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High-perfolmance liquid chromatographic quantitation of phenylthiocarbamyl muramic acid and glucosamine from bacterial cell walls

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ABSTRACT

A sensitive assay for the simultaneous determination of muramic acid and glucosamine from bacterial cell walls was developed. Cell wall skeleton (CWS) preparations were hydrolyzed with HCl to liberate free amino sugars, which were subsequently derivatized pre-column with phenylisothiocyanate. The phenylthiocarbamoyl (PTC) amino sugars were rapidly resolved by reversed-phase gradient HPLC and quantified with UV detection. With this assay, *Mycobacterium phlei* CWS samples were found to contain muramic acid levels comparable to those found with a specific enzymatic assay. However, a colorimetric total hexosamine assay gave values 20–30% higher than those obtained by the specific PTC amino sugar assay. The detection limit of each PTC amino sugar was 5 pmol.

INTRODUCTION

A unique feature of bacterial cell walls is their peptidoglycan component, which is composed of the amino sugars muramic acid (MA) and glucosamine (GlcN), and amino acids.

Since MA is unique to bacteria, the measurement of peptidoglycan amino sugars has relevance in several lines of research, including: bacterial cell wall biochemistry [1], cell wall immunomodulating and antitumor properties [2], and specific measurement of bacterial biomass and infection [3–5].

Peptidoglycan amino sugars have increasingly been quantified by chromatographic techniques, which potentially are more specific and sensitive than traditional colorimetric assays. GLC-MS has been successfully employed for the determination of bacterial cell wall MA [5–8]. HPLC analysis of MA and GlcN using pre-column derivatization reagents for amino acids, and hardware for amino acid analysis has resulted in low detection limits (10 pmol) and rapid sample preparation protocols [3,4,9].

This paper describes a more sensitive HPLC analysis of phenylthiocarbamyl (PTC) MA and GlcN after pre-column derivatization with phenylisothiocyanate (PITC), a widely employed reagent for amino acid analysis [10–12]. The method described involved a relatively simple sample preparation protocol and the detection limit for the PTC amino sugars was 5 pmol (signal-to-noise ratio of 3: 1).

EXPERIMENTAL

Reagents

HPLC-grade acetonitrile (MeCN), methanol, sodium acetate trihydrate, water and reagent-grade concentrated hydrochloric acid were obtained from J. T. Baker (Phillipsburg, NJ, USA). HPLC-grade glacial acetic acid and phosphoric acid were obtained from Fisher Scientific (Pittsburgh, PA, USA). H amino acid standard (H-std), sequanal grade PITC and HPLC-grade triethylamine (TEA) were obtained from Pierce (Rockford, IL, USA).

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L-Cysteic acid (CYA), D-glucosamine–HCl, muramic acid and disodium hydrogenphosphate were obtained from Sigma (St. Louis, MO, USA).

Sample hydrolysis

Cell wall skeleton (CWS) preparations (RibiImmunoChem Research, Hamilton, MT, USA; Nos. 014-104 to 014-107) were hydrolyzed *in vacuo* at 100°C for 12 to 42 h in 4 *M* HCl which contained 118.2 μmol CYA as an internal standard (2.00 mg CWS/ml acid solution).

Pre-column derivatization

Aliquots (100 μl) of sample hydrolysates in 50 × 6 mm borosilicate glass tubes were dried to < 125 mTorr (1 Torr = 133.322 Pa) on a Picotag (Waters, Milford, MA, USA) vacuum station, then dried again after the addition of 20 μl of redry solution (methanol-water-TEA, 2:2: 1). Finally, 20 μl of derivatizing solution (methanol-water-TEA-PITC, 7: 1: 1:1) was added to each tube, and the mixtures were held at atmospheric pressure for 5 min and then dried as stated above.

Calibration standard

Aliquots (20 μl) of a stock standard solution (which contained 591 μmol CYA, 928 μmol GlcN and 995 μmol MA) were subjected to the derivatization procedure as described above. Note: dry derivatized sample and standard preparations are stable for at least 3 weeks at -20°C.

