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Profiling and quantitation of bile acids in human biofluids by LC-TIMS-MS

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Introduction

The physiological relevance of bile acids (BA) resulted in extensive research and the recent discovery of **novel BA conjugates** shown to be related to **human health and the microbiome** [1]. BAs are derived from cholesterol and their chemical complexity arises from the occurrence of hydroxyl or carbonyl groups in different stereochemistries. This renders bile acids analysis challenging as several isobaric and isomeric BAs can´t be separated by RP-LC.

We optimized a **RP-LC-TIMS-MS method** providing trapped ion mobility separation (TIMS) capabilities orthogonal to RP-LC. This allows separating co-eluting unconjugated BAs, among others. This is crucial as they lack characteristic MS/MS fragments, rendering their annotation using common, low-specificity SIM LC-QQQs methods ambiguous.

The established method provides quantitation and profiling capabilities for BAs extracted from complex human biofluids.

Methods

Bile acid standards were obtained from Cambridge Isotope Labs (Tewksbury, USA), Steraloids (Newport, USA) and Medical Isotopes (Pelham, USA). NIST SRM 1950 human reference plasma was obtained from Sigma-Aldrich (Taufkirchen, Germany). C8-RP chromatographic conditions and plasma extraction were conducted following a protocol from Sarafian et al. 2015 [2]. LC-trapped ion mobility (TIMS) MS analyses were performed using an Elute UHPLC connected to a timsTOF Pro 2 system equipped with a VIP-HESI source operated in negative ion mode (Bruker Daltonics, Bremen, Germany). The resulting four-dimensional data (*m/z*, RT, mobility, and MS/MS) was processed using TASQ® 2023 and MetaboScape® 2023 (Bruker Daltonics, Bremen, Germany) for targeted quantitation and non-targeted profiling, respectively. In both software workflow solutions, raw data was automatically recalibrated for mass and mobility.

Results

1) LC-TIMS-MS method for profiling of bile acids in biological samples

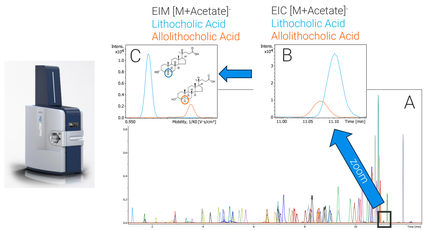

Fig. 1 LC-TIMS-MS analysis of >70 bile acid standards

In total **71 bile acids** can be separated by the LC-TIMS-MS method and hence confidently annotated utilizing the established assay. The optimized LC gradient and HRMS used in our LC-TIMS-MS analysis allowed many bile acid species to be separated by either LC or MS alone (see **Figure 1 A**). Even in this simplified system of 71 synthetic reference materials, co-elution of several isobaric bile acid pairs was observed. **Lithocholic acid** and **Allolithocholic acid** (see **Figure 1 B**), for example, were further separable using TIMS of their [M+Acetate]⁻ ion species as highlighted in **Figure 1 C**. This clearly demonstrates the additional benefit of TIMS for increasing the specificity of measurement and enhancing the confidence in annotation for these bile acids which can not be achieved by common LC-QQQ methods.

2) Quantitation and comparison to reference values

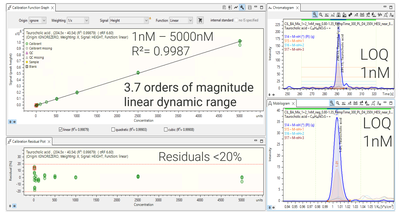

Fig. 2 Taurocholic acid quantitation

Table 1: Bile acid concentrations determined by LC-TIMS-MS are within the reference concentration range

Name	MEDM Location +/- Standard uncertainty [nmol/ml]; Bowden L, *et al.* 2017 [3]	LC-TIMS-MS determined concentration [nmol/ml] n=2
Chenodeoxycholic acid	0.30 +/- 0.11	0.28
Cholic acid	0.12 +/- 0.034	0.14
Deoxycholic acid	0.35 +/- 0.083	0.3
Glycochenodeoxycholic acid	1.1 +/- 0.18	1.03
Glycodeoxycholic acid	0.43 +/- 0.069	0.38
Glycoursodeoxycholic acid	0.15 +/- 0.024	0.17
Glycocholic acid	0.24 +/- 0.069	0.18
Taurocholic acid	0.026 +/- 0.0056	0.022
Ursodeoxycholic acid	0.11 +/- 0.024	0.11

Figure 2 shows the **calibration curve** for Taurocholic acid with a **linear dynamic range of 3.7 orders of magnitude** (1nM – 5000 nM) an R^2 value of 0.9987 and residuals below 20%. This demonstrates the quantitative capabilities of the established assay.
We extracted bile acids from human reference plasma (SRM 1950) and compared the determined concentrations to the MEDM Locations established by Bowden *et al.* [3]. **Table 1** lists the bile acid that were detected within the dynamic range of the LC-TIMS-MS method. All determined concentrations are within the range of the published standard uncertainty locations.

