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Note

Determination of simple phenols in faeces and urine by high-performance liquid chromatography

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Phenols are reported to be co-carcinogenic [1] and are neurologically active [2]. High levels of faecal *p*-cresol have been reported in a group of hyperactive children [3]. The simple phenols, phenol, *p*-cresol and 4-ethylphenol, are produced in the gut by microbial degradation of tyrosine [4]. In the course of dietary studies it was required to monitor the relationship between dietary tyrosine and the levels of the simple phenols in stool and urine samples.

Phenols may be determined by gas chromatography (GC) [5,6], but their polarity and volatility may contribute to reproducibility problems [6]. High-performance liquid chromatographic (HPLC) procedures have been used for the determination of a wide range of phenolics in environmental and industrial samples [7,8], but the conditions used, such as gradient elution or other methods of extraction, for aqueous samples with a simple sample matrix, were not readily adaptable to our more complex samples. A more recent HPLC procedure [9] reports the determination of cresol isomers in biological samples to monitor exposure to toluene. Since we were concerned only with *p*-cresol, derived from tyrosine, we opted for the present system.

For the determination of phenol, *p*-cresol and 4-ethylphenol in biological samples, we report here the combination of small-scale steam distillation of faecal and other samples with an HPLC procedure to quantify phenols in the distillate.

EXPERIMENTAL

Materials

Acetonitrile (Acetonitrile-190, Ajax Chemicals, Sydney, Australia) was HPLC grade. Water was from a Milli-Q water purification system (Millipore, Bedford,

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MA, U.S.A.). Acetic acid was analytical reagent grade (BDH, Melbourne, Australia). Reference phenols were at least 98% pure (Tokyo Kasei Kogyo, Tokyo, Japan). Aqueous standard solutions of the phenols were prepared to a concentration of 10 mmol/l and used to prepare working standards in water of individual phenols and mixtures. Standard solutions were stored at 4 °C.

Sample preparation

Faecal samples were manually freed from undigested solid food material, such as seed, homogenized, and 500 mg weighed into a screw-capped culture tube. Phosphate buffer (0.1 *M*, pH 5.5, 4.5 ml) was added and the capped tube shaken to obtain an even dispersion. Urine samples (1 ml) were mixed with 4 ml buffer. Although our interest was with free phenols, the procedure was used with acid-hydrolyzed urine prepared by mixing 1 ml urine with 3 ml water and adjusting to pH 1.0 with 2.5 *M* sulphuric acid. The mixture was heated in a screw-capped tube at 100 °C for 30 min, cooled and adjusted to pH 5.5 with 2.5 *M* sodium hydroxide. The total samples were introduced to the still with a glass syringe fitted with a wide bore, square-ended needle. Each sample tube was rinsed with a small volume of water, and rinse water was added to the still.

Steam distillation

The steam still featured a steam-jacketed sample chamber with a total volume of about 14 ml. Operating conditions were set to give 10 ml distillate in about 6 min. For each sample 10 ml distillate were collected in a 10-ml graduated cylinder, and the distillate was transferred to a screw-capped glass vial.

Chromatographic conditions

The chromatographic equipment (Perkin Elmer, Norwalk, CT, U.S.A.) consisted of a Sigma 3B pump module, an LC100 column oven, a Model 650-10S fluorescence spectrophotometer and a Sigma 15 data station. The column, 250 mm×4 mm, RP-8, mean particle size 5 μm, was obtained prepacked (Merck, Darmstadt, F.R.G.). The injection system was a Model 7105 injector valve (Rheodyne, Berkeley, CA, U.S.A.), fitted with a 175-μl loop. The mobile phase was water–acetonitrile–acetic acid (89:9:2, v/v/v). The flow-rate was 2.5 ml/min. The column oven temperature was 40 °C. These conditions gave an operating back-pressure of about 22 MPa. Detector excitation and emission wavelengths were 275 and 300 nm, respectively, with slit widths of 5 nm. The sensitivity setting was medium. Injection volume was 100 μl.

Quantification and identification

The peak areas of phenols from test samples were quantified using external distilled standards, and the results were expressed as nmol/g wet weight for faeces or nmol/ml for urine. Coincidence of peak retention time with standards was the basis of identification during chromatography. During development of the procedure, thin-layer chromatography with field desorption mass spectrometry of material eluted from spots was used, as previously reported [10], to confirm the identity and amount (to 1 μg) of the simple phenols in the samples.