Dilution prior to injection

Derivatized CWS and standard preparations were reconstituted in 100 μl of diluent (5 m*M* disodium hydrogenphosphate titrated to pH 7.5 with 5% phosphoric acid and mixed with MeCN; buffer: MeCN = 95:5, v/v). HPLC analyses of 10-μl aliquots of samples and 5-, 10-, 15- and 20-μl aliquots of calibration standards were conducted within 24 h after reconstitution with diluent.

Chromatography

Waters HPLC hardware was used: a WISP 700 autosampler, two 510 pumps, a Nova-pak 300 × 3.9 mm stainless-steel 4 μm C_{18} column (preceded by a 0.2-μm in-line filter) operated at 46°C inside a temperature control module, and a 440 spectrometer equipped with a 254-nm filter. The HPLC system was controlled via a system interface module (SIM, Waters) by Maxima software (version 3.3, Waters) run in a NEC SX20 computer.

TABLE I

SOLVENT GRADIENT CONDITIONS USED FOR THE PTC AMINO SUGAR HPLC ANALYSES

Time (min)	Flow (ml/min)	Percent eluent A	Percent eluent B	Curve type
0.0	1.0	100.0	0.0	
10.0	1.0	100.0	0.0	
10.5	1.0	0.0	100.0	Linear
11.0	1.5	0.0	100.0	
16.0	1.5	0.0	100.0	
16.5	1.5	100.0	0.0	Linear
29.0	1.5	100.0	0.0	
29.5	1.0	100.0	0.0	

Eluents

The solvent system consisted of two eluents. Eluent A was an aqueous buffer of 0.05 A4 sodium acetate trihydrate, containing 0.1% (v/v) TEA and titrated to the desired pH (the range tested was pH 4.3 to 6.4) with glacial acetic acid; the buffer was mixed (v/v) with MeCN (the range tested was 3 to 6% MeCN). Eluent B, which was used solely as a column washing solvent, was MeCN–water (60:40, v/v). The solvent gradient utilized is displayed in Table I.

Quantitution

Chromatographic data were collected via the SIM and processed by the Maxima software. Injections of the calibration standard were used to prepare standard curves, and internal standard quantitation was used to calculate analyte concentrations.

Optimization of chi omatographic conditions

The process was aided by preparing various H-std and calibration standard PTC derivatives as described above for the calibration standard preparation.

Standurd uddition experiments

CWS was treated as described above for samples, except that 4 *M* HCl spiked with GlcN (92.8 μmol),

MA (99.5 μmol) and CYA (118.2 μmol) was used. Recovery was evaluated by comparing peak areas of the spiked CWS samples to those of the non-spiked CWS samples.

Enzymatic MA and colorimetric total hexosamine assays

MA was measured in the CWS hydrolysates enzymatically as described by Tipper [13]. Total hexosamine was measured spectrophotometrically after CWS hydrolysis as described by Smith and Gilkerson [14].

RESULTS AND DISCUSSION

Chromatographic optimization was accomplished by starting with HPLC conditions similar to those employed for PTC amino acid analysis [10–12]. Specifically, standard and sample preparations were initially chromatographed on the Nova-Pak column using eluent A at pH 6.4 mixed 94:6 (v/v) with MeCN (gradient conditions as in Table I). Extensive interference was observed between the PTC amino sugars and amino acids. Therefore, the pH of eluent A was decreased incrementally until resolution of the major PTC amino sugar peaks was obtained in the pH range of 5.1-5.3. Further resolution was achieved by altering the MeCN content (between 3 and 6%, v/v) of eluent A (pH 5.1). The results of these tests indicated the optimal MeCN content to be 3.5% (v/v) (Fig. 1). Finally, the pH of eluent A with 3.5% (v/v) MeCN was decreased incrementally to pH 4.5 (Fig. 2). At a pH of 4.5 the internal standard (CYA) and major PTC amino sugar peaks eluted well before any possible interfering PTC amino acids, resulting in very rugged chromatographic conditions. Calibration standard and CWS analyses using eluent A adjusted to pH 4.5 and mixed 96.5:3.5 (v/v) with MeCN (10-μl injection volumes) are displayed in Fig. 3 (note the different y-axis scaling for each chromatogram).

