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Research Article





Comparison of liquid chromatography-high-resolution tandem mass spectrometry (MS^2) and multi-stage mass spectrometry (MS^3) for screening toxic natural products

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ABSTRACT

Background: Liquid chromatography-high-resolution mass spectrometry (LC-HR-MS) has emerged as a powerful analytical technology for compound screening in clinical toxicology. To evaluate the potential of LC-HR-MS³ in detecting toxic natural products, a spectral library of 85 natural products (79 alkaloids) that contains both MS² and MS³ mass spectra was constructed and used to identify the natural products. Samples were analyzed using an LC-HR-MS³ method and the generated data were matched to the spectral library to identify the natural products. Methods: To test the performance of the LC-HR-MS³ method in different sample matrices, the 85 natural product standards were divided into three groups to separate structural isomers and avoid ion suppression effects caused by co-elution of multiple analytes. The grouped analytes were spiked into drug-free serum and drug-free urine to produce contrived clinical samples.

Results: The compound identification results of the 85 natural products in urine and serum samples were obtained. The match scores using both MS^2 and MS^3 mass spectra and those using only MS^2 mass spectra were compared at 10 different analyte concentrations. The two types of data analysis provided identical identification results for the majority of the analytes (96% in serum, 92% in urine), whereas, for the remaining analytes, the MS²-MS³ tree data analysis had better performance in identifying them at lower concentrations.

Conclusion: This study shows that in comparison to LC-HR-MS (MS²), LC-HR-MS³ can increase the performance in identification of a small group of the toxic natural products tested in serum and urine specimens.

1. Introduction

Liquid chromatography-high-resolution mass spectrometry (LC-HR-MS) has emerged as a powerful analytical technology for compound screening in clinical toxicology. It is capable of determining the accurate mass and corresponding molecular formula of an analyte. Following a full scan, specific molecular ions can be selected for fragmentation, and the corresponding product ions can be monitored allowing for tandem mass spectrometry (MS/MS or MS²). This methodology shows excellent performance in the identification of compounds in complex biological matrices.[1-3] For some instrument platforms, additional generations of product ions can be produced via fragmentation, often referred to as multi-stage mass spectrometry (MSⁿ).[4] MS³ product ions can be generated from an MS² product ion. In MS³ analysis, as illustrated in Fig. 1, the fragmentation patterns of precursor ions and those of MS^2 product ions can be matched to a spectral library that contains both MS² and MS³ mass spectra for compound identification, providing structural information and confidence in the detection of targets. [5-9] LC-HR-MS³ could potentially enhance the specificity or detection limit for identifying compounds of clinical interest, [10] specifically in clinical toxicology applications.

Natural products are chemical substances produced by living organisms, mainly plants.[11] Many natural products have biological effects on humans and animals, and some of them are used as pharmaceuticals.[12,13] A comprehensive screening method for toxic natural products is necessary to aid in their identification and to better

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Abbreviations: LC-HR-MS, liquid chromatography-high-resolution mass spectrometry; MS/MS or MS², tandem mass spectrometry; MSⁿ, multi-stage mass spectrometry; DDA, data-dependent acquisition; MeOH, methanol; CAN, acetonitrile; HCD, high-energy C-trap dissociation.

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Fig. 1. Data acquisition and compound identification workflow of the LC-HR-MS³ method.

understand their potential toxic effects in clinical toxicology cases. However, the large number of toxic natural products, including many structural analogs, makes it challenging to develop a comprehensive set of conventional immunoassays to screen them. It is particular due to the fact that an immunoassay lacks a wide coverage of targets and has limited specificity to structural analogs.

Previously we described an LC-HR-MS (MS²) method for the identification of toxic natural products. [3,14] It is currently unknown whether utilizing LC-HR-MS³ provides enhanced performance for identifying small molecules in clinical toxicology cases in comparison to MS^2 . To evaluate the potential of LC-HR-MS³ in detecting toxic natural products, a spectral library of 85 natural products containing both MS² and MS³ mass spectra was constructed using a quadrupole-linear-ion-trap-Orbitrap tandem mass spectrometer. The spectral library focused on alkaloids (79 out of 85 natural products), which represent a major and commonly encountered group of toxic natural products.[15,16] As clinical toxicology tests are most often performed using blood and urine samples,[17] the study used serum and urine sample matrices spiked with the analytes. Samples were analyzed using an LC-HR-MS³ datadependent acquisition (DDA) method with the same fragmentation conditions as the spectral library construction. The generated data were matched to the spectral library to identify the natural products in two data processing mechanisms: [1] matching both MS² and MS³ mass spectra to the spectral library, and [2] matching only MS² mass spectra to the spectral library.

