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A sensitive liquid chromatography/mass spectrometry-based assay for quantitation of amino-containing moieties in lipid A

Thomas F. Kalhorn[1], **Anahita Kiavand**[2], **Ilana E. Cohen**[2], **Amanda K. Nelson**[2], and **Robert K. Ernst**[2,3,*]

[1]Department of Medicinal Chemistry, University of Washington, Seattle, WA 98195, USA

[2]Department of Medicine, University of Washington, Seattle, WA 98195, USA

[3]Department of Microbial Pathogenesis, University of Maryland Dental School, Baltimore, MD 21201, USA

Abstract

A novel sensitive liquid chromatography/mass spectrometry-based assay was developed for the quantitation of aminosugars, including 2-amino-2-deoxyglucose (glucosamine, GlcN), 2-amino-2-deoxygalactose (galactosamine, GalN), and 4-amino-4-deoxyarabinose (aminoarabinose, AraN), and for ethanolamine (EtN), present in lipid A. This assay enables the identification and quantitation of all amino-containing moieties present in lipopolysaccharide or lipid A from a single sample. The method was applied to the analysis of lipid A (endotoxin) isolated from a variety of biosynthetic and regulatory mutants of *Salmonella enterica* serovar Typhimurium and *Francisella tularensis* subspecies *novicida*. Lipid A is treated with trifluoroacetic acid to liberate and deacetylate individual aminosugars and mass tagged with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, which reacts with primary and secondary amines. The derivatives are separated using reversed-phase chromatography and analyzed using a single quadrupole mass spectrometer to detect quantities as small as 20 fmol. GalN was detected only in *Francisella* and AraN only in *Salmonella*, while GlcN was detected in lipid A samples from both species of bacteria. Additionally, we found an approximately 10-fold increase in the level of AraN in lipid A isolated from *Salmonella* grown in magnesium-limited versus magnesium-replete conditions. *Salmonella* with defined mutations in lipid A synthesis and regulatory genes were used to further validate the assay. *Salmonella* with null mutations in the *phoP*, *pmrE*, and *prmF* genes were unable to add AraN to their lipid A, while *Salmonella* with constitutively active *phoP* and *pmrA* exhibited AraN modification of lipid A even in the normally repressive magnesium-replete growth condition. The described assay produces excellent repeatability and reproducibility for the detection of amino-containing moieties in lipid A from a variety of bacterial sources.

Deoxy-aminosaccharides are ubiquitous components of connective tissue, chitin, cell membranes, and a variety of microstructures that are generally chemically and biochemically inert. These compounds have also been found as major components of lipid A, the hydrophobic anchor of lipopolysaccharide (LPS) present in the outer membrane of Gram-negative bacteria.[1–3] Species of lipid A from *Salmonella enterica* serovar Typhimurium (S. Typhimurium)[4–6] and *Francisella tularensis* subspecies *novicida* (*F. novicida*),[7–10] examples of which are shown in Figs. 1(A) and 1(B), respectively, have varying ratios of acylated and non-acylated aminosugars, fatty acids, and phosphates. The 2-amino-2-deoxyglucose (GlcN) dimer is the

*Correspondence to: R. K. Ernst, Department of Microbial Pathogenesis, University of Maryland Dental School, 650 W. Baltimore Street, Baltimore, MD 21201, USA. E-mail: E-mail: rkernst@umaryland.edu.

backbone structure, which anchors the fatty acid substituents and defines the compound. The presence of individual amino-containing residues, such as 2-amino-2-deoxygalactose (GalN), 4-amino-4-deoxyarabinose (AraN), and ethanolamine (EtN), are variable components of lipid A and are dependent on bacterial species and specific environmental growth conditions.[2,3,11,12] These amino-containing residues are bound to the GlcN core either directly through a 1-4′ glycosidic bond or as phosphate diesters. The structures of these amino-containing compounds and of the internal standard used in these analyses are shown in Fig. 1(C). These non-acylated free amines are normally protonated at physiological pH and affect the net charge of the molecule. They play a role in resistance of the bacteria to host innate immune killing mechanisms, which include cationic antimicrobial peptides (CAPs), and to antibiotics, and in the proinflammatory host response elicited by the bacteria.[13–16]

Here we describe the development and application of a novel sensitive analytical liquid chromatography/mass spectrometric-based assay for quantitation of amino-containing residues present in lipid A. Methods for the hydrolysis and derivatization of intact lipid A isolated from wild-type and genetically characterized lipid A biosynthesis mutants of *S.* Typhimurium and *F. novicida* were developed and used to determine the levels of the individual amino-containing compounds per lipid A molecule.

EXPERIMENTAL

Chemicals and reagents

Glucosamine hydrochloride, *N*-acetylglucosamine, 2-amino-2-methyl-1,3-dihydroxypropane, and ethanolamine hydrochloride were from Sigma (St. Louis, MO, USA), 2-galactosamine hydrochloride was from Calbiochem (Gibbstown, NJ, USA), and aminoarabinose was synthesized according to the procedure of Naleway *et al.*[17] by Svetlana Stekhova (University of Washington). The derivatizing reagent, 6-aminoquinolyl-*N*-hydroxylsuccinimidyl carbamate (AQHSC), was synthesized by the method of Cohen and Michaud[18] or purchased from Waters Corporation (AccQ•Tag™; Milford, MA). Trifluoroacetic acid (TFA) was from Spegranal (Riedel-deHaen, Germany). All high-performance liquid chromatography (HPLC) solvents and buffers were analytical grade.

Bacterial strains and growth conditions

Salmonella enterica serovar Typhimurium strains were obtained from Samuel I. Miller (University of Washington)[19–21] and the wild-type *Francisella tularensis* subspecies *novicida* U112 strain was obtained from Francis Nano (University of Victoria; Victoria, Canada)[22] (Table 1). *Salmonella* strains were cultured in Luria-Bertani broth or Lysogeny broth (LB) supplemented with 1 mM $MgCl_2$ or in N-minimal medium[23] supplemented with 0.1% casamino acids, 38 mM glycerol, and 10 μM, 1 mM, or 10 mM $MgCl_2$ at 37°C with aeration and harvested in stationary phase. The *Francisella* strain was cultured in tryptic soy broth (TSB) supplemented with 0.1% cysteine (TSB-C) at 37°C with aeration and harvested in the stationary phase.

LPS purification and lipid A isolation

LPS was isolated using a rapid small-scale isolation method for mass spectrometry analysis.[24] A 1.0 mL aliquot of Tri-Reagent (Molecular Research Center; Cincinnati, OH, USA) was added to a cell culture pellet (1–10 mL of an overnight culture), resuspended, and incubated at room temperature for 15 min. Chloroform (200 μL) was added and the samples were vortexed and incubated at room temperature for 15 min. Samples were centrifuged for 10 min at 13 400 *g* and the aqueous layers were collected. Water (500 μL) was added to the lower layers and vortexed. After 15–30 min, the samples were centrifuged as above and the aqueous layers again

collected. Two more aliquots of water were added to each sample for a total of four extractions. The combined aqueous layers were frozen and lyophilized.

The LPS was then hydrolyzed to lipid A by the addition of 0.5 mL 1% sodium dodecyl sulfate (SDS) in 10 mM sodium acetate buffer, pH 4.5, to the lyophilized powder.[25] Samples were incubated at 100°C for 1 h, frozen, and lyophilized. The dried pellets were washed in 100 μL of water and 1 mL of acidified ethanol (100 μL 4 N HCl in 20 mL 95% EtOH). Samples were centrifuged at 2300 *g* for 5 min and the supernatant discarded. The lipid A pellet was further washed (twice for a total of three washes) in 1 mL of 95% EtOH. The entire series of washes was repeated twice. Samples were resuspended in 500 μL of water, frozen, and lyophilized.[25]

For each lipid A sample, two vials were prepared, each containing 10 μg of lipid A and 5 ng of internal standard (IS), dissolved in 200 μL 1.0 M TFA. The first vial, used in the analysis of AraN, was heated at 90°C for 30 min, and the second vial, used in the analysis of GlcN, GalN and EtN, was heated at 90°C for 24 h. After heating, the individual samples were frozen and lyophilized before derivatization and analysis as described below.

