Optimization of Data Acquisition and Sample Preparation Methods for LC-MS Urine Metabolomic Analysis

Abstract: Nowadays, chromatographic methods coupled with mass spectrometry are the most commonly used tools in metabolomics studies. These methods are currently being developed and various techniques and strategies are proposed for the profiling analysis of biological samples. However, the most important thing used to maximize the number of entities in the recorded profiles is the optimization of sample preparation procedure and the data acquisition method. Therefore, ultra high performance liquid chromatography coupled with accurate quadrupole-time-of-flight (Q-TOF) mass spectrometry was used for the comparison of urine metabolomic profiles obtained by the use of various spectral data acquisition methods. The most often used method of registration of metabolomics data acquisition – TOF (MS) was compared with the fast polarity switching MS and auto MS/MS methods with the use of multivariate chemometric analysis (PCA). In all the cases both ionization mode (positive and negative) were studied and the number of the identified compounds was compared. Additionally, various urine sample preparation procedures were tested and it was found that the addition of organic solvents to the sample noticeably reduces the number of entities in the registered profiles. It was also noticed that the auto MS/MS method is the least efficient way to register metabolomic profiles.

Keywords: UHPLC, Q-TOF, MS/MS, fast polarity switching, metabolomics

1 Introduction

Metabolomics belongs to the so-called omics disciplines and, together with genomics, transcriptomics and proteomics is one of the basic tools used in the study of systems biology. This technique represents a holistic research strategy and complements the knowledge gained by other methods of information on low molecular weight molecules (< 1500 Da) found in the biological samples. Metabolomics primarily enables the capture of changes in the metabolic profile, which is mainly used to search for biomarkers of various disease processes especially in cancer research [1-3].

Nowadays, mass spectrometry (MS) coupled with chromatographic methods is one of the fastest developing and most commonly used techniques in metabolomics/metabonomics research. The use of modern hybrid and high resolution MS analyzers and advanced modes of spectral data acquisition as well as chemometric tools enables capture of a suitably specific metabolomic profile and identification of characteristic metabolites [3,4]. A commonly used strategy in the metabolomic analysis is the registration of the metabolic profiles in MS mode (preferably in high resolution – TOF), and next chemometric analysis and database searching, then performing additional analysis in targeted MS/MS mode in order to identify characteristic metabolites [5-7]. However, this procedure is time consuming and requires multiple repetitions of the analysis of the biological samples in MS and MS/MS mode as well as in different ionization modes (positive and negative). Therefore in currently reported metabolomics studies the more advanced data acquisition techniques such as fast/rapid polarity switching (FPS) or auto MS/MS mode are sometimes used [4,8,9]. In the case of FPS technique MS spectra are recorded in both polarizations at the same time, however MS/MS analysis should be finally performed separately. In auto MS/MS mode after MS scan MS/MS fragmentations are “on-line” performed for automatically selected precursor ions.
In this case there is no need to perform additional MS/MS analysis, however, the number of MS scans is lower than the other data acquisition modes. FPS and auto MS/MS data acquisition techniques are certainly more convenient and faster than the classical strategy of registration profiles, however, in such cases the lower selectivity of the MS method can be expected.

The aim of this study was to compare various spectral data acquisition methods available in the modern high resolution LC-MS equipment and to select the optimal strategy for urine metabolomic profiling. Additionally, various urine sample preparation procedures were also tested and all the obtained results were finally compared with the use of multivariate chemometric analysis.

2 Experimental

Gradient grade acetonitrile and methanol for LC-MS were purchased from Merck (Dramstadt, Germany). Formic acid for LC-MS was obtained from Fluka (Steinherin, Germany). Water for UHPLC-MS was purchased from Sigma-Aldrich (St. Louis, USA).

UHPLC-MS analysis was performed with the use of Agilent Accurate-Mass Q-TOF LC/MS G6520B system with dual electrospray (DESI) source and Infinity 1290 ultra-high-pressure liquid chromatography system consisting of: binary pomp G4220A, FC/ALS thermostat G1330B, autosampler G4226A, DAD detector G4212A, TCC G1316C module and Zorbax Eclipse-C18 (2.1 x 50 mm, dp = 1.8 μm) HD column (Agilent Technologies, Santa Clara, USA). A mixture of methanol (A) and water (B) with addition of 0.1% solution of formic acid in both media was used as a mobile phase. The gradient elution was carried out at constant flow 0.3 ml min⁻¹ from 5% A (95% B) 0 – 1 min and then 5% A to 90% A 1 – 9.5 min and next 90% A 9.5 – 10 min. 2 min post time was performed to return to initial condition. The injection volume was 1 μl or 3 μl for diluted samples (1:2 v/v). The column temperature was maintained at 35°C and the autosampler was kept at 4°C. Mass Hunter workstation software in version B.04.00 was used for the control of the system and data acquisition.

