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Matching Shotgun Metabolomic Ions from Urine Samples to Reference Standards and the HMDB Database for Metabolite Identification: It is Not as Straight-Forward as You Think.

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The analyst creed has always been "isolate, identify and quantify", and thus for a century or more chemistry and analytical science has been dedicated to these aims. Initially, we only isolated our desired molecule as single entities from a complex mixture and then quantified it. Often this was laborious and complex. Rapid and resource efficient techniques developed; but the need to evaluate multi-components and their relative balance, or imbalance, in complex bio-systems has become the expectation in medicine and other disciplines. The need to run multiple specific molecular tests is extremely resource intensive. However, as we progressed we realised that we could simultaneously isolate (resolve) multiple analytes in complex mixtures quickly and quantitate these resolved molecules (at least relatively) by rapid powerful technologies such as chromatography coupled mass spectrometry. This has led to the development of the analytical specialties of proteomics and metabolomics [1].

The expectation has been that identification of resolved molecules would also be rapid and straight forward, particularly as mass spectrometry should give characteristic mass and mass fragmentation spectra [2,3]. However, this has not proven to be the case and only resolved molecules that are shown to be of interest (because in complex mixtures the resolved peak changes are associated with a condition or biological phenomena) are identified [4-6].

Often we match a resolved molecule to characteristics of a pure standard and spike the standard into the sample to observe intensification of the peak in question. However, ultimately, only mass spectral fragmentation is considered conclusive identification [7,8]. In this respect the aim is to match the MS/MS pattern of molecular ions of your resolved molecules with online reference library databases and hence achieve identifications within minutes.

Although these are logical strategies, the biological matrix and biological processing within a sample when subjected to one or other mass spectrometry technique introduce an element of what is best described as a *Chaos effect* (from Chaos theory where multiple small components alter significantly the end pattern).

The distributions of the atomic nuclei of a compounds component atom's are generally well defined in a molecule; and even the more mobile electrons are within defined probability orbitals. In general these distributions of atomic molecular forces are stable and a given molecule will fragment, in a mass spectrometer, in a reproducible manner. However, fragmentation spectral databases are largely derived from pure standards, subject to mass analysis within a defined matrix and storage. The external forces acting on the same compound in a complex biological specimen are not the same. These may alter, in a minor way, the distribution of atomic forces and electron orbital distributions of the compound. Thus fragmentation may be effected and this could lead to characteristic spectra of a standard not quite matching the same molecule from a biological sample peak spectra. Thus, that identification of metabolites may, like weather forecasting, not be an exact matching science.

Experimental Examples

We have been examining metabolomic changes associated with maternal urine of those pregnant with an aneuploid fetus. Two principle component analytes, of *m/z* 114.07 and 315.2, were identified as major metabolomic marker peaks of fetal Down syndrome in maternal urine. We subsequently identified these as dihydrouracil (mass of 114.103 Da) and progesterone (mass of 314.46 Da) but this was not as straightforward as we hoped [9].

Dihydrouracil Matching

During our experiments in which samples were resolved by HILIC and Reverse Phase HPLC and subjected to electrospray ionisation, ion trap - time of flight (IT – ToF) MS/MS mass spectrometry following. The dihydrouracil standard was observed to form $[M+H]^+$ ions and hence, *m/z* = 115.05 was trapped in IT instead of *m/z* of 114.07 which is the molecular ion for what we now believe to be *in vivo* dihydrouracil resolved in urine samples. The MS/MS ions obtained from running standard Dihydrouracil (115.05 *m/z*) were compared with the Down syndrome aneuploidy principal component identified metabolomic marker with *m/z* 114.07.

The dihydrouracil standard, being a pure standard, ionised better than the proposed endogenous dihydrouracil present in the urine samples. This may explain why, during MS/MS fragmentation, standard preparation dihydrouracil appeared to more readily form $[M+H]^+$ ions whereas *in vivo* dihydrouracil in the urine samples formed abundance of molecular ions with a positive charge: Following MS/MS of dihydrouracil standard resolved by LC-MS, Sparidans et al. [10] reported obtaining product ions *m/z* – 73, due to loss of H_2CCO; *m/z*- 55 ,from the loss of NH_2CONH_2 and *m/z* – 56, due to the formation of $NH_2CONH_3^+$ and theses are therefore described as characteristic molecular ions of dihydrouracil in the HMDB database. Figure 1 shows possible fragments of dihydrouracil. A fragment ion with *m/z* of 57.3, observed in the urine sample as well as dihydrouracil

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standard analysis could also be due to a double charged dihydrouracil. An ion of *m/z* of 57 could also be observed if the molecule ionises into two equal fragments (C_2H_3NO). The formation of molecular cation from C_3H_5NO could produce fragments with a positive charge and *m/z* of 71.04 which was observed in the urine samples and is also listed within the MS/MS reference spectra of dihydrouracil within HMDB.

