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Determination of phenol in urine by high-performance liquid chromatography with on-line precolumn enzymatic hydrolysis of the conjugates

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Abstract

A precolumn enzyme reactor containing β-glucosidase immobilized on LC-NH_2 packed-material beads was used on-line with HPLC for determining the glucuronide/sulphate metabolites of benzene. After dilution with phosphate buffer (pH 6.8), the urine sample was injected into the HPLC system directly. Subsequently, after hydrolysis of the conjugates, phenol was produced in the enzyme reactor and was separated from other urinary components on a reversed-phase C_{18} column with fluorescence detection. A switching valve assembly was used to control the passage of the sample and the eluent into the reactor to prevent damage to the enzyme by the elution solvent. Factors affecting the enzymatic hydrolysis were investigated. The proposed method provides a simple and rapid procedure for urinary phenol determination. The calibration graph was linear in the range 0.25–5.0 ppm with a good correlation coefficient ($r = 0.999$), and in the range 0.05–1.0 ppm with $r = 0.981$. The detection limit was 10 ppb and the relative standard deviation was less than 2.27%. Application of the method is illustrated by the analysis of a urine sample collected from a gas station worker.

1. Introduction

Conjugation of phenols with D-glucuronic acid and sulphate ions is a common metabolic pathway of benzene in humans [1–4]. These compounds are regarded as part of the metabolic products of excretion in urine [5,6]. As about 30% of retained benzene appears as phenol *in vivo*, the urinary phenol excreted is applied in the evaluation of the biological exposure index (BEI) for benzene [7–10].

Angerer and Hörsch [11] recently reviewed methods for the determination of urine phenol. Generally the glucuronides or sulphates are hydrolysed either by acid [12,13] or enzymatically with the use of a β-glucuronidase [14–19]. The released phenol is then subjected to solvent extraction [14] or steam distillation [12,13] prior to chromatographic determination. However, the extraction methods were reported to have the disadvantage of insufficient separation of phenol from the other constituents of the urine, which results in a high analytical background [11], and the steam-distillation method required an enrichment procedure owing to the low benzene exposure levels. These multi-step determinations are very time consuming.

Enzymatic hydrolysis is preferred over acid

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hydrolysis because of the mild reaction conditions. However, if enzymatic hydrolysis is carried out with a soluble enzyme, disadvantages of high cost, long incubation time, introduction of contaminants and potential interferences in the chromatogram still occur. Immobilized enzymatic hydrolysis has been studied and found to be valuable by improving the selectivity in the chromatographic separation of substrates, and it has been considered to be an important analytical tool for the future [15–17]. Bowers and Johnson [18,19] used an enzyme reactor with immobilized β-glucosidase on controlled-pore glass (CPG) for the on-line cleavage of urinary estriol conjugates before HPLC analysis. Later, Dalgaard *et al.* [20] used an enzymatic post-column cleavage to determine glycosides after HPLC separation. Boppana *et al.* [21] used the same procedure to determine the glucuronide conjugates of fenoldopam. In all these studies CPG was used as the support, but it is expensive and often requires a specific reaction (silanization) design and a skilful operator for enzyme immobilization.

In this work, the popular commercially available LC-NH_2 packed-material beads were used to immobilize β-glucosidase directly according to a modified procedure [22–27] and used in a precolumn reactor with an HPLC system for the determination of urinary phenol.

