Novel Strategies for Metabolite Identification using HPLC-Ion Trap Mass Spectrometry

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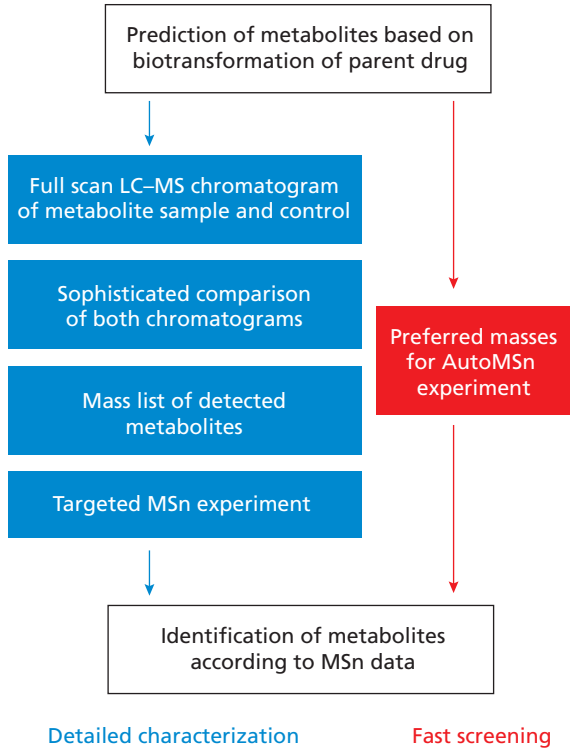
Introduction

The major task of metabolite identification is rapid and reliable elucidation of structural changes. This fact applies both to metabolism of xenobiotics and endogenous compounds. Mass spectrometry is a predominant tool in metabolic studies and especially multiple-stage MS capabilities are of great advantage in LC–MS experiments in case of MS/MS data characterized by non-specific fragmentations.

Figure 1: Strategies for metabolite identification.

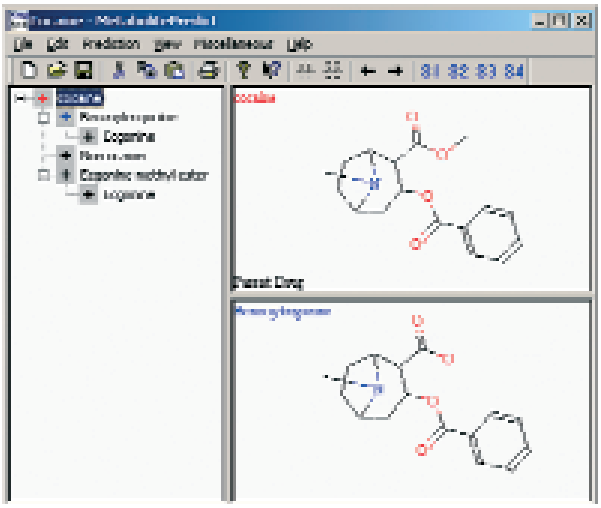


Ion trap mass spectrometers have proven excellent tools for metabolite identification, especially because of their capability of automated MS^n experiments. Our strategy for metabolite identification combines the prediction of possible metabolites with a powerful matrix filter for metabolite samples. Here we describe two alternative strategies for metabolite identification, both of them supported by the new Metabolite Tools software, used in combination with ESI-ion trap mass spectrometry.

Instrumentation

LC–MS analyses were performed using an HP 1100 liquid chromatography system (Agilent Technologies, Palo Alto,

Figure 2: Metabolite tree of cocaine. Cocaine metabolism is characterized by (1) hydrolysis of the ester moieties by esterases to ecgonine methyl ester (EME) and benzoylecgonine and (2) oxidation through cytochrome P450s to norcocaine.



California, USA) interfaced with an esquire3000 plus ESI-ion trap mass spectrometer (Bruker Daltonics). RP-HPLC columns (5 µm particles, 2.0 × 150 mm C18 material) and acetonitrile-water-formic acid gradients were applied for separation.