Lipid A structural modifications in individual samples were confirmed qualitatively by negative ion matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) analysis.[26,27]

Standard preparation

The primary standard mixture of the four analytes was prepared from stock solutions as a cocktail at a final concentration of 25 ng/mL/component. A set of eight standards ranging in concentration from 0.125 to 25 ng/mL was prepared by serial dilution from the primary standard mixtures. One mL of standard solution and 1.0 mL IS solution (5.0 ng/mL) were combined in a reaction vial, flash frozen, and lyophilized. The standards were then sealed and stored at −80°C until use. The IS, 2-amino-2-methyl-1,3-dihydroxypropane, was chosen because, like the other analytes, it is a polyhydroxy alkane with a primary amine. In addition, it is of purely synthetic origin and does not appear as a biological product.

For each run, standards were dissolved in 200 μL 1.0 M TFA, vortexed, and heated at 90°C for 30 min or 24 h. The samples were then frozen, lyophilized, and dissolved in 50 μL 0.2 M borate buffer, pH 8.8, prior to the addition of 50 μL of 1.0 mg/mL AccQ•Tag™ derivatizing reagent in acetonitrile. The samples were vortexed, incubated at room temperature for 15–30 min, then evaporated to dryness under nitrogen. Derivatized samples were reconstituted in 100 μL of distilled water, vortexed, and transferred to injection vials for analysis.

Chromatography and mass spectrometry

All mass spectra were collected using atmospheric pressure ionization in the positive electrospray mode (API-ES$^+$). Single ion monitoring (SIM) studies were performed on a Series 1100 MSD (Agilent, Palo Alto, CA, USA); the tandem mass spectrometric (MS/MS) and multiple reaction monitoring data were obtained using a Premier XE tandem quadrupole mass spectrometer (Waters, Milford, MA, USA). Separation was achieved using an 1100 HPLC (Agilent, Palo Alto, CA) or an Aquity ultra-performance liquid chromatography system (Waters, Milford, MA). Each system was equipped with solvent degasser, high-pressure gradient, and temperature-controlled injector. Mass spectra were obtained and ionization conditions optimized on samples purified by preparative HPLC using direct injection series (MSD) or infusion (Premier XE).

The derivatized analytes were separated using a Develosil 5u RP-Aqueous C-30 column (150 × 2.1 mm; Phenomenex, Torrence, CA, USA) using a 20 mM ammonium formate (pH 7.5)/acetonitrile (ACN) gradient. There was a linear increase from the initial 2% ACN to 5% at 0.5

min, 10% at 6.5 min and 20% at 17 min. The ACN was then increased to 80% at 18 min for a 1 min washout, returned to the initial 2% at 20 min and equilibrated for 8 min prior to the next injection. The flow was 0.4 mL/min throughout the run, temperature was uncontrolled, and the run time was 30 min. Eluent before 4.5 and after 16 min was diverted from the mass spectrometer.

The mass spectrometers were tuned to maximize the protonated molecular species equivalent to the [analyte molecular weight (MW) + 171]$^{+}$, generating *m/z* values of 232 (EtN), 320 (AraN), 350 (GalN & GlcN), and 276 (IS) for the compounds of interest. The observed signals were consistent with the formation of the *N*-quinoyl-*N′*-aminosaccharide urea as the derivative. The ionization conditions used in SIM (Agilent MSD) mode were: gas temp, 350°C; drying gas (nitrogen), 12 L/min, nebulizing pressure (nitrogen), 35 psi; and Vcap, 5500 volts (V). When running selected reaction monitoring (SRM; MicroMass Premier XE), the precursor > 171 transition was monitored. The conditions on the Premier XE were: capillary, 2.0 Kv; cone, 15 V; extractor, 3.0 V; desolvation gas (nitrogen) flow, 400 L/h; source temperature, 120°C; desolvation temperature, 400°C; and collision voltage, 30 eV.

Determination of repeatability and reproducibility

For repeatability experiments, five replicates of each individual analyte were run at three different concentrations: 1, 7, and 25 ng/sample, representing low, medium, and high concentrations. Found concentrations were derived from a standard curve run on the same day. The precision calculations were based on the standard deviation of the average concentration of each compound and the accuracy based on the deviation and bias of the data from the expected values. This experiment was repeated on each of five days to obtain the inter-observer reproducibility data.

Linearity and limits of detection

Lower limits of quantification were calculated as mass applied to the column producing a signal five times background noise and based upon a derivatized stock solution of 25 ng/mL equivalents of GlcN. Serial dilutions were made to 74 pg/mL. The upper limit of the linearity was also tested to 25 ng of each of the four analytes of interest, which is four times the amount normally used for routine assays of lipid A. Saturation of the detector was based on consistent negative deflection in the slope of the standard curves when all the points were used relative to the lower quadrant.

Kinetics of hydrolysis and analyte stability

Lipid A samples from *Salmonella* and *Francisella* were heated at 90°C in 1.0 M TFA and aliquots drawn at time points ranging from 15 min to 24 h. Samples were allowed to cool to room temperature, supplemented with IS, frozen, and lyophilized. Individual samples were then derivatized and analyzed as described above. The resulting data were used to define the kinetics of formation and degradation of free AraN, GalN, GlcN, and EtN from the lipid A molecule. The stability of the individual analytes was also assessed. The amino-containing compounds were dissolved in 1.0 M TFA and heated at 90°C for times from 15 min to 24 h. Samples were cooled to room temperature and processed as described above for lipid A-derived samples. Stability calculations were based on comparisons to identical samples that had been dissolved in water, lyophilized, and derivatized without heating.

RESULTS

Derivatization of amino-containing compound

To develop an assay to quantitate the individual amino-containing constituents of lipid A (GlcN, GalN, AraN, and EtN), the analytes were hydrolyzed at high temperature in 1.0 M TFA to liberate individual monosaccharides with free amine groups and subsequently derivatized using a commercially available labeling reagent (AccQ•Tag™). This reagent was developed by Waters Corporation to fluorescently label and quantitate the free amines in individual amino acids. We utilized this reagent to detect derivatized carbohydrate amines using mass spectrometry. The derivatization of the aminosugars was rapid and complete. We were able to ascertain that the response was linear up to 10 μg of lipid A/sample and that there was no change in response when twice the standard concentration of derivatizing reagent was used (data not shown), suggesting that the reaction went rapidly to completion without exhaustion of the derivatizing reagent. Finally, excess derivatizing reagent rapidly degrades in water to aminoquinoline (AMQ) and succinimide (NHS), two low molecular weight compounds (*m/z* 144 and 99, respectively) that do not interfere with MS detection.

Mass spectral characteristics of derivatized aminosugars

The mass spectrum and proposed fragmentation pathway of the derivatized GlcN isolated by preparative HPLC are shown in Fig. 2. The product ion scan of AQC-GlcN (*m/z* 350) is typical of the compounds of interest. In all of the precursor spectra of these compounds, the signal resulting from the molecular ion dominates. The product ion scan shows almost exclusive formation of the protonated aminoquinoylisocyanate ion (*m/z* 171). No other ion observed has an abundance greater than 10% at collision energies ranging from 20 to 40 eV.