2. Materials and methods

2.1. Chemicals and reagents

Among the 85 natural product standards, 9 (bufotenine, cathinone, coniine, cotinine, dihydrokavain, ephedrine, mitragynine, pseudoephedrine, yangonin) in either dry powder or solution state were purchased from Cerilliant (Round Rock, TX), and all the other 76 natural product standards in dry powder state were purchased from ChemFaces (Wuhan, China). The complete list of the 85 natural products can be

found in Table 1. LC-MS grade methanol (MeOH), acetonitrile (ACN), and water were purchased from Fisher Scientific (Waltham, MA). Pooled drug-free serum was purchased from Bio-Rad (Hercules, CA), and pooled drug-free urine was purchased from UTAK Laboratories (Valencia, CA).

2.2. Sample preparation

Natural product standards were dissolved in 1:1 ACN: dimethyl sulfoxide to 0.50 mg/mL and then diluted in a sample diluent (1:1:2 mixture of MeOH, ACN, and 5.0 mM ammonium formate in water, added with 0.05 % formic acid) to 1.0 μ g/mL for spectral library construction.

To test the performance of the LC-HR-MS³ method in different sample matrices, the 85 natural product standards were divided into three groups to separate structural isomers and avoid ion suppression effects caused by co-elution of multiple analytes, as indicated in Table 1. The grouped analytes were spiked into drug-free serum and drug-free urine to produce contrived clinical samples. A series of serum samples were made at analyte concentrations of 0.50, 1.0, 2.5, 5.0, 10, 25, 50, 100, 250, and 500 ng/mL, and a series of urine samples were made at analyte concentrations of 1.0, 2.5, 5.0, 100, 250, 500, and 1000 ng/mL.

For serum samples, 125 μ L serum was mixed with 375 μ L acetonitrile. The sample was centrifuged at 13000 rpm for 10 min, and 400 μ L supernatant was taken and dried under nitrogen flow at 37 °C. The sample was reconstituted in 50 μ L sample diluent. For urine samples, 100 μ L urine sample was mixed with 400 μ L sample diluent.

2.3. LC-HR-MS³ method

The LC-HR-MS³ method utilized an Orbitrap ID-X Tribrid mass spectrometer coupled with a Vanquish UHPLC (Thermo Fisher Scientific, San Jose, CA). LC separation was carried out using an Accucore C18 column (2.1 mm \times 100 mm, 2.6 μm particle) with gradient elution (mobile phase A: 5 mM ammonium formate in water with 0.05 % formic acid; mobile phase B: MeOH: ACN 1:1 with 0.05 % formic acid). The

Table 1

The compound identification results of the 85 natural products in urine and serum samples using the LC-HR-MS³ analysis. If a compound at a certain concentration was identified by both the MS²-MS³ tree data analysis and MS² data analysis, the corresponding cell is marked green. The cell is marked yellow if a compound was identified by only the MS²-MS³ tree data analysis, or it is blank if a compound was identified by neither of them. The difference between the match scores (MS²-MS³ tree data analysis) is written in each cell (blank if the compound was not identified).

| Annalitien | CAS | Typical Natural Source | Biological Activity
 | Compound Type
 | Molecular Formula | Analyte Group | 500
 | 250 | 10 | Conce
0 50 | ntratio
25 | n in Seru | m (ng/m)
5 | 2.5
 | 1 0. | 5 100 | 00 5 | 00 25 | 0 100
 | ntration
50 | in Urine (n
25 | z/ml)
10 5 | 2.5 | 1 |
|--|---|--

