Quantification of quorum sensing molecules and their interaction with polymyxin-B hemoperfusion in human plasma by LC-HRMS

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Overview

- A fast and sensitive **LC-HRMS (LTQ-orbitrap) method to measure QS molecules** in bacterial cultures and human plasma samples was developed.
- The method was compared with two more selective **neutral loss LC-TQMS (triple quadrupole)** methods to detect and quantitate the presence of Acyl homoserine lactones (AHLs) or hydroxyquinolones (HQs)
- One of the aims of this study was to compare different acquisition modes, mobile phases and stationary phases in order to **optimize known and unknown QS molecules detection**.
- The method was applied to the characterization of **wild type vs. mutant Pseudomonas aeruginosa strains** with different ability to produce QS molecules.

Introduction

Quorum sensing (QS), is a chemical communication phenomenon that has attracted wide concern over the last years. In this process, bacterial populations could act together by signaling molecules released in host biofluids, likewise in the social network phenomenology. QS pass through the bacterial envelope and regulate virulence gene expression and biofilm formation. It would be interesting to clarify if QS could bind the lipid A fraction of lipopolysaccharide (LPS) endotoxins.

Published studies[1] suggested that QS can also interact with eukaryotic cells exerting immunomodulatory effects and playing a key role in urinary tract infection. We evaluated the potential role of QS in sepsis-associated AKI (acute kidney injury) by studying their biological effects on human kidney tubular epithelial cells (TEC).[2]

In this work, our aim is the identification/quantification of specific biomarkers involved in the sepsis-related multi-organ failure associated with high mortality, with regards to AKI. The relationship between bacterial products such as the recently discovered QS and microvesicles (MV) could lead to the identification of new potential targets for medical intervention.

The project consists initially in the study of interaction of QS with polymyxin-B bound membranes for hemoperfusion then in the isolation of QS from plasma of septic patients and on bacterial cultures by different methods based on HPLC coupled to MS.

Methods

The study was initially carried out in neutral physiological solution and then extended to biofluids as bacterial culture supernatant or human plasma. Biological samples were extracted by LLE with dichloromethane. HPLC-HRMS analyses were accomplished on two instruments:

i) Dionex Ultimate 3000 LC system coupled via ESI interface with a Thermo Scientific LTQ-Orbitrap FT-MS.

ii) Shimadzu Nexera LC coupled via Turbo Ion Spray (TIS) interface with a Sciex 5500 Q-trap MS.

C18-RP columns were used for chromatographic separation. 3-oxo-C_{12} AHL QS from *P. aeruginosa* and C_7 HQ were purchased by Sigma Aldrich.

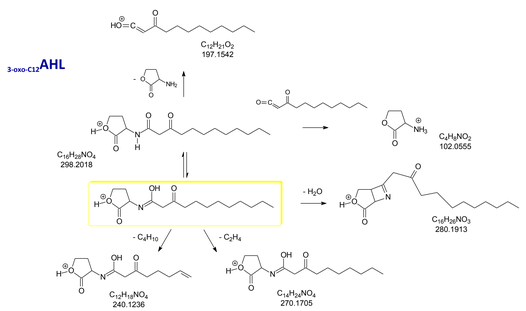
Experiments of interaction and hemoperfusion were achieved on a medical device where polymyxin-B, an antibiotic with high affinity for endotoxins, has been bound and immobilized to polystyrene fibers (Toraymyxin™).

LC: mobile phase (gradient conditions): formic acid 10 mM / methanol, from 85/15 to 0/100 in 50'. Column: Phenomenex Luna C18(2) 100 A, 150 × 2.1 mm.
ESI source conditions: source voltage 4.5 kV; capillary voltage 19 V; capillary temperature 270°C.
TIS source conditions: source voltage 5.5 kV; source temperature 500°C.