HPLC separation and retention times of derivatized aminosugars

The HPLC extracted ion chromatograms of the derivatized analytes are shown in Fig. 3. The separation was designed to retain the analytes for at least two column volumes in order to prevent non-specific ion quenching that can occur from salts in the sample, to give at least 80% baseline separation of both the α and β anomers of the two isobaric compounds (GlcN and GalN), and to separate excess derivatizing reagent products from the analytes in order to minimize the possibility of ion-quenching. Typical retention times for the individual analytes were: AraN (*m/z* 320) 11.5 min; GalN (*m/z* 350) 5.6 and 9.2 min; GlcN (*m/z* 350) 5.8 and 8.1 min; EtN (*m/z* 232) 14.6 min; and IS (*m/z* 276) 16.8 min. A contaminant (*m/z* 276) with a retention time of 9.1 min did not interfere with analyte or standard analysis. Two peaks were positively ascribed to each of the amino-hexoses, with the pyranose forms dominating to such an extent that the furanose forms were not detected. For the amino-pentose (AraN), only a single peak was observed, suggesting that there was no separation of the two anomers, that one anomer dominated to such an extent that the minor isomer could not be detected, or that the uncyclized form dominated when derivatized.

Detection limits of the assay: sensitivity and linearity

Routinely, the assay was running using SIM detection and standards run from 0.125 to 25 ng/sample. Typical standard curves of each of the analytes are shown in Fig. 4. Assuming 100% derivatization, the amount of the individual analytes at the lowest concentration represents 25 pg on-column. The absolute limit of quantitation for each analyte was approximately 20 fmol of GlcN, GalN, and EtN on column and 100 fmol of AraN. The reason(s) for the diminished sensitivity for AraN is unknown. The sensitivity using SRM was considerably better; we have applied sub-fmol quantities of analyte on-column and successfully integrated both anomers of GalN and GlcN for quantitation. These results suggest that this assay has a large linear range and can detect trace quantities of GalN or AraN in the presence of substantial quantities of

GlcN. Interestingly, ethanolamine was observed in all samples, including blanks, and resulted in poor precision at low concentrations (see next section). This precludes accurate measurement of EtN at levels below 5 pg/sample.

Repeatability and reproducibility of the assay

Reliability studies were designed to quantify experimental variability attributable to the repeatability (intra-day variation) and reproducibility (inter-day variation) of the assay. These results are shown in Table 2 and Table 3. Both repeatability and reproducibility were excellent, with variability for the former less than 5% and for the latter less than 10%. The lower concentrations of ethanolamine showed a higher variability and lower accuracy in both the repeatability and reproducibility studies. This may be due to ubiquitous low levels of EtN as a contaminant from the manufacture of plastics used in the processing of our samples. The accuracy for both the repeatability and reproducibility studies averaged greater than 94% of the actual values at the 7 and 25 ng analyte levels. The aminosugar quantitation at the 1 ng analyte level shows an average accuracy of 74 and 76% of the actual values for the repeatability and reproducibility studies, respectively, if EtN is excluded due to the elevated background described above. After derivatization, the analytes were extremely stable; stock solutions and individual lipid A samples were used to confirm chromatographic conditions in samples stored at 4°C for over 1 year.

The accuracy and robustness of the method allowed us to apply it confidently to the analysis of the aminosugars present in lipid A of the bacteria *S*. Typhimurium and *F*. *novicida*.

Hydrolysis conditions for lipid A

Hydrolysis of an intact lipid A molecule is required to generate monomers of the individual components and to remove the fatty acids attached to the glucosamine (GlcN), leaving a free amine group at the 2 position for derivatization (Fig. 1). It was observed in our initial experiments using purified AraN that the analyte was quickly destroyed during hydrolysis at high temperature, as compared to the other analytes (data not shown). Therefore, we were concerned that prolonged exposure to the hydrolysis conditions required for liberation of GlcN, GalN, and EtN from lipid A molecules could potentially result in the destruction of the AraN present at the 1 or 4′ position of lipid A.

To determine the respective hydrolysis times for the individual aminosugars present in lipid A, a time course experiment using lipid A (~10 μg) isolated from a wild-type *S*. Typhimurium strain and liberated from the intact LPS molecule by mild acid hydrolysis was carried out (Fig. 5(A)). The time course experiment clearly shows that AraN was rapidly hydrolyzed from the bulk of the molecule between 0.5 and 1 h of heating and in the following 4 h was degraded almost completely. The other components appeared more slowly, suggesting that extended incubation times were required for the complete hydrolysis of the intact lipid A molecule. GlcN, which must undergo several O-deacylations, an N-deacylation, and glycolysis, achieved a maximum concentration after 24 h of heating with no further change after an additional 12 h. The other two components, EtN and GalN, showed maxima at 18 and 24 h, respectively, of hydrolysis (data not shown).

Further work using purified analytes precisely defined the acid-catalyzed degradation of AraN. The results of a stability study under hydrolysis conditions over a 4 h period at 90°C confirmed the stability of GalN, GlcN, and EtN levels and the propensity of AraN to degrade quickly under the assay hydrolysis conditions (Fig. 5(B)).

Because of the observed instability of AraN under these hydrolysis conditions, we adopted the practice of hydrolyzing two aliquots of each lipid A sample. The first aliquot, heated for 30

min, is used to quantify the AraN content of the sample, and the second aliquot, heated for 24 h, is used to calculate the GlcN, GalN, and EtN in the sample. A schematic diagram for this hydrolysis and derivatization procedure is shown in Fig. 6. An application of this technique is demonstrated in the analysis of lipid A isolated from S. Typhimurium and *F. novicida* (shown in Fig. 7). Extracted ion chromatograms of lipid A isolated from *Salmonella* (Fig. 7(A)) clearly show the presence of GlcN and AraN, but not GalN. The *Francisella* lipid A sample (Fig. 7 (B)) contains both GlcN and GalN, but not AraN. The clear separation of both the α and β anomers of the isobaric GlcN and GalN isomers allows for unequivocal structural assignment in the *Francisella* lipid A sample.

Analysis of aminoarabinose in *S.* Typhimurium lipid A

In the environment, bacteria encounter dramatically different niches to which they must adapt. Expression of genes, such as the two-component regulatory system (PhoP-PhoQ) or biosynthetic enzymes (PmrE or PmrF) involved in the modification of lipid A in *Salmonella*, can be induced by modulating bacterial growth conditions (e.g., temperature, nutrient availability, medium osmolarity, and pH) or through defined genetic mutations. Using the assay, the extent of AraN modification per lipid A molecule isolated from a wild-type S. Typhimurium strain after growth under different magnesium concentrations (10 μM, 1 mM, and 10 mM) in N-minimal medium was determined. Growth conditions were chosen that would induce (10 μM) or repress (10 mM) the expression of genes required for the synthesis and attachment of AraN to lipid A at either the 1 and/or 4′ position (see Fig. 1). We observed ~10-fold induction in AraN levels in lipid A isolated from the magnesium-limited growth condition (77.0% of lipid A modified with AraN) as compared to the magnesium-replete growth condition (7.9% of lipid A modified with AraN) (Fig. 8(A)). Lipid A isolated after growth at an intermediate magnesium concentration (1 mM) showed ~3-fold induction of AraN (20.9% of lipid A modified with AraN). Addition of AraN to lipid A was confirmed using negative ion matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS (data not shown).

To further validate our assay, we determined the level of AraN per lipid A molecule isolated from previously characterized lipid A mutants of *S.* Typhimurium (Table 1) after growth in LB broth supplemented with 1 mM magnesium. This growth condition has been shown previously to repress the addition of AraN in a variety of bacterial backgrounds (data not shown).[4,5,28–30] As expected, AraN addition to lipid A was not observed in strains that carried null mutations in either *phoP* or the two genes (*pmrE* and *pmrF*) required for the synthesis of AraN (Fig. 8(B)). In contrast, the two constitutively activated gene mutants, $PhoP^c$ and $PmrA^c$, showed increased levels of AraN, with >50% of lipid A molecules containing AraN (Fig. 8(B)).