The product ions with *m/z* values of 93.31, 69.77 and 56.74 were obtained for the dihydrouracil standard and these matched with product ions of a precursor ion with *m/z* of 114.07 in urine sample. The ion with *m/z* of 56.4 and its ortholog $[M+H]^+$ ion with *m/z* of 57.4 were found to be a common fragment ion in endogenous urinary and standard dihyroruracil MS/MS spectra respectively. Dihydrouracil standard and the unknown ion with *m/z* of 114.07 in the urine samples had identical retention times in HILIC and RPLC. However, as demonstrated the parent ion and mass spectral patterns only partly matched in comparative experiments and that registered in the HMDB.

Progesterone Matching

The mechanism of MS/MS fragmentation of steroids in general and progesterone in particular, is complex and mainly involves σ-bond cleavages [11]. Figure 2 shows the possible fragmentation pattern that progesterone may undergo to form these ions.

In our study, the three major fragment ions observed in the urine samples and when a progesterone standard was run had *m/z* values of 109.07, 123.1 and 133.1. However, the most abundant ion in our comparative study was observed to be *m/z* of 255.2. The *m/z* of 255.2 and 133.1 are not registered in the HMDB as known progesterone MS/MS spectral fragments. Zhang et al. [12], using tandem MS conditions, reported that majority of fragmentations occurred by the cleavage of peripheral groups followed by the cleavage of the rings [12]. The cleavage of ring A with intact C=O and CH_3 groups may give the fragment with *m/z* of 109.07 and formula of $C_7H_9O^{\bullet}$. As suggested by Hammerum and Djerassi [13] ketene elimination and cleavage of ring