An additional test of eluent A at pH 4.3 was made which revealed that at this pH the minor PTC amino sugar peaks in samples could be resolved. Major/minor peak area ratios for PTC MA and GlcN were identical in calibration standard and sample analyses under these conditions, suggesting that use of only the major peak areas for calibration

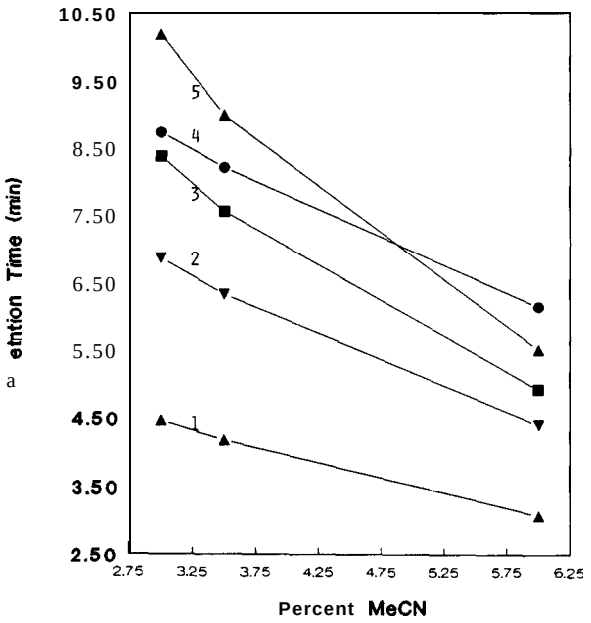

Fig. 1. The effects of eluent A MeCN content (percent, v/v) on the elution of PITC-derivatized CYA (I), MA (2), GlcN (4), aspartic acid (3) and glutamic acid (5).

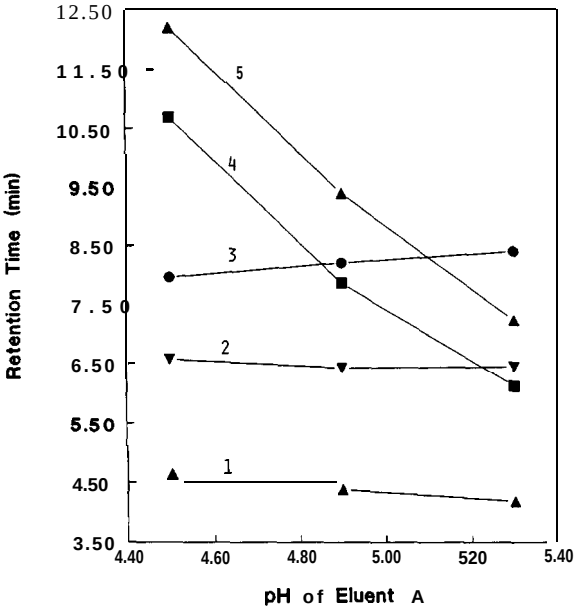

Fig. 2. The effects of eluent A pH on the elution of PITC-derivatized CYA (1), MA (2), GlcN (3), aspartic acid (4) and glutamic acid (5).

