APPLICATION NOTE

Liquid Chromatography/ Mass Spectrometry

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Identification of Unanticipated Prescription Drugs and Metabolites in Donor Urine Using Accurate Mass LC/MS

Overview

Donor urine obtained for a drug study was collected from donors reporting no use of prescription, over-the-counter or herbal preparations in the eight days prior to donation. However, LC/MS analysis showed that a number of non-endogenous compounds were detected in this urine. To identify the compounds, information from the mass spectral data set was used in a search of the Human Metabolome database.

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Methods

Reagents. Human urine, as specified in the Overview, was purchased commercially

Sample Preparation. ISOLUTE 400 µL 96-well SLE+ and sample collection plates were obtained from Biotage® (Uppsala, Sweden). 2 mL aliquots of urine, 20 µL of 5M ammonium acetate, pH 5 and 30 µL of β-glucuronidase (333.3 units/µL) were incubated in vials at 40 °C for 30 min. Samples were returned to room temperature then 20 µL of concentrated ammonium hydroxide was added to each vial and mixed. Aliquots of 400 µL of each sample were added to wells of the SLE+ plate on a Supelco® vacuum manifold, incubated and then eluted with two additions of ethyl acetate (600 µL each) which were allowed to flow by gravity, followed by a brief pulse of vacuum. Eluates were dried under a stream of nitrogen and reconstituted with 350 µL of 5% acetonitrile in water containing 0.1% formic acid.

Liquid Chromatography. UHPLC was performed with a Flexar FX-15 UHPLC pump, autosampler, and column oven (PerkinElmer, Waltham, MA) using a Brownlee™ SPP Phenyl-Hexyl column (2.1 x 100 mm, 2.7 µm, N9308485). Sample injection was 10 µL, column temperature was 45 °C and flow rate was 0.4 mL/min. Mobile phase A was water containing 0.1% formic acid. Mobile phase B was acetonitrile containing 0.1% formic acid. Gradient separation: 0.8 min. hold at 7% B, 7% to 60% B over 4 min., 60% to 100% B over 0.1 min., 1 min. hold at 100% B, 100% to 7% B over 0.1 min., 3 min. equilibration at 7% B.

Mass spectrometry (MS). MS analysis was performed with a PerkinElmer AxION® 2 TOF mass spectrometer fitted with an Ultraspray™ 2 Dual Probe electrospray source. The instrument was operated in positive pulse mode with 3 spectra per second acquisition rate. The capillary exit was set to 90 V and the skimmer to 25 V. Drying gas flow was 14 L/min. at 350 °C and the endplate heater was set to medium. The instrument was operated in lock mass mode with melamine (*m/z* 127.0727) and reserpine (*m/z* 609.2807) calibrants and a search span of 50 millimass units. The lock mass solution was delivered to the left ESI probe from the onboard pressurized calibration vials, with flow rate regulated to 30 µL/min. by connecting a length of PEEK tubing to provide backpressure.

Using FormulaFinder software for compound matching. FormulaFinder is a utility within the AxION® 2 TOF driver software which compares an experimental mass and isotopic pattern with those of formulas in a configured database. The result is a list of candidate matched chemical compounds, ranked by a score related to the mass accuracy and isotopic pattern fit.

In the FormulaFinder interface, selected measured *m/z* values, along with selections for a charge carrier moiety, and its charge are entered by the user (See example in Figure 3). Tolerances for the deviation between measured and calculated masses and the database to be searched are selected. Isotopic peak groups are chosen in the spectrum peak list within TOF driver. These measured *m/z* values and isotopic ratios are transferred automatically to the FormulaFinder interface when the utility is started from a software button.

The "Search database" button starts the search by calculating a monoisotopic mass for the molecule. This mass is compared with the pre-calculated masses for all the formulas in the database, to give a list of matched formulas within the selected mass range and theoretical isotopic pattern tolerance windows.

The final result is a list of candidate formulas ranked by a score based on the match of mass accuracy and isotope pattern to the experimental spectrum. Selecting any formula in the list shows the theoretical isotope pattern overlaid on the experiment isotopic profile for visual confirmation as well as links to additional information in the database, including a compound name and structure.

