Detecting Low Abundant Endogenous Cardiac Steroids from Biological Fluids Using Structure-Based MS^n Approach On an Orbitrap Tribrid Mass Spectrometer

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ABSTRACT

Purpose: Developing an unbiased discovery to targeted verification approach to detect low abundant endogenous cardiac steroids from biological fluids with high confidence on a Thermo Scientific™ Orbitrap ID-X™ Tribrid™ mass spectrometer.

Methods: Cerebrospinal fluid (CSF) samples from healthy participants were used as the model of biological fluids. High resolution MS and MS^2/MS^3 data were collected with an Orbitrap Tribrid mass spectrometer. The comprehensive structure relative fragment ion information from MS^2 and MS^3 was processed using Thermo Scientific™ Mass Frontier™ 8.0 software and Thermo Scientific™ Compound Discoverer™ 3.0 software for cardiac steroid annotation. A targeted PRM (parallel reaction monitoring) assay was developed based on the discovery phase results and used for confirming that the cardiac steroids annotated from the discovery phase could reproducibly detected across multiple CSF samples.

Results: Two endogenous cardiac steroids (Digoxigenin & Marinobufagenin) were detected with the discovery experiments and their existences across multiple CSF samples were confirmed with targeted verification experiments.

INTRODUCTION

Endogenous cardiac steroids are specific inhibitors of the sodium pump (Na+/K+-ATPase) and play important biological roles such as regulating cell growth, differentiation, apoptosis, fibrosis, immunity, carbohydrate metabolism, and nervous and mental functions. Detecting endogenous cardiac steroids from biological fluids is very challenging because of their low concentration (pg-ng/ml) ranges and lack of authentic standards. We have developed a novel structure-based MS^n discovery approach which enables annotation of cardiac steroids and related compounds based on basic steroid sub-structure identification using MS^2 and MS^3 spectral tree data. We applied this approach to cerebrospinal fluid (CSF) samples and were able to detect some low abundant endogenous cardiac steroids from the CSF samples. The detected cardiac steroids were further verified using a targeted MS^2 approach. The discovery and verification results are reported here.

MATERIALS AND METHODS

Sample Preparation

CSF sample from a 12 year old healthy male who had a ventricular shunt placed in infancy to treat hydrocephalus was used for the detection of cardiac steroids in the discovery phase. Nine mL of ethanol was combined with 1mL of CSF to precipitate proteins. The supernatant was removed and evaporated to dryness, then reconstituted into 200 ul of 10% methanol. Ventricular CSF samples from three other healthy individuals with longstanding shunts were used with the same sample preparation or filtered using a 3K centrifugal filter for confirmation of detected cardiac steroids in the targeted verification phase.

HPLC Conditions

A Thermo Scientific™ Vanquish™ UHPLC system performed separations. Mobile phase A was water with 0.1% formic acid and mobile phase B was methanol with 0.1% formic acid. The column was a Thermo Scientific™ Hypersil GOLD™ column (2.1 x 150mm, 1.9µm) that operated at 45 ° C and a flow rate of 260 µL/min. Separation of compounds was carried out with gradient elution profile as Table 1. The injection volume was 20 µL.

MS Conditions

All the data was collected on a Orbitrap ID-X Tribrid mass spectrometer. The mass spectrometer set up is shown in Table 2. For the precursor ion mass range between 300 – 550 m/z, data dependent HCD MS^2 scans were collected. For the precursor ion mass range between 550 – 1250 m/z, an intelligent product ion-dependent MS^n approach was used, in which an HRAM full MS scan was followed by CID MS^2 scans. The product ions generated from each MS^2 scan are monitored by instrument and a CID MS^3 scan is further triggered if one or multiple pre-defined neutral sugar molecules were detected from an MS^2 scan (Figure 1).

Data Analysis

Mass Frontier 8.0 software and Compound Discoverer 3.0 software were used for cardiac steroid detection. mzCloud™ spectral library and ChemSpider™ database were employed in the data processing workflow.

