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Simple high-performance liquid chromatographic analysis of phenol and *p*-cresol in urine and feces

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Abstract

Previous reports which present methods of analysis of phenol and *p*-cresol by HPLC are usually designed for the detection of these compounds in urine, can be complicated by the use of uncommon equipment or additional techniques such as steam distillation or derivatisation, or concentrate on the detection of phenol rather than *p*-cresol. In this paper we report a simple method suitable for the analysis of phenol and *p*-cresol in both urine and feces, based upon extraction into ether following acid hydrolysis and UV detection.

Keywords: Phenol; *p*-Cresol

1. Introduction

Phenols are toxic chemicals whose presence in biological systems is generally undesirable [1]. In humans, phenols can be detected in both urine and feces, with urine being the major route of excretion [2]. In the gut they originate from either the accidental ingestion of phenol, benzene and other aromatic compounds [3], or from the metabolism of food residues by bacteria, resulting in the in situ formation of phenols from aromatic amino acids [4].

We have been conducting a series of human studies into the effect of diet on the composition of large bowel contents, to identify compounds which are potentially toxic and act as markers of colonic health [5,6]. We are particularly concerned with measuring the amounts and types of phenols excreted in feces and urine. We have focussed on *p*-cresol and phenol because these are the major metabolites of tyrosine [7], and as they are both known promoters of skin tumours [1] it is possible that they may also be associated with other epithelial tumours.

There are many reports on the measurement of phenols (especially phenol) in urine. Some examples include colorimetry [8]—a non-specific method, gas chromatography (GC) [9–11], and high-performance liquid chromatography (HPLC) [2,12,13]. To our knowledge, only one report published in the last ten years details the measurement of phenols in feces [2]. The focus of this work was to apply existing methods, mainly developed for urine analysis, to feces using instrumentation available to our laboratory (that is, HPLC with UV detection). We aimed to

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maintain a simple extraction technique, without the use of enzyme digestion [14], distillation [2] or pre-column separation [15]. HPLC with UV detection is a reliable method for phenols in urine: Yoshikawa et al. [13] noted a correlation coefficient of 0.998 when he compared the detection of *p*-cresol in urine by HPLC and GC in the range 10 to 300 μg/ml. The method published by Murray and Adams [2] used HPLC, but was not suitable to our laboratory because it involved steam distillation, a column oven, developed a high back pressure in the column and used fluorimetric detection. For this reason we attempted to develop a simple HPLC protocol for the measurement of phenol and *p*-cresol in urine and feces, by adapting several existing methods [2,3,13].

2. Experimental

2.1. Materials

All reagents used were analytical grade quality except methanol which was HPLC grade (FSE, Homebush, Australia). Phenol, *p*-chlorophenol, sodium dihydrogen orthophosphate, diethyl ether and sodium hydroxide were supplied by BDH (Kilsyth, Australia) and *p*-cresol and hydrochloric acid were supplied by AJAX (Auburn, Australia). A stock standard solution of 200 μg/ml phenol and 1000 μg/ml *p*-cresol was prepared in water. This was diluted with water to a working standard of 20 μg/ml phenol and 100 μg/ml *p*-cresol. The internal standard solution consisted of 250 μg/ml *p*-chlorophenol in water.

2.2. Sample preparation

For a 24-h period urine was collected immediately after micturition and refrigerated, then stored frozen at −20°C until required for analysis. Following elimination, feces were immediately frozen on solid carbon dioxide and stored frozen at −20°C. Analyses were completed within three months of sample collection. Eadsforth and Coveney [12] reported that urinary phenol and phenol conjugates are stable for up to 72 days at 4°C.

Urine

To 5.0 ml of urine in a screwcapped glass tube, 2 ml of concentrated hydrochloric acid and 1 ml of the internal standard solution were added. This mixture was boiled for 60 min. After cooling, the phenols were extracted with 4 ml of diethyl ether by repeated inversion of the tube for 1 min. This was then centrifuged for 10 min at 500 *g* (Beckman TJ-6, Palo Alto, CA, USA). The organic phase was aspirated into 3 ml of 0.05 *M* NaOH in methanol. The resultant solution was evaporated to dryness under a stream of nitrogen, residues were dissolved in 0.5 ml of water, and this solution was then filtered through a syringe filter (Millipore HATF 0.45 μm, Bedford, MA, USA) prior to injection into the chromatograph. This protocol follows that of Yoshikawa et al. [13].

