

Cell-Based Metabolite Identification for Expanding the Human Metabolome MS/MS Database

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Overview

Purpose

To explore a cell-based metabolite identification approach for expanding the human metabolome MS/MS database.

Method

The test compound is added to a vial of human liver microsomes (HLM) and allowed to metabolize for a specified time, $t = 0.5 - 24$ h. The incubation is then stopped and sample clean-up performed. Each sample is analyzed by LC MS on the Esquire 3000+ Ion Trap. The molecular formula was verified with a FT ICR MS.

Results

Using this method 13 different metabolites have been identified from several different precursors. In some cases it was found that the metabolite profile changes with incubation time. One metabolite has been verified in human urine.

Introduction

Human metabolome identification is a challenging task. The goal of this project is to create a public MS/MS database of human metabolites (HMDB). The HMDB will consist of positively identified metabolites, their structures, MS and MS/MS data, experimental conditions and any other relevant information. Metabolite standards are needed to create this database. The standards may be commercially available, readily synthesized or newly identified from biological sources, such as human urine or blood. However, deducing the structures of unknown metabolites in human fluids is challenging. In this work, we report our research in exploring a cell-based metabolite identification approach for expanding the HMDB

HLM can be used to metabolize known compounds. The structures of the resulting metabolites can be determined using the structure of the known compound, comparing the MS/MS spectra and the masses of the known compound with that of the metabolite. Essentially this method is a simple and controlled way to create a library of standards for the HMDB.

Experimental

Pooled HLM were combined with NADPH regenerating system, potassium phosphate buffer and the primary test compound (example illustrated 2-Methylbutyrocarnitine (2-MBC)). The samples were allowed to incubate at 37°C for a specified time, between 30 minutes and 24 hours, and the reactions stopped by adding an aliquot of acetonitrile containing 5% acetic acid. Each sample was then vortexed to ensure the reaction was stopped and centrifuged to remove the microsomes and proteins.

The liquid was decanted into clean vials and stored at -20°C pending analysis. Controls were also prepared for each sample with buffer substituted in lieu of the test compound.

The samples were analyzed using LC MS and MS/MS, on an Esquire 3000+ ion trap MS, followed by analysis on a FT ICR MS for better mass accuracy to confirm the chemical formula.

The microsome samples are not as complex as urine or other biological fluids. However, to further simplify metabolite detection, two programs, MetabolitePredict© and MetaboliteDetect©, were used for the initial identification of possible metabolites in each sample. When a precursor structure is entered into the MetabolitePredict© software the program applies a set of defined rules to the compound and outputs a list of possible metabolite structures. Each metabolite has a slight structural change compared to the precursor. Each predicted metabolite also contains information on the chemical formula and isotopic mass. The rules applied to get a specific metabolite can be deduced from the metabolite's structural changes and the list of rules.

The MetaboliteDetect© software will subtract a reference chromatogram from the sample chromatogram to find the unique peaks in the sample. The software then compares each MS in the chromatogram to the predicted metabolite list and highlights any matching masses, i.e., the possible metabolites.

Once the possible metabolites are identified a MS/MS spectrum can be obtained to verify the metabolite's identity.

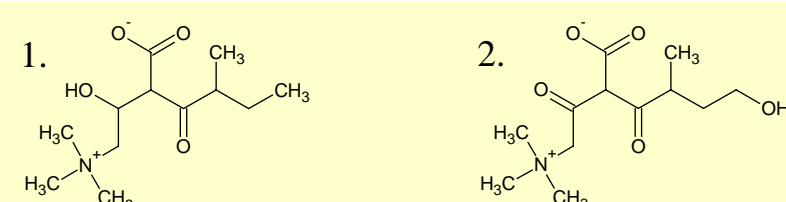
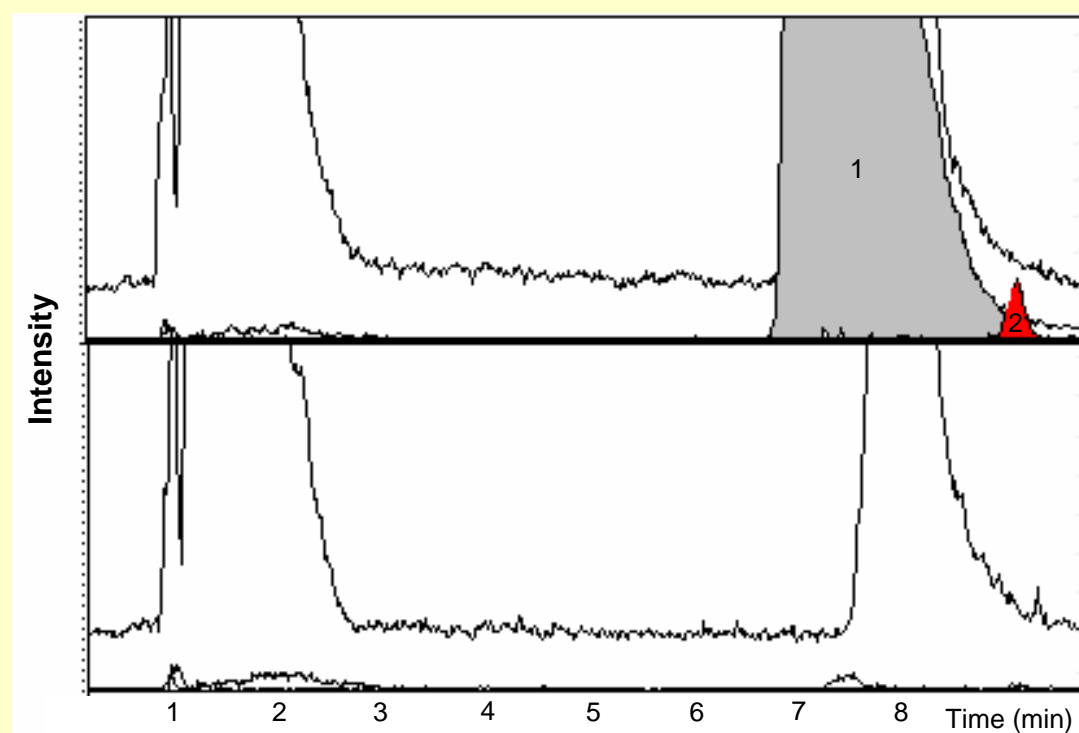