The optimization of the instrument conditions started with the proper tuning of Q-TOF detector in a positive and negative mode with the use of Agilent ESI-L tuning mix in extended dynamic range (2 GHz) and the following settings were applied: gas temperature 300°C, drying gas 10 l min⁻¹, nebulizer pressure 40 psig, capillary voltage 3000 V, fragmentor voltage 140 V, skimmer voltage 65 V, octopole 1 RF voltage 250 V.

Data acquisition was performed in centroids with the use of all the enabled modes: TOF (MS), fast polarity switching and auto MS/MS. Spectral parameters in the all cases were set as follows: mass range 70–1050 m/z, acquisition rate 1.2 spectra/s (for MS and MS/MS data). Collision energy (for auto MS/MS mode) was calculated with formula: 4 V (slope)*(m/z)/100 + 5 V (offset). Maximum 2 precursors per cycle were selected with an active exclusion mode after 1 spectra for 0.2 min, the threshold was set at 200.

To ensure accuracy in masses measurements, reference mass correction was used. Mass 112.985587 and 1033.988109 (negative ionization) and 121.050873 and 922.009798 (positive ionization) were used as lock masses.

Fresh human urine was used as a biological matrix for this study. Six simple sample preparation methods (with no extraction procedure) were tested: centrifugation at 14000 x g for 10 min. (method A), centrifugation at 14000 x g for 10 min and next the dilution of the supernatant with water at a volume ratio 1:2 (method B), centrifugation at 14000 x g for 10 min and next the dilution of the supernatant with methanol at a ratio 1:2 (method C), centrifugation at 14000 x g for 10 min and next the dilution of the supernatant with acetonitrile at a ratio 1:2 (method D), centrifugation at 200 000 x g for 10 min (method E) and centrifugation at 200 000 x g for 10 min and next the dilution of the supernatant with a mixture of methanol-acetonitrile (1:1, v/v) at a ratio 1:2 (method F). In all the cases the temperature of centrifuge was kept at 4°C. The samples were filtrated through a 0.22 μm nylon filters and then analyzed by LC-MS.

Molecular feature extraction algorithm (MFE) from the Mass Hunter Qualitative Analysis software version B.06.00 (Agilent) was used for data back-ground ion noise cleaning and to extract the list of the potential compounds. The MFE parameters were optimized and the following settings were applied: single charge state of the analyzed ions, more than 5000 counts for compound filter, isotope model: common organic molecules with peak spacing tolerance 0.0025 m/z.

In order to perform multivariate chemometric analysis the obtained results were next exported to the Mass Profiler Professional (MPP) software version 12.6 (Agilent and Strand Life Sciences Pvt. Ltd.). Using this software the data was normalized and aligned and next the principal component analysis (PCA) was performed.

Additionally, the accurate masses of the extracted features (by MFE) were searched by the use of the METLIN database [10] in order to acquire the quantitative comparison of the used spectral data acquisition methods.
3 Results and Discussion

UHPLC system coupled with Q-TOF accurate mass spectrometer was employed for the comparison of urine metabolomic profiles obtained by the use of various spectral data acquisition methods. All the chromatographic profiles were obtained in the reversed-phase ultra high performance liquid chromatography system with the use of broad range gradient elution in order to achieve retention for a wide range of compounds. The column used and methanol as modifier was assumed a priori, whereas the gradient profile was optimized against the number of separated peaks. The between-injection variability was satisfactory and the number of compounds did not differ visibly. For further comparison we have used example chromatograms. The chromatograms (TIC) obtained in TOF (MS) mode are shown in Fig. 1. Compared to HPLC system, UHPLC allowed the registration of specific profiles in a relatively short period of time (10 min), however, this methodology, characterized by short elution time and narrow peaks, requires a special optimization of Q-TOF method (acquisition rate, range, number of precursors per cycle for auto MS/MS method etc.).