--
---|---|---
--|---|-----|---
---|---|---|--|---|---
--|--|---|---|---|--|-----|--|
| Moontune | 302-27-2 | Aconitum species | Cardiovascular Toxicity / Neurotoxicity / Gastrointestinal
Toxicity
 | Alkaloid
 | C34H47ND11 | 2 | 0
 | 0 | 0 | 0 | 0 | 0 | 0 | 0
 | 0 0 | 0 | 0 | 0 0 | 0
 | 0 | 0 | 0 0 | 0 | 0 |
| Ajmaline | 4360-12-7 | Rauvolfia verticillata | Cardiovascular Toxicity / Neurotoxicity
 | Alkaloid
 | C20H26N2O2 | 1 | 0
 | 0 | 0 | 0 | 0 | 0 | 0 |
 | | 0 | 0 | 0 0 | 0
 | 0 | | | | |
| Allomatrine | 641-39-4 | Sophora alopecuroides | Neurotoxicity / Cardiovascular Toxicity
 | Alkaloid
 | C15H24N2O | 3 | 0
 | 0 | 0 | 0 | 0 | 0 | 0 | 0
 | 0 | 0 | 0 | 0 0 | 0
 | 0 | 0 | 0 0 | | |
| Anagyrine | 486-89-5 | Cytisus scoparius (Common Broom) / Lupinus species (Lupine) | Neurotoxicity / Cardiovascular Toxicity
 | Alkaloid
 | C15H20N2O | 3 | -4
 | -2 | - 3 | -5 | -7 | 0 | 0 | 0
 | | -7 | 7 | 0 0 | 0
 | 0 | 0 | | | |
| Arecoline | 63-75-2 | Areca catechu | Cardiovascular Toxicity / Neurotoxicity / Gastrointestinal
Toxicity
 | Alkaloid
 | C8H13ND2 | 2 | 1
 | | | | | | |
 | | 3 | 3 | 6 6 | 18
 | 40 | | | | |
| Atropine | 51-55-8 | Atropa belladonna | Neurotoxicity / Cardiovascular Toxicity
 | Alkaloid
 | C17H23NO3 | 1 | -1
 | | | | | | |
 | | 0 | | 1 0 | 0
 | -1 | -1 | -1 0 | 0 | 0 |
| Berberine | 2086-83-1 | Coptis chinersis (Chinese Goldthread); Corydalis yanhusuo;
Hydrastis canademis (Goldenseal) / Berberis species | Gastrontestinal Toxicity / Cardiovascular Toxicity / Neurotoxicity
/ Henatotoxicity
 | Alkaloid
 | C20H18NO4 | 1 | 0
 | 0 | 0 | 0 | 0 | 0 | 0 |
 | | | | |
 | | | | | |
| Boldine | 476-70-0 | Litsea glutinosa | Hepatotoxicity
 | Alkaloid
 | C19H21NO4 | 1 | -1
 | -3 | -1 | . 0 | 0 | -3 | 0 | 3
 | 2 | 0 | 0 | 0 -3 | 3 -1
 | -1 | 1 | 4 8 | 10 | |
| Brucine | 357-57-3 | Strychnos nux-vomica | Neurotoxicity / Rhabdomyolysis / Nephrotoxicity
 | Alkaloid
 | C23H26N2O4 | 1 | 0
 | 0 | 0 | 0 | 0 | 0 | 0 | 0
 | | 0 | • | 0 0 | 0
 | 0 | 0 | 0 0 | 0 | |
| Bufotenine | 487-93-4 | Bufo gargarizans (Asiatic Toad) | Gastrointestinal Toxicity / Cardiovascular Toxicity /
Neurotraticity
 | Alkaloid
 | C12H16N2O | 3 | 0
 | -1 | -1 | . 0 | -1 | 0 | -3 |
 | | 0 | • | 0 -1 | L 0
 | 0 | 2 | 0 | | |
| Camptothecin | 7689-03-4 | Camptotheca acuminata | Hematologic Toxicity / Gastrointestinal Toxicity
 | Alkaloid
 | C20H16N2O4 | 3 | 0
 | 0 | 0 | 0 | 0 | | |
 | | 0 | • | 0 0 | 0
 | 0 | 0 | 0 | | |
| Catharanthine | 2468-21-5 | Catharanthus roseus (Rose Periwinkle) | Neurotoxicity / Hematologic Toxicity / Gastrointestinal Toxicity /
Hair Loss
 | Alkaloid
 | C21H24N2O2 | 1 | 0
 | 2 | 1 | 17 | 15 | 17 | 2 | 14 1
 | 2 15 | 5 2 | 2 | 1 1 | 10
 | 16 | 14 | 18 26 | 38 | |
| Cathinone | 71031-15-7 | Catha edulis (Khat) | Cardiovascular Toxicity / Neurotoxicity
 | Alkaloid
 | C9H11NO | 1 | 0
 | 0 | 0 | 0 | | | |
 | | 0 | 0 | 0 0 | 0
 | | | | | |
| Cannabidiol | 13956-29-1 | Cannabis sativa | Inactive Constituent of Cannabis sativa
 | Terpenoid
 | C21H3002 | 3 | 0
 | 0 | 21 | 3 0 | | | |
 | | 31 | 1 | 0 |
 | | | | | |
| Cannabinol | 521-35-7 | Cannabis sativa | Constituent of Cannabis sativa
 | Terpenoid
 | C21H26O2 | 1 | 0
 | 0 | 0 | | | | |
 | | 0 | • | 0 |
 | | | | | |
| Cephalotaxine | 24316-19-6 | Cephalotaxus fortunei | No Reported Toxicity
 | Alkaloid
 | C18H21NO4 | 2 | 0
 | 0 | 0 | 0 | 0 | 0 | |
 | | 0 | • | 0 0 | 0
 | 0 | 0 | | | |
| Chelerythrine | 34316-15-9 | Chelidonium majus | Hepatotoxicity
 | Alkaloid
 | C21H18NO4 | 2 | 0
 | | | | | | |
 | | | | |
 | | | | | |
| Chelidonine | 476-32-4 | Chelidonium majus | Hepatotoxicity
 | Alkaloid
 | C20H19NO5 | 2 | 0
 | 0 | 0 | 0 | 0 | 0 | 0 | 0
 | 0 0 | 0 | 0 | 0 0 | 0
 | 0 | 0 | 0 0 | 0 | 0 |
| Onchonine | 118-10-5 | Cinchona ledgeriana | Cardiovascular Toxicity / Neurotoxicity (presumably / found with
guinine)
 | Alkaloid
 | C19H22N2O | 2 | 0
 | 0 | 0 | 0 | 0 | 0 | 0 | 0
 | D | 0 | 0 | 0 0 | 0
 | | | | | |
| Cinnamoylcocaine | 521-67-5 | Erythroxylum coca | Toxicity Not Described
 | Alkaloid
 | C19H23NO4 | 2 | 0
 | 0 | 0 | 0 | 0 | | |
 | | 0 | • | 0 0 | 0
 | 0 | 0 | 0 | | |
