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A Liquid Chromatography High-Resolution Tandem Mass Spectrometry Method to Quantify QS-21 Adjuvant and Its Degradation Products in Liposomal Drug Formulations

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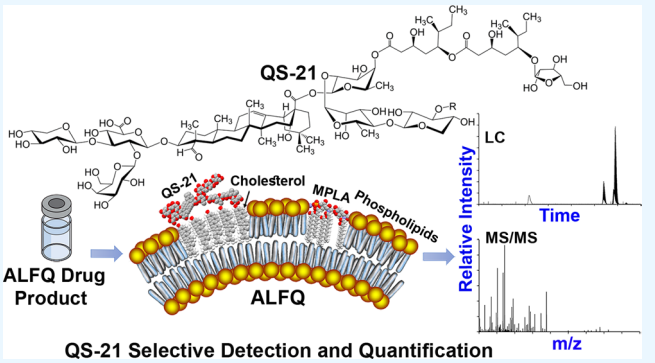
ABSTRACT: Identification and quantification of an active adjuvant and its degradation product/s in drug formulations are important to ensure drug product safety and efficacy. QS-21 is a potent adjuvant that is currently involved in several clinical vaccine trials and a constituent of licensed vaccines against malaria and shingles. In an aqueous milieu, QS-21 undergoes pH- and temperature-dependent hydrolytic degradation to form a QS-21 HP derivative that may occur during manufacturing and/or long-term storage. Intact QS-21 and deacylated QS-21 HP elicit different immune response profiles; thus, it is imperative to monitor QS-21 degradation in vaccine adjuvant formulation. To date, a suitable quantitative analytical method for QS-21 and its degradation product in drug formulations is not available in the literature. In view of this, a new liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and qualified to accurately quantify the active adjuvant QS-21 and its degradation product (QS-21 HP) in liposomal drug formulations. The method was qualified according to the FDA Guidance for Industry: Q2(R1). Study results showed that the described method presents good specificity for QS-21 and QS-21 HP detection in a liposomal matrix, good sensitivity characterized by the limit of detection (LOD)/limit of quantitation (LOQ) in the nanomolar range, linear regressions with correlation coefficients, $R^2 > 0.999$, recoveries in the range of 80−120%, and precise detection and quantification with % relative standard deviation (RSD) < 6% for QS-21 and < 9% for the QS-21 HP impurity assay. The described method was successfully used to accurately evaluate in-process and product release samples of the Army Liposome Formulation containing QS-21 (ALFQ).



INTRODUCTION

QS-21 is a potent adjuvant from the bark extract of *Quillaja saponaria Molina* tree, endemic in Chile,[1−3] and is currently involved in several human vaccine trials.[4−6] The structure of QS-21 is characterized by two groups of heterogeneous sugar moieties (i.e., linear and branched), a triterpenoid fragment, and an acyl chain (Figure 1), all of which are believed to be essential in its immune-stimulating activity.[4,6−8] The QS-21 extract contains four (4) isomeric mixtures, which mainly differ in the terminal sugar unit of the linear carbohydrate group (Z = apiose/xylose) and in the linkage of the acyl chain in the fucosyl unit (X/Y).[9−11] This molecule has undergone extensive research and development in adjuvant formulations[4,6] and has been used in multiple clinical trials, and synthetic variants are being developed.[4,8,12−17] Several studies have shown that QS-21 induces both cellular and humoral immune responses and was effective in various clinical and preclinical studies.[4,18−23]

The currently available commercial QS-21 extract contains multiple isomers, which are difficult to separate by reversed-phase high-performance liquid chromatography (RP HPLC). In aqueous media, in addition to the QS-21 isomers, QS-21 1 and QS-21 2, small quantities of other derivatives such as QS-21 R1 and QS-21 R2 (Figure 1) were also observed and detected. Most likely, these derivatives were formed during purification and coelute with the isomeric constituents of the QS-21 natural product. The heterogeneity of the currently available QS-21 extract complicates the formulation and challenges our understanding of the QS-21 adjuvant mechanism of action.

As a stand-alone adjuvant, QS-21 exhibits a dose-limiting toxicity due to its hemolytic property that causes local erythema.[4,5] Its strong interaction with cholesterol resulted in

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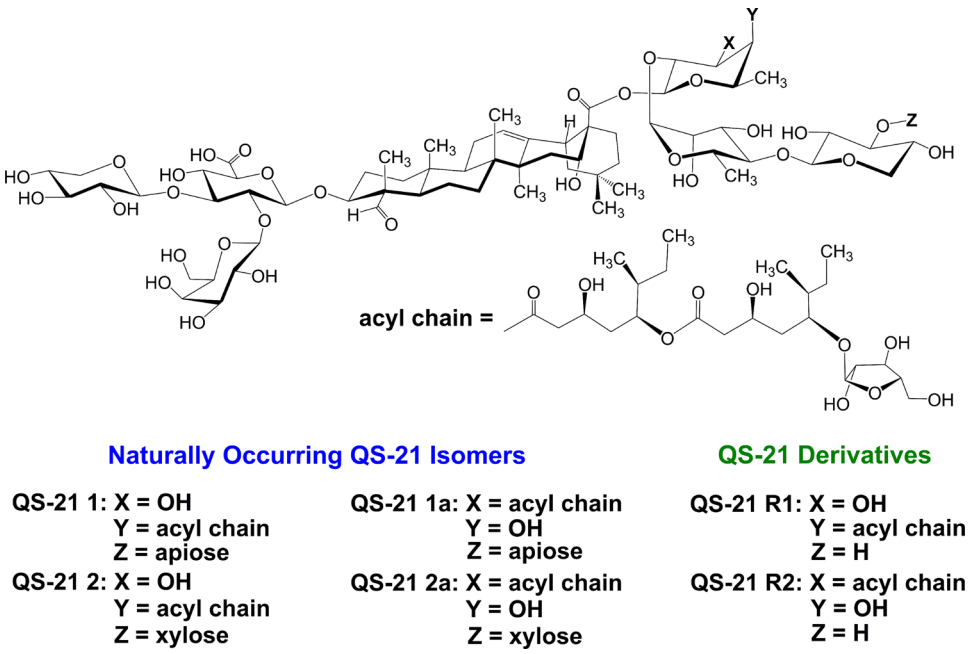