Figure 1. The EIC of 1) 2-MBC m/z 246 and 2) metabolite m/z 260 are identified in the chromatogram of sample and control after incubation with microsomes for 3 hours.

When a possible metabolite is determined using MetabolitePredict© and MetaboliteDetect© the identity must be verified. All metabolites were confirmed using MS/MS analysis and comparing the metabolites' MS/MS spectra with that of the parent compound. Figure 2 is the MS/MS spectral comparison of the 2-MBC metabolite (b), initially identified in Figure 1, to that of the primary compound, 2-MBC (a).

The possible 2-MBC metabolite is verified by identifying common fragment ions and/or common neutral losses in the two MS/MS spectra. In this particular example there is one common fragment ion with m/z 85, as well as several common neutral losses of 161 and 59 Da. Thus the identified compound is a 2-MBC metabolite.

Results and Discussion

This procedure was repeated for each of the identified metabolites listed in Table 1.

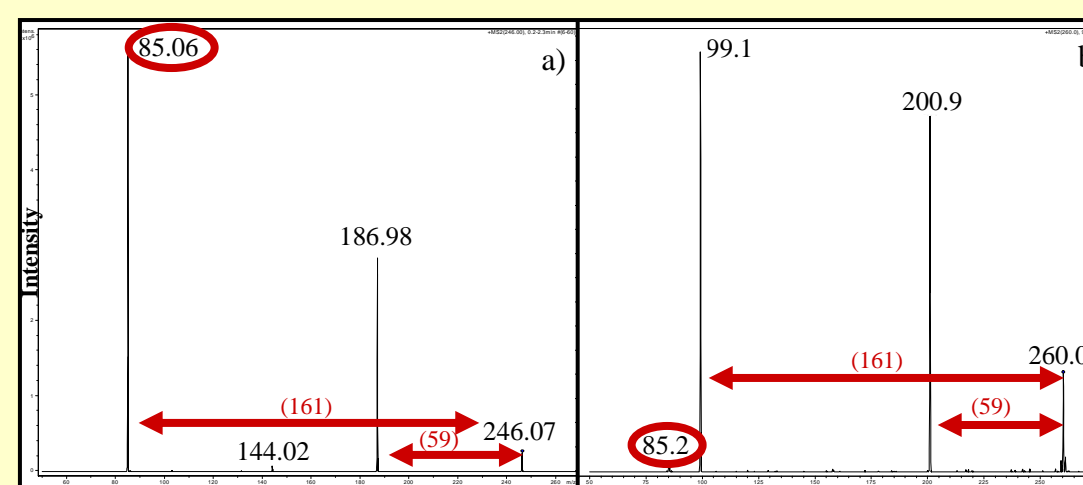


Figure 2. MS/MS Spectra of a) 2-MBC and b) 2-MBC metabolite

The structure of the 2-MBC metabolite (structure 2 in Figure 1) was initially predicted by MetabolitePredict©. This structure has also been confirmed by the fragmentation pattern in the MS/MS spectra.

The MetabolitePredict© software also gives details about metabolism. The 2-MBC metabolite is a generation 2 metabolite, hence, two metabolic reactions were performed on the 2-MBC molecule to get the resulting 2-MBC metabolite. The software also reports that this particular metabolite was formed by CYP450 hydroxylation and alcohol dehydrogenase of the 2-MBC molecule.

