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Identification of metabolites in human plasma using GC-Orbitrap-MS after online derivatization

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Keywords

Q Exactive GC Orbitrap GC-MS/MS, on-line sample preparation, metabolites, high resolution, Orbitrap technology, gas chromatography, high resolution metabolites library

Introduction

Gas chromatography–mass spectrometry (GC-MS) is an established analytical tool used successfully to determine the metabolic profile of biological samples.[1] It provides distinct advantages of chromatographic resolution, peak capacity, wide metabolite coverage and access to comprehensive spectral libraries.[2] However, this extensive metabolite coverage is only possible with a derivatization step to convert the polar/semi-polar metabolites into volatile analogues, thus making them more amenable to GC separation. Classic derivatization protocols involve alkylation and silyation of samples, with the latter preferred as it enables the derivatization of complex molecules such as sugars.[3] Silyation introduces a substituted silyl group (R_3Si) to target compounds by replacing the active hydrogen to generate a less polar and more volatile derivative. There are many published procedures for off-line derivatization, however, the trimethylsilyl (TMS) derivatives are unstable and a source of significant variability throughout an analytical batch. Much of this variation comes from the differing amount of time that samples experience from the time of derivatization to injection and analysis. A solution to this inherent problem is to have an automated on-line derivatization procedure so that samples are derivatized shortly before injection into the GC inlet.

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In this study, a high resolution accurate mass metabolomics library was used for the identification of metabolites in human plasma. Analysis was performed using a bench top, high resolution accurate mass Orbitrap GC-MS in combination with online sample derivatization. Compound identification was enabled by matching deconvoluted spectra against a high resolution accurate mass metabolomics library containing over 850 metabolites.[4]

Experimental

Sample preparation and derivatization

A single unspiked human plasma sample was used throughout the study. For sample preparation a volume of 20 µL plasma was extracted with 300 µL of pure methanol. The eight extracts were placed on a shaker for 10 min (1400 rpm) and then centrifuged for 5 min (10000 rpm). Following this, 200 µL of the supernatant was transferred into a GC vial with inserts and dried down under a constant stream of nitrogen (at 40 °C). To each dry vial 50 µL of 20 mg/mL methoxyamine-HCl solution in pyridine were added and vortexed for 15 seconds before heating the samples for 15 min at 80 °C. After cooling, the liquid was taken to dryness under nitrogen (40 °C). The dry samples were than capped and the following derivatisation step was performed on eight replicates. The on-line protocol allowed the samples to be prepared individually and immediately prior to injection. To each vial, 50 µL of MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide) was added followed by 30 seconds of vortexing. Silylation was performed by incubating the vials at 80 °C for 15 minutes with continuous shaking. After a 5 minute cool down to room temperature, 5 µL of trimipramine-d3 (10 µg/mL) were added to check for column/instrument performance. Finally, 1 µL of sample was injected into a hot injector with a 5:1 split ratio.

Instrumental analysis

In the experiments described, a Thermo Scientific™ Q Exactive™ GC Orbitrap™ GC-MS/MS system was coupled to a Thermo Scientific™ TRACE™ 1310 GC system, and a Thermo Scientific™ TraceGOLD™ TG-5SilMS 30 m × 0.25 mm I.D. × 0.25 µm film capillary column (P/N:26096-1420) was used for GC separation. On-line derivatization and injection of liquid samples was performed automatically using a Gerstel® MultiPurpose Sampler MPS (Gerstel, Muehlheim, Germany) operated using Gerstel® Maestro software. The Q Exactive GC Orbitrap mass spectrometer was tuned and calibrated in under one minute using PFTBA to achieve the best ion transmission and sub-ppm mass accuracy. The mass spectrometer was operated in full-scan using 60,000 mass resolution (measured as FWHM at *m/z* 200). Lockmass corrected data was processed with Thermo Scientific™ TraceFinder™ software. Additional details regarding the GC and MS conditions are given in Tables 1 and 2.

Table 1. GC and injector conditions.

TRACE 1310 GC System Parameters	
Injection volume (µL):	1
Liner:	Split
Inlet (°C):	250
Carrier gas, (mL/min):	He, 1.0
Split ratio:	5:1
Oven Temperature Program	
Temperature 1 (°C):	70
Hold time (min):	4
Temperature 2 (°C):	320
Rate (°C/min):	20
Hold time (min):	8

Table 2. Mass spectrometer conditions.

Q Exactive GC Orbitrap GC-MS/MS Parameters	
Transfer line (°C):	250
Ionization type:	EI
Ion source (°C):	230
Electron energy (eV):	70
Acquisition mode:	Full-scan
Mass range (Da):	50-650
Resolving power (FWHM at *m/z* 200):	60,000
Lockmass, column bleed (*m/z*):	207.03235

Data processing

Data was acquired using TraceFinder software. This single platform software allows instrument control, method development functionality, and qualitative and quantitation-focused workflows. TraceFinder software also contains spectral deconvolution and spectral matching functionality. The Thermo Scientific™ High Resolution Metabolomics Library, containing spectra for over 850 metabolites, was used for compound identification.