Table 1. HPLC Gradient

Time	% A	%B
0	95	5
1	95	5
15	50	50
22	5	95
25	5	95
25.1	95	5
30	95	5

Table 2. Orbitrap ID-X instrument set up

ESI source	Orbitrap-ID-X
Sheath gas 35	Pos Ion (150-1200 amu)
Aux gas 5	MS: R=60K (FWHM at m/z 200)
Spray volt. 3.4 kV	MSn: R=15K (FWHM at m/z 200)
RF-Lens 40	Cycle time: 1.2 second
Cap. temp. 300°C	MS^2 Isolation width: 1.6 Da
Heater temp. 300°C	MS^n Isolation width: 1.6 Da (MS2) → 2.0 Da (MSn)

RESULTS

MS^n workflow development for cardiac steroids detection

Cardiac steroids are a class of compounds with a steroid nucleus and a five-membered lactone ring (cardenolides) or a six-membered lactone ring (bufadienolides) attached in the C17 position. If the cardiac steroids also contain sugar residues in C3 position, they are called cardiac glycosides. Currently, only a few endogenous cardiac steroids have been reported from biological samples such as plasma and urine[1,2]. Because human cerebrospinal fluid (CSF) reflects pathology in the brain and inhibits the activity of the Na+/K+ pump in human red blood cells, there is a high research interest to identify if any endogenous cardiac steroids exist in human CSF and can be used as biomarkers for early heart related disease diagnoses.

However, it is very challenging to detect the endogenous cardiac steroids from CSF because of their low concentration range and limited spectra references in the spectral libraries. In order to address this challenge, we developed a structure-based MS^n workflow aimed to detect the compounds which contain the common cardiac steroid structure with or without exact spectral library match. So we can focus on these putative cardiac steroid class compounds for structure annotation using MS^2 and complementary MS^3 fragment ion information. In order to use instrument time more efficiently, we developed a product ion dependent workflow which collects MS^2 data on the precursor ions constantly and only collects MS^3 data if a sugar neutral loss is detected from the MS^2 data (Figure 1, A). The MS^n spectra tree generated from each unknown compound is searched against the mzCloud and custom MS^n spectral libraries to detect which compound contains the common cardiac steroid sub-structure (Figure 1 B) with Mass Frontier 8.0 software. The detected compounds that had full or partial cardiac steroid library match can be classified into cardiac steroid class. For those that had only sub-structure identification via the partial MS^n spectra tree match, the completed structure candidates can be proposed by searching the ChemSpider database with Compound Discoverer 3.0 software. If multiple structure candidates were proposed, the FISh score (number of matching fragments / total number of fragments above S/N threshold x 100%) can be used for structure ranking (Figure 1C).

Applying the structure-based MS^n workflow to CSF sample for detecting the endogenous cardiac steroids

The developed structure-based MS^n workflow was applied to the CSF sample for evaluating if any endogenous cardiac steroid compounds could be detected from the CSF sample with this approach. Figure 2 shows the extracted base peak chromatograms for MS, MS^2 and MS^3 acquired from the CSF sample. The collected MS^3 spectral tree data were searched against mzCloud and custom spectral libraries using Mass Frontier 8.0 software. Two cardiac steroids (digoxigen and marinobufagenin) were successfully detected from the CSF sample via exact MS^n spectral tree match (Figure 1 & Figure 2). No cardiac glycosides were detected from the CSF sample and no further data processing with Compound Discoverer 3.0 software was needed.

Verifying the existence of the two detected endogenous cardiac steroids across multiple CSF samples using targeted PRM assay

In order to verify that the identity of the two annotated cardiac steroids are true and they can be detected from other CSF samples reproducibly, a targeted PRM assay to collecting MS^2 data only on digoxigen and marinobufagenin was developed using digoxigen and marinobufagenin reference standards. The identity of two detected cardiac steroids were confidently confirmed using PRM assay with the same CSF sample used in the discovery phase.

Figure 1. Cardiac steroid structure annotation workflow – a commercially available standard of Digoxin (C41H64O14; MW: 780.4296) is used as an example for demonstrating the workflow