Configuring a Database for FormulaFinder. The default database for searches is downloaded from PubChem (pubchem.ncbi.nlm.nih.gov) and contains over 68 million entries of characterized chemical compounds. Because compounds in this study were expected to be either endogenous or of pharmaceutical origin, the Human Metabolome database (HMDB) was used in preference to PubChem. HMDB contains 42,000 compounds identified in human urine[1, 2, 3]. The database information was downloaded from www.hmdb.ca as an sdf format file, which was then converted to a CSV file of formulas and names within PerkinElmer ChemBioFinder software. This file is used by FormulaFinder; on first installation of a new database, the neutral masses for all the formulas are calculated and stored.

Results

The LC/MS analysis of a blank urine extract detected a number of compounds (Figure 1). The major ions for each peak in the separation were used to determine the compound eluting at that time. Early eluting peaks were identified as caffeine, which is commonly detected in urine after the consumption of coffee or tea, and the pain reliever acetaminophen. Later eluting peaks were determined to be prescription drugs or their metabolites present in the urine.

Drug Identification

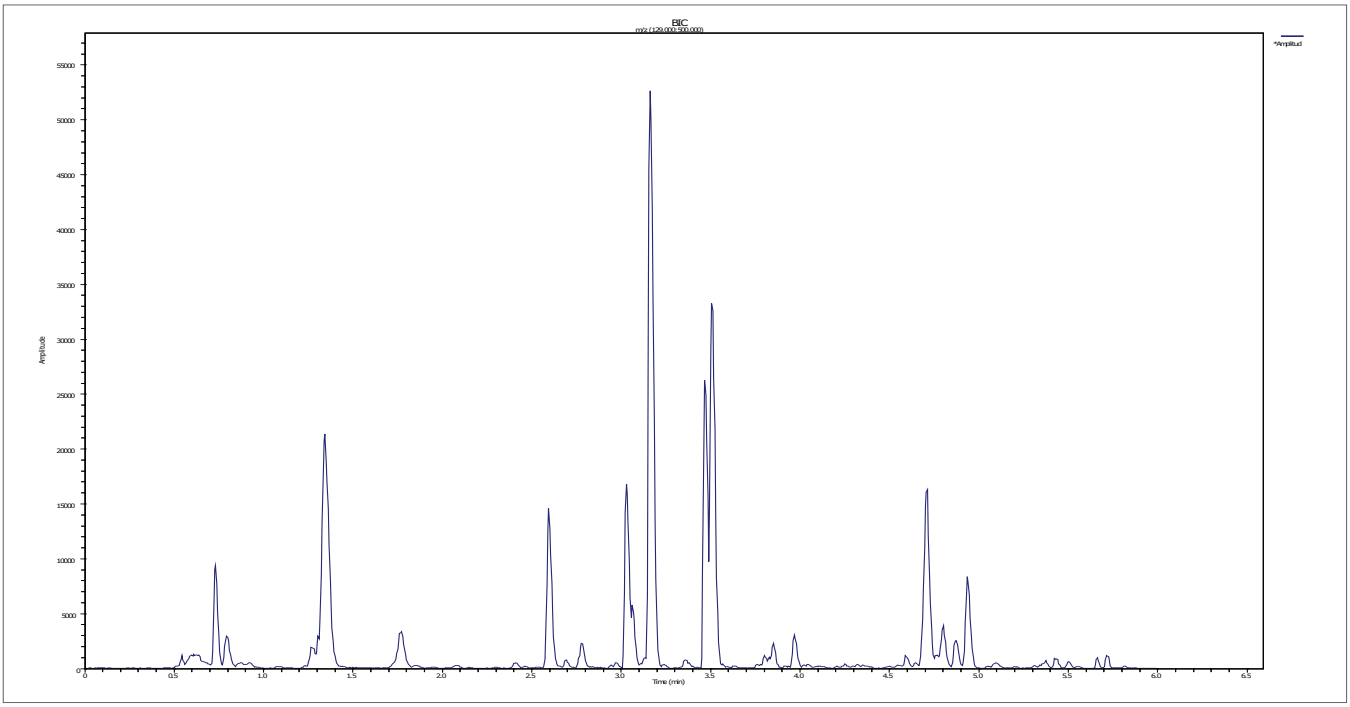


Figure 1. Base Peak Ion chromatogram for the 6 min. separation of compounds in a single donor urine sample.

