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# Bioanalysis of *Pseudomonas aeruginosa* alkyl quinolone signalling molecules in infected mouse tissue using LC–MS/MS; and its application to a pharmacodynamic evaluation of MvfR inhibition

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ABSTRACT

Alkyl quinolone molecules 2-heptyl-4-quinolone (HHQ) and 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) are important quorum sensing signals, which play a mediatory role in the pathogenesis of acute and chronic *Pseudomonas aeruginosa* infection. A targeted approach inhibiting the bacterial 'multiple virulence factor regulon' (MvfR) protein complex, offers the possibility to block the synthesis of MvfR-dependant signal molecules. Here, a high throughput bioanalytical method was developed using LC–MS/MS detection for the selective determination of HHQ and PQS in mouse tissue homogenate, over a sensitive range of 1–5000 and 10–5000 pg/mL, respectively. Chromatographic peak distortion of the iron chelator PQS was overcome with the applied use of a bidentate chelator mobile phase additive 2-Picolinic acid at 0.2 mM concentration, giving an improved separation and response for the analyte, whilst maintaining overall MS system robustness.

Following thigh infection with *P. aeruginosa* strain 2-PA14 in mice, the concentration and time course of HHQ and PQS (4-hydroxy-2-alkyl-quinolone (HAQ) biomarkers) residing in the biophase were evaluated, and exhibited a low level combined with a substantial inter-individual variability. Quantifiable levels could be obtained from approximately 15 h post infection, to the study termination at 21–22 h. A dose dependant reduction in HAQ tissue concentrations at selected time points were obtained following MvfR inhibitor administration versus drug vehicle ($p < 0.01$, Kruskal–Wallis—one way ANOVA) and meta-analyses of several studies enabled an inhibitory concentration ($IC_{50}$) of 80 nM free drug to be determined. However, due to the experimental limitations a defined time profile for in-vivo HAQ production could not be characterised. Microsomal stability measurements demonstrated a rapid metabolic clearance of both alkyl quinolone biomarkers in the bacterial host, with a hepatic extraction ratio greater than 0.96 (the measurable assay limit). High clearance underpinned the low concentrations present in the well-perfused thigh tissue. Along with method development and validation details, this paper considers the kinetics of in-vivo HAQ bio-synthesis during *Pseudomonas* infection; and risks of biomarker over-estimation from samples which contain an exogenous population of bacteria.



## 1. Introduction

*Pseudomonas aeruginosa* (Pa) is an opportunistic gram-negative bacterium known to increase the morbidity and mortality of several lung disease states [1], burn injuries [2] and urinary tract infections [3] in human patients. The severity and persistence of Pa infection is characterised by the metabolic adaptability of the bacteria and

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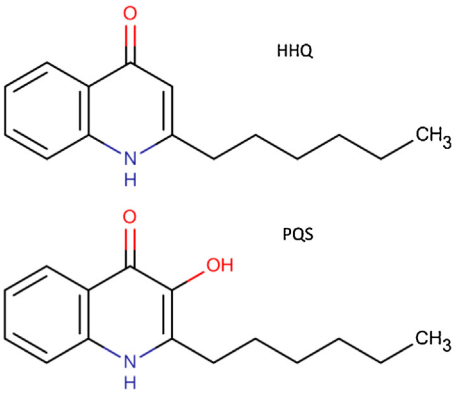


**Fig. 1.** Chemical structures of HHQ and PQS.

are aided by a coordinated virulence factor release (transcriptional regulators, proteases, toxins and lipopolysaccharides) capable of modulating the local environment to confer properties of adaptive resistance to immune defence, and antibiotic treatment. Due to the difficulty in treating Pa, it is one of the most common respiratory tract infections in hospitalised patients [1]. The bacterial communication network operates through 'quorum sensing' signals; small multifunctional molecules able to diffuse from the intracellular compartment into the extracellular space and regulate cell behaviour in neighbouring colonies. The phenotypic properties affected include bacterial motility, biofilm formation, and population density control.