DISCUSSION

Biological importance of AraN modification of lipid A

Addition of AraN to lipid A results in increased resistance to cationic antimicrobial peptides.[20,28,29,31] Cationic antimicrobial peptides (CAPs), which are produced by the innate immune system and also used clinically as antibiotics, kill bacteria by binding to and disrupting bacterial cell membranes.[13–16] Electrostatic interactions between the positively charged CAPs and negative charges contributed to the lipid A molecule by phosphate substituents facilitate binding of CAPs to the bacterial cell surface. Modification of lipid A with AraN, which is positively charged, decreases its negative charge, leading to increased bacterial CAP resistance as a result of reduced CAP binding. Bacteria that are unable to add AraN to their lipid A exhibit increased sensitivity to CAPs, including polymyxin B. Thus, the presence or absence of AraN

on the lipid A of a patient-derived bacterial isolate can serve as an indication of the infecting organism's resistance to polymyxin antibiotics, perhaps guiding therapy choices.

Limitations of previous methods for aminosugar detection

Most of the current studies relating to the lipid A structure make use of ^{13}C- and ^{1}H-NMR for a qualitative description of the aminosugar constituents[32] or emphasize the effects of variables on the genes central to aminosugar synthesis, transport, and/or addition within the organism.[33,34] To date, quantitative analysis of the AraN content of lipid A is limited to one published report by Conrad *et al.*[35] That procedure included hydrolysis, high-voltage paper electrophoresis, N-acylation, borohydride reduction, and O-acylation prior to GC analysis. Generally, quantitative analysis of aminosugars is broadly based on two different derivatization-based methodologies. One set of procedures for non-nitrogenous sugars generally involve isolation of the analyte followed by gas chromatographic separation of the trimethylsilyl,[36,37] alkylated,[38] or acylated derivatives,[39,40] followed by analysis using either mass spectrometry or flame ionization detection. Alternative methods are based on HPLC separation. Underivatized compounds can be separated using ion-exchange chromatography using far UV (λ_{190}), refractive index, or amperometric detection.[41–43] Other methods derivatize the amine using isocyanates or acid chlorides to produce ureas or amides, respectively. The N9-fluorenylmethyl ureas, *N*-phenylureas,[44] *N*-phenylthiocarbonyl ureas,[42,45,46] N-naphthylureas,[47] *N*-aminocarbonylquinoline ureas,[48,49] and dansylamides[50] have all been used for aminosugar analysis. The resulting compounds can be separated by reversed-phase HPLC, and the derivative greatly enhances sensitivity using conventional UV or fluorescent detection. All of these methods lack selectivity, which, in practice, often results in long run times, and makes the analysis of minor components impossible.

Advantages of the described assay

The assay method described here allows for the simultaneous analysis of the three aminosugars and ethanolamine present in lipid A using AQHSC as a derivatization reagent. The optimal parameters for this reaction have been well described and the derivatization reagents are available commercially for the fluorescent analysis of amino acids (Water's AccQ•Tag™). The individual amino-containing components of lipid A form *N*-aminoquinoylcarbonyl (AQC) ureas, three of which have distinct molecular weights equivalent to the $MW_{(aminosugar)} + 171$. The separation system described gives excellent baseline separation of the two isobaric compounds GlcN and GalN, which are therefore also easily identified and quantitated.

The aminoquinoline carbonyl derivative has been well described as an effective label used in HPLC separation with fluorescent detection of primary amines, especially amino acids. However, it lacks the selectivity required when quantitating low concentrations of analyte. In addition, fluorescence intensity is dependent on chromatographic variables such as fraction of organic solvent, pH, buffer concentration, and temperature, making such a system less rigorous and reproducible than desirable. The method described here is much less prone to errors attributable to the variables listed while providing quantitation into the subfemtomole range when using SRM. This sensitivity will make possible analysis of LPS/lipid A structures from minor populations and/or minor constituents that may have immunological importance.

Optimization of hydrolysis

A critical aspect of this study has been standardization of the ideal conditions for the hydrolysis of different aminosugars from the lipid A core. Originally, we used methanolic hydrochloric acid in an attempt to simultaneously perform fatty acid methyl ester analysis of the lipid fraction of the lipid A molecule, while liberating the aminosugars for derivatization and HPLC analysis. However, this method completely degraded AraN and resulted in partial methylation of GalN and GlcN. Using aqueous TFA, a milder method of hydrolysis, with two separate samplings,

more accurate calculations of AraN/GlcN or GalN/GlcN are possible, and thus the average fraction of the lipid A substituted with AraN or GalN can be calculated.

The assay described here exhibits increased selectivity and sensitivity when compared to previous methods of quantitation of aminosugars and allows the accurate and reproducible determination of aminosugar levels in lipid A isolated from a variety of bacterial strains. We have applied this method to the analysis of lipid A from *Francisella* and *Salmonella*, including defined biosynthetic and regulatory mutants. The application of this method to other bacterial isolates could allow a correlation between genetic variation and/or growth conditions and the final aminosugar levels present in lipid A. It would also make time course studies possible, which, used in combination with established fatty acid analytical methodology, would more fully define the intermediate species of lipid A biosynthesis. The ability to quantify AraN levels in lipid A using this assay could prove useful in predicting the resistance of clinical bacterial isolates to polymyxin antibiotics. Additionally, this method has the potential for use in detecting endotoxin contamination in a variety of samples.

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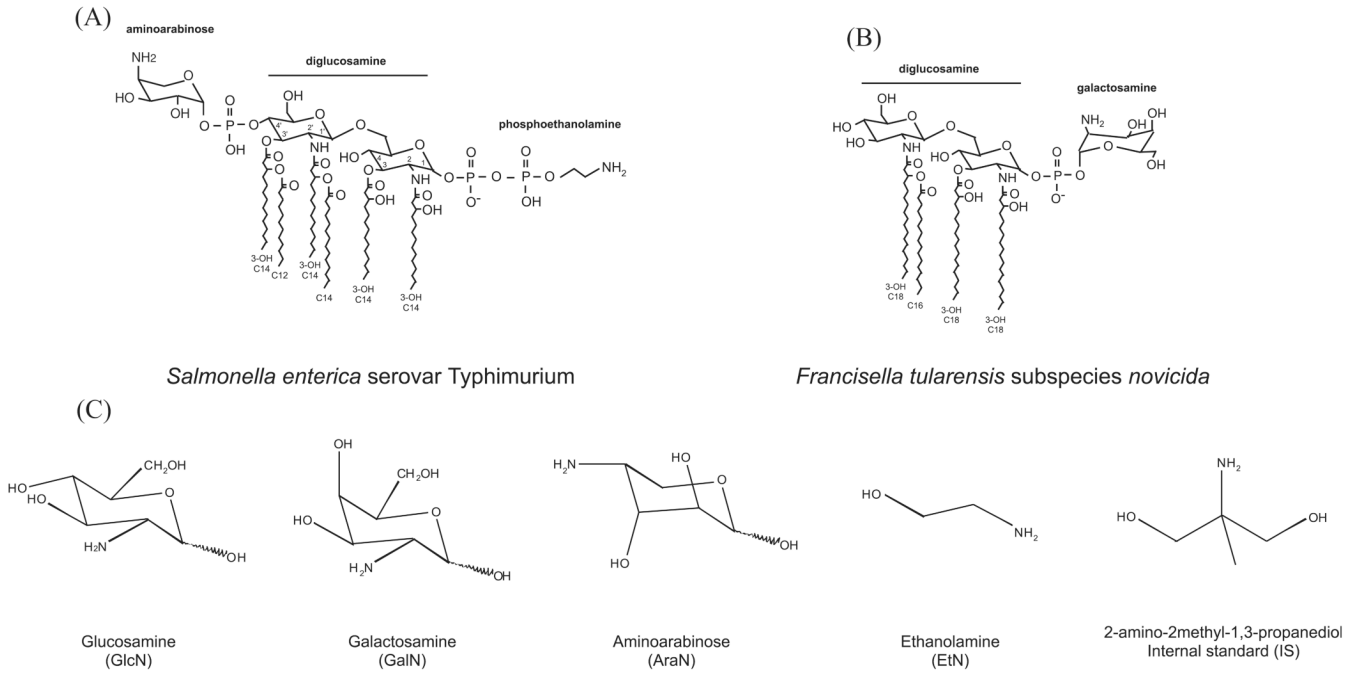

Figure 1.