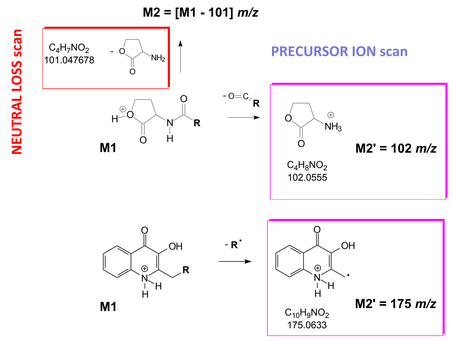
LTQ-orbitrap MS analyzer conditions: full scan FTMS positive ion mode. 300-1000 *m/z* @ 30000 resolution (500 *m/z* FWHM). MS/MS precursor ions: 3-oxo-C_{12} AHL 298 *m/z* (MH^+; collision energy, CE = 30) ; C_7 HQ 260 *m/z* (CE = 30).
AB Sciex 5500 MS analyzer conditions: MS/MS positive ion mode. 150-350 m/z.
SRM 3-oxo-C_{12} AHL 298 *m/z* → 197 *m/z*, CE = 21 V; 298 *m/z* → 102 *m/z*, CE = 15 V (quantitation);
Neutral loss scan (NL) AHL 150-350 m/z , CE = 15-25 V, ΔM 101 Da.
Precursor ion scan (PI) AHL 150-350 m/z , CE = 15-25 V, → 102 *m/z*; HQ 150-350 m/z , CE = 35-45 V , → 175 *m/z*.

Blood samples were obtained by the Blood Bank of Molinette Hospital, Turin. Bacterial cultures were kindly provided by Dr Viviana Orlandi from University of Insubria, Varese, Italy.

Scheme 1: QS analytes with main fragmentation pathways observed for protonated molecular ions



Scheme 2: TQ MS MS selective acquisition modes



Scheme 3: Ions formed by previously identified AHLs herein investigated

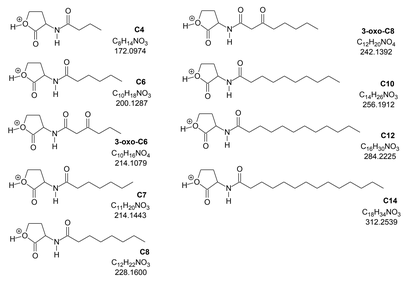


Table 1: Linearity and LLOQ of quantitative analysis method

	3-oxo-C_{12} AHL	C_7 HQ
HRMS calibration curve	y = 118 x + 8 E5; r^2 = 0.9999	---
HRMS LLOQ (ppb)	1.0	---
$HRMS^2$ calibration curve	y = 47.5 x + 3 E5; r^2 = 0.9999	---
$HRMS^2$ LLOQ (ppb)	1.0	---
SRM MS/MS calibration curve	y = 985.45 x – 190642; r^2 = 0.9997	y = 296.45 x – 7873; r^2 = 0.9997
SRM LLOQ (ppb)	0.050	0.050
NL MS/MS calibration curve	y = 5372.8 x – 1 E6; r^2 = 0.9992	---
NL LLOQ (ppb)	5.0	---
PI MS/MS calibration curve	y = 7069.3 x – 3 E6; r^2 = 0.9995	y = 122 x – 97182; r^2 = 0.9998
PI LLOQ (ppb)	5.0	0.050

Figure 1: Hemoperfusion filter operation scheme

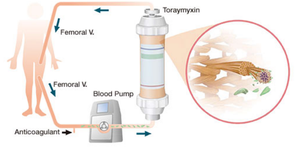
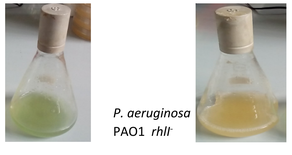