Preparation of samples for quality control

Phenols were added to faecal samples containing no detectable phenols or small quantifiable amounts to give a range of standards. The samples were processed by the complete procedure and amounts recovered expressed as nmol/g wet weight after allowing for phenols present before the addition of standard phenols. Urine samples with added phenols were processed by the procedure for urine. Although our interest was with free phenols, the effect of acid hydrolysis on the free phenols was assessed by adding phenols before and after hydrolysis to aliquots of the same urine and comparing recoveries. Phenol conjugates would be present normally in urine but were not available as standards for this study. Inter-day reproducibility and recovery data were obtained by comparing results obtained for a concentration range of spiked faecal and urine samples analyzed each day for five days.

Sensitivity

The sensitivity of the procedure was estimated as the smallest amount of a phenol in a 100-μl injection volume that was twice the signal-to-noise ratio calculated from the chromatogram.

RESULTS AND DISCUSSION

Fig. 1A is a chromatogram of a distillate of a standard containing 1 nmol each of phenol, *p*-cresol and 4-ethylphenol. The respective retention times were 4.7 ± 0.1, 9.2 ± 0.2 and 19.9 ± 0.3 min. Indole (I), 5 nmol, was included in the stan-

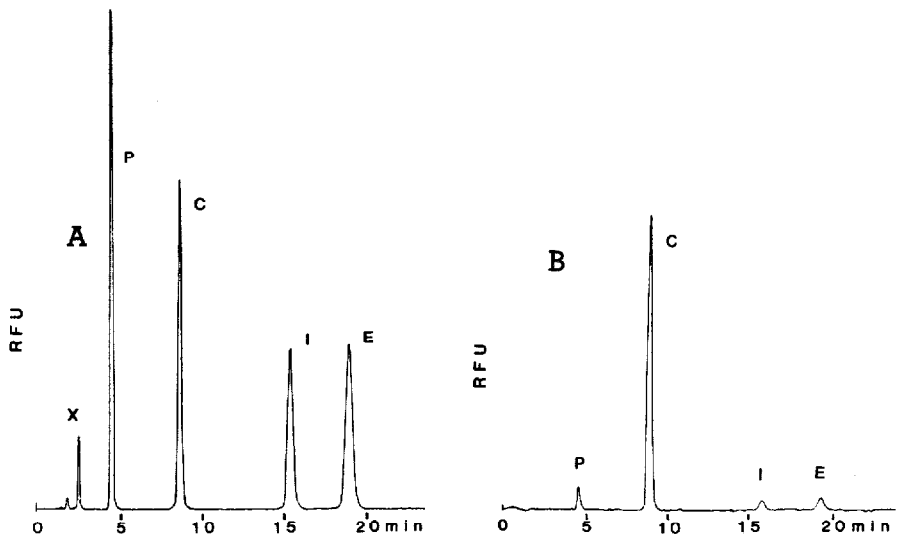

Fig. 1. (A) Chromatogram of distillates of standard phenols: phenol (P), *p*-cresol (C) and 4-ethylphenol (E), 1 nmol of each phenol, and indole (I), 5 nmol; × = impurity associated with the indole used. Retention times for P, C, I and E are 4.7 ± 0.1, 9.2 ± 0.2, 16.3 ± 0.2 and 19.9 ± 0.3 min, respectively. (B) Chromatogram of phenols in faecal sample from adult on high meat diet. Concentrations are 9.9, 189.3, 57.6 and 14.0 nmol/g wet weight for P, C, I and E, respectively. RFU = relative fluorescence units.

dard since most samples contained a component having the retention time of indole (16.3 ± 0.2 min). Fig. 1B is a chromatogram from a faecal sample from an adult on a high meat diet. Phenols concentrations were 9.9, 189.3 and 14.0 nmol/g wet weight for phenol, *p*-cresol and 4-ethylphenol, respectively. Fig. 2A is a chromatogram of phenols present in a faecal sample from an infant on breast milk. The amounts calculated for phenol and *p*-cresol were 0.1 and about 0.02 nmol/g wet weight, respectively. Fig. 2B was obtained from a faecal sample from a child on a high tyrosine diet. Phenol, *p*-cresol and 4-ethylphenol values were 2.9, 154.1 and 4.9 nmol/g wet weight, respectively. 4-Ethylphenol was detected in less than 1% of samples from infants who were mainly on a milk diet, whereas 4-ethylphenol was often present at appreciable levels in samples from adults. Fig. 3A is a chromatogram obtained from distilled but unhydrolyzed urine. Values for phenol, *p*-cresol and 4-ethylphenol were 9.2, 14.2 and 2.1 nmol/ml, respectively. Fig. 3B is a chromatogram of the same urine as Fig. 3A but after acid hydrolysis. Values were 53.5, 272.1 and 7.9 nmol/ml for phenol, *p*-cresol and 4-ethylphenol, respectively.