Feces

An aliquot of 0.5 g feces was accurately weighed (to four decimal places) into a screwcapped glass tube, and vortex-mixed with 5 ml of phosphate buffer (0.1 *M*, pH 5.5) [2] and 50 μl of internal standard solution. The mixture was then centrifuged at 500 *g* for 10 min. The top layer was decanted into another tube containing 2 ml of concentrated hydrochloric acid, and boiled for 60 min. After cooling the phenols were prepared as above, with the exception that after evaporation with nitrogen the sample was made up with 0.25 ml of water.

2.3. Chromatographic conditions

The HPLC apparatus consisted of a Waters 501 pump (Milford, MA, USA), Rheodyne 7125 injector (Cotati, CA, USA) fitted with a 50-μl loop, and a Chrom-A-Scope rapid scanning UV detector with computerised integration software (Barspec, Hart Analytical, Collingwood, Australia). The column dimensions were 250 mm × 4.6 mm, packed with 5-μm particles of Econosil RP-18 (Alltech, Deerfield, IL, USA). The mobile phase was methanol with 0.02 *M* phosphate

buffer pH 4.0 (48:52, v/v). The flow-rate was 0.7 ml/min and the UV detection setting was 270 nm. Under these conditions the column back-pressure was 13.8 MPa. The volume of sample injected was 50 μl. These conditions were similar to that of Schaltenbrand [3].

3. Results

A range of standard solutions containing phenol and *p*-cresol levels appropriate to the expected levels from earlier reports [2,3,7,8,10,16] in urine (10–100 μg phenol and 50–500 μg *p*-cresol) and feces (0.5–4 μg phenol and 2.5–20 μg *p*-cresol) were analysed. From this a calibration curve was established. The correlation (r) between the relative area of phenol and *p*-cresol to the internal standard, and the amount of phenol or *p*-cresol extracted, was 0.999 ($y = 0.009x - 0.003$) and 1.000 ($y = 0.006x + 0.017$), respectively, for the urine, and 0.996 ($y = 0.165x + 0.034$) and 0.997 ($y = 0.119x - 0.029$) for the feces. In these equations the peak area relative to the internal standard is represented by y and the amount (μg) of extracted compound is represented by x.

Replicated analyses ($n = 4$) of standard solutions were conducted to obtain estimates of analytical variation. For the higher concentration standard (250 μg *p*-chlorophenol, 60 μg phenol, 300 μg *p*-cresol) we obtained an R.S.D. of 1.6% and 1.4% for phenol and *p*-cresol, respectively, and for the lower concentration standard (12.5 μg *p*-chlorophenol, 2 μg phenol and 10 μg *p*-cresol) the values were 4.7% and 3.2%. Replicated ($n = 4$) analyses of a urine sample (5 ml urine, 250 μg *p*-chlorophenol) gave an R.S.D. of 9.9% for phenol and 2.4% for *p*-cresol. Corresponding values for feces (0.5 g feces, 12.5 μg *p*-chlorophenol) were 24.0% and 9.4%.

The minimum level of detection, calculated as three times the height of the baseline long term noise [17], was calculated as 0.2 μg of phenol and 0.8 μg *p*-cresol.

Spiked samples were run to determine the efficiency of the extraction method in recovering the phenols. We achieved a recovery of 98% for phenol (spike of 40 μg) and 105% *p*-cresol (spike of 200 μg) in the urine, and 103% for phenol (spike of 2 μg) and 101% *p*-cresol (spike of 10 μg) in the feces.