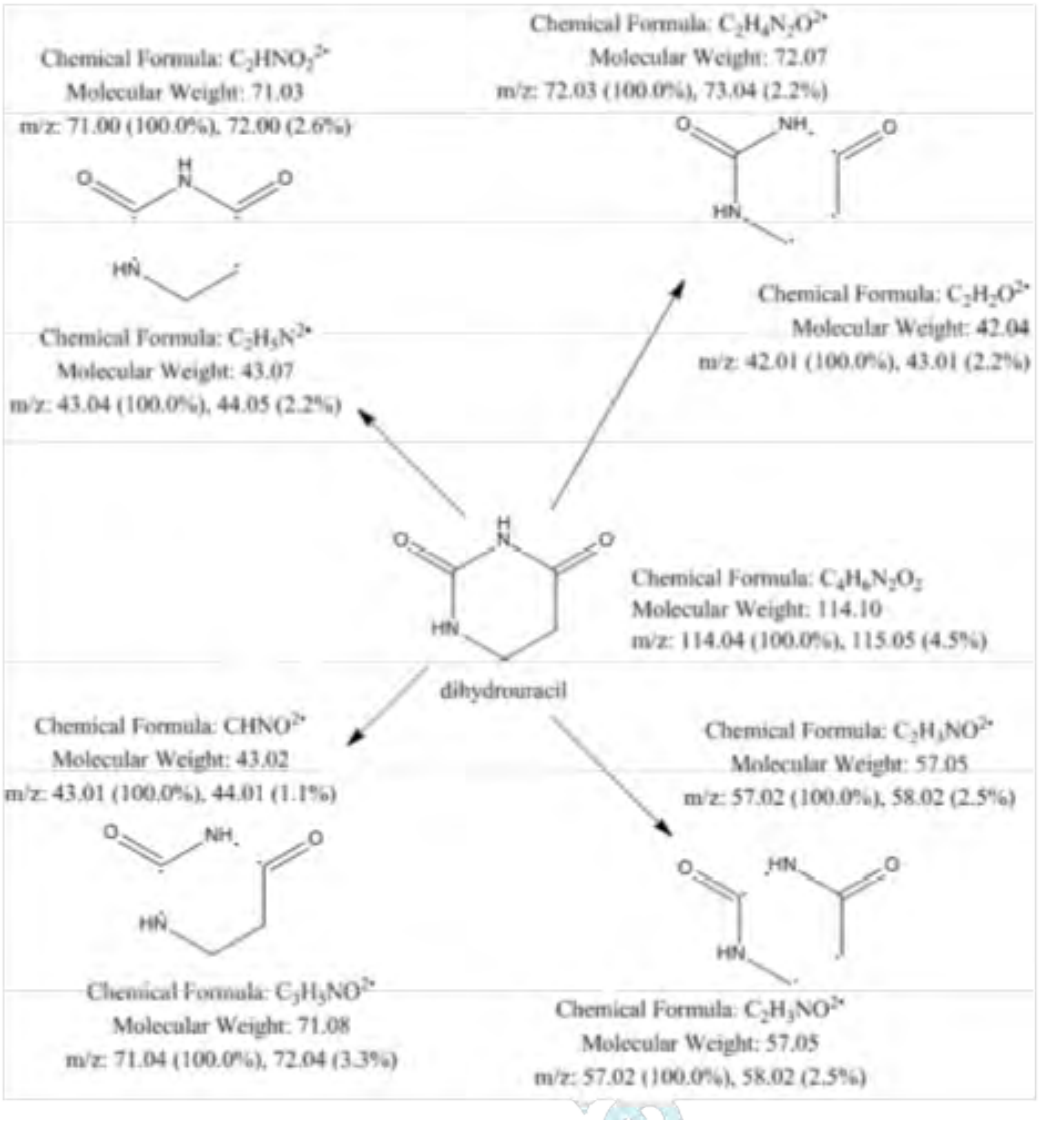

Figure 1: Proposed MS/MS fragmentation of endogenous dihydrouracil from a urine matrix

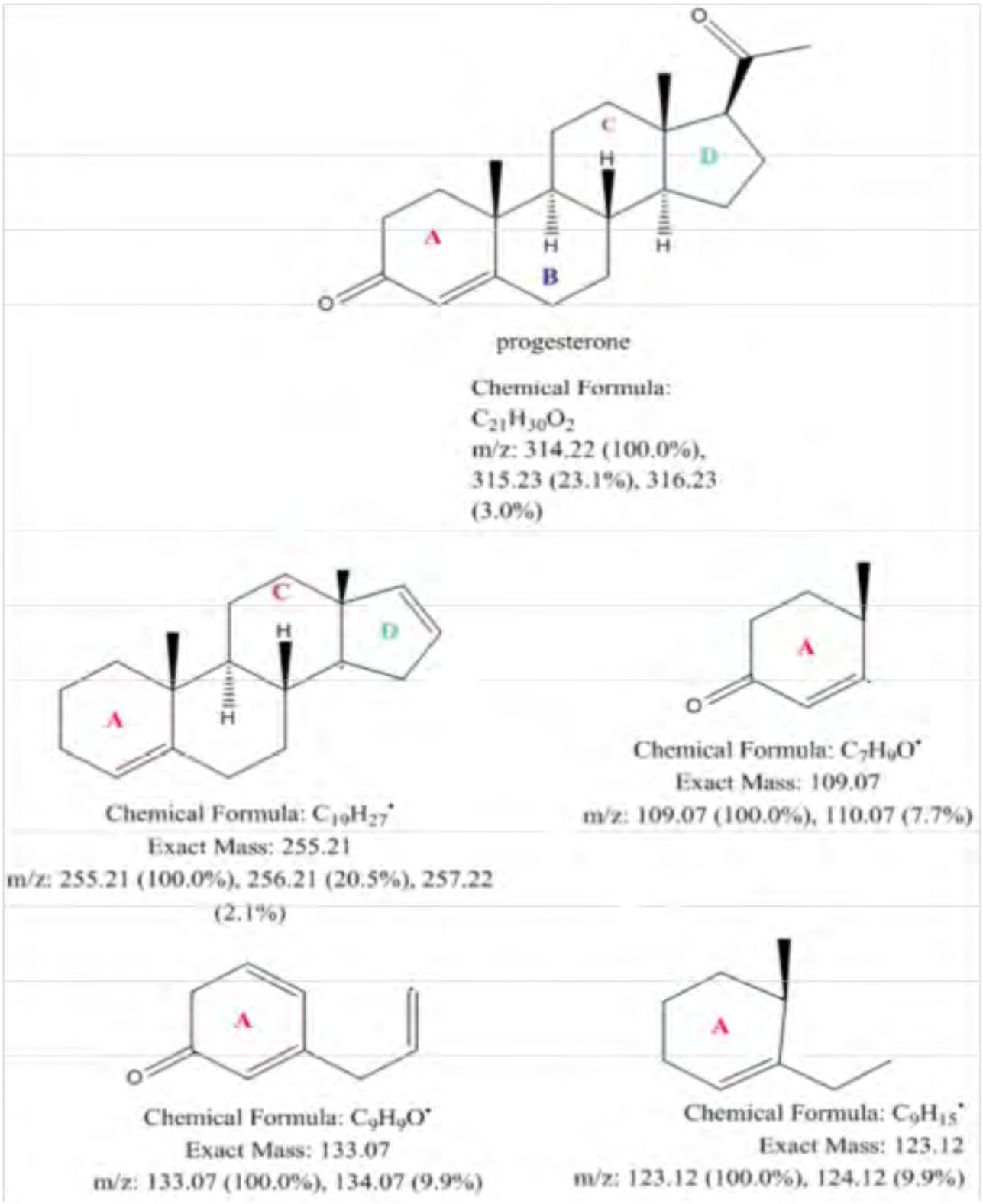

Figure 2: Proposed MS/MS fragmentation of endogenous Progesterone from urine matrix

B seems to be fragmentation mechanism evident in formation of ion with *m/z* of 109.07 as well as *m/z* of 123.1. Partial loss of ring B and total loss of ring C and D from the intact progesterone could produce the fragment with *m/z* of 133.1. However, the extent to which hydrogen rearrangement takes place within progesterone is unknown and how a complex matrix will effect that re-arrangement is entirely speculative.

