (2 scans). Each time window held a three dimensional data structure defined by the sample, chromatographic, and mass spectral dimensions (figure 1).

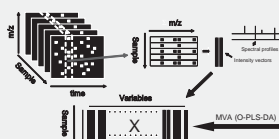


Figure 1. An overview of the pre-processing step

Summing of these three dimensional "data cubes" in the chromatographic dimension produced a two dimensional data table defined by the sample dimension and the summed spectral dimension. The data table for each sample was further compressed down to a small number of latent variables by means of alternating regression (AR).

By combining the "concentration profiles" created from the AR from all time windows (figure 1), a final "reduced" data table (X) was obtained, which was subject to further multivariate analysis (MVA). All data analysis was performed using Matlab software 7.0 (Mathworks, Natick, MA, USA) and Simca-P+ 11 (Umetrics AB, Umeå, Sweden). The orthogonal partial least squares discriminant analysis (O-PLS-DA)^[6] was performed using software provided by Dr Olivier Cloarec^[7].

Chromatography

Triplicate sample injection runs were performed using UPLC columns C8, Phenyl, and C18 (all 2.1 x 100 mm) with the following gradient: 1-20% of 0.1% formic acid in acetonitrile (ACN) (B) over 0-4 minutes (99-80% 0.1% formic acid in Aqueous (A)), 20-40% of B 4-6 minutes, 40-95% B 6-9 minutes, the composition was held at 95% B for 4.5 minutes, and returned to 1% B at 14.50 minutes, the composition was kept at 1% B for a further 4.5 minutes before the next injection. Flow rate = 0.5 ml/min, injection volume = 2 µl.

MS

Source temp = 120 °C, cone gas = 10 L/hr, desolvation temp = 300 °C, nebulization gas = 600 L/hr. The capillary voltage = 3 kV for positive ion mode, cone voltage = 0 V, scan time = 0.1 s, DRE mode activated.

Results & Discussion

Pooled plasma samples were extracted using different solutions of methanol (400 µl of 100%, 90%, 80%, 70% and 50% methanol added to 100 µl plasma). The dried extracted samples were re-constituted in the equivalent methanol solution (e.g. 100% added to plasma = 80% methanol), the small re-constitution volume (50 µl) was required in order to get the low level compounds above the detection limit. This however meant that only the 100% and 90% methanol extraction mixes managed to fully re-constitute the concentrated extract (figure 2). On inspection of the raw TIC's, and due to previous analogous work using GC/MS^[8], the 100% methanol was selected as the plasma extraction solution (80% overall).

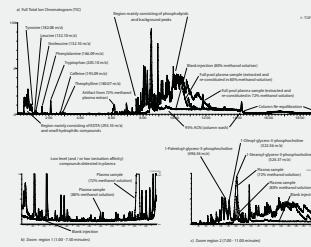


Figure 2. Raw total ion chromatogram (TIC) UPLC/MS spectra of blank sample injection (80% methanol solution), full pool extracted plasma with 80% methanol extraction solution, and full pool extracted plasma with 72% methanol extraction solution injected through a C18 (2.1 x 100 mm) UPLC column. Figure 1a) Full TIC, b) zoom region 1 (1.00 - 7.50 minutes) showing very low level small compound positive ions detected in the plasma, and c) zoom region 2 (7.00 - 11.00 minutes) consisting of phospholipids, larger, more lipophilic molecules in general and other peaks also detected in the blank injection (background peaks).

The three UPLC columns tested displayed similar patterns of separation with the plasma samples, showing elution of amino acids and small aromatic compounds early in the gradient, and phospholipid / larger lipophilic molecules eluting at high %B (ACN + 0.1% FA) in the mobile phase. The C18 column retained much lipophilic material that could not be completely washed off the column before the next injection, even with extended "ACN wash time" in the gradient (data not shown).

The long run times (3 x 20 sample injections + blank and standard mix injections) gave rise to "instrument drift". Changes in the sample profiles over time could be seen using PCA analysis (figure 3).