Amino-containing moieties present in the lipid A component of bacterial lipopolysaccharide. Specific modifications present on *Salmonella enterica* serovar Typhimurium (A) and *Francisella tularensis* subspecies *novicida* lipid A (B) are shown and include glucosamine (GlcN), galactosamine (GaIN), aminoarabinose (AraN), and ethanolamine (EtN). Structures of the individual analytes (GlcN, GaIN, AraN, and EtN) and IS (2-amino-2-methyl-1,3-dihydroxypropanediol) quantitated in this study are shown (C).

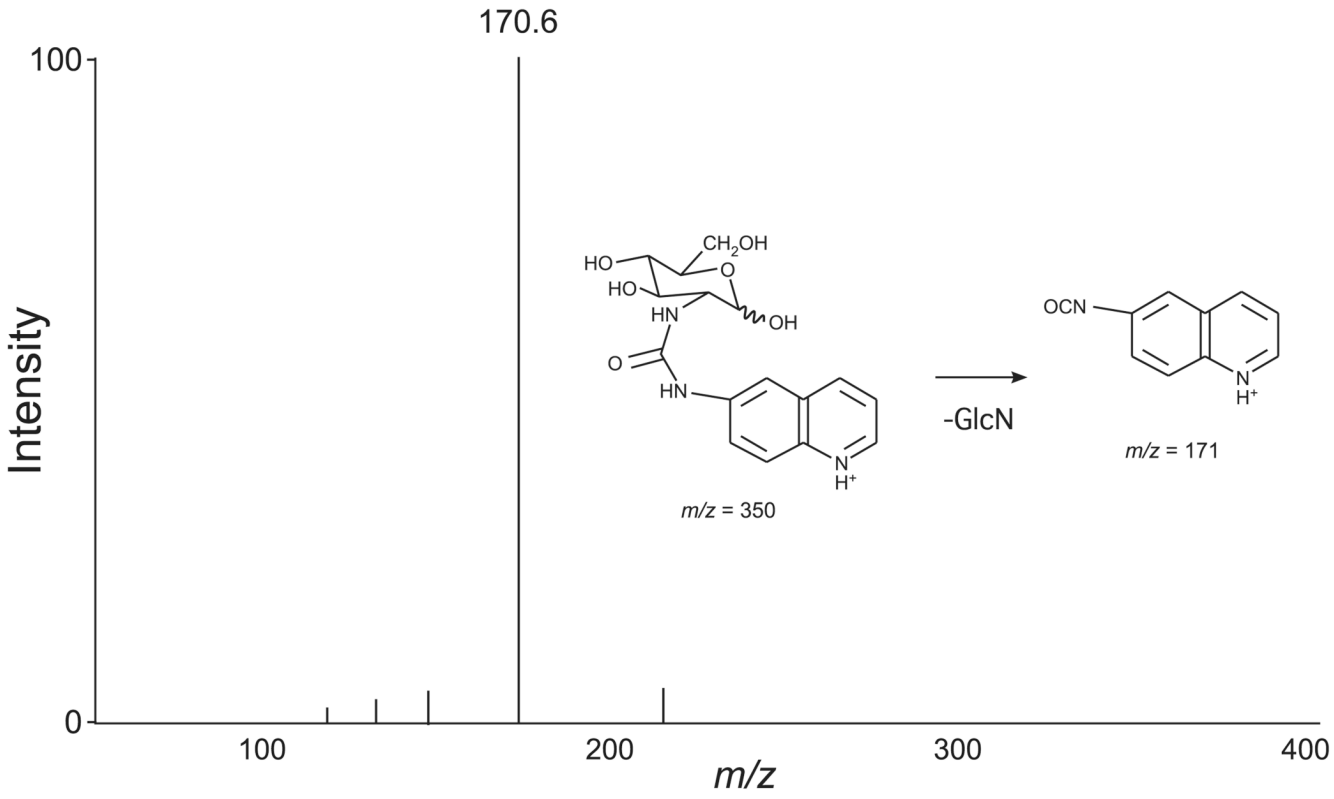

Figure 2.
Product ion spectrum of the protonated AQC-GlcN *m/z* 350 isolated by preparative HPLC. The protonated aminoquinoylisocyanate ion (*m/z* 171) dominant signal as shown in the proposed fragmentation scheme of the derivatized GlcN molecule.