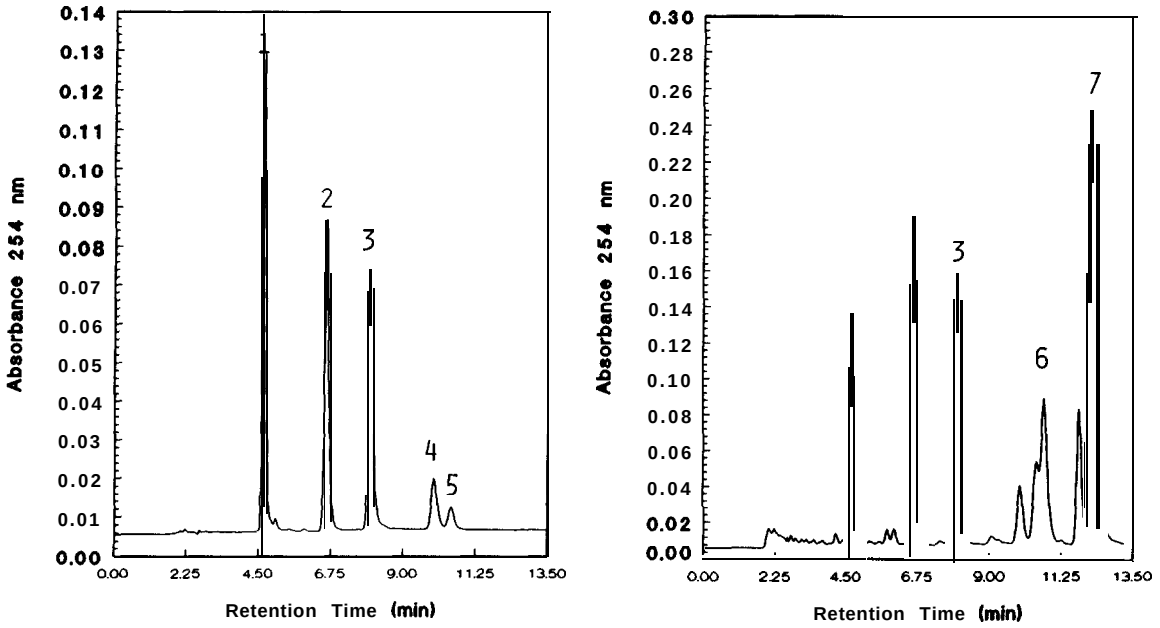

Fig. 3. Calibration standard (left) and CWS (right) chromatograms representing 10-μl injections of reconstituted sample are shown. Note the differences in r-axis scaling for each chromatogrdm. I = CYA; 2.4 = MA: 3,5 = GlcN; 6 = aspartic acid; 7 = glutamic acid.

and quantitation was sufficient. Eluent A should not be adjusted below pH 4.5 for routine analyses, however, since resolution of MA and GlcN decreases as pH is lowered (Fig. 2).

After the chromatographic conditions were optimized, the duration of sample hydrolysis at 100°C resulting in maximal release of MA and GlcN was determined. Hydrolysis times from 12 to 42 h were tested twice, with comparable results indicating maximal release of MA (one-way analysis of variance, ANOVA p = 0.0060) after 30 h at 100°C and qual release of GlcN (ANOVA p = 0.1085) throughout (Fig. 4). In subsequent analyses of CWS, 30 h of hydrolysis at 100°C was used.

Day-to-day analytical variability was tested by preparing and analyzing samples independently four times. Variability in MA and GlcN response was low, with individual daily results having relative standard deviations (R.S.D.s) below 4% and the overall average R.S.D. below 6% (Table II).

CWS samples were spiked with standards in standard addition experiments to test for possible sample matrix effects on standard recovery. However, no matrix effects were observed, since the recoveries

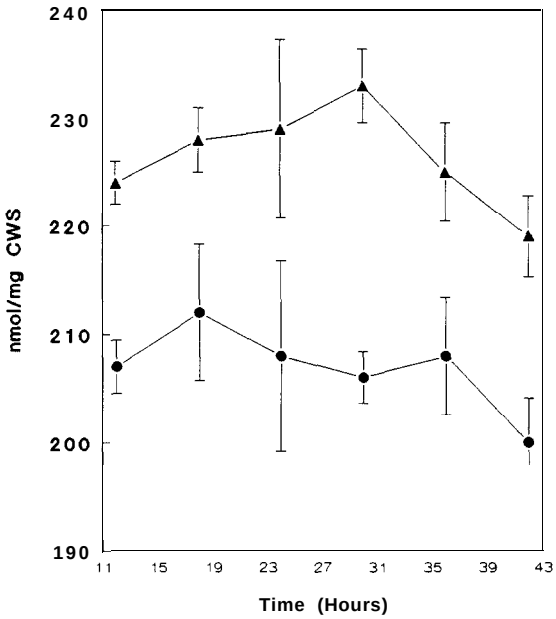

Fig. 4. The release of MA and GlcN from CWS as a function of heating time in 4 M HCl at 100°C is displayed. Each data point represents the mean of four samples, and the error bars represent one standard deviation of the mean. A = Muramic acid; ● = glucosamine.