| Colchicine | 64-86-8 | Colchicum autumnale (Autumn Crocus) | Gastrointestinal Toxicity / Hematologic Toxicity / Cardiovascular
Toxicity
 | Alkaloid
 | C22H25NO6 | 1 | 0
 | 0 | 0 | 0 | 0 | | |
 | | 0 | 0 | 0 0 | •
 | | | | | |
| Conline | 3238-60-6 | Conium maculatum (Poison Hemlock); Oenanthe javanica (Java
Waterdropwort) | Neurotoxicity / Cardiovascular Toxicity
 | Alkaloid
 | C8H17N | 3 | 0
 | 0 | 0 | 0 | 0 | 0 | |
 | | 0 | 0 | 0 0 | 21
 | 0 | 0 | 0 | | |
| Coptisine | 3486-66-6 | Chelidonium majus; Corydalis yanhusuo | Cardiovascular Toxicity / Gastrointestinal Toxicity /
Hepatotoxicity
 | Alkaloid
 | C19H14NO4 | 2 | 0
 | 0 | 0 | | Γ | Γ | | _
 | | Г | | |
 | Γ | | | | |
| Corydaline | 518-69-4 | Corydalis ambigue; Corydalis yanhusuo | CYP2C19 and CYP2C9 Inhibitor
 | Alkaloid
 | C22H27NO4 | 1 | -1
 | 0 | -1 | 0 | 0 | -1 | 0 | 0
 | 2 -2 | 2 -1 | 1 | 0 0 | 1
 | 0 | 1 | 0 0 | 10 | 18 |
| (+)-Corynoline | 18797-79-0 | Corydalis ambigua; Corydalis incisa | CYP3A4 Inhibitor
 | Alkaloid
 | C21H21NO5 | 3 | 0
 | -1 | -1 | . 0 | 0 | 0 | 0 | 0
 | 0 0 | -1 | 1 | 0 0 | 0
 | 0 | 0 | 0 1 | 0 | 0 |
| Cotinine | 486-56-6 | Nicotiana tabacum (Tabacco) | Nicotine Metabolite
 | Alkaloid
 | C10H12N2O | 1 | 0
 | 0 | -1 | 0 | 0 | 0 | |
 | | 0 | • | 0 0 | 0
 | 0 | 0 | \Box | | |
| Crotaline | 315-22-0 | Crotalaria pallida | Hepatotoxicity
 | Alkaloid
 | C16H23NO6 | 2 | $^{(1)}$
 | -1 | -1 | 0 | 0 | 0 | 0 | 0
 | 3 | -1 | 1 | 0 0 | 0
 | 0 | 0 | 0 0 | | |
| Cytisine | 485-35-8 | Genista tinctoria (Dyer's Greenweed) | Neurotoxicity / Cardiovascular Toxicity / Gastrointestinal
Toxicity
 | Alkaloid
 | C11H14N2O | 2 | 0
 | 0 | 0 | 0 | 0 | 0 | 0 | 46
 | | 0 | D | 0 0 | 0
 | 0 | 6 | 0 | | |
| Dihydrocapsaicin | 19408-84-5 | Capsicum annuum (Chili Peppers) | Neurotoxicity / Irritant
 | Alkaloid / Capsaicinoid
 | C18H29NO3 | 3 | -2
 | -1 | -1 | -2 | -4 | -5 | 0 | 0
 | | -1 | 1 | 0 -3 | 0
 | • | 0 | 0 0 | | |
| Dihydrokavain | 587-63-3 | Piper methysticum (Kava) | Hepatotoxicity
 | Lactone
 | C14H16O3 | 1 | -4
 | -1 | 0 | 0 | -4 | 0 | 0 | 0
 | 0 0 | 0 | D I | 0 -1 | ı o
 | 0 | 0 | 0 0 | 0 | 0 |
| Dihydrosanguinarine | 3606-45-9 | Chelidonium majus | Cardiovascular Toxicity
 | Alkaloid
 | C20H15NO4 | 2 | | | | |
 | | | | | | |
 | | • | Þ | 0 |
 | | | | | |
| Ephedrine | 299-42-3 | Ephedra species | Cardiovascular Toxicity / Neurotoxicity / Hepatotoxicity
 | Alkaloid
 | C10H15NO | 1 | -1
 | -1 | -1 | . 0 | 0 | 0 | - 1 | 2
 | | -1 | 1 | 1 0 | 0
 | 1 | 1 | 4 | | |
| Evodiamine | 518-17-2 | Tetradium ruticarpum | Hepatotoxicity / Cardiovascular Toxicity
 | Alkaloid
 | C19H17N3O | 3 | -1
 | -1 | -1 | -1 | -1 | 0 | -1 | 0
 | 0 0 | -1 | 1 | 4 -1 | -1
 | -1 | 0 | -1 -1 | -1 | 1 |
| Galantamine | 357-70-0 | Lycoris radiata | Neurotoxicity / Cardiovascular Toxicity
 | Alkaloid
 | C17H21NO3 | 2 | -2
 | -2 | -1 | -1 | -1 | -2 | -2 | -2
 | | -3 | 3 | 3 4 | 6
 | 3 | | | | |
| Glaucine | 475-81-0 | Corydalis yanhusuo | Neurotoxicity
 | Alkaloid
 | C21H25NO4 | 1 | 0
 | 0 | 0 | 0 | 0 | 0 | 0 | 13 1
 | 4 14 | ¢ 0 | • | 0 0 | •
 | 0 | 0 | 0 13 | 15 | 15 |
| Harmalidine | 109794-97-0 | Peganum harmala (Wild Rue) | Neurotoxicity / Hernatologic Toxicity / Cardiovascular Toxicity
 | Alkaloid
 | C16H18N2O | 3 | 0
 | 0 | 6 | 0 | 0 | | |
 | | 0 | • | 0 0 | •
 | 0 | 12 | | | |
| Harmaline | 304-21-2 | Peganum harmala (Wild Rue) | Neurotoxicity / Hematologic Toxicity / Cardiovascular Toxicity
 | Alkaloid
 | C13H14N2O | 1 | 0
 | 0 | 0 | 0 | 0 | 0 | 0 | 0
 | 0 0 | 0 | • | 0 0 | 0
 | 0 | 0 | 0 0 | 0 | 0 |
| Harmane | 486-84-0 | Peganum harmala (Wild Rue) | Neurotoxicity / Hernatologic Toxicity / Cardiovascular Toxicity
 | Alkaloid
 | C12H10N2 | 3 | 0
 | -1 | 0 | 0 | 0 | 0 | 0 |
 | | ٥ | 0 | • |
 | | | | | |
| Harmine | 442-51-3 | Peganum harmala (Wild Rue) | Neurotoxicity / Hematologic Toxicity / Cardiovascular Toxicity
 | Alkaloid
 | C13H12N2O | 1 | 0
 | 0 | 0 | 0 | 0 | 0 | 0 | 0
 | 0 0 | 0 | • | 0 0 | 0
 | 0 | 0 | 0 4 | 4 | 4 |
| Hordenine | 539-15-1 | Dendrolobium triangulare | Toxicity Not Described
 | Alkaloid
 | C10H15NO | 2 | 0
 | 0 | 0 | 0 | 0 | 1 | -4 | 5
 | | 0 | • | 1 3 | 13
 | | | | | |