Strategy 1: LC–MS and LC–MS/MS Characterization of Metabolites

Prediction of metabolites: Cocaine was chosen as the model compound to demonstrate a new strategy for metabolite identification: The molecular structure of the target compound is subjected to a set of biotransformation rules to intelligently predict possible metabolites. The molecular weight information of these metabolites can subsequently be used for a more focused MS experiment. The generated structures are an additional interpretation help for MS/MS spectra.

A metabolite tree of cocaine is presented in Figure 2. A list of masses of the possible metabolites, taking identical molecular compositions into account, is generated and can be used (e.g., as preferred mass list in an AutoMS^n experiment).

Experimental: Plasma samples from rats were spiked with cocaine and metabolites. Samples were treated as previously described by Singh et al.[1] and analysed by LC–MS and LC–MS/MS using ESI in the positive mode for ionization.

Detection of Metabolites

Figure 3 shows the comparison of two full scan LC–MS chromatograms of rat plasma samples. Differences between the

Figure 3: Comparison of plasma spiked with cocaine and metabolites and blank plasma.

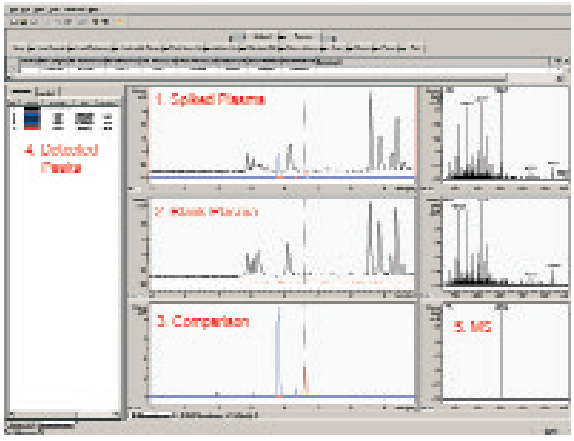
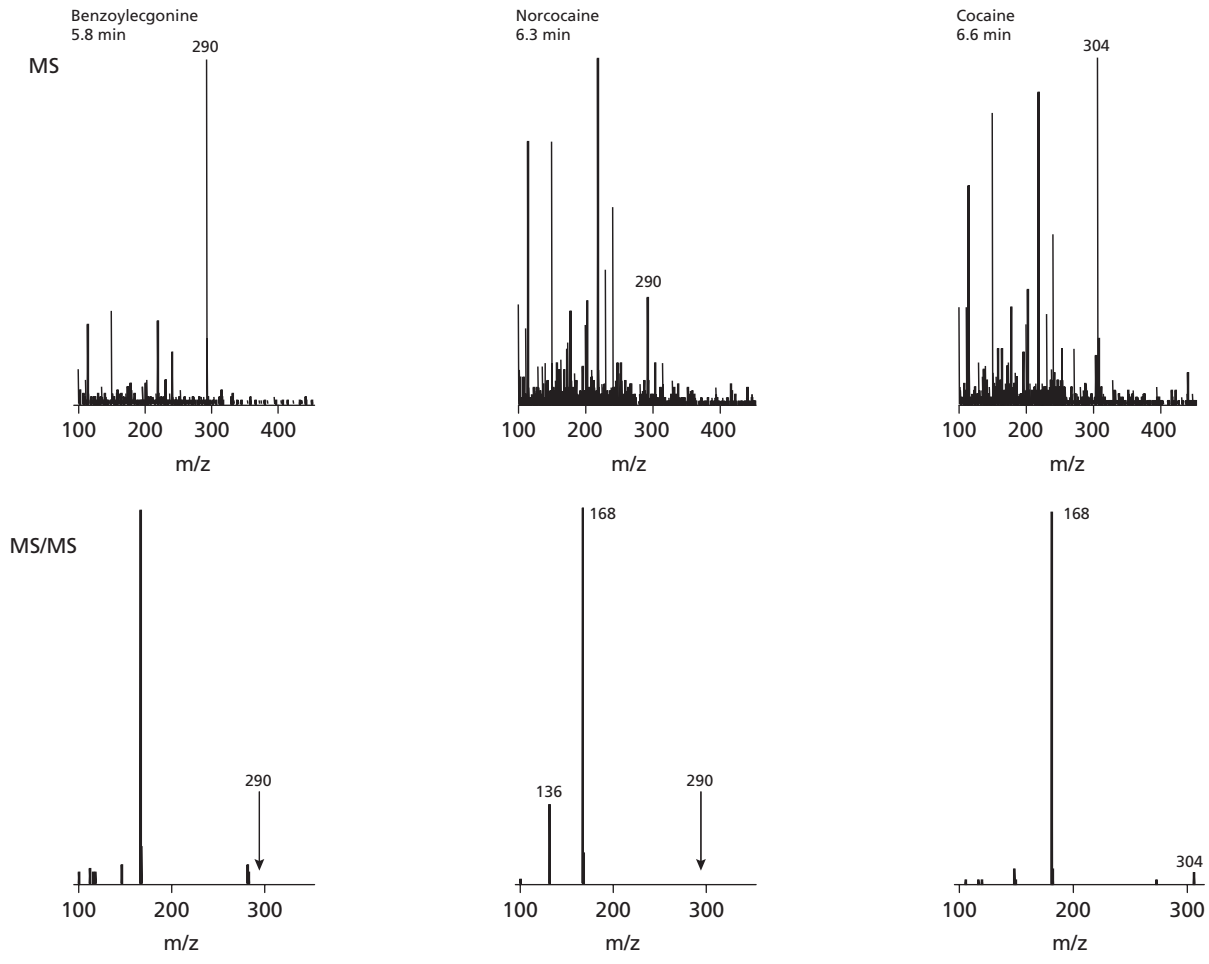