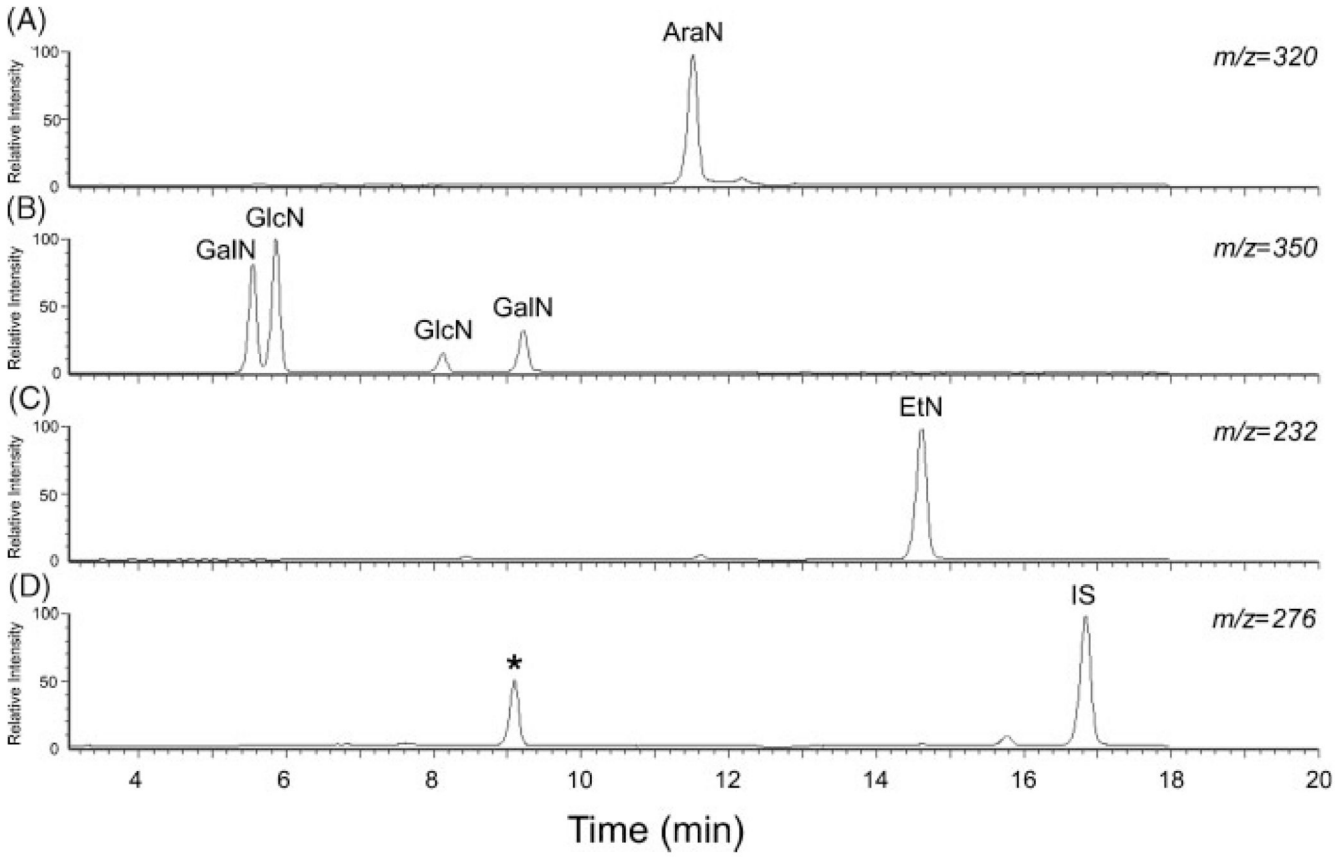

Figure 3.

Extracted ion chromatograms of individual amino-containing moieties. Scan of derivatives and signal intensity for the individual compounds. (A). *m/z* 320 (AraN); (B) *m/z* 350 (GaIN and GlcN); (C) *m/z* 232 (EtN); and (D) *m/z* 276 (IS). Typical retention times for the individual compounds are as follows: AraN (11.5 min), GaIN (5.6 and 9.2min), GlcN (5.8 and 8.1 min), EtN (14.6 min), and IS (16.8 min). On-column sample concentrations: individual analytes: 350 pg, IS: 250 pg. * indicates a contaminant (*m/z* 276) with a typical retention time of 9.1 min.

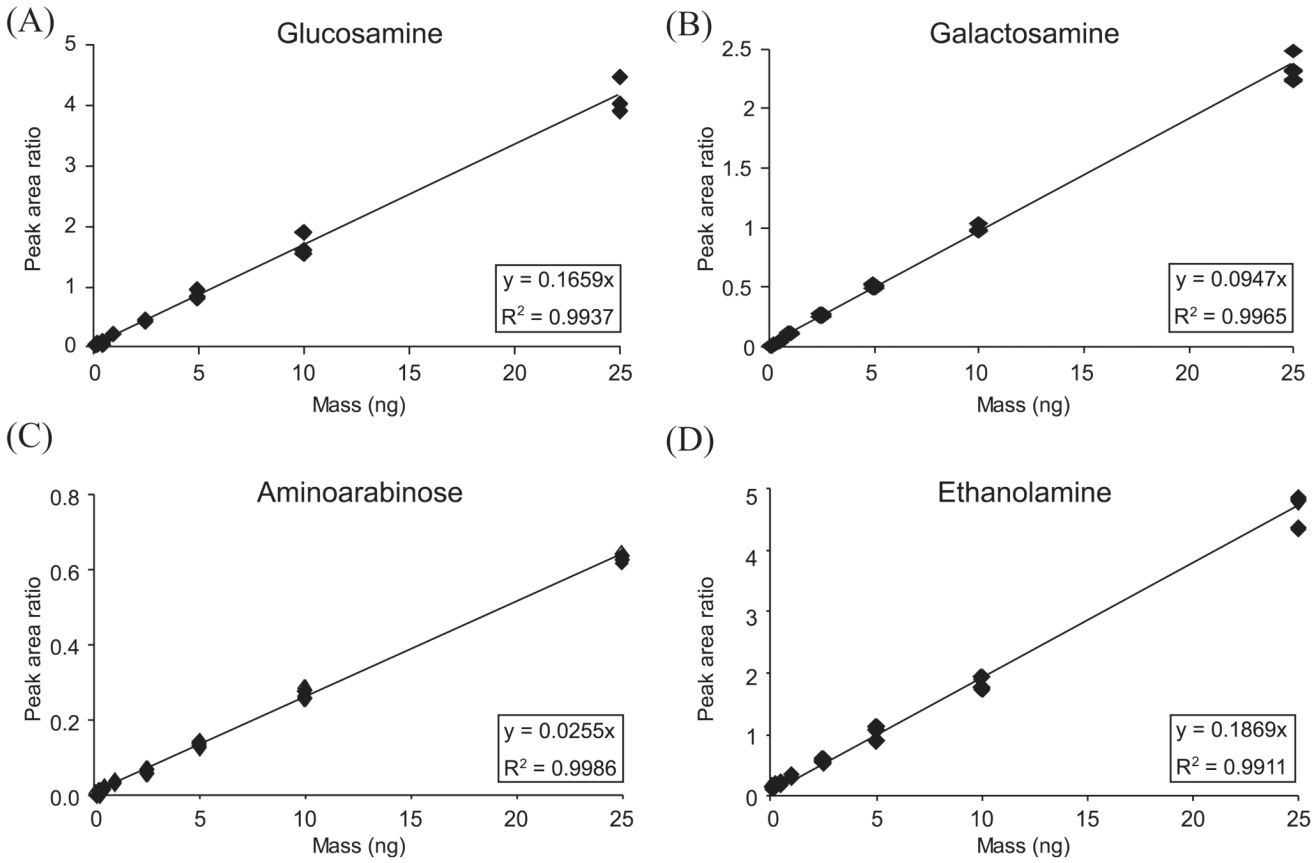

Figure 4.

Calibration curves for the quantitation of aminosugars. (A, B, C, and D) GlcN, GalN, AraN, and EtN show a wide linear range and reproducibility. The upper limit of linearity was tested to 25 ng of each of the four analytes. Three replicates are shown for each analyte.