2. Experimental

2.1. Apparatus

The HPLC system was obtained from Gilson (Villiers-le-Bel, France) and included two Model 302.5 SC single-piston pumps, a Model 811 dynamic 1.5-ml mixer, a Model 802C manometric module and a Shimadzu (Kyoto, Japan) RF-535 fluorescence detector. A Linear (Reno, NV, USA) UVIS-206 multiple-wavelength detector was also used. A J & W (Folsom, CA, USA) reversed-phase ODS column (15 cm × 4.6 mm I.D., particle size 5 μm) was used for separation. A Rheodyne (Cotati, CA, USA) Model 7125 injector with a 1.0-ml external loop was used for sample introduction. A Rheodyne Model 7010 switching valve was used to control the elution. An HP-3390A integrator (Hewlett-Packard, Avondale, PA, USA) was used to obtain the chromatogram and perform data calculations. An empty stainless-steel column (5.0 cm × 4.6 mm I.D.) was used to pack immobilized enzyme by using a Model 124A slurry-packing apparatus (Chemco, Osaka, Japan). The immobilized enzyme reactor (IMER)–HPLC system was assembled as shown in Fig. 1.

2.2. Reagents

Distilled, deionized water was used to prepare all solutions. The HPLC eluent was prepared from HPLC-grade methanol (Mallinckrodt, St. Louis, MO, USA) and water [30% (v/v) methanol in pH 6.8 phosphate buffer]. The eluent was filtered through a 0.45-μm PVDF membrane filter and degassed ultrasonically.

β-Glucuronidase, sulphatase (type H-2 from *Helix pomatia*), glutaraldehyde, phenyl β-D-glucuronide, *p*-nitrophenyl glucuronide, (3-aminopropyl)diethoxysilane and Trisma were all obtained from Sigma (St. Louis, MO, USA). LC-NH_2 supports (Supelclean LC-NH_2) were obtained from Supelco (Bellefonte, PA, USA); these are silica-based monomerically bonded packings with 40-μm particles and 60-Å pores. All other chemicals were of analytical-reagent grade from commercial suppliers.

Enzyme in buffer solution was prepared by dissolving 100 mg of β-glucuronidase (H-5, containing, sulphatase) in 100 ml of phosphatase buffer solution (pH 6.8) and stored in a refrigerator (4°C).

2.3. Immobilization of enzymes

A solution of 25 ml of glutaraldehyde (1.0%) in water was reacted with the LC-NH_2 support (1.0 g) with gentle stirring for 1 h at room temperature to generate aldehyde functional groups on the LC-NH_2. Excess of glutaraldehyde was removed by rinsing with water and the