Blanks were prepared by adding aliquots of water to tubes and extracting in the same way as the fecal and urine samples. These gave no observable absorbance at 270 nm and at times

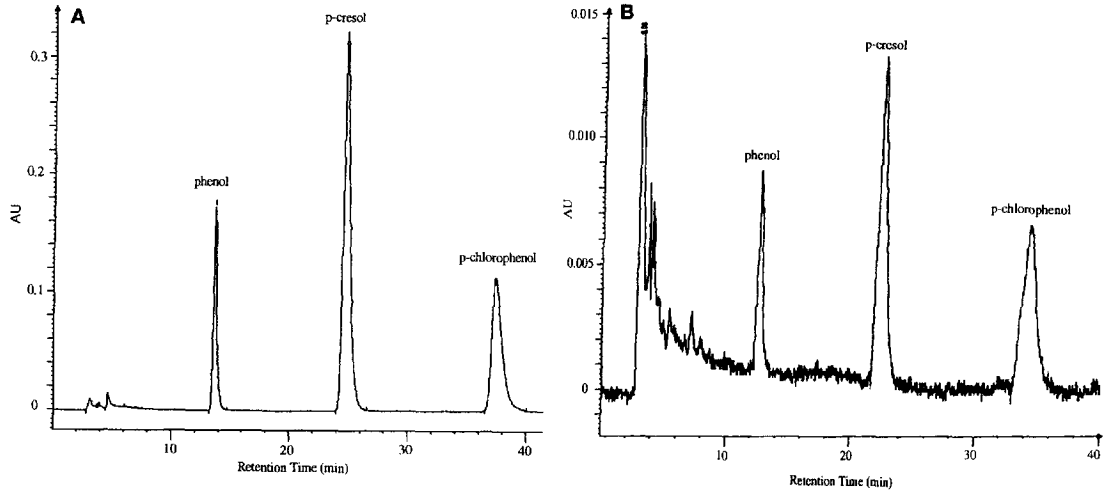

Fig. 1. (A) Separation of standard phenols at physiological concentrations found in urine (12 μg/ml phenol, 60 μg/ml *p*-cresol and 250 μg/ml *p*-chlorophenol). Measured at 270 nm. (B) Separation of standard phenols at physiological concentrations found in feces (4 μg/g phenol, 20 μg/g *p*-cresol and 25 μg/g *p*-chlorophenol). Measured at 270 nm.

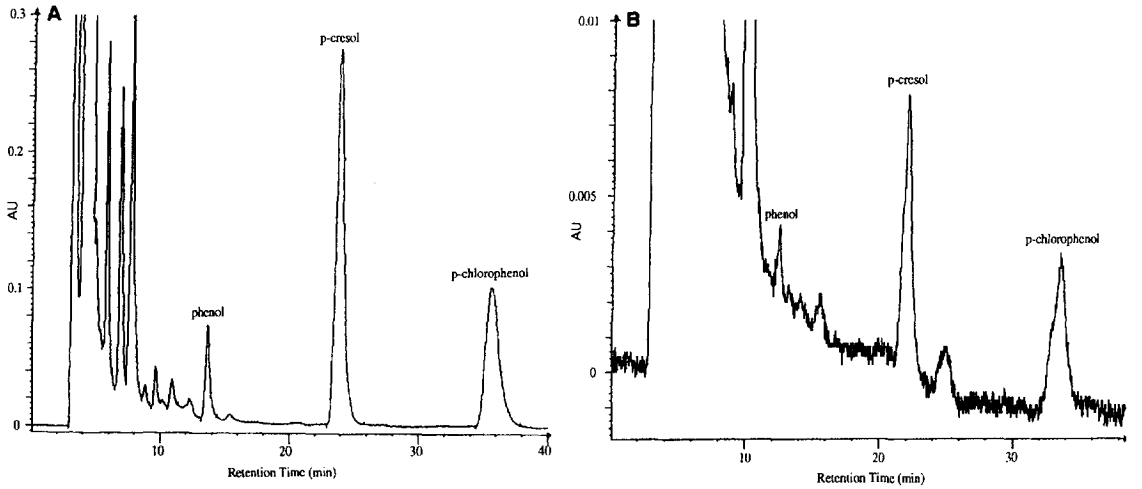

Fig. 2. (A) Chromatogram of phenols extracted from a pooled 24-h human urine sample (4.8 μg/ml phenol, 43 μg/ml *p*-cresol and 250 μg/ml *p*-chlorophenol). Measured at 270 nm. (B) Chromatogram of phenols extracted from a pooled 24-h human fecal sample (1.4 μg/g phenol, 18.1 μg/g *p*-cresol and 25 μg/g *p*-chlorophenol). Measured at 270 nm.