Results and Discussion

The initial step was to deconvolute the full-scan data and to then screen the resulting spectra against a high resolution metabolomics spectral library. The list of tentative hits returned for each peak were scored based on a combination of a classical search index (SI) score and high resolution filtering (HRF) value.[5,6] An example is shown in Figure 1 where pyroglutamic acid 2TMS is identified with a SI 903, HRF 99.4% and a combined score of 97.8%. The high resolution spectral library enables the confidence in matched compounds to be extremely high and the list of possible compounds much lower than if a nominal mass library was used. The high mass accuracy enables fast detections to be made with confidence. As Table 3 indicates the example metabolite base peaks were detected with sub 1 ppm mass accuracy.

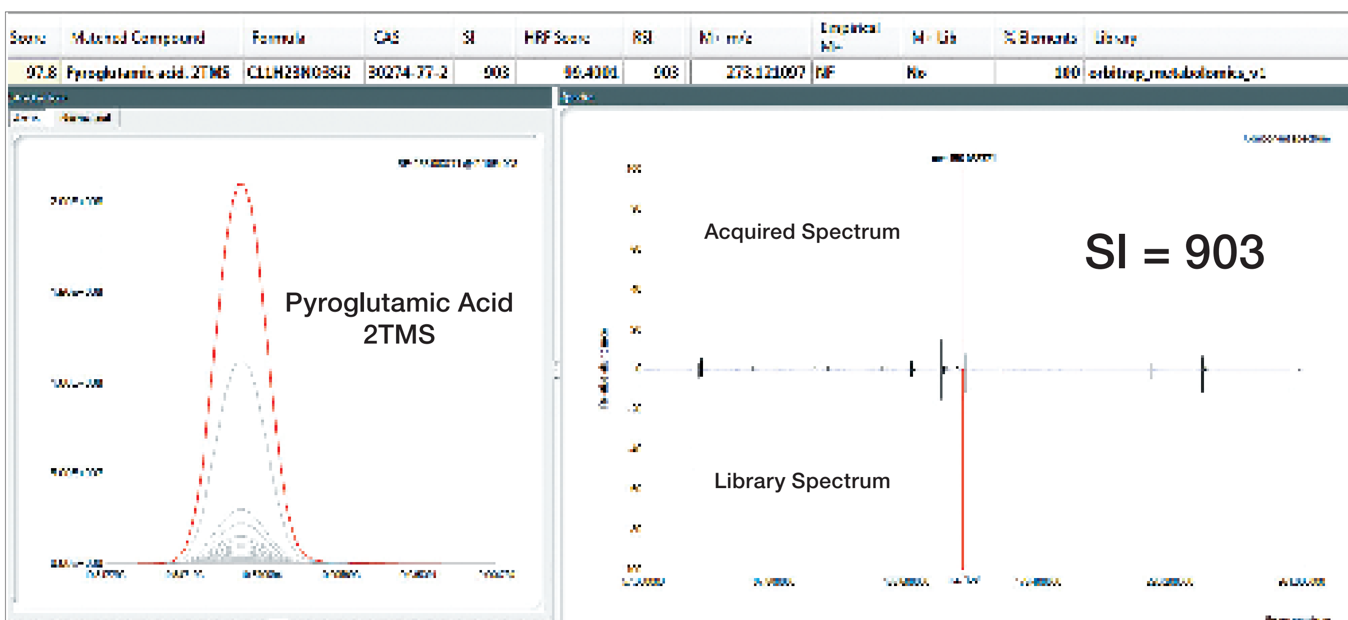

Figure 1. Identification of pyroglutamic acid 2TMS using spectral deconvolution and library searching against a high resolution metabolomics library.

Table 3. Summary of the peaks and the tentative identification of example metabolites in human plasma.

Compound	Retention time (min)	Base peak (*m/z*)	Mass accuracy (ppm)	On-line derivatization RSD % (n=8)
Ribose 4TMS	10.4	217.10745	0.5	12
Pyroglutamic acid 2TMS	10.6	156.08392	0.9	9
Palmitoleic acid TMS	13.4	117.03663	1.0	8
Myoionsitol 6TMS	13.6	217.10704	0.5	11
Stearic acid TMS	14.3	117.03663	0.9	5
Palmitic acid ester	15.8	283.26315	0.3	7
Cholesterol TMS	18.1	131.08859	0.7	8

Having made peak identifications, it was then possible to assess the repeatability of the derivatization protocol for specific compounds. The total number of deconvoluted features in the derivatized plasma was around 200 features present above a relatively high peak area of > 1,000,000. The repeatability of peak area was assessed by evaluating the relative standard deviation (%RSD) over 8 replicate samples. An RSD value of less than 15% is generally considered acceptable. Table 3 shows an example of seven metabolites and the mean peak area response and RSD% across the eight replicates. The %RSD for the seven compounds ranged from 5-12%. It was concluded that the on-line derivatization procedure showed acceptable reproducibility. The automated derivatization enabled samples to be injected at the same stage of derivatization, whereas off-line samples would have to be derivatized and then wait on the autosampler tray to be injected. This delay may increase the variability in response because compounds are degraded or continue to form whilst awaiting analysis. Therefore, on-line procedures are preferred as they enable the analyst to have more control over the derivatisation and increase the throughput through automated overlapping.

Conclusions

The results of this study demonstrate that the Q Exactive GC Orbitrap mass spectrometer in combination with automated on-line sample derivatization is an excellent analytical system that can be used for the detection and identification of plasma metabolites. Deconvolution software in combination with a high resolution metabolomics library enabled fast and confident identification of metabolites. The automated on-line derivatization demonstrated acceptable reproducibility of metabolite peak areas across the replicate samples which is essential in metabolomic studies.

References

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