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AMPEROMETRIC DETECTION OF MURAMIC ACID IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH A POST-COLUMN REACTION

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SUMMARY

Highly sensitive detection of muramic acid has been developed by using an amperiometric detector in high-performance liquid chromatography. The reducing ability of muramic acid was coupled with the redox reaction of bis(1,10-phenanthroline)copper(II), CBP, as mediator. CBP, reduced by muramic acid in a post-column reaction, was reoxidized with the amperometric detector, resulting in a highly sensitive and selective detection. A doublet peak was obtained, due to the α- and β-anomers of muramic acid. This was merged into a single peak by adding phosphoric acid to the sample solution. Addition of phosphoric acid to serum, spiked with muramic acid, also clarified the chromatogram. The detection limit was 0.05 ppm (4 pmol of muramic acid injected) in serum samples.

INTRODUCTION

Reducing carbohydates have been detected in high-performance liquid chromatography (HPLC) by refractive index[1,2], colorimetric[3–5] or fluorimetric methods[6–8]. These techniques often suffered from drawbacks, such as insufficient sensitivity or selectivity, and instability or corrosiveness of reagents. Therefore, an alternative detection method was sought which would be more sensitive and highly selective.

Recently, a novel detection method for reducing carbohydrates in HPLC with an amperometric detector has been developed[9]. Coupling the redox reaction of a copper complex as a mediator with the reducing ability of sugars provides the following principle of detection:

$$Cu(phen)_2^{2+} \xrightarrow[OH^-,\ \text{heat, post-column}]{\text{reducing sugars}} Cu(phen)_2^{+} \xrightarrow[\text{oxidation, } +40\ \text{mV } \textit{vs.}\ \text{Ag/AgCl}]{\text{electrochemical}} Cu(phen)_2^{2+}$$

Bis(1,10-phenanthroline)copper (CBP) was utilized as the mediator. The great advantage of this method is that the applied potential is low enough to produce high

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selectivity and maintain a low background current and stability of the working electrode. The new method was applied to the determination of carbohydrates in human serum and proved to be highly selective and sensitive and to give excellent reproducibility and stability[10].

In the present paper I describe the detection of muramic acid added to human serum. Muramic acid is an amino sugar and one of the constituents of the cell walls of bacteria and blue-green algae, which is not found elsewhere in nature. Hence, the detection of muramic acid in human serum may provide evidence for the presence of bacterial cell wall debris, aiding in the elucidation of the possible etiologic rôle of bacteria in certain human diseases.

Colorimetric analysis of lactate, released from muramic acid by alkaline hydrolysis, is rather simple but non-specific[11,12]. Gas chromatography with flame ionization detection does not offer enough sensitivity[13,14]. Gas chromatography–mass spectrometry permits highly sensitive detection, but requires tedious derivatization[15]. Liquid chromatographic analysis with an amino acid analyzer is easier to accomplish and is now preferred, but it lacks sensitivity and specificity[16–19].

Muramic acid has reducing ability, like other simple carbohydrates. Hence, it was anticipated to be amenable to amperometric detection by CBP.

EXPERIMENTAL

Materials

All chemicals were reagent grade. Muramic acid and N-acetylmuramic acid were obtained from Sigma and Aldrich, respectively, and used as received. Stock standard solutions of muramic acid and N-acetylmuramic acid (1 mg/ml) were prepared by dissolving the solutes in distilled water, and stored in a refrigerator. CBP was prepared according as described[20]. It was recrystallized once from distilled water and dried at 80°C for 2 h under vacuum.

High-performance liquid chromatography

The HPLC apparatus was equipped with a Constametric IIG constant-flow pump (Milton Roy, Philadelphia, PA, U.S.A.) used at a flow-rate of 0.35 ml/min, a strong cation-exchange column (IEX 510K, silica base, 5 μm, 30 cm × 4 mm I.D.; Toyo Soda, Japan), sample injector with 20-μl loop (Model-7120; Rheodyne, U.S.A.) and an amperometric detector (VMD-101; Yanaco, Kyoto, Japan). The reagent solution containing CBP was delivered at a flow-rate of 0.80 ml/min by a constant-flow pump (TRI ROTAR-II; Japan Spectroscopic, Tokyo, Japan) and mixed with the column eluate by means of a simple stainless-steel. PTFE tubing (5 m × 0.5 mm I.D.) was used as the reaction coil, which was immersed in a water-bath, temperature-controlled to 95 ± 0.1°C. The reaction mixture was then passed through a cooling coil (30 cm × 0.25 mm I.D.) immersed in water before reaching the detector.