TABLE II

DAY-TO-DAY ANALYTICAL VARIABILITY OF THE PTC AMINO SUGAR ASSAY

One preparation of CWS (Ribi No. 014-107) was analyzed independently for MA and GlcN content on four separate dates. NA = Not applicable.

Analysis date	Average MA (R.S.D., %) (nmol/mg CWS)	Average GlcN (R.S.D., %) (nmol/mg CWS)	*n*
March 30, 1992	238 (2.24)	207 (3.44)	4
March 31, 1992	236 (1.88)	235 (1.12)	4
April 3, 1992	252 (1.06)	216 (1.69)	4
April 21, 1992	239 (0.63)	226 (1.44)	4
Overall Average	241 (3.02)	221 (5.49)	NA

of standards added to CWS samples (two times, independently) prior to acid hydrolysis approximated 100% (Table III).

The PTC amino sugar assay was compared to a specific enzymatic (lactate release) assay for MA [13]. Both assays yielded nearly identical results for MA content when four different CWS preparations were tested (Table IV). The total MA and GlcN content as determined by the PTC assay was also compared to a colorimetric total hexosamine assay [14]. This experiment revealed 20–30% lower results for the PTC-based assay (Table IV).

The ratio of MA/GlcN was found to be 1.09 with the PTC amino sugar assay (using the overall average values from Table II), which is very close to the theoretical MA/GlcN ratio of 1 .O expected for bacterial CWS peptidoglycan. This ratio determined with the PTC assay and the agreement between the

TABLE III

AVERAGE PERCENT RECOVERIES OF MA AND GlcN ADDED TO SUBSAMPLES OF CWS (RIB1 No. 014-107) DETERMINED INDEPENDENTLY TWO TIMES

Analysis date	Recovery MA (R.S.D., %)	Recovery GlcN (R.S.D., %)	*n*
March 31, 1992	102 (2.65)	105 (3.1 I)	4
April 3, 1992	102 (2.01)	102 (5.48)	4

TABLE IV

SAMPLES FROM FOUR CWS PREPARATIONS ANALYZED AFTER ACID HYDROLYSIS WITH THREE ASSAYS [PTC AMINO SUGAR (PTC); ENZYMATIC MA (ENZ); COLORIMETRIC TOTAL HEXOSAMINE (TOT HEX)] IN ORDER TO COMPARE THEM

Values are listed as μmol/mg CWS.

CWS No.	MA (PTC)	MA (Enz)	MA + GlcN (PTC)	Tot Hex
014-104	0.20	0.24	0.38	0.60
014-105	0.23	0.23	0.45	0.62
014-106	0.25	0.23	0.49	0.58
014-107	0.24	0.22	0.46	0.57

MA values derived from the PTC and enzymatic methods suggest that the total hexosamine values from colorimetric assay were erroneously high, possibly due to sample matrix interferences.

The PTC amino sugar assay was more versatile than the enzymatic assay [13], and was shown to be superior to the colorimetric assay [14] owing to its greater specificity. The PTC assay exhibited a lower detection limit (5 pmol per injection vs. 10 pmol) than other published HPLC assays for CWS amino sugars [3,9], and a limit comparable to that of GLC-MS analysis [8]. The PTC assay described had the advantages of a relatively simple sample preparation protocol and very rugged chromatographic characteristics. Finally, in addition to pure bacterial CWS, this assay should be applicable to whole-cell amino sugar analysis.

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