Drug 1. The largest peak in the LC/MS run, observed at 3.2 mins. gave a monoisotopic peak at *m/z* 408.1247 (Figure 2). A FormulaFinder search gave a single match within HMDB to a drug of formula $C_{16}H_{16}F_6N_5O$, calculated *m/z* 408.1255 listed as sitagliptin (Figure 3). This prescription drug is used to manage blood sugar levels in Type II diabetes patients. A search in the larger PubChem database also gave this same compound as a prominent match. Other possible formulas in PubChem come from patent literature, and were unlikely to be present in a donor urine sample.

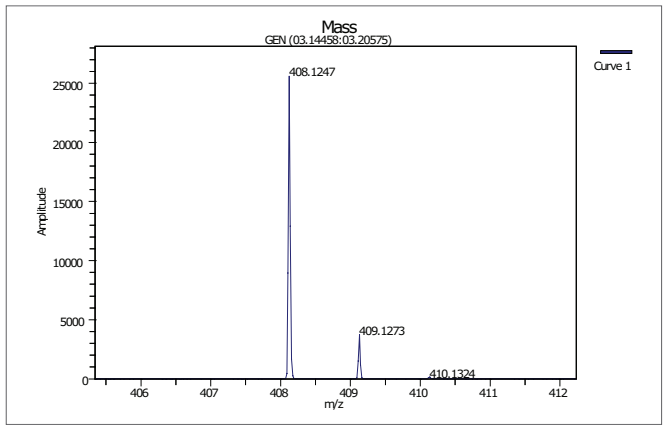
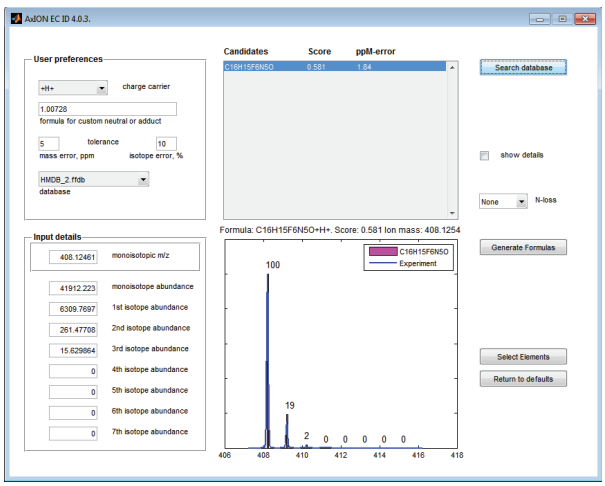
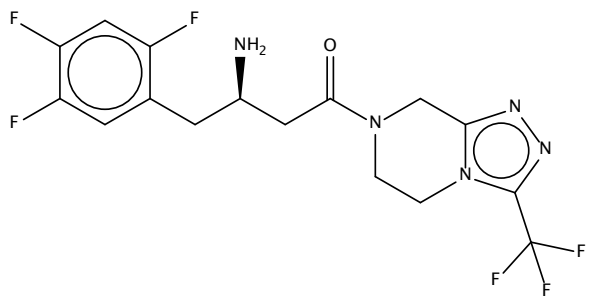


Figure 2. Isotope pattern of the major ions in the averaged spectrum at 3.2 min., for a compound identified as sitagliptin.





Chemical Formula: $C_{16}H_{15}F_6N_5O$

m/z: **407.12 (100.0%), 408.12 (19.4%), 409.12 (1.9%)**

Figure 3. FormulaFinder results for *m/z* 408.1247 – a single match in the HMDB to sitagliptin, a drug of formula $C_{16}H_{15}F_6N_5O$.

Drug 2. Another peak in the urine analysis at retention time 3.5 min. gave a major ion at *m/z* 373.1583 (Figure 4), which matched in HMDB to the formula $C_{20}H_{24}N_2O_3S$, calculated *m/z* 373.1579 for O-deacetyldiltiazem (Figure 5). This compound is known to be a significant metabolite in urine of the hypertension medication diltiazem. A search in the larger PubChem database, gave a large number of potential candidate formulas, but all unlikely to be present in a donor urine sample.

The major ions for other peaks in the separation could not be identified from a search of HMDB or PubChem databases. These may be other drug metabolites, which are not present in the database.