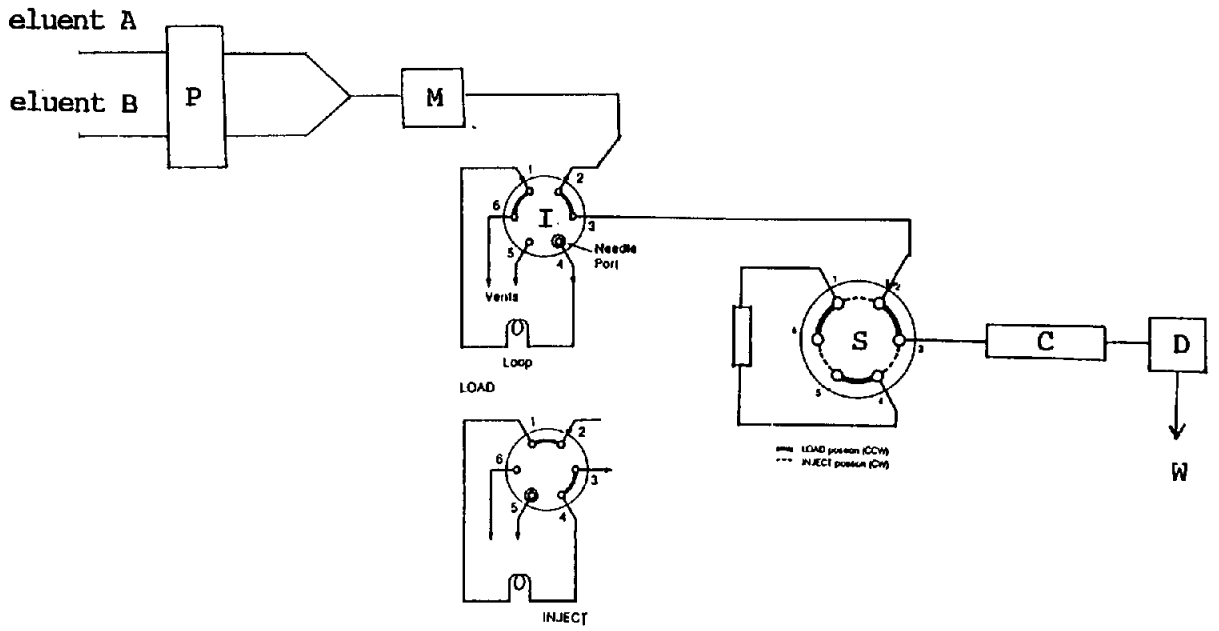

Fig. 1. Schematic diagram of the IMER–HPLC system. P = Pump; M = mixer; I = injector; C = column; S = switching valve; D = detector; W = waste. Eluent A = 0.04 *M* phosphate buffer (pH 6.8); eluent B = methanol–0.04 *M* phosphate buffer solution (pH 6.8) (30:70).

phosphate buffer (pH 6.8). The β-glucuronidase (in 25 ml of pH 6.8 solution) was attached at 4°C in the phosphate buffer for 24 h. The immobilized enzyme beads were then washed with water and 0.1 *M* NaCl to remove any absorbed or entrapped enzymes. The immobilized β-glucuronidase was stored by soaking in 0.1 *M* NaCl solution and kept at 4°C in a refrigerator.

2.4. Preparation of immobilized enzyme reactor (IMER)

Immobilized enzyme beads (0.5 g) was placed in a slurry reservoir, which was then filled with pH 6.8 phosphate buffer solution. The stainless-steel column (5.0 cm × 4.6 mm I.D.) was packed by compressing the slurry of immobilized enzyme beads using high-pressure nitrogen. If no sample was run, the reactor was removed from the HPLC system, rinsed with phosphate buffer (pH 6.8) and stored in 0.1 *M* NaCl solution at 4°C in a refrigerator.

2.5. Collection and pretreatment of urine samples

The urine samples were collected in 100-ml Pyrex glass flasks, frozen immediately and stored in a freezer at −20°C until analyzed. When the sample was analysed, it was first thawed at room temperature and the suspension was removed by centrifugation at 6000 g for 10 min. Finally, the sample was filtered through a 0.45-μm PVDF membrane filter and was then ready for chromatographic analysis.

3. Results and discussion

β-Glucosidase was immobilized on LC-NH_2 packed-material beads to give an on-line pre-column IMER. In order to find the optimum conditions for this IMER-HPLC system in urinary phenol determination, the following factors were studied: the IMER installation, the time for the hydrolysis of phenol conjugates and the

effect of methanol and urine concentrations on the IMER activity.

3.1. Installation and operation of IMER–HPLC system

The IMER was prepared as described earlier and installed as a precolumn via a switching valve to prevent the solvent from damaging the immobilized enzyme (Fig. 1). After the sample had been loaded in the sample loop, an eluent containing 6% (v/v) methanol was used to bring the samples into the IMER by controlling the switching valve, and then through the separation column. Note that the phenol conjugates were hydrolysed in the IMER and their products were concentrated on the inlet of the separation column. After switching the valve to turn off the IMER, the hydrolysis products were eluted with an eluent containing 30% (v/v) methanol in 0.04 *M* phosphate buffer (pH 6.8). The results showed that a 10-min on-line time with the IMER was optimum for the enzyme hydrolysis. With a shorter on-line time, part of the hydrolysis products were still retained on the IMER, and a longer on-line time would cause the diffusion of phenol in the separation column.