| Hyoscyamine | 101-31-5 | Atropa belladonna | Neurotoxicity / Cardiovascular Toxicity
 | Alkaloid
 | C17H23NO3 | 3 | 0
 | 0 | -1 | . 0 | -1 | -1 | 0 | -1 -
 | 1 2 | -1 | 1 | 0 -1 | i -1
 | -1 | -1 | -1 0 | 0 | 0 |
| Hypaconitine | 6900-87-4 | Aconitum species | Toxicity / Cardiovascular Toxicity / Gastrointestinal
Toxicity
 | Alkaloid
 | C33H45NO10 | 2 | 0
 | 0 | 0 | 0 | 0 | 0 | 0 | 0
 | 0 0 | 0 | • | 0 0 | •
 | 0 | 0 | 0 0 | 0 | 0 |
| Ibogaine | 83-74-9 | Ervatamia hirta | Cardiovascular Toxicity / Neurotoxicity
 | Alkaloid
 | C20H26N2O | 2 | 0
 | 0 | 0 | 0 | 0 | 0 | 0 | 0
 | | 0 | • | 0 0 | 6
 | 5 | | | | |
| Lappaconitine | 32854-75-4 | Aconitum species | Cardiovascular Tolicity / Neurotoscity / Gastromescinal
Toxicity
 | Alkaloid
 | C32H44N2O8 | 3 | 1
 | 6 | 1 | 0 | 0 | 0 | 0 | 1
 | 0 0 | 2 | 2 | 1 0 | 0
 | 6 | 0 | 0 1 | 0 | 0 |
| Leonurine | 24697-74-3 | Leonurus heterophyllus | Neurotoxicity
 | Alkaloid
 | C14H21N3O5 | 3 | 0
 | 0 | -1 | -1 | -1 | -1 | 0 | 0
 | 2 1 | . 0 | • · | 4 4 | L 0
 | -1 | 0 | -1 0 | -4 | 0 |
| Lobelin | 90-69-7 | Lobelia sessilifolia | Neurotoxicity / Cardiovascular Toxicity
 | Alkaloid
 | C22H27NO2 | 1 | 0
 | 0 | 0 | 19 | 0 | 0 | 19 | 20 2
 | 1 23 | 1 16 | 6 : | 14 0 | 19
 | 20 | 20 | 19 20 | 19 | |
| Lupanine | | |
 |
 | | | 0
 | 0 | | | | | 0 |
 | | | | |
 | | | | | |
| | 550-90-3 | Lupinus species (Lupine) | Neurotoxicity / Cardiovascular Toxicity
 | Alkaloid
 | C15H24N2O | 1 |
 | | 0 | Ť | | 0 | Ť |
 | | 0 | • | 0 0 | 0
 | 0 | 0 | | | |
| Lycorine | 550-90-3
476-28-8 | Lupinus species (Lupine)
Lycoris radiata | Neurotoxicity / Cardiovascular Toxicity
Gastrointestinal Toxicity
 | Alkaloid
 | C15H24N2O
C16H17ND4 | 1 | 0
 | 0 | 0 | 0 | 0 | 0 | 0 | 0
 | | 0 | • | o o | 0
 | 0 | 6 | | | |
| Lycorine
Matrine | 550-90-3
476-28-8
519-02-8 | Lupinus species (Lupinė)
Lycoris radiata
Sophora Japonica | Neurotoxicity / Cardiovascular Toxicity
Gastrointestinal Toxicity
Neurotoxicity
Neurotoxicity
 | Alkaloid
Alkaloid
Alkaloid
 | C15H24N2O
C16H17NO4
C15H24N2O | 1
2
2 | 0
 | 0 | 0 | 0 | 0 | 0 | 0 | 0
 | | 0 | o
o | 0 0
0 0 |
 | 0 | 0
6
0 | 0 0 | | |
| Lycorine
Matrine
Mesaconitine | 550-90-3
476-28-8
519-02-8
2752-64-9 | Lupinus species (Lupine)
Lycoris radiata
Sophora japonica
Accentum species | Neurotoxicity / Cardiousscular Toxicity Gastrointestinal Toxicity Neurotoxicity Neurotoxicity / Cardiousocular Toxicity / Gastrointestinal Neurotoxicity / Cardiousocular Toxicity / Gastrointestinal Cardiousouclar Unity / Neuromatricity / Gastrointestinal
 | Alkaloid
Alkaloid
Alkaloid
Alkaloid
 | C15H24N2O
C16H17ND4
C15H24N2O
C33H45N011 | 1
2
2
2 | 0
0
0
 | 0
0
0 | 0 | 0 | 0 | 0 | 0 | 0
0
 | 0 0 | 0
0
0 | o
o
o | 0 0
0 0
0 0 |
 | 0
0
0 | 0
6
0 | 0 0
0 0 | 0 | |
| Lycorine
Matrine
Mesaconitine
N-Methylcytisine | 550-90-3
476-28-8
519-02-8
2752-64-9
486-86-2 | Lupinos species (Lupino)
Lipcoris radiata
Sephora Japonica
Acontium species
Sephora Rinescens | Neurotaxichy / Cardiovascular Texicity
Gastrointestinal Texicity
Neurotaxichy / Cardiovascular Toxicity / Gastrointestinal
Cardiovascular Toxicity / Reartoxicity / Gastrointestinal
Toxicity
 | Akaloid
Akaloid
Akaloid
Akaloid
Akaloid
 | C15H24N2O
C16H17ND4
C15H24N2O
C33H45NO11
C12H16N2O | 1
2
2
2
1 | 0
0
0
 | 0 | 0 | 0 | 0 | 0 0 0 0 0 0 0 0 | 0
0
0 | 0
0
 | 0 0 | 0
0
0
0
0 | 0 0
0 0
0 0 | 0 0
0 0
0 0
0 0 | · 0
· 0
· 0
 | 0
0
0
0 | 0
6
0
0 | 0 0
0 0 | 0 | |
| Lycorine
Matrine
Mesaconitine
N-Methylcytisine
Methylisopelletisrine | 550-90-3
476-28-8
519-02-8
2752-64-9
486-86-2
18747-42-7 | Lupinos species (Lopend)
Lycotis radata
Sophera Japonca
Acothum species
Sophera Barvecens
Sophera Barvecens
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Mangena pacidas (Krastun)
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22669-628-8 | Lapina space lipsiha)
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Lephas species
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Fig. 2. The MS² and MS³ mass spectra acquired from the precursor ion of atropine in the LC-HR-MS³ analysis. The colored circles denote the corresponding MS² fragment and MS³ mass spectrum.