corresponding to the retention times of the analytes.

Fig. 1A represents a chromatogram for the higher concentration standards and Fig. 1B represents a chromatogram for the lower concentration standards. For each peak a UV spectrum was obtained and both retention time and UV spectra were used to identify the compounds in the biological samples.

Fig. 2A represents an extraction from urine of a healthy individual. Fig. 2B represents phenols extracted from a fecal sample from a healthy individual. Sample chromatograms such as these can be used to calculate the daily output of phenol and *p*-cresol, if 24-h collection samples are available.

Analysis of urine samples from 23 subjects produced a range in the 24 h excretion of urinary phenol and *p*-cresol of 1.4–8.3 and 1.3–145.0 mg/day, respectively. The analysis of phenol and *p*-cresol excreted in feces over 24 h ranged from 0.1 to 1.1 and 2.4 to 19.1 mg/day, respectively.

4. Discussion

The results presented here show that we have developed a simple, efficient and reasonably precise HPLC technique for the measurement of phenol and *p*-cresol in urine and feces. The run time of this method is relatively longer than obtained with many other published methods, including those for urine which we have adapted [2,3,13]. Fecal samples, however, contain many compounds which would potentially interfere with the separation and detection of phenol and *p*-cresol under our conditions if the run time was considerably shorter, especially when using a simple ether extraction procedure.

Our recovery calculations were based on the addition of simple phenols to urine and feces. However, in humans phenols are conjugated to sulphates and glucuronides by the normal functioning liver and also by colonic epithelial cells (predominantly as sulphates) [18]. Eadsforth and Coveney [12] demonstrated that 84% of phenyl sulphate was recovered from urine, after being hydrolysed with hydrochloric acid in a boiling bath for 60 min and extracted with dibutyl ether. As this procedure was similar to the one reported in this paper, we expect that greater than 80% of the phenols would be recovered, and are sufficiently high to enable us to make statistically valid comparisons between individuals whose output of phenols varies with diet.

The low levels of phenol detected in the fecal

samples approached the limits of detection of this system (as reflected by the large R.S.D.). However, this is unlikely to be a major problem when measuring levels of phenols in human fecal samples as *p*-cresol is the major tyrosine metabolite, making up more than 80% of the total phenols present [2].

With this method, detection of phenol and *p*-cresol was conducted at 270 nm. This is in contrast to the wavelength used by some other workers of 200–210 nm [19,20]. Although the minimum level of detection is lower at 270 nm, the higher wavelength was chosen to reduce possible interference due to light absorption by other extracted compounds with retention times near phenol.

The concentration of phenol and *p*-cresol measured in the urine and feces during the present study are comparable with other published reports using colorimetry, GC and HPLC [2,3,7,8,10,16]. Unfortunately few reports have published fecal phenol and *p*-cresol output data, with which to draw comparisons.

Earlier studies investigating the impact of fermentation on bacterial metabolism and metabolite production in humans assume that the levels of phenols in urine reflect protein fermentation in the colon. We have recently shown that the output of phenols in urine and the excretion of phenols in feces do not correlate with each other ($r = -0.180$, $n = 20$) (unpublished). Therefore urine may not be a sensitive indicator of events occurring in the colonic environment, and phenols should be measured directly in feces.

In conclusion we have presented a simple HPLC technique which is appropriate for human urine and feces samples. We feel that this method can be used for studies designed to measure the response of the production of phenols to dietary change, especially the major tyrosine metabolite *p*-cresol.

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