Figure 1. Structures of naturally occurring QS-21 isomers and derivatives. QS-21 1 and QS-21 2 isomers, and QS-21 R1 and QS-21 R2 derivatives were detected in the commercially available QS-21 extract under our experimental conditions. These derivatives were likely formed during the purification process.

the formation of pores in the lipid bilayer of cell membranes, leading to hemolysis.[24,25] In an aqueous solution, QS-21 undergoes a pH-dependent hydrolytic cleavage of the acyl chain fragment, producing QS-21 HP (Figure 2),[11] a derivative that elicits a different immune response.[26,27] Intact QS-21 is relatively stable at an intermediate pH < 5.0.[9,11] However, vaccines are normally formulated at pH ∼ 7.4, where QS-21 undergoes a spontaneous degradation to generate a deacylated product QS-21 HP. While it is known that QS-21 stimulates a balanced Th1/Th2 immune response, the QS-21 HP derivative only retains its Th2 capability.[26,27] These complications further challenge the adjuvant formulation stability and its storage. The above QS-21 liabilities were addressed by incorporating QS-21 in heterogeneous matrices such as in liposomal formulations,[24,25,28] surfactant emulsions,[3] or immune-stimulating complexes,[29] which does not only abrogate its hemolytic activity but also stabilizes the QS-21 by constraining and burying the labile acyl-fucose ester linkage in the hydrophobic environment.[11] This strategy has been used by our laboratory in developing QS-21-containing liposomes called the army liposome formulation with QS-21 (ALFQ), which contains the saturated phospholipids, dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG), monophosphoryl lipid A (MPLA) as a co-immunostimulant, and cholesterol, which irreversibly binds with QS-21 to eliminate its hemolytic activity.[24,30−32] ALFQ has been used in several phase I vaccine clinical trials targeting malaria antigens,[33] *Campylobacter*,[34] SARS-CoV-2,[35] and HIV.[36] A similar approach and derivative liposomal formulation containing QS-21 are found in the recently licensed vaccines for malaria (Mosquirix) and shingles (Shingrix).

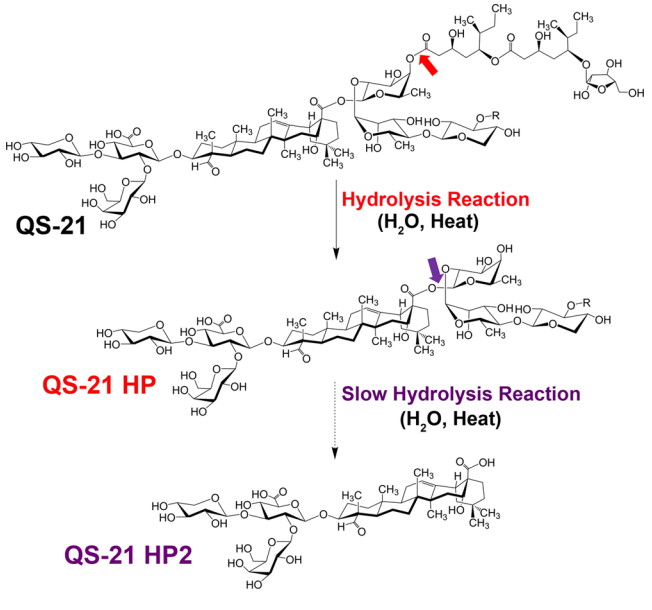

Figure 2. Hydrolytic decomposition pathway of intact QS-21. The second hydrolysis reaction was not observed under our reaction conditions. QS-21 HP2 (m/z 955.4549) was detected as one of the ionization products of QS-21 under MS conditions. R = apiose/xylose, H.

According to the FDA guidance for industry: drug stability guidelines, the analytical method for the stability study should be sufficiently specific to differentiate between unaltered drugs and any possible degradation products, and it should be supported by accuracy and precision.[37] In the case of the QS-21 adjuvant in a drug product matrix, the challenges include not only the development of a sensitive analytical method to accurately quantify the total concentration of all intact QS-21 isomers/derivatives and the degradation product QS-21 HP but also the generation of their appropriate working standards. To date, there are no reported accurate and reliable quantitative analytical methods for quantifying QS-21 isomers and their degradation product, QS-21 HP, in complex matrices. Most of the analytical techniques described in the

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literature for the detection of QS-21 derivatives in various sample matrices are for qualitative purposes. HPLC has been commonly used for the purification and detection of QS-21,[38−40] and stand-alone MS together with ^{1}H and ^{13}C NMR have been employed for the identification and for understanding the plausible fragmentation of QS-21.[10,41,42] All of these methods suffer from the inability to resolve the QS-21 isomers and derivatives and/or matrix interferences that limit their utilization for QS-21 accurate quantitative work in drug products.