3.2. Stability of LC-NH_2-bound β-glucosidase

Boppana *et al.* [21] tested the stability of CPG-bound β-glucosidase with various percentages of methanol and found that the activity of the immobilized enzyme was 100% retained if the methanol content was less than 25%. To confirm the stability of the IMER in the elution stage, the LC-NH_2-bound β-glucosidase was incubated at 30°C with 0.04 *M* phosphate buffer (pH 6.8) containing 10% (v/v) of methanol. After a 108-h test, the LC-NH_2-bound β-glucosidase retained completely its activity to hydrolyse 10 ppm of *p*-nitrophenylglucuronide. It was obvious that the methanol content (6%) in the eluent, which was applied to carry the sample through the IMER, did not damage the immobilized enzyme. With elution at a flow-rate of 1.0 ml/min, the IMER retained 90% of its activity after a 32-day elution. Concerning the stability of the IMER used for the hydrolysis of urinary phenol, 94.3% (R.S.D. = 2.7%, $n = 5$) of its activity was retained after 300 injections of urinary samples on testing with 10 ppm of *p*-nitrophenylglucuronide.

3.3. Effect of urine concentration

The complicated matrix of urine is toxic to enzymes and causes a decrease in enzyme activity. Because it is difficult to remove the toxic matrix, the dilution method was investigated to retain the enzyme activity. Phosphate buffer (0.04 *M*, pH 6.8) was used to dilute 5 ml of pretreated urine sample 1 + 0, 1 + 1, 1 + 2, 1 + 3, 1 + 4 and 1 + 5 (urine + buffer). Each diluted urine sample was then injected into the IMER–HPLC system. Fig. 2 shows the relationship between the peak area and the urine concentration. As can be seen, the peak area of phenol increased with increasing dilution, reached an optimum at fourfold dilution and decreased with further dilution. Obviously, the IMER was poisoned by the urine matrix, and an appropriate dilution was needed in order to maintain the enzyme activity. Hence a 1 + 3 dilution of urine samples was adopted before enzymatic hydrolysis to avoid the matrix toxicity towards the IMER.

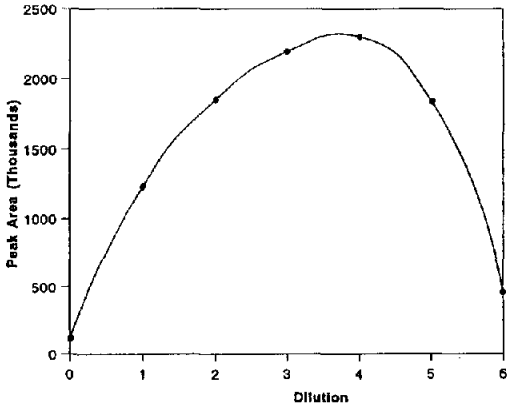

Fig. 2. Influence of urine matrix on enzyme activity.

3.4. Chromatographic separation of urinary phenol

Owing to the relatively high content of *p*-cresol and UV-detectable species compared with phenol conjugates in urine, the highly selective and sensitive fluorescence detector was used to detect the separated species. Fig. 3c shows the chromatogram of a urinary phenol sample analysed with the IMER–HPLC–fluorescence detection system. The separated species was compared with a reference sample (2.0 ppm of phenyl β-D-glucuronide injection) as shown in Fig. 3a and b and revealed the same retention behaviour, and was also verified by using a photodiode-array UV detector at 215 and 270 nm by scanning the peaks between 200 and 360 nm. Peak 4 in Fig. 3c was found to agree well with phenol and peaks 1–3 were other fluorescent species in hydrolysed urine sample. Comparison with other oxidation products of phenol (hydroquinone, resorcinol, catechol and 1,4-benzoquinone) did not show any correspondence. In this study, because only phenol was interest for use as the BEI of benzene, the unidentified peaks 1–3 were not investigated further.

