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GAS–LIQUID CHROMATOGRAPHY OF SIMPLE PHENOLS FOR URINALYSIS

SUSAN M. DIRMIKIS* and A. DARBRE

Department of Biochemistry, King's College, London WC2R 2LS (Great Britain)

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SUMMARY

Methods for determining simple phenols in urine by using gas–liquid chromatography were examined. It was shown that repeated on-column aqueous injections of phenols led to errors associated with peak tailing and normal ghosting. Injection of urines led to hydrolysis of conjugated forms of phenols left *in situ* in the top of the column. This gave rise to a form of ghosting not previously reported. Retention time data are reported with various column packings and stationary phases. A reproducible quantitative method for the analysis of simple phenols in urine is described, and compared with some other published methods.

INTRODUCTION

The principal simpler phenols reported to be present in human urine are phenol, *p*-cresol, catechol, resorcinol and quinol[1,2]. Many other phenolic compounds have been reported, such as phenolic acids[3], alcohols[4] and amines[5]. Phenols may be derived not only from the dietary intake of proteins[6], fats[7], smoked foods such as meat[8] and water[9] but also from a wide variety of exogenous sources such as tobacco smoke[10], mouth washes and ointments[11] and analgesics[12]. Exposure to benzene, which is of particular interest because of its known haemopoietic toxicity, may give rise to the formation and increased excretion of phenols[1,2,13] and this may be used as a measure of assessing the degree of exposure of humans[14].

Williams[1,15] reviewed the metabolism of phenols. They are excreted in the free state or conjugated with either glucuronic acid, or sulphuric acid[16]. The relative amounts of these may vary with the individual. Abnormal patterns of phenol excretion may arise from changes in the flora of the gut due to antibiotic intake[6], gastrointestinal disorders[6,17,18] and liver or kidney diseases[19].

Urinary phenols have been widely studied using a variety of colorimetric methods. However, Goren-Strul *et al.*[20] in their review of these methods pointed to the confusion which exists in the literature concerning the specificity of the reactions

* Formerly S. M. Maciver. Present address: Department of Pharmacology, The University, Sheffield S10 2TD, Great Britain.

involved. The analysis of phenols by gas–liquid chromatography (GLC)[21], which offered the advantages of specificity and sensitivity was used for urinalysis[14,18,19,22–24].

A survey of the literature showed that there is no general agreement on the level of normal phenol excretion in humans and comparisons between quoted results were difficult because some were given as rates of excretion and others as total amounts. Some results were corrected for the specific gravity of urines and others were not. In addition, values for free and total phenol were often obtained by different methods and the conditions used for the hydrolysis of glucuronide- and sulphate-conjugated phenols varied considerably (see Docter and Zielhuis[25]).

We report here on the development of a GLC method for the specific estimation of simple phenols in urine. This was part of a long-term project for the study of "normal" excretion values, possible diurnal variations and the effects of diet and exposure to benzene.

MATERIALS AND METHODS

Reagents

Phenols, benzoic and phenylacetic acids were obtained from BDH (Poole, Dorset, Great Britain) and ethylphenols from R. N. Emmanuel (Alperton, Middlesex, Great Britain). Purity was checked by GLC. Hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) were obtained from Hopkin and Williams (Chadwell Heath, Essex, Great Britain). N,O-Bis-(trimethylsilyl)-acetamide (BSA) and trifluoroacetic anhydride (TFAA) were purchased from BDH. TFAA was distilled under $CaCl_2$ drying tube immediately prior to use. Trimethylsilylimidazole (TMSIM) was obtained from Applied Science Labs. (State College, Pa., U.S.A.) and Silyl-8 was a trial sample from Pierce (Rockford, Ill., U.S.A.). Phenyl-β-D-glucuronide monohydrate was a gift from the Esso Research Centre (Abingdon, Berks., Great Britain) and was later purchased from Koch-Light (Colnbrook, Bucks., Great Britain). *o*-Hydroxyphenyl (catechol) β-D-glucuronide and *p*-hydroxyphenyl (quinol) β-D-glucuronide were gifts from Chugai, Tokyo, Japan. Diethyl ether was freshly distilled after shaking with aqueous ferrous sulphate and before use tested for the presence of peroxides. Hydrochloric acid, orthophosphoric acid and sodium sulphate were of Analar grade. Standard solutions for GLC were made up in dry chloroform and stored in the dark.

Enzymes

β-Glucuronidase, type H-2 (from *Helix pomatia* and containing arylsulphatase) and β-glucuronidase type II (from *E. coli*) were obtained from Sigma (St. Louis, U.S.A.).