In the present work, we have generated and isolated appropriate working standards and optimized analytical conditions for UPLC-MS/MS-based sensitive detection and accurate quantification of all intact QS-21 isomers/derivatives and its degradation product QS-21 HP in liposomal drug formulations. The designed method was qualified following the FDA Guidance for Industry: Q2(R1) Validation of Analytical Procedures. Qualification results demonstrated that the method has good analyte specificity, measurement sensitivity, accuracy, and precision, appropriate for quantifying QS-21 and QS-21 HP in liposomal drug products. The method has been utilized for the in-process sample and product release testing of cGMP-manufactured ALFQ adjuvants at the Walter Reed Army Institute of Research (WRAIR) for use in clinical studies.

RESULTS AND DISCUSSION

Method Development. The described UPLC-MS/MS method was designed to accurately determine the concentrations of QS-21 and its degradation product QS-21 HP in liposomal formulations. The development of the method was based on the following: (i) LC for target analyte separation and enhancement of sensitivity and selectivity of the method and (ii) tandem MS for establishing parallel reaction monitoring (PRM) transitions specific to target analytes for accurate and sensitive quantification. Several instrumental parameters of the LC and MS/MS components were explored during method optimization. Since QS-21 is prone to hydrolysis, solvent compatibility of the calibration standards was also investigated.

Working and Calibration Standards. To date, there are no commercially available QS-21 and QS-21 HP standards; thus, both standards for UPLC-MS/MS quantitative work were generated in-house. The QS-21 working standard was prepared from HPLC-purified QS-21 from the vendor Indena (through Desert King), while that of QS-21 HP was generated from intentional degradation of intact QS-21 with triethylamine (Et_3N), followed by HPLC purification, isolation, and lyophilization. The purity of in-house HPLC-purified intact QS-21 compounds and QS-21 HP were found to be >98% by a full-scan UPLC-MS/MS analysis. We found that commercially available QS-21 contains isomers and derivatives, namely, the major component QS-21 1 and minor components QS-21 2, QS-21 R1, and QS-21 R2 (Figure 1), which were observed to coelute in the preparative HPLC column. The concentrations of the calibration standards refer only to the major component QS-21 1. In this case, the total concentration of all QS-21 in test samples was determined by factoring the percent (%) peak area of other detected QS-21 isomer QS-21 2 and derivatives QS-21 R1 and QS-21 R2 relative to the total peak area (vide infra).

At room temperature, intact QS-21 degraded readily via hydrolysis to generate QS-21 HP (Figure 2).[11] The working standard stock solutions (1.0 mg/mL QS-21 and 1.0 mg/mL QS-21 HP) were prepared in methanol and stored at −80 °C. We found that these compounds are resistant to hydrolysis decomposition for more than a year. The ability to prepare relatively pure QS-21 and QS-21 HP and the resistance of intact QS-21 to degradation in appropriate conditions (i.e., solvent and storage temperature) provided us the opportunity to develop an LC-MS/MS-based method that can accurately quantify QS-21 and QS-21 HP in a liposomal drug product. We found that the accuracy for quantifying QS-21 and QS-21 HP in a liposomal formulation was not influenced by the matrix effect; thus, the working standard mixture containing QS-21 and QS-21 HP was prepared in methanol in the absence of the liposomal matrix.

LC Separation. LC conditions for separation were investigated using different mobile phases and gradient conditions, column temperature, and injection volume. Both QS-21 and QS-21 HP are polar molecules consisting of hydroxy groups and several sugar moieties; thus, the choice of a nonpolar C18 column was considered. The final method uses an Agilent Zorbax Plus C18 column (4.6 mm ID × 50 mm, 1.8 mm particle size), as described in the Experimental Section. The mobile phases were water and methanol acidified with 0.1% formic acid. Under these conditions, the isomers QS-21 1 and QS-21 2 and derivatives QS-21 R1 and QS-21 R2 were well separated, as shown in the total ion chromatogram (TIC) in Figure 3A. Further shown in the extracted ion chromatograms (EIC) are the different QS-21 isomers and derivatives eluting at different retention times, demonstrating the specificity of the method. The inclusion of 0.1% formic acid in the mobile phases facilitated the ionization of target analytes. Unlike other systems, neither tailing nor fronting of analyte peaks was observed at these chromatographic conditions; thus, buffer additives such as ammonium salts of acetate and formate were not added. Both QS-21 and QS-21 HP were detected under negative ionization mode; thus, to facilitate efficient ionization, mobile phases were kept from acidic additives. After appropriate separation conditions were established, injection volumes in the range of 1−5 μL were evaluated based on the associated peak area of each detected ion (Supporting Information, Table S2). The 5 μL injection volume was utilized in the following optimization experiments and in the final method qualification.

Tandem Mass Spectrometry. Electrospray ionization (ESI) coupled with quadrupole-orbitrap tandem mass spectrometry was employed to establish PRM transitions of target analytes for specific and accurate quantification. The appropriate spray voltage in the range of −2.0 to −3.5 kV was explored to optimize MS/MS detection sensitivity. Although a higher spray voltage at −3.5 kV resulted in a higher number of ions detected, as shown in the peak area (Table S3), an application of −2.5 kV was chosen to avoid a possible corona discharge or rim emission that may result in unstable MS signals. The precursor ions of the target analytes QS-21 isomers/derivatives and QS-21 HP were determined by a full-scan MS. As shown in Figure 4, QS-21 isomers and derivatives, and deacylated QS-21 HP were detected as $[M - H]^-$ ions with characteristic precursor ions of m/z 1987.92 for QS-21 1 and QS-21 2 isomers, m/z 1855.88 for QS-21 R1 and QS-21 R2 derivatives, and m/z 1511.65 for QS-21 HP, consistent with the negative ionization mode of detection. Based on these detected precursor ions, the analytical conditions provided excellent specificity with mass errors in the range of 0.16−2.16 ppm (Figure 4), lower than the instrument threshold detection error