An important class of quorum sensing molecules identified through anti-infective research are the 4-hydroxy-2-alkylquinolone (HAQ) congener series (Fig. 1) [4]. HAQ analogues vary in both the length and degree of saturation of the side chain that stem from C2 of the hydropyrid-4-one ring, the hydroxylation status at position C3 [4], and a further array of *N*-oxide derivatives (position C1) formed from the cognate molecules [5]. Despite the wide diversity of exogenous signalling molecules identified, HAQ's most frequently associated with gene regulation in acute infection are the Pa quinolone signal ((PQS)—2-heptyl-3-hydroxy-4(1*H*)-quinolone) and its metabolic precursor 2-heptyl-4-quinolone (HHQ). PQS and HHQ bind to the lysine type transcriptional regulator (LTTR) superfamily as co-ligands to form a transcription factor unit with multiple virulence factor regulation (MvfR). Once activated MvfR is able to bind to a series of target promoter regions to control the gene expression and release of multiple virulence factors such as elastase, pyocyanin, lectins and hydrogen cyanide [5], as well as regulate iron usage within cells [6]. By acting as auto-inducers to accentuate their own synthesis, PQS and HHQ can rapidly promote colony biofilm development and inflammation in the early stages of acute infection. In addition, MvfR plays a pivotal role in the pathogenesis of chronic human infection and the development of antibiotic resistance. The significance of MvfR induced virulence is highlighted in several infectious disease models using mutant (knock-out) MvfR$^-$ strains which exhibit greatly reduced pathogenicity [5] and HAQ production. Clinical evidence from pulmonary infection patients suggests that Pa cell to cell signalling is a necessary process for virulence to develop [7] and a deficiency in one or more of the networks, leads to reduced pathogenicity [1].

The quantitation of HHQ and PQS as both mechanistic and clinically diagnostic biomarkers in infectious disease states has been demonstrated through in vivo sample analyses, which indicate the molecules have a positive association with disease status in both cystic fibrosis patients [8] and human burn wounds [9], and that detection is also linked to the quantitative load of Pa at the start of pulmonary exacerbation [8]. However, significantly a large number of negative values in these studies (below the assay LLOQ) were observed in the plasma, sputum and urine bio fluids tested [8,10], indicating that in vivo concentrations in human are very low, and potentially reduced further by i.v. administration of the antibiotics designed to treat the infection itself. Previous LC–MS/MS methods developed to quantify HAQ production in Pa cell cultures [4,11] did not require significant analytical sensitivity, as measured concentrations were in the micro molar range, and methods were also focused primarily upon analyte identification. However, the same studies monitored the kinetics of PQS and HHQ biosynthesis in vitro, and demonstrated a time-lag between the logarithmic growth phase of bacterial density and maximal HAQ production [11]. Moreover, midway through the plateau phase of the population growth curve, PQS levels began to decline. The signal mechanisms which regulate Pa virulence in culture are complex [12] and as part of a hierarchical signalling cascade, PQS is regulated both positively by the LasR gene transcription factor and negatively by the RhLR gene transcription factor. In vivo bacterial host systems can be assumed to have the added complexity of HAQ distribution and clearance kinetics. In this context, to fully characterise the pharmacodynamic relationship of HAQ activity against measured colony forming units within an infection model, it is necessary to quantify both these aspects over time and in the presence of vehicle or drug treated groups.

This paper describes the analytical challenges and limitations identified with HHQ and PQS biomarker quantitation in mouse tissue, during drug discovery evaluation of a novel MvfR inhibitor. It provides practical guidance on the sample handling, preparation steps and optimisation of LC–MS/MS conditions for the presented bioanalytical method, and how the assay data was applied as part of a preclinical pharmacokinetic/pharmacodynamic (PKPD) evaluation.

## 2. Materials and methods

### 2.1. Chemicals and reagents

2-Heptyl-3-hydroxy-4(1*H*)-quinolone, >96% (PQS) and 2-heptyl-4-quinolone, >98% (HHQ) were purchased from Sigma Aldrich (Gillingham, UK), in addition to chemicals trichloroacetic acid, formic acid and 2-Picolinic acid. Acetonitrile (ACN—LC–MS grade), dimethyl sulfoxide (DMSO), ethyl acetate, *tert*-butyl methyl ether and 0.5 M Borate buffer (pH10) were obtained from Fisher Scientific (Loughborough, UK). Filtered deionised water was prepared through a Millipore Elix 5 purification system. DMSO Stock solutions of PQS and HHQ were prepared at 1 mg/mL (adjusted for the percentage purity) in amber glass vials and stored frozen at 4 °C.

Control mouse CD-1 biological tissues (thigh homogenates, lung homogenates) and fluids (plasma, urine) were obtained from Evotec's anti-infective research unit (Manchester, UK). All material infected with Pa was handled in a minimum class I safety cabinet. Microsomal fractions were purchased from Becton Dickinson (Oxford, UK).

### 2.2. Working solution and analytical standard preparation

DMSO Stock solutions were thawed at room temperature and serially diluted to make a series of combined analyte (HHQ & PQS) working solutions in acetonitrile, on the day of analysis. Positive displacement pipettes were used for all liquid transfer steps. Final working solution concentrations ranged from 0.1 to 500 ng/mL for calibration standards and 0.4 to 400 ng/mL for quality control (QC) standards. The biological matrix volume used in the assay (tissue homogenate or bio-fluid) was 250 μL. In each analytical batch, standard and QC samples were prepared alongside any unknown