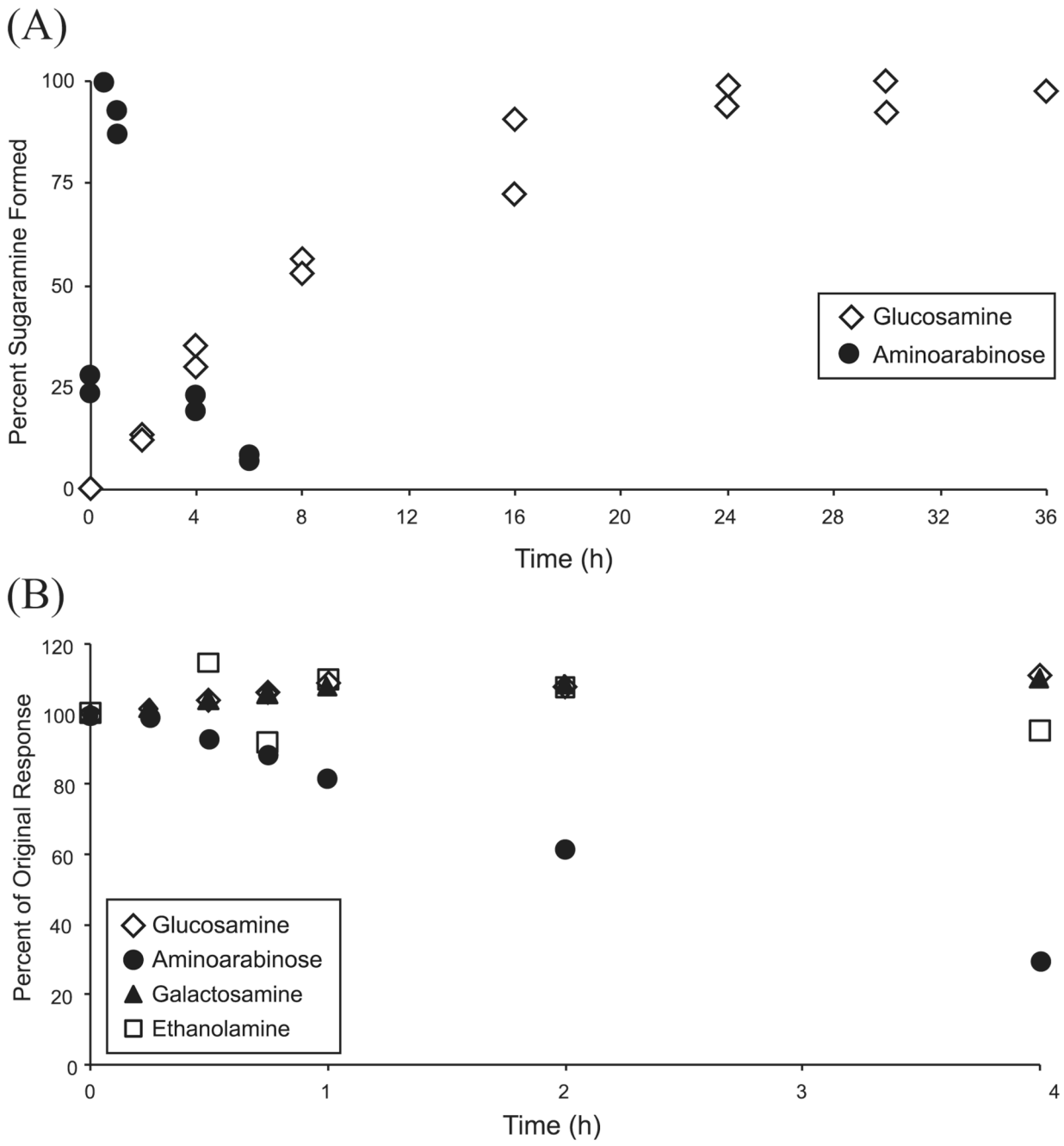

Figure 5.

Release and degradation of aminohydroxy compounds from lipid A under hydrolysis conditions (A) and stability of purified aminosugars under hydrolysis conditions (B). (A) 10 μg of lipid A isolated from wild-type *S*. Typhimurium was subjected to hydrolysis conditions (1.0 M TFA, 90°C) and the production of individual aminosugars monitored. AraN peaks at 30 min and is subsequently degraded over the next 5 h. GlcN is fully hydrolyzed after 24 h and is stable thereafter. (B) Purified GlcN, GaIN, EtN, and AraN were subjected to hydrolysis conditions (1.0 M TFA, 90°C) over a period of 4 h and their degradation monitored.

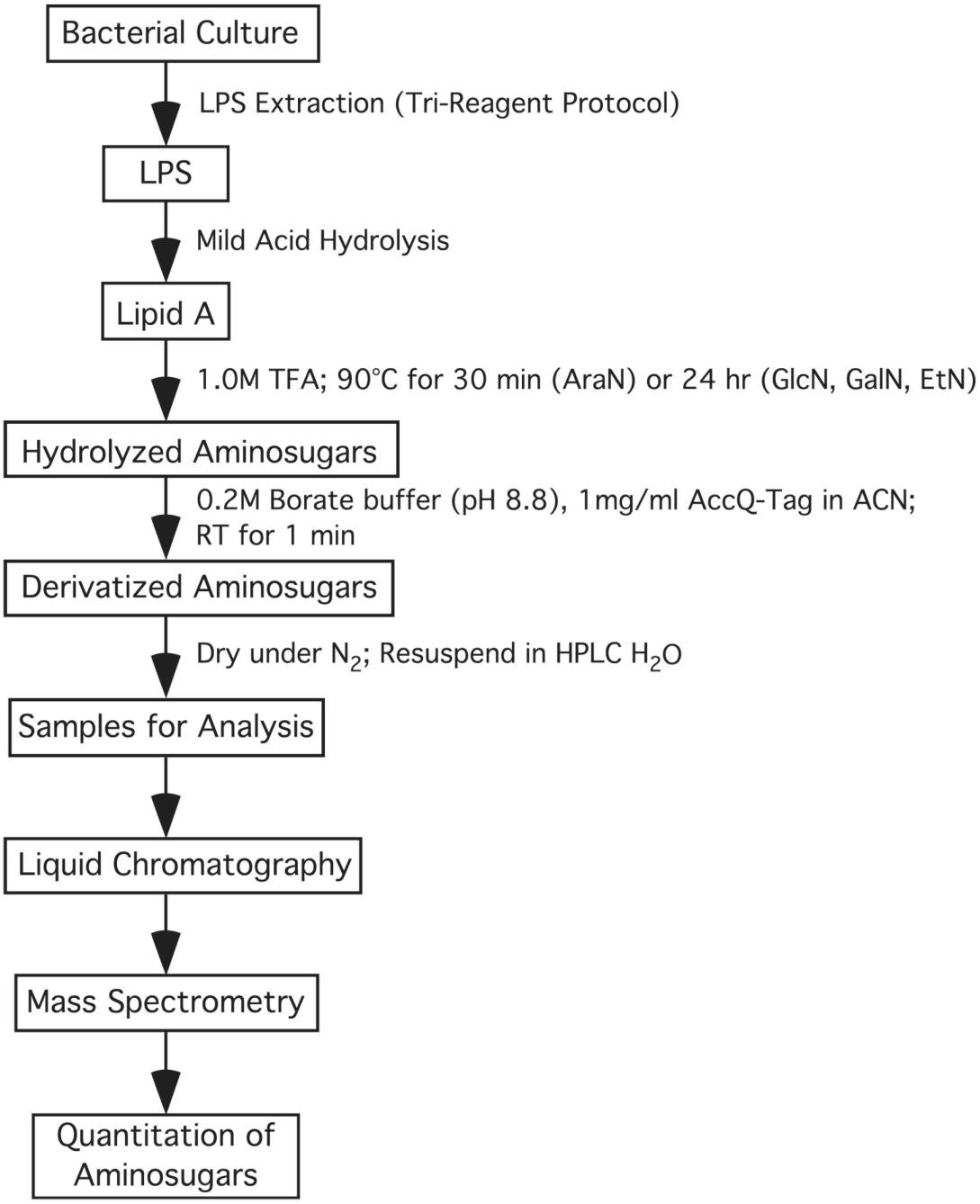

Figure 6.
Flow-chart for the analysis of amino-containing residues in bacterial lipid A samples.