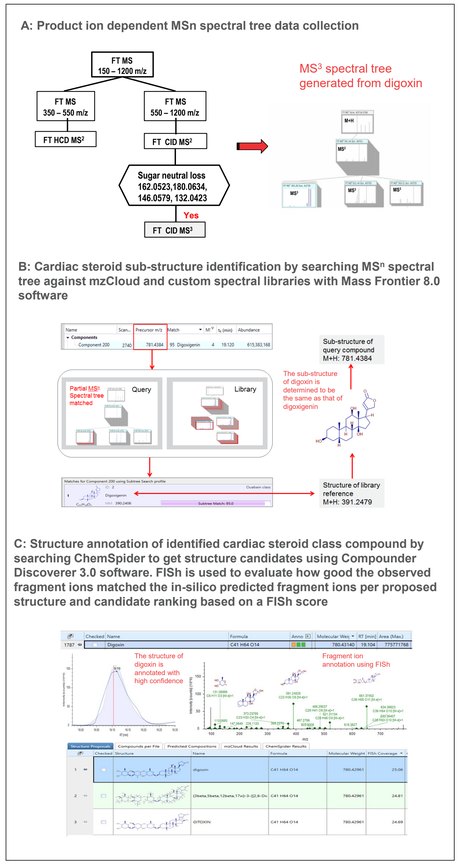


Figure 2. Extracted base peak chromatograms of CSF sample

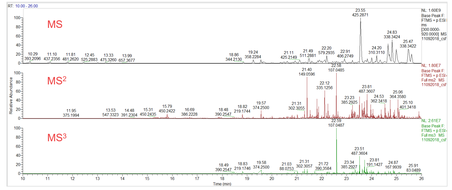


Figure 3. Annotation result of Digoxigen with the CSF sample

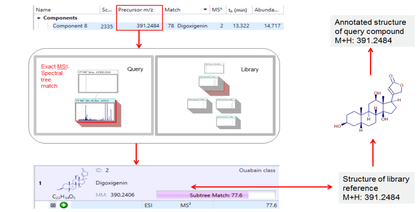
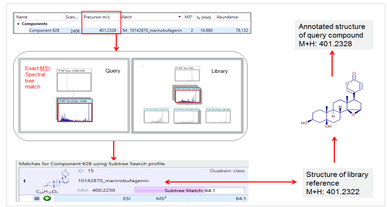


Figure 4. Annotation result of Marinobufagenin with the CSF sample



The benefits of using PRM compared to full MS are increased selectivity and sensitivity, resulting from utilizing instrument time for filling the Orbitrap analyzer with only relevant fragment ions from the targeted precursor ions (Figure 5). Because the concentration ranges of the two detected cardiac steroids are different across different CSF samples, the lower abundant cardiac steroids in some CSF samples can be detected only with PRM assay. Figure 6 shows one example where digoxigenin was only detected from a CSF sample with PRM approach.

Figure 5. Scheme of PRM (parallel reaction monitoring)

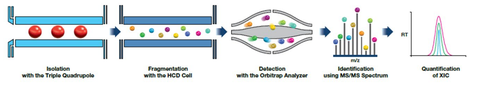
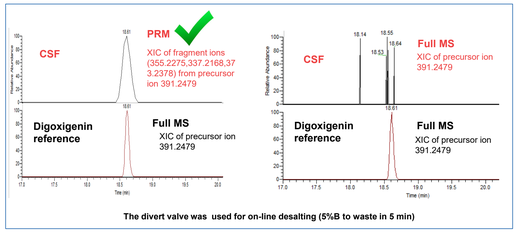


Figure 6. The detection of digoxigenin from a CSF sample which was simply filtered with a 3K centrifugal filter. The targeted PRM approach provided higher sensitivity and selectivity for detecting the digoxigenin from the CSF sample confidently compared to the full MS approach



The divert valve was used for on-line desalting (5%B to waste in 5 min)

The targeted PRM assay was applied to 5 CSF samples from different healthy humans. The CSF samples were prepared either with simple protein precipitation or filtered using a 3K centrifugal filter.

Both digoxigenin and marinobufagenin were successfully detected from the five CSF samples with the PRM targeted approach.

CONCLUSIONS

- **A structure-based MS^n workflow on an Orbitrap ID-X mass spectrometer was developed for endogenous cardiac steroid discovery from the biological fluid samples**
- **The workflow successfully detected two endogenous cardiac steroids from human CSF sample**
- **The existence of two detected cardiac steroids across multiple CSF samples were verified using a highly sensitive and highly selective targeted PRM assay**

REFERENCES

1. O. Aizman, et. al., Ouabain, a steroid hormone that signals with slow calcium oscillations, PNAS, November 6, (2001), vol. 98, no. 23, 13421-13424
2. C. Lenaerts, et. al., Revealing of endogenous Marinobufagin by an ultra-specific and sensitive UHPLC-MS/MS assay in pregnant women, Talanta 187 (2018) 193–199

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TRADEMARKS/LICENSING

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