As in mass spectrometry analysis the spectral information is most important, beside the optimization of MS analyzer, the selection of data acquisition method is crucial here. Therefore, first of all, it was decided to compare the most often used method of registration of spectral data – MS (TOF) mode with the FPS MS and auto MS/MS methods. In the case of the first method, all the analysis have to be carried out twice, one time in the negative polarity and one time in the positive polarity. This methodology often requires repeating the analysis often several times, especially when in a further step the targeted MS/MS analysis should be performed. In the FPS technique MS spectra are recorded in both polarizations at the same time, which allows a reduction in the number of repetitions of the analysis. However, in this case we also do not have information about MS/MS fragmentation. Therefore, it seems that the third method (auto MS/MS) is the most powerful technique. MS spectra is recorded in parallel to the MS/MS spectra, however, as in the first method (MS) the spectra are recorded only in one polarization. In order to get a simple comparison of the above Q-TOF data acquisition method, the registered profiles from the same urine sample were treated by MFE algorithm and the total number of generated compounds was summarized. Additionally, the accurate masses of the extracted features were searched with the use of the METLIN database (Table 1). It was observed, that regardless of the mode of ionization, the TOF mode is the most efficient way to register urine profiles. With the use of this method, the largest number of generated substances generated from the registered profiles, as well as the matched metabolites were obtained (Fig. 2). The advantage of the TOF mode over the other methods is beyond dispute, however, more false positive results can be expected here [11,12]. FPS and auto MS/MS modes are more adducible and faster than the MS mode, but the number of the potential metabolites identified by these methods is several times
lower, especially in the case of the auto MS/MS method. In this method it can be seen that most of the work time of the analyzer is dedicated to the fragmentation MS/MS and this drastically reduces the number of MS scans. Some important compounds were not found by the use of auto MS/MS. The most important examples to mention are: adenosine, allysine, caffeine, norleucine, uridine (positive ionisation) and ribulose, sebacic acid, kynurenic acid, methylxanthine (negative ionisation). Although for FPS method, additional compounds were found, comparing to auto MS/MS (norleucine, uridine, methyxanthine), the TOF method is significantly better for compound identification.

Additionally, in this study various urine sample preparation procedures were tested and the impact of this process on the registered profiles was evaluated similarly as in the case of spectral data acquisition methods (Table 1). It was observed that the use of ultracentrifugation (method E and F) did not improve the recorded urine profiles. Moreover, the addition of the organic solvents to the urine sample, which is quite often used, noticeably reduces the number of entities in the registered profiles. Only in the case of auto MS/MS/-/ more entities were registered comparing to the sample preparation method without organic solvent. In this situation, the easiest method without additional modification (method A) was found as the most effective method of the urine sample preparation for metabolomics studies.

In order to perform the multivariate chemometric analysis of all chromatographic profiles (n = 30) obtained by the use of various acquisition and sample preparation methods, all the profiles were aligned with MPP software and finally 776 entities were received. On this set of data the principal component analysis was performed and a visible categorization was observed. In the case of the comparison of sample preparation methods (Fig. 3) the methods without the addition of an organic solvent (A, B, E) are clearly grouped however, while method B (dilution with water) is at the periphery of the group. In this PCA analysis the first three components (PC1 – 3) explained 73.98% of the total variance. The comparison of the Q-TOF acquisition methods shows that the auto MS/MS mode

| Preparation Method | Data Acquisition Method | FPS /-/ | FPS /+/ | MS /-/ | MS /+/ | MS/MS /-/ | MS/MS /+/ |
|--------------------|-------------------------|---------|---------|--------|--------|-----------|-----------|
| A                  | 46 (33)                 | 71 (30) | 231 (71) | 286 (60) | 31 (23) | 29 (11)   |
| B                  | 39 (25)                 | 69 (27) | 233 (68) | 291 (68) | 29 (21) | 30 (13)   |
| C                  | 43 (28)                 | 54 (21) | 203 (64) | 233 (46) | 27 (18) | 28 (9)    |
| D                  | 36 (25)                 | 56 (20) | 206 (59) | 230 (39) | 34 (22) | 29 (8)    |
| E                  | 44 (30)                 | 70 (30) | 235 (74) | 281 (58) | 34 (25) | 28 (14)   |
| F                  | 44 (33)                 | 70 (21) | 219 (58) | 254 (38) | 41 (30) | 35 (10)   |

Figure 2: Comparison of the number of the generated compounds by MFE and matched by METLIN database obtained for sample preparation method A (sum for both polarizations).
Figure 3: Comparison of the sample preparation methods by PCA.

Figure 4: Comparison of the data acquisition methods by PCA (for FPS the summary data of both polarizations was used).
definitely stands out from the other modes, while the FPS mode is grouped between MS/MS and TOF modes (Fig. 4). In this case the first three components explained 96.35% of the total variance.

4 Conclusions

Based on the performed comparisons, it was found that the most effective spectral data acquisition method for urine metabolic profiling is TOF (MS) mode. Although, this procedure is time consuming and requires multiple repetitions of the analysis, the registered profiles are much more specific than the profiles obtained by the use of more advanced modes.

On the other hand, the least relevant strategy seems to be the auto MS/MS method, because too much information from the MS scans is lost and the number of potential metabolites drastically decreases.

The profiles registered in the fast polarity switching mode in quantitative terms show intermediate properties between TOF and auto MS/MS methods. However, the FPS technique seem to be useful for rapid profiling or the analysis of unstable biological samples.

It was found that the simplest urine preparation method, with no addition of solvents, was the most effective method for urine metabolomic profiling. The addition of organic solvents to the sample noticeably reduces the number of entities in the registered profiles.

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