Table 1. MS/MS data for the parent compound and each of its metabolites.

Compound *	m/z	MS/MS peaks (m/z)	Common Neutral losses	Metabolic rules used by MetabolitePredict©
2-MBC (1)	246	187, 144, 85		
Metabolite (2)	260	201, 99, 85	59, 161	CYP450 Hydroxylation Alcohol Dehydrogenase
PAGME	208	176, 148, 120, 91, 76		
Metabolite	195	176, 148, 120, 90	118	CYP450 Hydroxylation
PC	400	341, 257, 239, 151, 144, 137, 125, 123		
Metabolite	372	313, 229, 211, 144, 137, 123	59, 143, 161	N-Demethylation N-Demethylation
Metabolite	414	355, 321, 301, 271, 253, 235, 217, 189, 171, 161, 147, 135, 121	59, 143, 161	CYP450 Hydroxylation N-Demethylation
Metabolite	416	357, 339, 321, 301, 269, 255, 237, 219, 163, 157, 149, 135, 121	59, 161, 84, 102, 190, 204, 218	CYP450 Hydroxylation
Metabolite	416	339, 321, 301, 271, 255, 235, 217, 193, 161, 135, 121	161, 84, 204, 218	CYP450 Hydroxylation
Metabolite	386	327, 255, 151, 144, 137, 123	59, 161	N-Demethylation
MMOHA	224	206, 192, 164, 135		
Metabolite	210	192, 135	18	O-Demethylation
CapG	202	184, 156, 127, 109, 76		
Metabolite	259	241, 184, 156, 127	75	Glycine Transferase

Table 1. Continued

Compound *	m/z	MS/MS peaks (m/z)	Common Neutral losses	Metabolic rules used by MetabolitePredict©
SebA	203	185, 167, 157, 149, 139, 131, 121		
Metabolite	199	195, 181, 163, 153, 145, 135, 121 , 109, 93	18, 36, 46, 54, 64	Beta-oxidation hydrogenation
SebA (neg)	201	183, 139		
Metabolite	215	197, 153	18, 62	beta-oxidation hydroxylation Hydroxylation (or) hydroxylation alcohol dehydrogenase (or) beta-oxidation hydroxylation CYP450 Epoxidation (or)
APA	281	263, 235, 221, 200, 175, 166, 120		
Metabolite	166	149, 120	46	Amidase
APA (neg)	279	261, 244, 217, 200, 190, 175, 147, 113		
Metabolite	309	291, 265, 247	62, 44, 18	CYP450 Hydroxylation CYP450 Hydroxylation Alcohol Dehydrogenase

* Phenylacetylglycine methyl ester (PAGME); Palmitoylcarnitine (PC); Methyl α -methoxyhippuric Acid (MMOHA); Capryloylglycine (CapG); Sebatic Acid (SebA); Aspartyl Phenylalanine (APA).

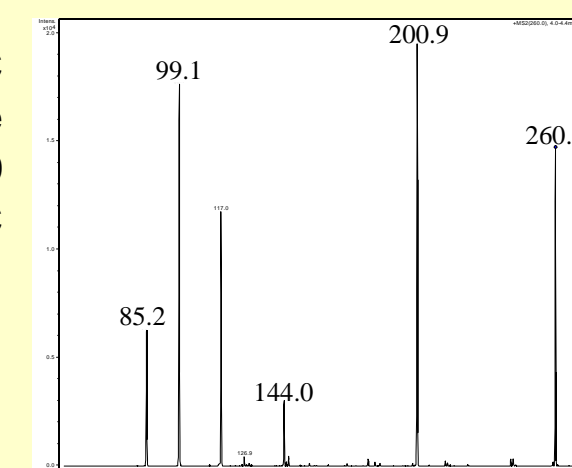


Figure 3. MS/MS of the 2-MBC metabolite (m/z 260) from urine sample

The 2-MBC metabolite (m/z 260) identified in urine has a nearly identical MS/MS spectrum and the same retention time as the HLM sample. In addition the precursor (2-MBC) was also identified in the same urine sample at the same retention time as the HLM sample. See Figure 1 structure 2.

Conclusions

Using human liver microsomes 13 different metabolites have been identified from several different precursor compounds. One of these metabolites has also been successfully identified in urine.

The FT MS data still needs to be collected on several metabolites to confirm their chemical formulas.

Future work for this project includes building up the metabolite database by repeating this procedure on other precursor compounds to identify their metabolites and to use this database to identify these compounds in biological fluids such as urine and blood.

The current approach only allows for phase I metabolism, therefore a future goal is to design an approach for phase II metabolism. This would make this method more similar to an *in vivo* system.

Acknowledgments

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