column temperature was set at 35 °C. The sample injection volume was set at 5 μ L, and the autosampler temperature at 10 °C. For MS analysis, ESI positive ion mode was selected, the spray voltage was set at 3.4 kV, the ion-transfer capillary temperature at 300 °C, sheath gas flow rate at 40, aux gas flow rate at 10, aux gas heater temperature at 375 °C, RF lens level 45.

DDA was implemented in scan cycles, each of which consisted of the following steps: (1) a full-scan with a scan range m/z 100–1000 at 120 K resolution, (2) higher-energy C-trap dissociation (HCD) fragmentation of a precursor ion followed by MS² product-ion scan with auto mass range at 30 K resolution, and (3) HCD fragmentation of a MS² product ion followed by MS³ product scan with auto mass range at 7.5 K resolution. In step (2), the top 10 abundant precursor ions in a full-scan were selected with an isolation window of 1.5 m/z and assisted collision energy optimization was employed to automatically pick a normalized HCD energy value from 20, 35, 45, 55, and 65. The assisted collision energy optimization was implemented in the linear ion trap in the mass spectrometer, which had a higher scan speed than the Orbitrap mass analyzer. An inclusion list with the mass-to-charge ratios of all the analytes was referenced during the full-scan to increase the detection capability for the compounds of interest. The inclusion list can be expanded if more compounds are added to the spectral library, but it should be noted that if more than 10 analytes co-elute in the LC-HR-MS³ method, only the 10 most abundant analytes will be selected from a fullscan for fragmentation. In step (3), the top three MS^2 product ions were selected with an isolation window of 2 m/z and a normalized HCD energy of 30 was used. To ensure high mass accuracy, EASY-IC internal mass calibration was employed.