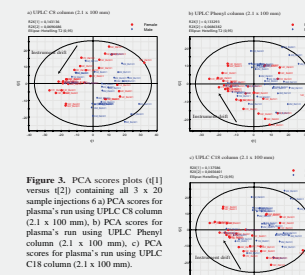


Figure 3. PCA scores plots (t1) versus (t2) containing all 3 x 20 sample injections 6 a) PCA scores for plasma's run using UPLC C8 column (2.1 x 100 mm), b) PCA scores for plasma's run using UPLC Phenyl column (2.1 x 100 mm), c) PCA scores for plasma's run using UPLC C18 column (2.1 x 100 mm).

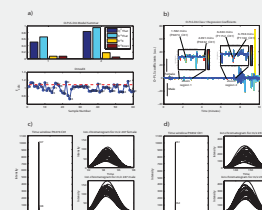


Figure 4. O-PLS-DA analysis of male and female plasma samples analysed by UPLC/MS using a UPLC C8 column (2.1 x 100 mm). Figure 4 a) O-PLS-DA model summary, b) O-PLS-DA regression coefficients, c) plot of time window P0274_C01 and resulting intensities of male and female plasma for 247 m/z, d) plot of time window P0432_C01 and resulting intensities of male and female plasma for 261 m/z.

The PCA analysis clearly shows the effects of "instrument drift" over the course of a study, therefore it is vital that samples are injected in a random order, therefore allowing us to see differences between sample groups without considering the normal changes experienced over time.

O-PLS-DA analysis was performed on the C8 column dataset (figure 4), allowing optimisation of the separation between male and female plasma, and also an accurate overview of the significant variables that separate the two sample classes. Tables 1 and 2 show the corresponding raw data for each sample injection of the ions found in the O-PLS-DA analysis in figure 4.

Table 1. Raw data summed ion counts between scans 266 and 279 (1.54 - 1.62 minutes) for positive ion 247.13 m/z (figure 4 c)

Sample	Intensity	Sample	Intensity	Sample	Intensity	Sample	Intensity
1	1000	11	1000	21	1000	31	1000
2	1000	12	1000	22	1000	32	1000
3	1000	13	1000	23	1000	33	1000
4	1000	14	1000	24	1000	34	1000
5	1000	15	1000	25	1000	35	1000
6	1000	16	1000	26	1000	36	1000
7	1000	17	1000	27	1000	37	1000
8	1000	18	1000	28	1000	38	1000
9	1000	19	1000	29	1000	39	1000
10	1000	20	1000	30	1000	40	1000

Table 2. Raw data summed ion counts between scans 423 and 441 (2.45 - 2.56 minutes) for positive ion 261.145 m/z (figure 4 d)

Sample	Intensity	Sample	Intensity	Sample	Intensity	Sample	Intensity
1	1000	11	1000	21	1000	31	1000
2	1000	12	1000	22	1000	32	1000
3	1000	13	1000	23	1000	33	1000
4	1000	14	1000	24	1000	34	1000
5	1000	15	1000	25	1000	35	1000
6	1000	16	1000	26	1000	36	1000
7	1000	17	1000	27	1000	37	1000
8	1000	18	1000	28	1000	38	1000
9	1000	19	1000	29	1000	39	1000
10	1000	20	1000	30	1000	40	1000

Conclusion

UPLC/MS can be used to perform metabolic profiling analysis on human plasma. Sample preparation is a vital part of the analysis that needs to be optimised, understood and performed consistently for results to be valid, here an 80% methanol solution was selected to perform protein precipitation on the plasma. The UPLC C8 column was selected as the best column out of the 3 tested for metabolic profiling analysis on plasma. The O-PLS-DA analysis is a powerful tool, allowing a view of the class distinctive variables, with a random sample injection order, the analysis is able to deal with the effects of "instrument drift" e.g. decreasing sensitivity that may occur during large experimental runs.

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