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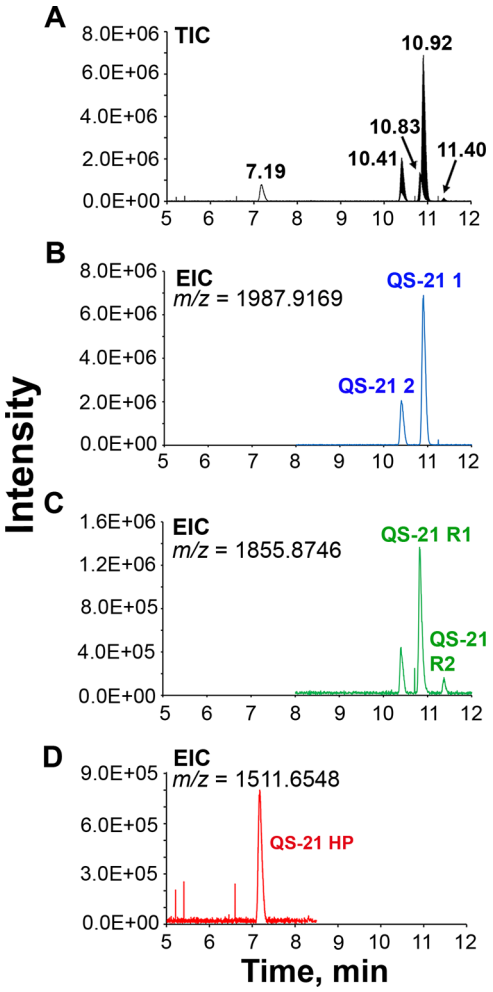

Figure 3. Chromatogram trace of cGMP-manufactured ALFQ showing separated peaks of all QS-21 isomers in (A) total ion chromatogram (TIC) and (B–D) extracted ion chromatograms (EIC) for each analyte.

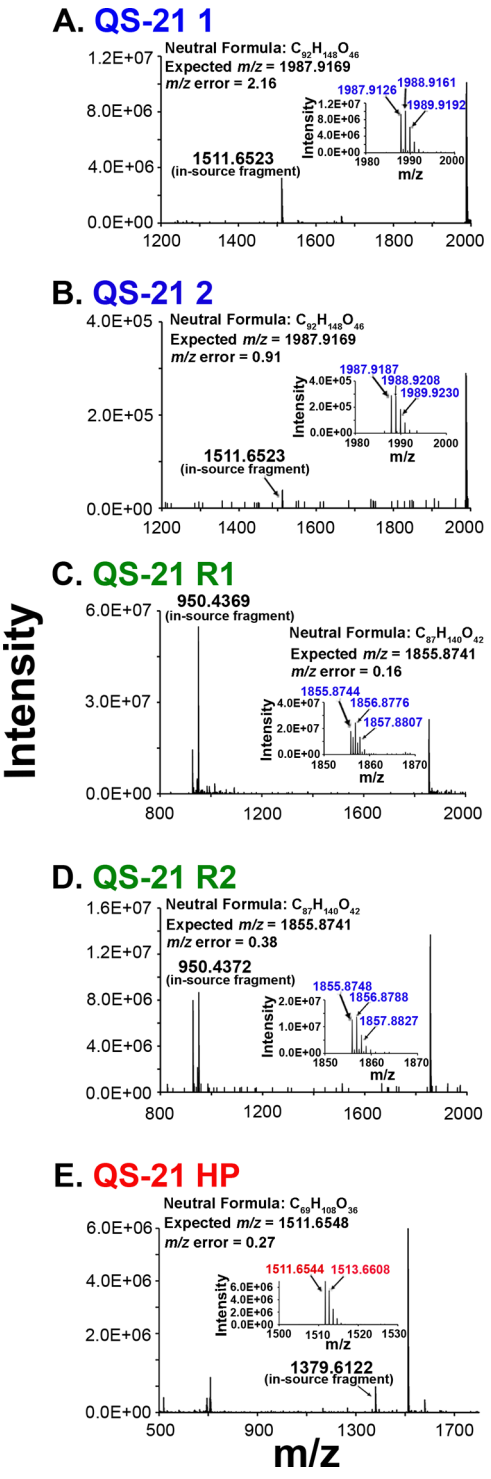

Figure 4. HRMS spectra of the detected QS-21 isomers and derivatives. (A) QS-21 1 (inset: zommed-in of m/z 1980–2000), (B) QS-21 2 (inset: zommed-in of m/z 1980–2000), (C) QS-21 R1 (inset: zommed-in of m/z 1850–1870), (D) QS-21 R2 (inset: zommed-in of m/z 1850–1870), and (E) QS-21 HP (inset: zommed-in of m/z 500–1700), showing the precursor ions and their associated mass detection errors relative to the expected ions.

of 5.0 ppm. The established precursor ions were used to set a PRM mode of detection, which has been shown to have a very specific and sensitive quantification. Under PRM, an appropriate normalized collision energy (NCE) was applied to promote fragmentation characteristics of each target analyte, where confirming ions were determined to establish relevant PRM transitions. The optimized NCE is presented in Table 1.