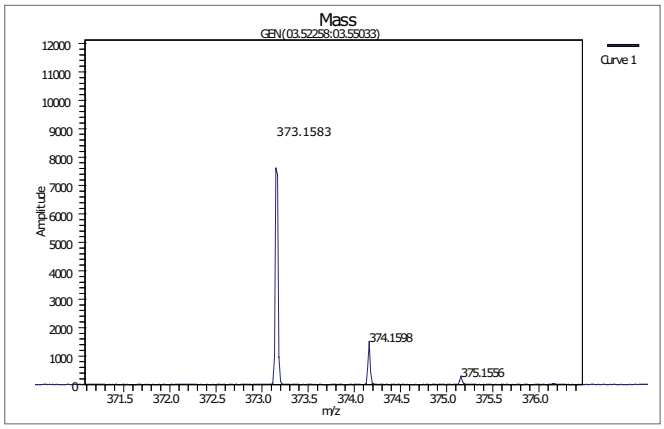


Figure 4. Isotope pattern of the major ions in the averaged spectrum at 3.5 min., for a compound identified as O-deacetyldiltiazem.

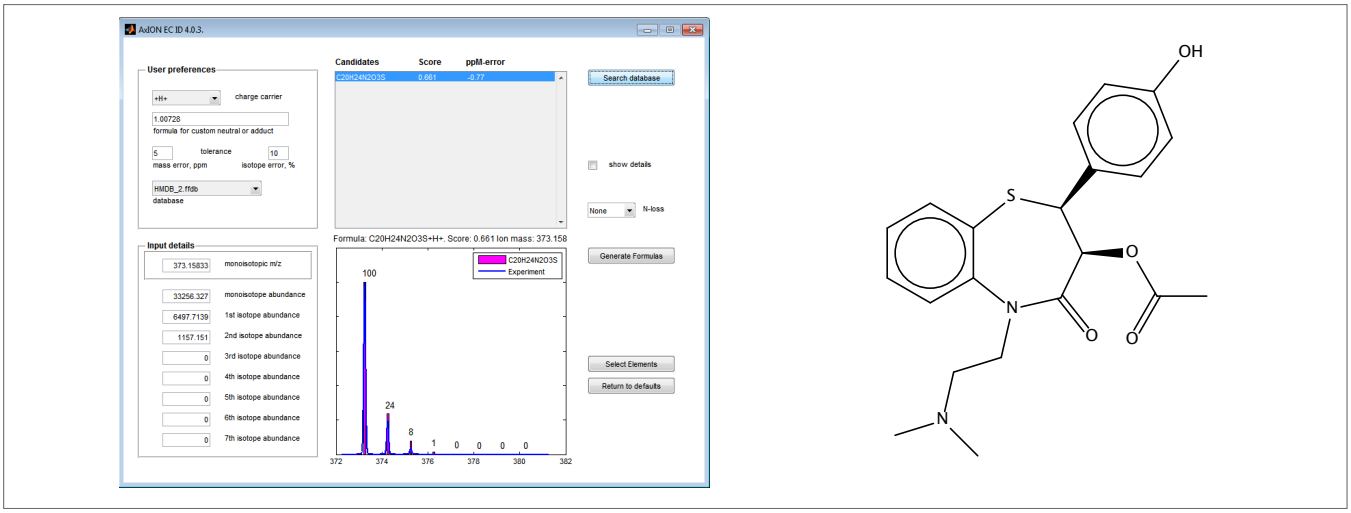


Figure 5. FormulaFinder results for *m/z* 373.1583 – a single match in HMDB to monodesmethyldiltiziam.

Further use of FormulaFinder functionality to interrogate data. Diltiazem has been reported[4] to be metabolized by deacetylation, as well as N- and O-demethylation. In urine, the major metabolite is monodesmethyldiltiazem (MA), with lower levels of deacetyl-diltiazem (M1), deacetyl-N-monodesmethyl-diltiazem (M2), deacetyl-O-desmethyl-diltiazem (M4) and deacetyl-N,O-desmethyl-diltiazem (M6). These minor metabolites are not included in PubChem or HMDB, so were not matched in a search.