Figure 2: *Pseudomonas aeruginosa* bacterial cultures



P. aeruginosa PAO1(wild-type)

P. aeruginosa PAO1 *rhlI*⁻

Results

In the early method development we optimized the chromatographic separation of several lipophilic QS compounds on RP-HPLC columns and studied the sensitivity of different ionization/acquisition modes. As far as AHLs are concerned, MS/MS study evidenced common losses useful for precursor ion analysis (generation of gamma-butyrolactone-2-ammonium ion) or neutral loss analysis (elimination of 2-amino-gamma-butyrolactone) of N-acyl homoserine lactone QS derivatives (Scheme 1 and 2).
For HQ only a potential fragmentation to a characteristic product ion was identified (Scheme 2).
A pre-validation of analytical performances involved linearity, accuracy, precision, LLOQ and recovery of determination. Correlation equations and LLOQ values are shown in Table 1.

Subsequently we investigated the interaction of studied QS (homoserinelactones) with gram- bacterial endotoxins as lipopolysaccharides (LPS) and polymyxin-B, chemically bound to the filtering cartridge for hemoperfusion. An evidence of QS-LPS and QS-polymyxin B hydrophobic interaction is presented. Figure 3 shows how 3-oxo-C_{12} AHL concentration drops down after incubation (static experiment) in the presence of hemoperfusion membrane with covalently bonded polymyxin-B or by filtration (dynamic experiment) upon the same membrane.
Finally QS molecules were determined in bacterial cultures and human plasma samples from AKI patients under hemoperfusion treatment. Only the results of bacterial cultures analyses are shown herein.
Interestingly, both HRMS-orbitrap and NL/PIS MS/MS acquired on the Q-trap instrument relealed the presence of AHL molecules in *Pseudomonas aeruginosa* cultures broths. As expected the concentration of AHL in wild-type PAO1 samples was higher than in mutant PAO1 *rhlI*⁻ samples. Eventually, the presence of C_4 AHL was evidenced again in the case of the wild-type sample only. Neutral loss scan seems to be more sensitive than precursor ion scan in the microbiological study (Figure 4).

Figure 3: Standard AHL quantitation after incubation/filtering using toraymyxin™ hemoperfusion filter

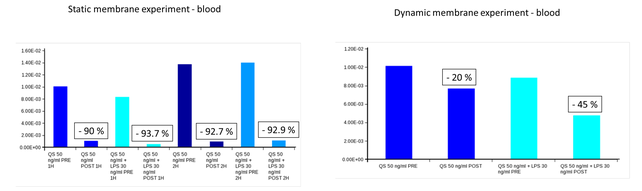
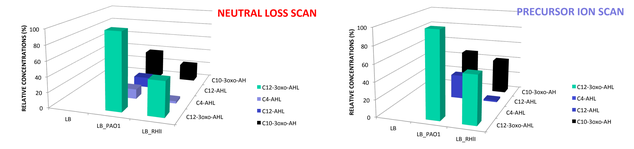


Figure 4: Quantitation of AHL produced by wild-type vs. mutant *P. aeruginosa* PAO1 in lysogeny broth (LB) medium



Conclusions

Both HRMS and NL or PI MS/MS methods show to be fast and selective for the rapid quantification of bacterial small molecules in biological fluids. Preliminary experimental data indicate that the method is suitable for the analysis of biofluids and evidence the ability of PMX-B bound membrane to interact with AHL QS molecules.

References

1. P. Boontham *et al*. Significant immunomodulatory effects of *Pseudomonas aeruginosa* quorum-sensing signal molecules: possible link in human sepsis. *Clinical Science* (2008) **115**, 343–351.
2. V. Cantaluppi *et al*. Quorum Sensing (QS) Molecules Released by Gram Negative Bacteria induce tubular injury interacting with lipopolysaccharide (LPS): a new potential mechanism of sepsis-associated AKI. Presented at *19th International Conference on Advances in Critical Care Nephrology* (San Diego, 2014).
3. T.R.I. Cataldi *et al.* Occurrence of N-acyl-L-homoserine lactones in extracts of some Gram-negative bacteria evaluated by gas chromatography–mass spectrometry. *Analytical Biochemistry* (2007) **361**, 226–235.