The relative abundance of *m/z* 255.2 could be due to the easier fragmentation of the peripheral functional groups in most of the progesterone present in the urine matrix samples and spiked standard. Thus, under these conditions along with loss of peripheral groups on rings A and D and with loss of hydrogen on ring D, hydrogen rearrangement may take place leading to formation of positively charged molecule ($C_{19}H_{27}^{\bullet}$) with *m/z* of 255.2. **5.**

Conclusion

As more data from *in vivo* metabolomic samples and perhaps more sophisticated adaptive matching software, are added to the HMDB, rapid identification of marker metabolites will be made with higher degrees of confidence. Until then analytical skill, experience and judgment are critical to the speciality. On balance we are reasonably certain that the metabolomic markers of Down syndrome identified in our study are endogenous dihydrouracil and progesterone; but with only marginally more confidence than the supercomputer, chaos theory modelled, weather forecast for the next day.

References

1. Trivedi DK, Iles RK (2014) Do not just do it, do it right: urinary metabolomics--establishing clinically relevant baselines. Biomed Chromatogr 28: 1491-1501.
2. Dettmer K, Aronov PA, Hammock BD (2007) Mass spectrometry-based metabolomics. Mass Spectrom Rev 26: 51-78.
3. Liesenfeld DB, Habermann N, Owen RW, Scalbert A, Ulrich CM (2013) Review of mass spectrometry-based metabolomics in cancer research. Cancer Epidemiol Biomarkers Prev 22: 2182-2201.
4. Trivedi DK, Iles RK (2012) The application of SIMCA P+ in shotgun metabolomics analysis of ZIC® HILIC-MS spectra of human urine-experience with the Shimadzu IT-TOF and profiling solutions data extraction software. J Chromatogr Sep Tech 3: 1-5
5. Trivedi DK, Jones H, Shah A, Iles RK (2012) Development of Zwitterionic Hydrophilic Liquid Chromatography (ZIC®HILIC-MS) Metabolomics Method for Shotgun Analysis of Human Urine. J Chromat Separation Techniq 3: 144.
6. Trivedi DK, Iles RK (2014) HILIC-MS-based shotgun metabolomic profiling of maternal urine at 9–23 weeks of gestation – establishing the baseline changes in the maternal metabolome. Biomed Chromatogr 29: 240-245.
7. Sana TR, Gordon DB, Fischer SM, Tichy SE, Kitagawa N, et al. (2013) Global mass spectrometry based metabolomics profiling of erythrocytes infected with Plasmodium falciparum. 8: e60840.
8. Wood PL (2014) Mass spectrometry strategies for clinical metabolomics and lipidomics in psychiatry, neurology, and neuro-oncology. Neuropsychopharmacology 39: 24-33.
9. Trivedi DK, Iles RK (2014) Shotgun metabolomic profiles in maternal urine identify potential mass spectral markers of abnormal fetal biochemistry–dihydrouracil and progesterone in the metabolism of Down syndrome, Biomed Chromatograph.
10. Sparidans RW, Bosch TM, Jorger M, Schellens JHM, Beijnen JH (2006) Liquid chromatography-tandem mass spectrometric assay for the analysis of Uracil, 5,6-dihydrouracil and ß-ureidopropionic acid in urine for the measurement of the activities of the pyrimidine catabolic enzymes. Journal of Chromatography B 839: 45-53.
11. Jeilani YA, Cardelino BH, Ibeanusi VM (2011) Hydrogen rearrangement and ring cleavage reactions study of progesterone by triple quadrupole mass spectrometry and density functional theory. J Mass Spectrom 46: 625-634.
12. Zhang Y, Wang Z, Ma Z, Cheng Y (2008) Characterization of progesterone derivatives by LC-DAD-ESI/MSn and its application to the identification of impurities in Flurogestone acetate. Chromatographia 68: 903-909.
13. Hammerum S, Djerassi C (1975) Mass spectrometry in structural and stereochemical problems – CCXLIV: The influence of substituents and stereochemistry on the mass spectral fragmentation of progesterone. Tetrahedron 31: 2391-2400.