3.5. Calibration graphs and detection limits

In order to test the applicability of the method for the determination of phenol in hydrolysed urine, calibration graphs were constructed by IMER–HPLC analysis of phenyl β-D-glucuronide over the concentration ranges 0.25–5.0 and 0.05–1.0 ppm (as phenol). The correlation coefficients of the linear relationships between the peak areas (y) and the injected quantities concentrations (x, ppm) are 0.999 ($y = -201.1 + 856.4x$, $S_b = 217.8$, $S_m = 82.3$, $n = 5$) and 0.981 ($y = -87.3 + 688.8x$, $S_b = 69.4$, $S_m = 134.8$, $n = 5$, where S_b is the standard deviation of the intercept and S_m is the standard deviation of the slope), respectively. The reproducibilities were examined with five replicate IMER hydrolyses of 0.6 and 2.0 ppm of phenyl β-D-glucuronide. The within-day R.S.D.s were 2.27% and 0.59% and the between-day R.S.D.s (measured every other day) were 2.1% and 1.97%, respectively. The

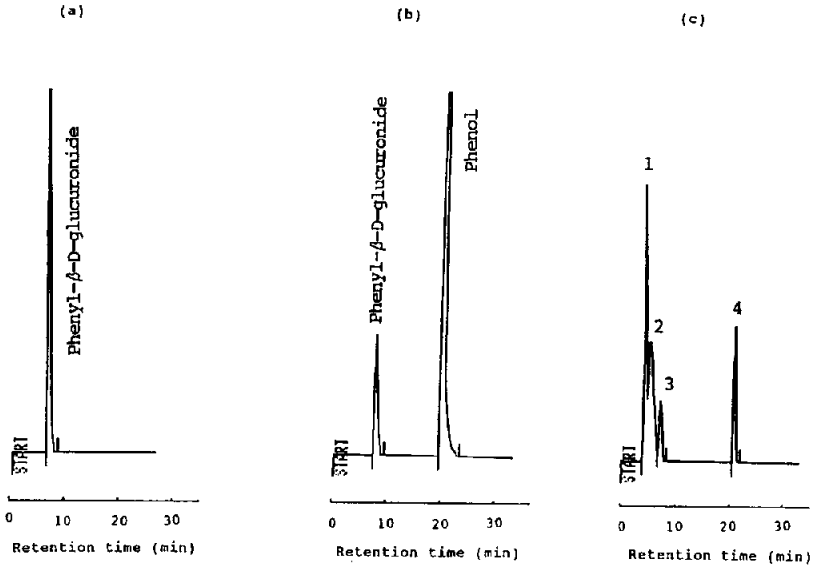

Fig. 3. Chromatograms of samples obtained by on-line IMER–HPLC with fluorescence detection: (a) phenyl β-D-glucuronide (2.0 ppm), not through IMER; (b) phenyl β-D-glucuronide (2.0 ppm), through IMER; (c) urine sample from a gas station worker. Peaks 1–3 = unknowns; 4 = phenol. Elution: isocratic elution with 6% aqueous methanol (pH 6.8) for the first 10 min, then increased to 30% methanol within 5 min, kept at this concentration until 30 min, then back to 6% methanol. Flow-rate, 1.0 ml/min. Detection with excitation at 270 nm and emission at 300 nm.

instrumental detection limit was 10 ppb based on three times the average background noise level and the method detection limit was 50 ppb. For an accuracy test, 2.0 ppm of phenyl β-D-glucuronide were added to a phenol-free urine sample and then determined by the proposed method. The recovery was 97.7% (R.S.D. 2.8% for five replicate determinations).

3.6. Analysis of a urine sample from a gas station worker

A urine sample collected from a gas station worker was analysed by the standard addition method using the proposed IMER–HPLC–fluorescence detection system under the optimum experimental conditions. The phenol concentration in the urine was found to be 0.27 ppm (R.S.D. 3.6% for five determinations). Compared with the creatinine content (83.7 mg per 100 ml of urine), the phenol content was 0.32 mg per gram of creatinine.

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