The sensitivity for the phenols was better than 10 pg. Distilled standards were compared with undistilled standards to evaluate distillation efficiency. For the range 0.1, 10, 100, 1000 and 2000 nmol ($n=3$ for each amount) the recoveries were 99.4–101.9% with coefficients of variation of 1.5–2.5%. Inter-day recovery and reproducibility data for phenols added to faecal samples are summarized in Table I. The recovery data for urine samples for the range 1.0, 10, 100 and 1000 nmol/ml ($n=5$ for each concentration) were 100.9–102.0% with coefficients of variation of 1.5–2.6%. For the same range of urine samples it was found that acid hydrolysis caused no loss of free phenols, and the recoveries were 100.1–101.7% with coefficients of variation of 1.4–2.6%. Table II lists the range of values and means for faecal phenols for subjects with no diagnosed clinical condition. In one class of subjects with defined behavioural abnormalities, the range of values for *p*-cresol was significantly different [3]. Table II also includes a range of values for free phenols obtained from urine samples collected from adults. Because of the difficulty in obtaining urine samples from infants, no data are given here.

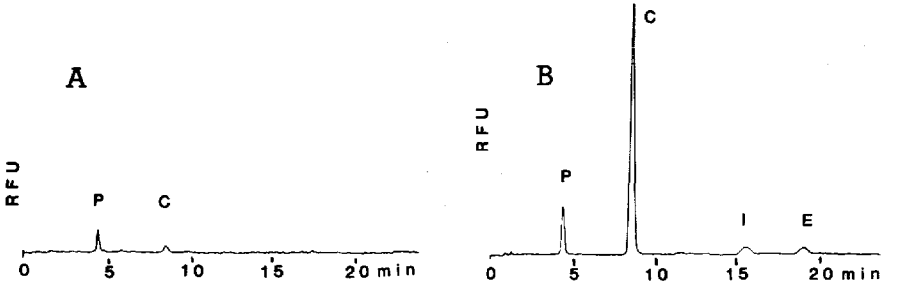

Fig. 2. (A) Chromatogram of distillates of faecal sample from breast-fed infant. Concentrations are 0.1 and about 0.02 nmol/g for phenol (P) and *p*-cresol (C), respectively. (B) Chromatogram of faecal sample from infant on cow's milk. Concentrations are 2.9, 154.1, 4.5 and 14.9 nmol/g for P, C, indole (I) and 4-ethylphenol (E), respectively. RFU = relative fluorescence units.

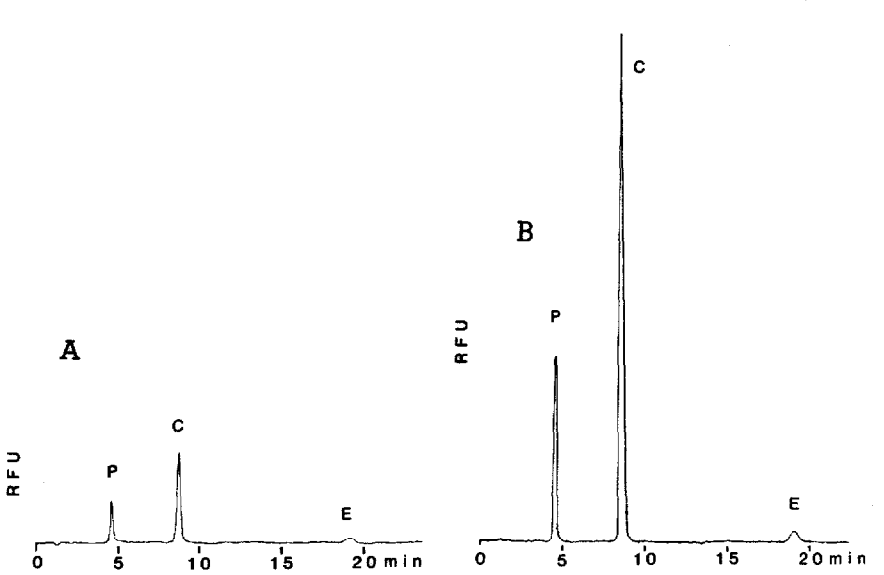

Fig. 3. Chromatograms of (A) unhydrolyzed infant urine and (B) the same urine but acid-hydrolyzed. Concentrations for phenol (P), *p*-cresol (C) and 4-ethylphenol (E) are 9.2, 14.2 and 2.1 nmol/ml (A) and 53.5, 272.1 and 7.9 nmol/ml (B). RFU = relative fluorescence units.