Gas–liquid chromatography

Two gas chromatographs were used: Microtek MT-220 (Techmation, Edgware, Middlesex, Great Britain) and Pye Series 104, Model 24 (Pye-Unicam, Cambridge, Great Britain) each fitted with dual flame ionisation detectors (FID). Nitrogen (99.9%, "white spot" from British Oxygen, Wolverhampton, Staffs., Great Britain) was used as carrier gas. Integration of peak areas was carried out with an Instron Integrator (Instron, High Wycombe, Bucks., Great Britain) and with a Kent Chromalog-2

digital integrator (Kent Instruments, Luton, Beds., Great Britain) used in conjunction with a 1-mV 1-sec chart recorder (Leeds & Northrup, Birmingham, Great Britain) and a 10-mV 1-sec chart recorder (Honeywell-Brown), respectively.

Support materials. Chromosorb 101, 60–80 mesh and Chromosorb T, 40–60 mesh, were obtained from Johns-Manville (Manville, N.J., U.S.A.); Anakrom ABS 80–90 mesh from Fison's (Loughborough, Leics., Great Britain); Celite 560 from Koch-Light; high-performance (HP) Chromosorb W, 80–100 mesh, from Perkin-Elmer (Beaconsfield, Bucks., Great Britain) and Bentone 34 from Berk (London, Great Britain).

Stationary phases. PEG 6000 and PEG 20M were obtained from Union Carbide (London, Great Britain); Carbowax 20M terephthalic acid and neopentyl glycol succinate (NPGS) from Applied Science Labs.; Free Fatty Acid Phase (FFAP) from Phase Separations (Queensferry, Cheshire, Great Britain); tri-*o*-(cresyl) phosphate (TCP) from BDH and tris-(2,4-xylenyl) phosphate (TXP) from Pye-Unicam.

The coating of support material was carried out using a flat-bottomed dish as previously described[26]. When coating with TXP the evaporation of the chloroform used as solvent was carried out below 35° with the aid of a water pump. The use of higher temperatures resulted in a marked loss of efficiency and resolution.

For the analysis of monohydric phenols in urine extracts, glass columns, 2 m × 2.5 mm I.D. were filled as previously described[26] with HP Chromosorb W 80–100 mesh coated with TXP 0.5% (w/w). The inlet heater was maintained at 20° above the column temperature of 110–116° and the section of column in the inlet heater zone was packed with deactivated glass thread. This thread was changed periodically as it became contaminated with non-volatile compounds from urine extracts. Dihydric phenols in urinary extracts were chromatographed as their trimethylsilyl (TMS) derivatives on identical columns, but the solid support was coated with TXP 5.0% (w/w).

Collection and storage of urine samples

Most urine samples were analysed for both free phenol and total phenol so that strong acid could not be added as a preservative because of the possible hydrolysis of labile sulphate conjugates. Bacteriostatic agents were not used because of their possible effects on enzyme assays. Toluene was found to inhibit the β-glucuronidases used for the determination of glucuronide conjugates. Because urines contain β-glucuronidase and arylsulphatase, specimens were stored at −20° in 20-ml "Sterilin" bottles (Sterilin, Richmond, Surrey, Great Britain) or polythene screw cap jars within 30 min after collection and thawed immediately before analysis. Control samples showed no change after several weeks.

Colorimetric methods

Phenol analyses by both the Folin and Ciocalteu and 4-aminoantipyrine methods were as described[27].

Phenyl glucuronides. A 5-ml urine sample was adjusted to pH 7.2 using 1.0 *M* HCl and 5 ml buffer (0.05 *M* Tris–HCl, pH 7.2) added. With urines of specific gravity 1.020 or greater, 10 ml buffer were added. One millilitre buffered enzyme solution (0.05 *M* Tris–HCl, pH 7.2, containing 0.029 I.U. Type II β-glucuronidase) was added and incubation carried out at 37° for 24 h in a glass-stoppered tube.

Phenyl sulphates. A 5-ml urine sample adjusted to pH 1.0 with concentrated HCl was refluxed on a boiling water-bath under a condenser for 1 h. These conditions kept the hydrolysis of glucuronides to a minimum.

Total phenols. A 5-ml urine sample was adjusted to pH 5.0 with 1.0 *M* HCl and 5 ml buffer (0.1 *M* acetic acid–sodium acetate, pH 5.0) added. With urines of specific gravity 1.020 or greater, 10 ml buffer were added. *Helix pomatia* juice type H-2 (0.1 ml containing approximately 0.58 I.U. *β*-glucuronidase and 16.7 I.U. arylsulphatase) was added and the mixture incubated at 37° for 48 h in a glass-stoppered tube. The time-course of the reaction was followed.