For intact QS-21, an application of 40 V NCE resulted in a similar fragmentation pattern of all isomers (Figure 5A) with a characteristic base peak of m/z 485.33 (triterpenoid fragment) due to the cleavage of the central glycosyl ester, and the glycosidic linkage between the branched trisaccharide and triterpene domain. Another prominent peak with m/z 955.46 (fragment consisting of triterpene and trisaccharide moieties) due to the hydrolysis of the central glycosyl ester linker was observed. The observed fragmentation patterns of QS-21 under our analytical conditions are consistent with the previous report on the structural elucidation of a QS-21 derivative from *Quillaja brasiliensis* using a stand-alone MS corroborated with NMR spectroscopy.[41] Under this optimized NCE, all intact QS-21 isomers and derivatives showed similar fragmentation sites and patterns, resulting in a common confirming ion (m/z 485.33; Table 1).

In the case of deacylated QS-21 HP, an application of 35 V NCE in PRM resulted in a similar fragmentation pattern with that of intact QS-21 1, with a difference in the intensity of the fragments. Its fragmentation pattern shows a base peak with m/z 955.46 (Figure 5B; fragment consisting of a branched

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Table 1. PRM Inclusion List and Transition

analyte	transition (m/z)	NCE (V)
QS-21 1	1987.9126 → 485.3272	40
QS-21 2	1987.9126 → 485.3272	40
QS-21 R1	1855.8747 → 485.3272	40
QS-21 R2	1855.8747 → 485.3272	40
QS-21 HP	1511.6544 → 955.4549	35

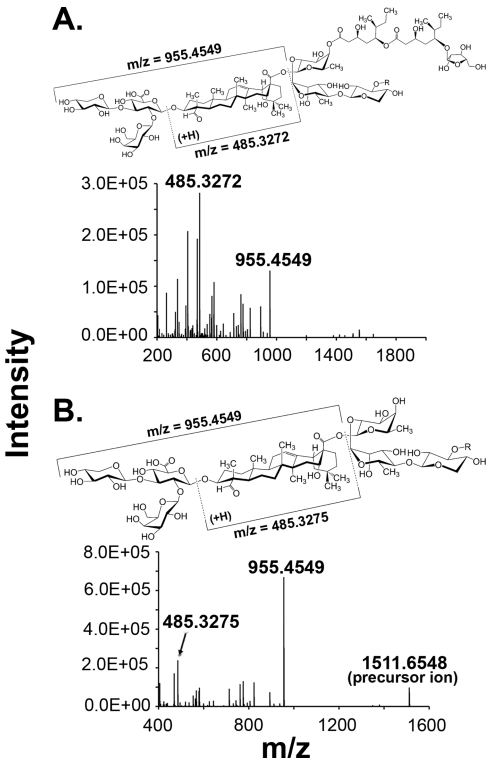

Figure 5. Plausible fragmentation patterns of (A) the major isomer QS-21 1 and (B) QS-21 HP. Other intact QS-21 isomers and derivatives exhibit the same fragmentation patterns. R = apiose/xylose, H.

trisaccharide and triterpene domain) due to the cleavage of the central glycosyl ester bond. Another less intense fragment with m/z 485.33 (triterpene domain) was also observed.

Method Qualification. The described method was qualified based on detection sensitivity (limit of detection (LOD)/limit of quantitation (LOQ)), linearity/dynamic range, selectivity/specificity, and measurement accuracy and precision, following the FDA Guidance for Industry: Q2(R1) Validation of Analytical Procedures.[43] Initial system suitability was first established to ensure that the analytical instrumentation system was appropriate with the method, demonstrating the consistency of signals and chromatographic retention time (RT). System suitability was evaluated based on the variance of the peak area and RT of ten (10) samples, with % RSD of <5 and <1%, respectively. The tabulated RT and integrated peak area of QS-21 and QS-21 HP can be found in the Supporting Information (Table S4).

Selectivity/Specificity. The quantification of QS-21 and QS-21 HP in the described method utilized specific PRM transitions characteristic of each target analyte under MS/MS detection. The specificity of the method was exemplified using freshly prepared lab-grade ALFQ and cGMP-manufactured ALFQ, containing a liposomal matrix, in comparison to a neat system suitability solution that only contains QS-21 and QS-21 HP standards in methanol. In both samples, with or without the relevant matrix, no other peaks were detected, resolved, or identified other than the target analytes QS-21 and QS-21 HP (Figure 3A and Table 2). Further, all negative blanks revealed that there were no interferences present that could lead to a false positive identification (Figure S1). Verification of selectivity was further demonstrated by spiking the liposomal drug samples (vide infra) with QS-21 HP. Results showed a quantitative amount of QS-21 HP in the liposomal matrix within 80−120% recovery (Table 5). Overall, these results demonstrate that the sample matrix and solvent blank did not interfere with analyte detection.

Linearity/Dynamic Range and LOD/LOQ. The linear/dynamic range of an analytical procedure is a range of concentrations where the detector response is proportional to the analyte concentrations. At this range, the analytical method should exhibit suitable precision, accuracy, and linearity. The method linearity/dynamic range was evaluated from five (5) sets of fourteen (14) calibration concentrations in the range of 160−0.02 μg/mL for QS-21 and 80−0.01 μg/mL for QS-21 HP. Based on the regression coefficient (R^2 > 0.995) and required % recoveries (80−120%) at each calibration point, the described method exhibits a linear range of 0.039−5.0 μg/mL for QS-21 and 0.02−2.5 μg/mL for QS-21 HP, characterized by the R^2 in the range of 0.9994−1.0 (Table 3). Beyond these concentration ranges, the analyte concentration−detector response exhibits a quadratic relationship.

Table 3. Linearity/Dynamic Range

analyte	conc. range (μg/mL)	curve fit (weighing scheme)	R^2	S/N, LL[a]	% recovery
QS-21	0.039−5.0	linear (equal)	0.9994−1.0	458	80−120%
QS-21 HP	0.02−2.5	linear (equal)	0.9995−1.0	300	80−120%

[a]LL = lower limit; S/N = signal-to-noise ratio.