The mobile phase was 0.03 *M* potassium dihydrogenphosphate, adjusted to pH 2.70 with phosphoric acid. The reagent solution contained 1 m*M* CBP in 0.2 *M* disodium hydrogenphosphate, adjusted to pH 10.90 with 2 *M* sodium hydroxide. The potential applied to the working glassy carbon electrode was fixed at +40 mV *vs.* Ag/AgCl.

Pooled serum was spiked with muramic acid, then deproteinized by mixing

with an equal volume of acetonitrile, followed by centrifugation at 2000 *g*. The supernatant was directly injected into the HPLC apparatus.

RESULTS AND DISCUSSION

Since muramic acid is amphoteric, as shown in Fig. 1, it can be chromatographed under conditions similar to those employed for amino acids. Usually, it is chromatographed on a strong cation-exchange column with an eluent of low pH[16–18]. The retention of muramic acid increases as the salt content of the mobile phase is lowered. The retention time rapidly increases when the concentration of KH_2PO_4 in

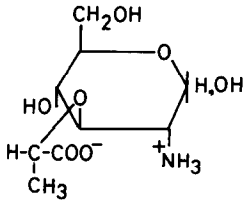

Fig. 1. Structure of muramic acid.

the eluent is less than 0.02 *M*. Lowering the pH of the mobile phase also results in an increase of retention time but to a lesser extent. According to a previous study[9], the pH of the mixture in the reaction coil should be maintained around 11. Hence, it is desirable to choose a low buffering capacity eluent, that is, a low salt content. An eluent of 0.03 *M* KH_2PO_4 and pH 2.70 was employed as a compromise between analysis time and separation of muramic acid from most of the amino acids and other contaminants. At such a low salt content the mobile phase is weak. When serum samples are injected, the column must therefore be purged with a stronger eluent (such as 0.2 *M* phosphate buffer, pH 6.80) at the end of each working day. Otherwise, unidentified late eluates may cause problems in subsequent chromatograms.

The rate of reduction of CBP by sugars depends greatly on the pH and temperature. The higher the temperature, the faster is the rate for muramic acid, as is the case for simple sugars[9]. Most simple sugars show the greatest response at pH 11.1–11.3, as reported previously[9]. The pH of the reagent solution was optimized at 11.20 for water as the eluent[9] and at 11.70 for 0.1 *M* borate buffer (pH 8.98)[10]. However, the maximum response of muramic acid occurs at a lower pH. The dependence of response on the pH of the reagent solution is shown for muramic acid and glucose in Fig. 2. Glucose showed its maximum response at a slightly higher pH than in our earlier study, reflecting the low pH of the eluent. The pH for the maximum response of muramic acid was significantly lower than that for glucose. This situation is favourable for the determination of muramic acid in serum, since the response of glucose as the major interfering component can be suppressed in relation to that of muramic acid. Thus, the reagent solution was adjusted to pH 10.90.

Fig. 3 shows the chromatogram obtained for a standard sample of muramic acid prepared in distilled water. The doublet corresponds to the α- and β-anomers of muramic acid. As the peak splitting complicates the chromatogram and decreases the sensitivity, it is better to devise a condition for obtaining a single peak. Acidifi-

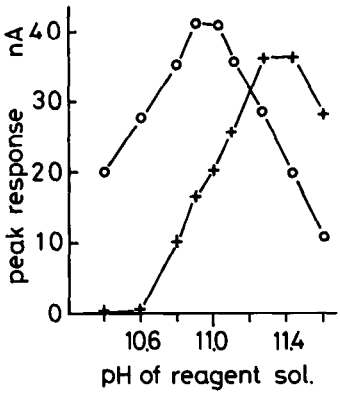

Fig. 2. Dependence of peak response on the pH of the reagent solution (O) muramic acid; (+) glucose.