Figure 4: MS and MS/MS spectra of cocaine and metabolites.



base peak chromatograms (BPC, black) are not easily detected. However, a sophisticated algorithm comparing the two chromatograms yields the detection of compounds exclusively present in the spiked sample. The respective extracted ion chromatogram (EIC) traces are depicted in the third window. The mass spectra corresponding to the green time line are presented on the right side. The detected mass list contains the retention time and *m/z* values that are present only in the sample. The mass spectra of benzoylecgonine, norcocaine and cocaine are given in Figure 4. Even norcocaine and cocaine not showing detectable peaks in the BPC are easily filtered out by the algorithm. Based on the comparison data, MS/MS spectra were obtained in the respective time segments (see Figure 4). Base peak in all spectra is 168 *m/z*. Benzoylecgonine and norcocaine having the same molecular weight are easily distinguished because of characteristic fragments at 150 *m/z* and 136 *m/z*.

Strategy 2: Fast Metabolite Screening with AutoMS^n

Fatty acid glucuronides in human urine: The cytochrome P450 and soluble epoxide hydrolase (sEH) dependent metabolism of linoleic acid (LA), (see Figure 5), yields various cytotoxic products. The following experiments focus on the products 9,10-dihydroxy-12-octadecenoic acid (LTXD) and 12,13-dihydroxy-9-octadecenoic acid (iso-LTXD), which are generated by neutrophils. LTXD/iso-LTXD is furthermore substrate for UDP-glucuronyltransferase (UDPGT).

The target was the development of a fast procedure for the simultaneous identification of glucuronides and the respective aglycons from human urine within a single chromatographic run. The procedure was used to investigate which isomers of linoleic acid metabolites are primarily excreted in human urine.

Experimental: Human urine was buffered to pH = 5 with acetic acid and extracted twice with ethylacetate. The solvent was removed in vacuo and the residue reconstituted in methanol. Samples were measured using ESI in the negative mode. The preferred masses for the AutoMS^n experiment were calculated from the molecular weight of the predicted fatty acid metabolites.

The AutoMS^n Experiment: The AutoMS^n function allows an intelligent precursor selection using hierarchical decision criteria. The included mass range represents the highest priority. The next priority is the absolute intensity a mass peak needs to exceed to be considered for an MS^n experiment. Preferred masses can be selected as additional selection criterion.