TABLE I

INTER-DAY REPRODUCIBILITY DATA FOR FAECAL PHENOLS

At each concentration $n = 5$.

Compound	Concentration added (nmol/g wet weight)	Mean concentration found (nmol/g wet weight)	Coefficient of variation (%)	Recovery (%)
Phenol	0.1	0.1	2.5	102.0
	1.0	1.0	2.5	99.2
	10	10.0	2.6	100.4
	100	99.7	2.3	99.7
	1000	1013.2	2.1	101.3
	2000	2014.9	2.5	100.7
p-Cresol	0.1	0.1	2.6	100.0
	1.0	1.0	2.7	100.3
	10	10.1	2.7	101.1
	100	100.5	2.4	100.5
	1000	1013.3	2.2	101.3
	2000	2031.7	2.3	101.6
4-Ethylphenol	0.1	0.1	2.7	100.0
	1.0	1.0	3.0	100.7
	10	10.2	2.3	102.0
	100	98.6	2.4	98.6
	1000	1018.7	2.2	101.9
	2000	2035.8	3.2	101.8

TABLE II

FAECAL PHENOLS IN HEALTHY INFANTS AND ADULTS AND FREE PHENOLS IN ADULT URINES

Values (nmol/g wet weight for faeces or nmol/ml for urine) are the ranges with means in parentheses. N.D. = < 0.01 nmol.

Sample	*n*	Concentration		
		Phenol	*p*-Cresol	4-Ethylphenol
Faeces (infant)	19	N.D.-74.8 (16.8)	N.D.- 761.5 (180.6)	N.D.-2.9 (0.2)
Faeces (adult)	31	0.1-241.3 (73.0)	N.D.-1624.4 (392.6)	N.D.-7.3 (0.6)
Urine (adult)	34	5.6-184.0 (36.3)	7.1-802.4 (138.8)	0.1-2.3 (0.12)

Interferences

The *o*- and *m*-isomers of cresol are reported to occur in the urine as a result of exposure of an individual to toluene [5] and not as a result of the microbial degradation of tyrosine [4]. Consequently, the occurrence of *o*- and *m*-cresol would be expected to be rare, except in those individuals subjected to such exposure. The retention times of *m*- and *o*-cresols were 8.9 ± 0.2 and 9.3 ± 0.2 min, respectively, and would, therefore, if present in a urine, interfere with *p*-cresol. In preliminary studies, urine and faecal samples were screened for the presence of the isomers by supplementary gas chromatography [11]. No sample analyzed contained detectable amounts of *o*- or *m*-cresol. Other phenols, such as hydroquinone or resorcinol, often present in faecal samples, did not occur in the distillate. Other volatiles that would be present in the distillate, including some amines and short-chain fatty acids, were not detectable by our system. As well as indole, skatole was expected to be present in faecal samples and in the distillates. Both compounds are microbial degradation products of tryptophan. The retention time for skatole was found to be 36.2 ± 0.3 min. The indoles did not interfere with the phenols of interest. At recommended detector settings, detector sensitivity was about 200 times less for skatole than for indole. For several samples shown to contain skatole by UV detection, the skatole was not detected with the recommended fluorimeter settings.

The procedure was applied successfully to determine the phenols in spent microbial culture media, distilling 1 ml culture supernatant with 4 ml buffer. The 10-ml distillate volume was chosen as the most convenient for measurement. At the recommended distillation rate it was determined that the phenols distill essentially completely within the first 2 ml of distillate. A few sera were processed using 0.1 ml serum with 1 ml buffer and collecting the first 2 ml distillate. No recovery or reproducibility studies were made on sera or microbial culture samples, but chromatograms were visually free from significant interferences.

Steam distillation minimized sample manipulation and reduced the possibility of interferences that might arise with extraction procedures using polar solvents. The use of a fluorimetric detector gave high selectivity for the phenols and permitted detection of as little as 10 pg of the phenols of interest. The procedure has

proven particularly valuable for evaluating the large numbers of samples obtained during dietary studies.

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