Ion peaks corresponding to these metabolites, together with the parent drug, could be detected as minor peaks in the 2.8 - 4 min. retention time range. The GenerateFormula utility of FormulaFinder was used to calculate potential formulas for unknown compounds, based on user-allowed permutations of allowed elements. To reduce the number of candidate formulas, GenerateFormula allows a base elemental formula to be defined as a repeating unit. Here the core of the diltiazem structure [$C_{13}H_{11}NS$] was assumed to be unchanged during metabolism, and set as a base unit. With this limitation on potential formulas, the peak at *m/z* 359.1417 gave one candidate result, $C_{19}H_{22}N_2O_3S$, corresponding to a deacetylated and demethylated metabolite. Other peaks were assigned to known diltiazem metabolites from unique formulas in the same way (Table 1).

Further confirmation of metabolite identifications resulted from the presence of an ion at *m/z* 178.0352 in the spectrum of each diltiazem related peak. This ion, formula C_9H_8NOS, is produced by in-source fragmentation of the identified metabolites.

Table 1. Table of detected metabolites of diltiazem. Note the species at 3.06 and 3.46 min. are isobaric, and so cannot be unambiguously identified without reference standards.

Retention time (min.)	*m/z*	Formula	Diltiazem Metabolite
2.94	331.1097	$C_{17}H_{18}N_2O_3S$	O-deacetyl-N,N-desmethyl, O-desmethyldiltiazem
3.03	345.1261	$C_{18}H_{22}N_2O_3S$	O-deacetyl-N,O-desmethyldiltiazem
3.06	359.1417	$C_{19}H_{22}N_2O_3S$	O-deacetyl-O-desmethyldiltiazem or O-deacetyl-N-desmethyldiltiazem
3.34	387.1373	$C_{20}H_{22}N_2O_4S$	N,O-desmethyl- or N,N desmethyldiltiazem
3.46	359.1417	$C_{19}H_{22}N_2O_3S$	O-deacetyl-O-desmethyldiltiazem or O-deacetyl-N-desmethyldiltiazem
3.50	373.1583	$C_{20}H_{24}N_2O_3S$	O-deacetyldiltiazem
3.80	401.1517	$C_{21}H_{24}N_2O_4S$	N-desmethyldiltiziam
3.85	**415.1671**	$\mathbf{C_{22}H_{26}N_2O_4S}$	**diltiazem parent drug**
3.96	458.1527	$C_{23}H_{25}N_2O_6S$	Unknown diltiazem metabolite

Data for drug 1, assigned as sitagliptin ($C_{16}H_{15}F_6N_5O$) was also reviewed. Sitagliptin is excreted mainly as the parent drug,[5] with only 13% metabolites in urine. No known metabolites of sitagliptin were detected in this urine sample.

Conclusion

A number of compounds were detected by LC/MS analysis of an extracted urine sample. Two compounds were assigned as known prescription drugs by comparing the measured accurate masses and isotopic patterns with those of formulas in a database of human metabolites in urine. Other compounds, not present in the database, were determined to be metabolites of one of the drugs by comparison of their calculated formulas to known metabolites.

This method could be used during the LC/MS identification of many drugs, drug metabolites, degradants and impurities. This study highlights the value of performing these studies using a TOF mass spectrometer. In contrast to the use of triple quadrupole MRM approaches, where only anticipated components are detected, TOF data may be interrogated post-acquisition to permit detection and identification of unknown or unexpected components, such as those demonstrated in this study.

References

1. Wishart DS, Tzur D, Knox C, et al., *HMDB: the Human Metabolome Database.* Nucleic Acids Res. 2007
2. Wishart DS, Knox C, Guo AC, et al., *HMDB: a knowledgebase for the human metabolome.* Nucleic Acids Res. 2009
3. Wishart DS, Jewison T, Guo AC, Wilson M, Knox C, et al., HMDB 3.0 — *The Human Metabolome Database in 2013.* Nucleic Acids Res. 2013
4. Studies on the metabolism of diltiazem in man, Sugihara J, Sugawara Y, Ando H, Harigaya S, Etoh A, Kohno K., J Pharmacobiodyn. 1984 Jan;7(1):24-32.
5. Metabolism and excretion of the dipeptidyl peptidase 4 inhibitor [14C]sitagliptin in humans, Vincent SH1, Reed JR, Bergman AJ, Elmore CS, Zhu B, Xu S, Ebel D, Larson P, Zeng W, Chen L, Dilzer S, Lasseter K, Gottesdiener K, Wagner JA, Herman GA. Drug Metab. Dispos. 2007 Apr;35(4):533-8.

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