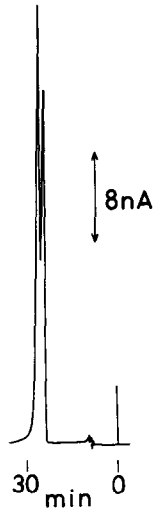

Fig. 3. Chromatogram of authentic muramic acid.

cation of the sample solution with HCl, $HClO_4$, or CCl_3COOH was partly effective in this. A single peak grew at increased retention time with concomitant loss of the doublet, but a considerable amount of the doublet still remained. Complete convergence of the doublet was obtained by adding H_3PO_4 to the sample solution. Fig. 4 shows the effect of additing of H_3PO_4 (0.03 *M*). The retention increased with decreasing pH of the sample, accompanied by a growth of the second peak and a reduction of the first peak. The doublet finally converged to single peak at pH less than 1.2. When the sample, once acidified, was neutralized with alkali, the original doublet was restored. This behaviour aids in the identification of an unknown peak as muramic acid.

Fig. 5a shows the chromatogram obtained for a pooled serum, spiked with muramic acid (0.25 ppm in serum). The doublet is indicated by arrows. Glucose was eluted slightly after the solvent front, followed by uric acid. There were other, un-

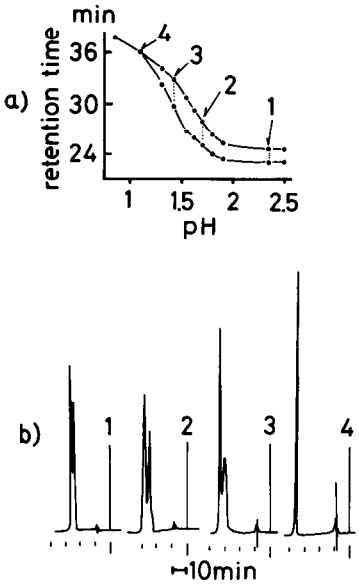

Fig. 4. Effect of H_3PO_4, added to the sample solution. (a) Dependence of retention times of the doublet on the pH of the sample solution; (b) changes in the chromatogram, produced by H_3PO_4. The numbers correspond to those in (a).

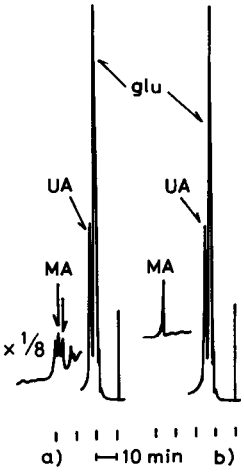

Fig. 5. Chromatograms of muramic acid in serum. (a) Concentration of muramic acid 0.25 ppm; (b) after addition of H_3PO_4 to (a). MA = Muramic acid; glu = glucose; UA = uric acid.

known, minor peaks. Addition of H_3PO_4 to the serum sample also clarified the chromatogram by smearing out minor peaks and merging the muramic acid doublet, as shown in Fig. 5b.

Muramic acid is found in peptidoglycan as the N-acetyl derivative. The peak response obtained with N-acetylmuramic acid was about one tenth of that of muramic acid. Therefore it is preferable to detect muramic acid after elimination of the

N-acetyl group. N-acetylmuramic acid was boiled in 2 *M* HCl solution in a leak-tight micro-vessel under reduced pressure. The cleavage of N-acetyl was quantitatively completed within 1 h.

Amino acids did not interfere, since most of them did not respond to the detection method used in this study. Only tyrosine gave a small response, but it did not interfere, because of its stronger retention. Glucosamine, glutathione and ergothionine gave noticeable responses, but all of them were separated from the single peak or doublet of muramic acid.

The detection limit, *i.e.* the minimum detectable concentration in serum, was estimated to be 0.05 ppm (*ca.* 4 pmol injected). The linear range of response extended up to 10 ppm. The detector sensitivity increased linearly with the CBP content of the reagent solution[9]. The concentration of 1 m*M* CBP employed in this preliminary study is quite low. Hence, an additional improvement in the detection limit may be expected to a level as low as 0.01 ppm by using a concentration of 5 m*M* CBP.

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