2.4. Data analysis

The data analysis for the LC-HR-MS³ results was performed in Mass Frontier software (Thermo Fisher Scientific, San Jose, CA). The results of the LC-HR-MS³ were analyzed in two data processing mechanisms for comparison. In the MS²-MS³ tree data analysis, data were searched against the spectral library constructed using the 85 natural product standards. Extracted ion chromatograms were generated using joint component detection (JCD) mechanism with built-in mass tolerance based on the mass accuracy of the data. The retention time window was set at \pm 0.1 min for candidate ion extraction.[18] A candidate ion was scored by matching the precursor ion mass, MS² mass spectrum, and MS³ mass spectrum to the corresponding data for a particular compound in the spectral library. In the MS² data analysis, the candidate ion was also scored by matching only the precursor ion mass and MS² mass spectrum to the spectral library. The match scores were generated by proprietary fitting algorithms. A compound was called positive if the match score was over a threshold of 80, which was determined by systematically analyzing the match scores of known analytes.

3. Results and discussion

When implementing the LC-HR-MS³ method, MS^2 and MS^3 mass spectra were generated using the Orbitrap ID-X Tribrid mass spectrometer. The MS^2 and MS^3 mass spectra acquired from one precursor ion are organized in a tree structure in the data analysis software, as shown in Fig. 2. Based on the dissociation reaction mechanisms in mass spectrometry, the in-silico fragmentation prediction tool in the software can annotate the mass peaks in a mass spectrum with predicted chemical structures of product ions. This function can be used to interpret specific features in mass spectra.

Using the LC-HR-MS³ method, the compound identification results of the 85 natural products in urine and serum samples were obtained, as shown in Table 1. The match scores using both MS^2 and MS^3 mass spectra (MS^2-MS^3 tree data analysis) and those using only MS^2 mass spectra (MS^2 data analysis) were compared at 10 different analyte concentrations. Additional information about the natural products, including natural sources and biological activities, is also provided in Table 1.(3) Using a match score of 80 as the threshold for compound identification, if a compound at a certain concentration was identified by both the MS^2-MS^3 tree data analysis and MS^2 data analysis, the



Fig. 3. Two examples showing that the MS² mass spectra contained significant background noise while the MS³ mass spectra did not. (Left) For natural product vindoline in urine (2.5 ng/ml), the MS² mass spectrum of the precursor ion of m/z 457.234 and the MS³ mass spectrum of the MS² fragment of m/z 188.107. (Right) For natural product scopolamine in serum (1.0 ng/ml), the MS² mass spectrum of the precursor ion of m/z 304.154 and the MS³ mass spectrum of the MS² fragment of m/z 138.091.

corresponding cell is marked green. The cell is marked yellow if a compound was identified by only the MS²-MS³ tree data analysis, or it is blank if a compound was identified by neither of them. Five compounds in serum (cannabidiol, cytisine, lobeline, nuciferine, reserpinine) and 11 compounds in urine (arecoline, boldine, catharanthine, cannabidiol, coniine, lobeline, methylisopelletierine, nuciferine, nonivamide, reserpinine, vindoline) at certain concentrations were identified by only the MS^2 - MS^3 tree data analysis. No compound was identified by only MS^2 data analysis. The difference between the match scores (MS^2-MS^3) tree data analysis minus MS² data analysis) is written in each cell (blank if the compound was not identified). In addition, there were three compounds in serum (dihydrosanguinarine, neferine, tetrandrine) and five compounds in urine (berberine, chelerythrine, coptisine, olaquindox, sparteine) not identified at any concentration, probably due to matrix effect in the screening method. This issue is not uncommon in compound screening and can potentially be solved by establishing specific analytical methods with optimized sample preparation protocols. Besides these compounds, Table 1 shows that the two types of data analysis provided identical identification results for the majority of the analytes (96 % in serum, 92 % in urine), whereas, for the remaining analytes, the MS²-MS³ tree data analysis had better performance in identifying them