Analysis of urine using AutoMS^n: Figure 6 shows mass spectra obtained from the AutoMS^n experiment. The MS spectrum shows 489.4 *m/z* as base peak which is selected for the MS/MS experiment. The base peak of the MS/MS spectrum is 313.3 *m/z*, the [M-H]- of LTXD/iso-LTXD. Fragmentation of this peak leads to the third spectrum with 200.9 *m/z* as highest intesity. This spectrum clearly identifies the peak at 12.0 min as glucuronide of LTXD. An identification of the individual glucuronide isomers has not been performed as suitable standards for retention time comparison were not available.

Figure 5: Metabolite tree of linoleic acid.

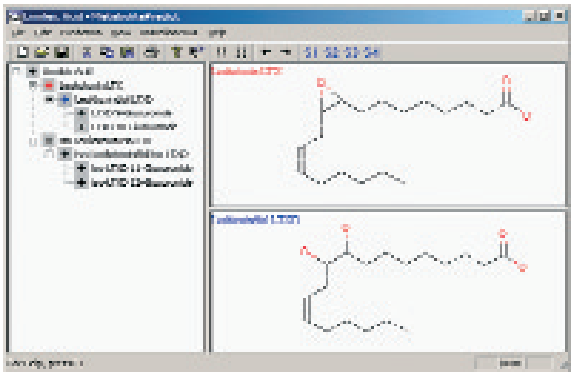


Figure 6: Identification of LTXD-glucuronide according to the AutoMS3 data.

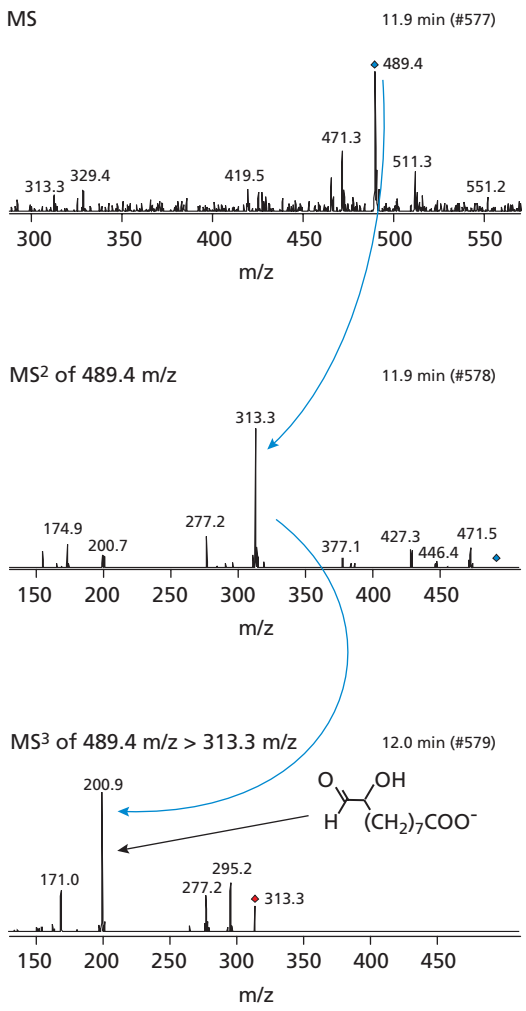


Table 1: Identification of C18 fatty acid metabolites in human urine.

	RT[min]	m/z	Compound
1	12.0	489.4	LTXD-glucuronide
2	12.5	489.4	LTXD-glucuronide
3	13.1	491.3	Dihydroxystearic acid-GLUC
4	13.6	491.4	Dihydroxystearic acid-GLUC
5	16.5	315.4	Dihydroxystearic acid

Conclusion

Both metabolite identification strategies using a prediction of possible metabolites prior to a detailed characterization or a fast screening were successfully demonstrated. These new tools will significantly facilitate metabolite identification in the future.

Acknowledgement

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Reference

1. *Anal. Chem.*, **71**, 2021–2027 (1999).

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