3) Intertechnology and interlaboratory CCS value comparability

We measured **bile acid reference mixtures** using two LC-TIMS-MS setups. The same MS method was applied, but different LC gradients and column chemistries were used (a 15 min gradient in Bremen, Germany and a 12.5 min gradient in Billerica, US). **Table 2** shows the CCS values deviated on average by only 0.1% permitting confident interlaboratory annotations.

The average **deviation of 0.3% in CCS values** determined by TIMS vs. Drift Tube (DT) technology underlines the ability to match CCS values acquired by TIMS to reference values in public repositories like the Unified CCS Compendium [4].

Table 2–TIMSCCS values are comparable between laboratories and to reference DTCCS values

Name	CCS (Å²) [M-H]- Reference Poland C., *et al.* 2020 [5]	TIMS Bremen [n=6]	TIMS Billerica [n=6]	% CCS deviation TIMS Bremen vs. Poland et al. [5]	TIMS Bremen vs. Billerica
Glycolithocholic acid	199.5 +/- 0.2	198.8 +/- 0.1	199.1 +/- 0.2	0.3	-0.1
Glycodeoxycholic acid	199.9 +/- 0.2	199.2 +/- 0.1	199.4 +/- 0.1	0.4	-0.1
Glycoursodeoxycholic acid	201.1 +/- 0.1	200.5 +/- 0.1	200.6 +/- 0.1	0.3	0.0
Glycocholic acid	202.2 +/- 0.1	201.6 +/- 0.0	201.8 +/- 0.1	0.3	-0.1
Taurolithocholic acid	206.4 +/- 0.1	206.0 +/- 0.1	206.2 +/- 0.2	0.2	-0.1
Taurochenodeoxycholic acid	207.2 +/- 0.2	206.7 +/- 0.1	206.9 +/- 0.1	0.2	-0.1
Tauroursodeoxycholic acid	207.6 +/- 0.1	207.2 +/- 0.1	207.4 +/- 0.2	0.2	-0.1
Taurocholic acid	207.6 +/- 0.2	207.0 +/- 0.1	207.3 +/- 0.1	0.3	-0.2
Average				0.3	0.1

4) Bile acid profiling in SRM 1950

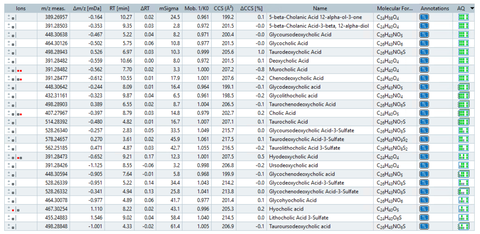

Fig. 3 Bile acids annotated in SRM 1950 human reference plasma using MetaboScape

Figure 3 shows that in total **25 bile acids were annotated** in the human plasma extract using the Target List for 71 bile acids (containing, name, molecular formula, retention time and CCS value). Annotation quality (AQ) was assessed based on AQ score, a visual tool in MetaboScape incorporating multiple molecular identifiers (e.g., CCS and *m/z*).

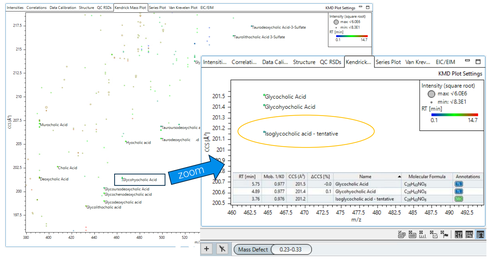

Fig. 4 Discovery and assignment of Bile acids using interactive vizalizations in MetaboScape

The use of the **Kendrick Mass Defect plot within MetaboScape** readily enabled mining of the data for possible bile acids that were not annotated by the Target List. **Figure 4** shows a zoom into the mass and mobility region for Glycocholic acid and Glycohyocholic acid. A feature was spotted with a similar mass and mobility (in the plot below the two annotated bile acids). The retention time for this potential bile acid is lower compared to the other two Glycine conjugated bile acids. A likely annotation for this bile acid is Isoglycocholic acid. Isoglycocholic acid contains a 3 beta -OH orientation compared to Glycocholic acid. This difference in orientation renders Isoglycocholic acid more polar and can lead to an earlier reversed phase elution.

Conclusion

- LC-TIMS-MS based method for 71 bile acid leverages CCS as a comprehensive criterium for annotation confidence.
- The LC-TIMS-MS method provides quantitative results covering up to 3.8 orders linear dynamic range.
- Determined bile acid concentrations in SRM 1950 human reference plasma are within the range of published reference values.
- Untargeted Profiling using the MetaboScape solution enabled to readily pinpoint and annotated a bile acid not contained in the custom Target List of 71 standards.

LC-TIMS-MS

References

[1] DOI:10.1038/s41586-020-2047-9
[2] DOI:10.1021/acs.analchem.5b01556
[3] DOI:10.1194/jlr.M079012
[4] https://mcleanresearchgroup.shinyapps.io/CCS-Compendium
[5] DOI: 10.1021/jasms.0c00015