The sensitivity of the method was characterized by the limit of detection (LOD) and the limit of quantification (LOQ).

Table 2. Specificity of the PRM Method

sample	analyte	PRM transition (m/z)	area	RT	S/N
system suitability	QS-21	1987.91690 > 485.3272	3 175 238	10.91	6260
	QS-21 HP	1511.65475 > 955.4549	8 964 144	7.19	7891
lab-grade ALFQ	QS-21	1987.91690 > 485.3272	5 205 721	10.91	21 223
	QS-21 HP	1511.65475 > 955.4549	512 383	7.19	776
cGMP-grade ALFQ	QS-21	1987.91690 > 485.3272	3 598 131	10.91	13 931
	QS-21 HP	1511.65475 > 955.4549	582 175	7.19	953

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The LOD is the lowest quantity of an analyte that can be distinguished from the blank, with a signal-to-noise ratio of (S/N ≥ 3), while the LOQ is the lowest amount of an analyte in test samples, with a signal-to-noise ratio of (S/N ≥ 10), which can be quantitatively determined with suitable precision and accuracy. LODs and LOQs of the method for detecting QS-21 and QS-21 HP are summarized in Table 4. These values were determined from five (5) sets of linear calibration standards following eqs 2 and 3 (Experimental Section). Sensitivity studies showed that the method can detect QS-21 and QS-21 HP in the liposomal matrix in the nanomolar range.

Table 4. Summary of Method LOD and LOQ

	analyte	
parameter	QS-21	QS-21 HP
mean ± SD LOD (μg/mL)	0.026 ± 0.011	0.006 ± 0.003
mean ± SD LOQ (μg/mL)	0.080 ± 0.03	0.018 ± 0.01

Accuracy and Precision. Freshly prepared lab-grade ALFQ containing 200 μg/mL of total QS-21 in liposomal formulation with virtually no QS-21 HP and cGMP ALFQ manufactured in 2016, which contains both QS-21 and a significant amount of the degradation product QS-21 HP were utilized to establish accuracy and precision of the described method. Table 5 shows that at all dilution conditions (1:50, 1:100, and 1:200), QS-21 measurements exhibit % recoveries in the range of 90−110%, which are within the acceptable limit. In the case of QS-21 HP quantitation, the % recoveries are in the range of 97−120%, for 5, 10, and 20% spiked levels in liposomal test samples. Overall, the described method exhibits good accuracy for measuring intact QS-21 and a good impurity assay for the presence of the degradation product QS-21 HP in liposomal drug formulation.

Table 5. Percent Recoveries Associated with QS-21 and QS-21 HP Measurements in Freshly Prepared Lab-Grade ALFQ[a]

Accuracy Studies for QS-21 Measurements			
	QS-21 conc. (μg/mL)		
dilution of test samples	calculated	theoretical	% recovery
1:100	202.53 ± 12.91	200	101.26 ± 6.46
1:200	207.94 ± 26.14	200	103.97 ± 13.07
1:50	212.71 ± 8.03	200	106.35 ± 4.02
Accuracy Studies for QS-21 HP Measurements			
	QS-21 HP conc. (μg/mL)		
QS-21 HP spiked level (%)	calculated	theoretical	% recovery
20	39.40 ± 0.40	40	98.50 ± 1.00
10	20.77 ± 0.68	20	103.83 ± 3.40
5	11.13 ± 0.76	10	111.33 ± 7.57
0	N.D.		

[a]N.D. = not detected.

The method precision was established in terms of within-run and between-run repeatability based on the % RSD of the calculated concentrations and chromatographic RT associated with the analysis of ten (10) liposomal test samples. The within-run sample preparation repeatability (N = 10) for quantifying QS-21 and QS-21 HP was characterized by % RSD < 3 and <10%, respectively, while the RT repeatability was associated with % RSD < 1% for both analytes (Table 6).

A tighter variance was observed in the within-run injection repeatability (N = 10, 20) with % RSD < 4% for QS-21 and QS-21 HP measurements and % RSD < 1% with respect to the RT. The method precision was further demonstrated in the between-run variability evaluated from three (3) independent measurements for QS-21 on different days, with % RSD < 6% (Table 7).

Table 7. Summary of the Between-Run Repeatability of QS-21 Measurements in Freshly Prepared Lab-Grade ALFQ

	QS-21 conc. (μg/mL) in test Samples		
day	preparation 1	preparation 2	preparation 3
day 1	233.39	237.36	200.67
day 2	236.96	216.36	210.76
day 3	229.78	223.98	222.94
mean ± SD	233.38 ± 3.59	225.90 ± 10.63	211.46 ± 11.15
% RSD	1.54	4.71	5.27

Application in Real Samples. The proposed method was used to accurately evaluate in-process samples for ALFQ manufacturing and the release of the final product at WRAIR. The method is currently used to monitor the stability of cGMP-manufactured ALFQ.