at lower concentrations. In other words, at higher concentrations none of the analytes required adding MS^3 mass spectra to identify them, but adding MS^3 mass spectra extended the identification to lower concentrations for a small number of analytes (three compounds in serum, six compounds in urine). In the latter cases, the difference can solely be attributed to the employment of MS^3 spectral matching, where the two-level fragmentation in MS^3 analysis provided in-depth structural information of analytes to increase the match score. On the other hand, as the contribution of MS^3 mass spectra was limited for the majority of the analytes, it was indicated that in general the one-level fragmentation in MS^2 analysis could provide sufficient specificity in compound identification.

There were some examples when the MS^3 mass spectra allowed for enhanced identification when MS^2 mass spectra contained significant background noise. Two examples are shown in Fig. 3. For the natural product vindoline in urine (2.5 ng/ml), the MS^2 mass spectrum of the precursor ion of m/z 457.234 contained a significant background peak at m/z 281.172 which resulted in a lower match score, while the MS^3 mass spectrum of the MS^2 product ion of m/z 188.107 provided a good match score to the spectral library. For the natural product scopolamine in serum (1.0 ng/ml), the MS^2 mass spectrum of the precursor ion of m/z



Fig. 4. Differentiation of matrine and lupanine using the MS^3 mass spectra of the MS^2 fragment of m/z 247.180.

304.154 had multiple background peaks which resulted in a lower match score, while the MS^3 mass spectrum of the MS^2 product ion of m/z 138.091 matched well to the spectral library. The background peaks in these examples might be fragment ions from other co-eluting substances that formed precursor ions at the same nominal m/z as the analyte. This observation help explain the differences between the match scores (MS^2-MS^3 tree data analysis minus MS^2 data analysis), which were 20 for vindoline and 19 for scopolamine.

The employment of MS³ spectra in data analysis also increased the depth of structural characterization and enhanced the differentiation of structural isomers. For example, matrine and lupanine are structural isomers and analogs, and the MS² mass spectra of the two analytes contained mass peaks at the same m/z values; a single mass peak may correspond to different product ions with various structures but an identical chemical formula. By checking the MS³ mass spectra of the MS² product ion at m/z 247.180, different MS³ fragmentation patterns were observed, and the two analytes could be differentiated, as shown in Fig. 4. The software interpreted that the MS^2 product ions at m/z247.180 in the two MS² mass spectra actually had different structures, as denoted in the graphs in Fig. 4. The MS³ fragmentation successfully revealed the structural difference between the two MS² product ions with the same m/z value. Although in this study matrine and lupanine were separated into different groups for LC-HR-MS³ analysis, this example demonstrated the effectiveness of MS³ fragmentation patterns in differentiating structural isomers. Given that structural isomers may co-exist in actual samples, the use of MS³ can be particularly beneficial in solving clinical toxicology cases.

Since the mass spectrometer is able to acquire further fragmentation patterns from MS^3 product ions, the use of MS^4 mass spectra for compound identification was tested on some natural products but no benefit was identified. This is likely due to the following facts: (1) MS^3 product ions mostly represent simple structures at low m/z and further fragmentation did not provide additional value for compound identification; and (2) the scan cycle time in DDA was significantly increased to include MS^4 fragmentation, which might reduce the data quality of low-concentration analytes, i.e., missing the chromatographic peak summit or collecting fewer ions for MS^n analysis. Thus, in the current experiment settings, it is unnecessary to employ MS^4 or higher-stage mass spectra for compound screening.

In clinical toxicology cases, accurate identification of toxins significantly aids in patient management and decreases the need for additional diagnostic evaluations. The potential of MSⁿ has been largely unexplored for the identification of toxins in clinical cases. This study shows that in comparison to LC-HR-MS (MS²), LC-HR-MS³ can increase the performance in identification of a small group of the toxic natural products tested in serum and urine specimens. Further investigations are warranted to fully understand the potential advantages of utilizing MS³ mass spectra for compound identification in clinical toxicology, such as testing other types of toxic natural products and synthetic compounds.

In a broader scope of clinical applications, it is clear that the depth of structural characterization would enrich the information content of clinical markers and significantly enhance the performance of clinical tests. Successful application of MS³ has been reported in clinical research, i.e., tissue imaging and pharmacokinetics.[19,20] In addition, MSⁿ can be particularly useful for analyzing clinical specimens in which metabolites are not included in the spectral library or co-eluting substances are present. By providing in-depth structural information about analytes, MSⁿ has the potential to complement the current mainstream tandem mass spectrometry and enhance the performance of mass spectrometry in clinical applications.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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