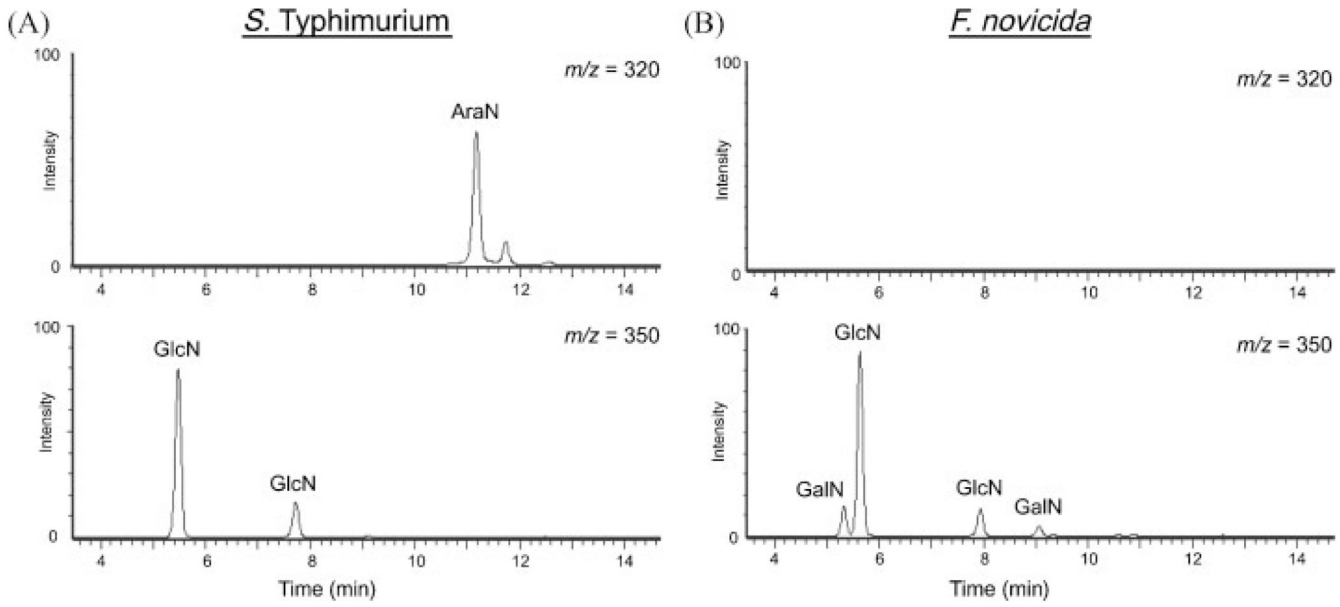

Figure 7.

Analysis of aminosugars in lipid A of *S*. Typhimurium and *F*. *novicida*. SIM chromatograms of AraN, GlcN, and GalN in *Salmonella* (A) and *Francisella* (B). (A) Lipid A isolated from S. Typhimurium PhoP^c strain after growth in LB broth supplemented with 1 mM $MgCl_2$ was heated at 90°C for 30 min to measure AraN and 24 h to measure GlcN and GalN. Modification of lipid A with GalN was not observed in this sample. (B) Lipid A isolated from wild-type *F*. *novicida* strain U112 after growth in TSB-C was heated at 90°C for 30 min to measure AraN and 24 h to measure GlcN and GalN. Modification of lipid A with AraN was not observed in this sample.

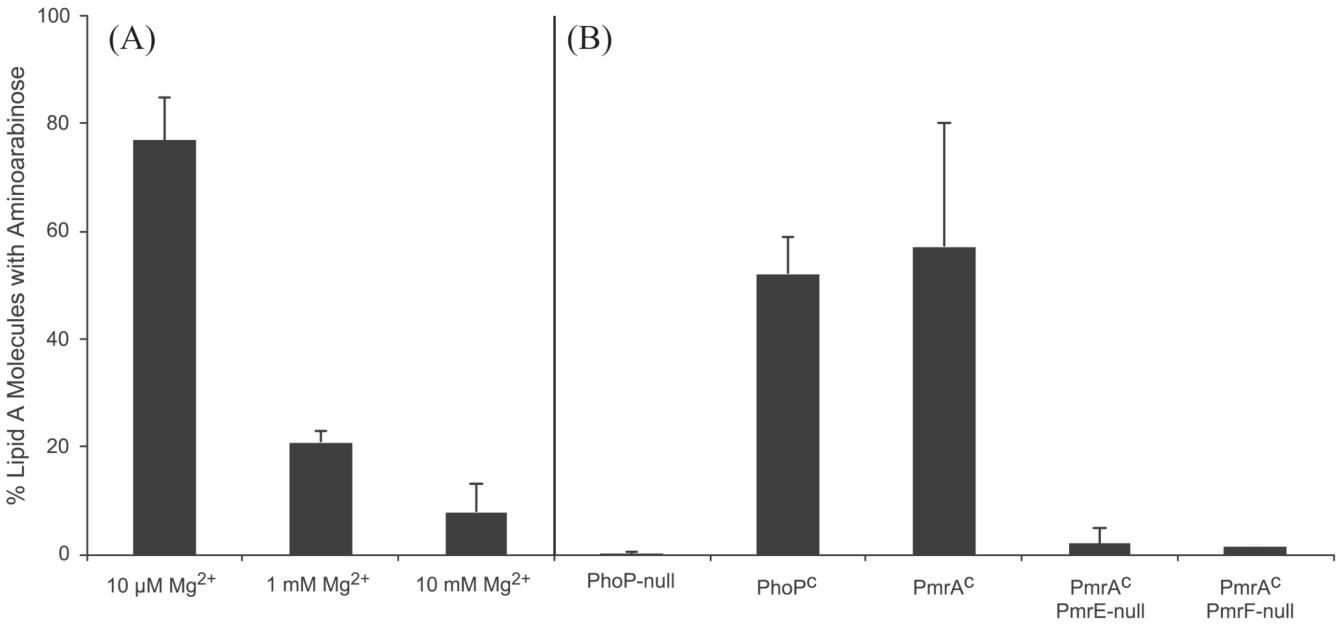

Figure 8.

Detection of modification of *S*. Typhimurium lipid A with AraN. (A) Quantitation of AraN in *S*. Typhimurium: role of magnesium-limited growth. Growth in medium that is limited for magnesium activates the PhoP-PhoQ system, which leads to addition of AraN to lipid A. (B) Quantitation of AraN in *S*. Typhimurium: role of PhoP and Pmr biosynthetic genes. Bacteria were grown in magnesium-replete medium and the level of AraN modification in lipid A (AraN per two GlcN residues) was calculated for the individual samples. All results are the average of three independent lipid A extractions. All experiments repeated in triplicate.

Table 1

Bacterial strains used in this study and relevant properties

Strain	Name	Genotype or relevant phenotype	Source	Reference
Salmonella Typhimurium	ATCC 14028s	Wild-type	S. I. Miller (University of Washington)	
	PhoP - constitutive ($PhoP^c$)	*pho*-24	S. I. Miller (University of Washington)	21
	PmrA - constitutive ($PmrA^c$)	14028s *pmrA*505 zjd::Tn10d-cam	S. I. Miller (University of Washington)	19
	$PmrA^c$ PmrE-null	$pmrA^c$ *pmrE1*::Tn10d	S. I. Miller (University of Washington)	20
	$PmrA^c$ PmrF-null	$pmrA^c$ *pmrF1*::Tn10d	S. I. Miller (University of Washington)	20
Francisella novicida	U112	Wild-type	F. Nano (University of Victoria)	22

Table 2

Repeatability in measurement of AraN, GalN, GlcN, and EtN

	Calculated pg/sample (SD); N = 5 samples			
pg/sample	**AraN**	**GalN**	**GlcN**	**EtN**
1	0.96 (0.03)	1.40 (0.02)	1.35 (0.02)	1.62 (0.03)
7	6.72 (0.21)	6.87 (0.20)	6.91 (0.23)	6.53 (0.28)
25	25.9 (0.76)	24.9 (0.99)	24.2 (0.94)	26.2 (0.70)

Values are the average of five measurements of a single sample at each concentration (low, medium, and high). The standard deviation (SD) is given in parentheses.

Table 3

Reproducibility in measurement of AraN, GalN, GlcN, and EtN

	Calculated pg/sample (SD); N = 5 samples			
pg/sample	**AraN**	**GalN**	**GlcN**	**EtN**
1	1.26 (0.15)	1.23 (0.04)	1.19 (0.23)	0.75 (0.52)
7	7.15 (0.68)	7.53 (0.57)	7.16 (0.56)	6.53 (1.23)
25	26.2 (4.2)	26.2 (2.0)	25.2 (3.1)	26.0 (2.2)

Values are the average of five independent samples at each concentration (low, medium, and high), processed and measured on separate days. The standard deviation (SD) is given in parentheses.