SUMMARY

The new UPLC-MS/MS-based method demonstrated promising analytical characteristics for quantifying QS-21 and QS-21

Table 6. Summary of Within-Run Sample Preparation and Injection Repeatability for QS-21 and QS-21 HP Measurements in cGMP-Manufactured ALFQ

	QS-21		QS-21 HP	
	conc. (μg/mL)	RT (min)	conc. (μg/mL)	RT (min)
Sample Prep. Repeatability				
N = 10				
mean ± SD	167.75 ± 4.26	10.90 ± 0.00	4.36 ± 0.36	7.18 ± 0.01
% RSD	2.54	0.04	8.26	0.14
Injection Repeatability				
N = 10				
mean ± SD	165.51 ± 1.85	10.90 ± 0.00	4.57 ± 0.07	7.18 ± 0.01
% RSD	1.12	0.04	1.53	0.14
N = 20				
mean ± SD	166.87 ± 1.42	10.91 ± 0.01	5.63 ± 0.19	7.18 ± 0.01
% RSD	0.85	0.09	3.38	0.14

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HP in liposomal drug products. To the best of our knowledge, this is the first report of a quantitative method for this purpose. The method qualification results following the FDA Guidance for Industry: Q2(R1) for validation showed that the designed method exhibits good analyte specificity/selectivity and good analyte measurement sensitivity, accuracy, and precision. The measurements of target analytes, QS-21 and QS-21 HP, were not influenced by the sample matrix; thus, this method has the potential to accurately determine QS-21 and its degradation product in QS-21-containing solutions and adjuvant formulations. The proposed method was used to accurately evaluate in-process samples for ALFQ manufacturing and the release of the final drug product at WRAIR. The applicability of the method will be demonstrated in the stability studies of cGMP-manufactured liposomal drug products.

EXPERIMENTAL SECTION

Chemical Reagents and Instrumentation. All solvents and reagents used in mobile phases (methanol, water, and formic acid) were Optima LC-MS grade and were purchased from Fisher Chemicals. DMPC, DMPG, 3D-PHAD (synthetic monophosphoryl lipid A, MPLA), and cholesterol for liposomal preparation were purchased from Avanti Polar Lipids Inc. and were used without further purification. Triethylamine (Et_3N) used for the hydrolysis reaction was purchased from Sigma-Aldrich (Saint Louis, Missouri). The QS-21 working standard was prepared from in-house HPLC-purified QS-21 purchased from the vendor Indena (through Desert King). The QS-21 HP working standard was generated from the purified product of the base-mediated hydrolysis of QS-21.

ALFQ was prepared following the established procedure.[31] cGMP-grade ALFQ was provided by the Pilot Bioproduction Facility (PBF) at the WRAIR.

Purification of QS-21 and QS-21 HP was done using a Shimadzu UltraFast Liquid Chromatograph (UFLC; LC-6AD) equipped with a Shimadzu Fraction Collector (FRC-10A). Quantitative analyses were done using a Thermo Scientific Vanquish Flex UHPLC system coupled with a Q-Exactive Quadrupole-Orbitrap Mass Spectrometer, controlled by Xcalibur software version 4.4. The data were processed using Thermo Scientific TraceFinder 5.1.

Working and Calibration Standards. To produce the in-house QS-21 working standard, commercial QS-21 from the vendor Indena (through Desert King) was purified using preparative HPLC (Supporting Information). The purity of the fractions was confirmed by a full-scan UPLC-MS/MS experiment. The pure fractions from purification were collected and lyophilized to generate a white and fluffy solid product. A 1 mg/mL QS-21 in methanol was prepared as a stock solution and stored in a −80 °C freezer to prevent hydrolysis decomposition.

The working standard for the QS-21 hydrolysis decomposition product, QS-21 HP, was generated from an intentional base-mediated hydrolysis reaction of intact QS-21 with excess Et_3N (∼5 equiv). Briefly, QS-21 (∼10 mg) was dissolved in 50/50 H_2O/methanol and added with ∼5 equiv Et_3N. The reaction mixture was stirred for ∼72 h at room temperature. QS-21 HP was isolated using preparative HPLC in a similar manner as described for the purification of the intact QS-21 working standard (Supporting Information), and the product purity was confirmed by a full-scan UPLC-MS/MS experiment. Pure fractions were collected and lyophilized to generate a white solid product. A 1 mg/mL QS-21 HP in methanol stock solution was prepared and stored in a −80 °C freezer.

From the above stock solutions, a working standard mixture containing 5 μg/mL QS-21 and 2.5 μg/mL QS-21 HP was prepared. A calibration standard mixture with QS-21 in the range of 5.0−0.02 μg/mL and QS-21 HP in the range of 2.5−0.01 μg/mL was prepared by serial dilution of the standard in methanol. A fresh set of calibration standards was made for every analysis. The calibration standards were not prepared in a liposomal matrix containing DMPC, DMPG, 3D-PHAD, and cholesterol in Sorensen phosphate buffer saline (SPBS), as the matrix effect was not observed under our analytical and experimental conditions.

Liposomal Test Samples. To have a better understanding of the limitations and applicability of the method, both non-cGMP and cGMP-manufactured ALFQ samples were utilized for the development and qualification of the described analytical method for quantifying QS-21 and QS-21 HP in the liposomal matrix. Different levels of sample dilutions in LC-MS-grade methanol, 1:50 (low), 1:100 (mid), and 1:200 (high), were investigated to establish the appropriate assay dilution condition. The 1:100 assay dilution was employed in the final and optimized method.

Optimized UPLC-MS/MS Conditions. Quantitative analysis of QS-21 and QS-21 HP was done using a Thermo Scientific Vanquish UHPLC coupled with a Q-Exactive Quadrupole-Orbitrap detector. The separation was carried out in an Agilent Zorbax Eclipse Plus C18 column (4.6 mm ID × 50 mm, 1.8 μm particle size), using water with 0.1% formic acid (A) and methanol with 0.1% formic acid (B) as mobile phases with a constant flow of 0.5 mL/min at a controlled column temperature of 35 °C. The UPLC gradient used is described in Table S5. The injection volume was set at 5 μL. All data were acquired using negative electrospray ionization (ESI) in parallel reaction monitoring (PRM) mode. The electrospray and source settings were as follows: 2.5 kV (capillary voltage), 320 °C (capillary temperature), 25 AU (sheath gas flow rate), 10 AU (Aux gas flow rate), and 300 °C (Aux gas temperature).

Intact QS-21 1 and QS-21 2 were detected as $[M - H]^-$ with a PRM transition of m/z 1987.9169 > 485.3272 at 10.91 and 10.41 min (chromatographic RT), respectively. QS-21 R1 and R2 derivatives eluted at 10.83 and 11.40 min, respectively, were detected using the m/z 1855.8746 > 485.3268 PRM transition. The degradation product QS-21 HP was detected using a PRM transition of m/z 1511.6548 > 955.4549 at 7.19 min. Quantification was done using an external calibration method with an equal weighting scheme in TraceFinder 5.1 (Thermo Scientific, Waltham, MA).

Quantitation of the Total QS-21 Concentration. The concentration of QS-21 1 in the liposomal drug product was obtained by interpolation from the calibration curve (i.e., QS-21 1 peak area vs concentration), followed by a dilution factor multiplication. Since the calibration peak area accounts only for the major component QS-21 1, further correction was done to include other QS-21 isomers and derivatives (i.e., QS-21 2, QS-21 R1, and QS-21 R2). Other QS-21 isomers/derivatives have been accounted for in the final/total QS-21 concentration following eq 1.

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$$[QS-21]_{total} = [QS-21\ 1] + [QS-21\ 1] \frac{\sum(\%\ \text{area of other QS}-21\ \text{Derivatives})}{100} \quad (1)$$

Analytical Method Qualification. The development of the method was followed by qualification studies to establish the selectivity/specificity, sensitivity, linearity, precision, and accuracy of the method to quantify QS-21 and QS-21 HP, following the ICH quality guidelines for validation.[43]

System Suitability. Initial system suitability exploratory studies were conducted to determine appropriate concentrations of the system suitability solutions that will be employed to ensure the suitability of the equipment during the assay. A mixture of 1.25 μg/mL QS-21 and 0.63 μg/mL QS-21 HP provided good signals for both analytes and was utilized as a standard system suitability solution. The overall assay suitability was evaluated based on the coefficient of variation (CV) of the chromatographic RT and the peak area of the six (6) injections at the beginning and at the end of the sequence.

Specificity. Standard solutions containing only QS-21 and QS-21 HP in methanol, and freshly prepared lab-grade and cGMP-manufactured ALFQ were utilized to establish the ability of the method to detect the target analytes selectively and specifically in the presence of other components, matrix, and solvents. This parameter was established using PRM transitions for detection and quantification.

Linearity and Dynamic Range. Wide concentration ranges of QS-21 (160−0.02 μg/mL) and QS-21 HP (80−0.01 μg/mL) were explored to determine the linear range of the calibration curve. The linearity and linear range were evaluated based on the regression coefficient ($R^2 > 0.995$) and the % difference of the calculated concentrations relative to the theoretical calibration concentrations (% difference < 20%) from five (5) sets of calibration standards with the defined concentration ranges.

Limit of Detection (LOD)/Limit of Quantitation (LOQ). The sensitivity of the developed method was evaluated by determining the LOD and LOQ. These parameters were extracted from the linearity studies. The LOD was established from the standard error of the *y*-intercept (σ) and the slope (S) of the linear calibration curve using eq 2. The signal-to-noise (S/N) ratio of the LOD should be ≥3.

$$\text{LOD} = \frac{3.3\sigma}{S} \quad (2)$$

The LOQ, on the other hand, was determined using eq 3. The LOQ should exhibit an S/N ≥ 10. Both the LOD and LOQ were established from five (5) independent sets of calibration standards.

$$\text{LOQ} = \frac{10\sigma}{S} \quad (3)$$

Accuracy. The accuracy was established to better understand the ability of the method to provide the calculated and determined concentrations of the target analytes relative to the expected/true values and to verify if any of the excipients in the liposomal formulations affect the analyses of the target analytes. Three (3) levels of assay dilution conditions were explored: 1:50, 1:100, and 1:200, and the accuracy was established based on the percent (%) recoveries at each dilution relative to the nominal concentration (200 μg/mL) of QS-21 in ALFQ test samples. The accuracy of the method to quantitate the degradation product QS-21 HP was established based on the % recoveries relative to the theoretical values at 5, 10, and 20% QS-21 HP spike levels.

Precision. The precision of the analytical system was evaluated in terms of the repeatability of ten (10) sample preparations of cGMP-manufactured ALFQ at appropriate assay dilution within a single run. Injection repeatability was also established from one sample preparation injected 10× and another sample preparation injected 20×. The between-run method variability was established from three (3) independent measurements of QS-21 concentrations in the same ALFQ test sample performed on three (3) different days by the same analyst. The precision of the method to quantify QS-21 and QS-21 HP was defined based on the % relative standard deviation (RSD) of the calculated concentrations and chromatographic RT, associated with sample preparation and sample injection within a single run and % RSD of the calculated QS-21 concentrations analyzed in multiple runs performed at different days.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c01877.

Details of the HPLC purification of QS-21 and QS-21 HP working standards; comparison of blank and extracted ion chromatograms; data on optimization of injection volume and spray voltage; and complete system suitability of the final UPLC-MS/MS method (PDF)

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The manuscript was written through contributions of all authors. The preparation of QS-21 and QS-21 HP working standards, and analytical method development and qualification were performed at the Laboratory of Adjuvant and Antigen Research (LAAR), WRAIR. All authors have given approval of the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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