Preparation of urinary extracts

The urine sample at pH 1.0 was extracted three times with two volumes diethyl ether in a stoppered separating funnel. Since vigorous shaking caused emulsification, the extraction was standardised by inverting the mixture by hand sixty times. The combined ether extracts were dried over anhydrous Na_2SO_4 for 30 min with occasional mixing. Chloroform (containing 0.1 mg acenaphthene as internal standard) was then added to the combined dried extracts. The extracts in a round-bottomed flask were reduced to about 5 ml on a rotary evaporator, using a water-bath at 25–30°, transferred to a 10-ml stoppered tube and further concentrated to approximately 0.1 ml. Losses of free phenols occurred if the extract was allowed to reach complete dryness. Aliquots of this extract were then injected on to the column of the gas chromatograph.

Conversion of phenols to their TMS derivatives

Urinary monohydric and dihydric phenols were converted to their TMS derivatives by adding to the concentrated ether extract 100 *μl* of a mixture of chloroform–HMDS–TMCS in the ratio 9:3:1. The reaction was allowed to proceed at room temperature in a stoppered tube for 5 h and an aliquot of the reaction mixture was then injected into the gas chromatograph. TMS *p*-cresol was used as internal standard.

RESULTS AND DISCUSSION

Gas–liquid chromatography of urine samples

Attempts were made to chromatograph urinary phenols both by the direct injection of urine on to the column, and by the injection of a concentrate prepared by extracting urinary phenols with an organic solvent.

Silicone-type stationary phases. Using a column with 20% Apiezon L, Lebbe *et al.*[23] carried out the analysis of phenols by direct injection of acid-hydrolyzed urine, but the results are subject to criticism because Hermann and Post[28] showed that the presence of water, although not detectable on the GLC record, interfered with the response of the FID. Both injections of urine and urinary extracts gave severely tailing solvent peaks with these stationary phases as shown by the solid line in Fig. 1, where an ether extract prepared from urine was injected on to a GLC column with 10% Apiezon L stationary phase. The dotted line shows the response obtained with pure phenol dissolved in dry ether.

It was reported that the difficult separation of *m*- and *p*-xylenes could be achieved with SE-30 coated on to Bentone 34 (ref. 29) and this was investigated for the separation of *m*- and *p*-cresol, but no peaks were obtained. While they have the advantage

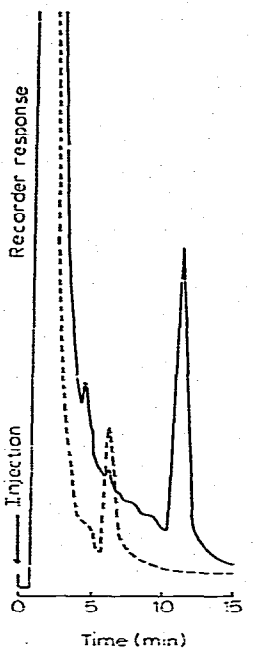

Fig. 1. GLC of a urine extract with silicone stationary phase. Conditions: Microtek MT-220, dual FID. Glass column, 2 m × 2.5 mm I.D., packed with Anakrom ABS 80–90 mesh coated with Apiezon L (10% w/w). Inlet temperature, 150°. Oven temperature, 120°. Nitrogen flow-rate, 20 ml/min. Attenuation, 1×10^{-11} A for f.s.d. Sample injected, 1 μl. ——, Ether extract from 100 ml urine, concentrated to approximately 0.1 ml. – – –, Phenol in dry ether.

of temperature stability and relative chemical inertness, all packings coated with silicone-type stationary phases deteriorated in use with urine analyses, as shown by increased solvent tailing and worsening separation. On the silicone-type phases investigated, *m*- and *p*-cresols were not separated. The *o*-cresol was eluted before the *m*- and *p*-isomers. This is an example of the well-known "ortho" effect[30].

Polyester-type phases. NPGS was used for urinary phenols[23] and to reduce peak tailing NPGS with added phosphoric acid was used for phenol derivatives[31]. Urine was injected on to a pre-column filled with borosilicate glass powder heated at temperatures between 140° and 220°. The column with mixed stationary phase (NPGS 3% and H_3PO_4 1% w/w) was at 115°–120°. Despite the use of matched columns a stable baseline was not easily obtained with the attenuation (1.6×10^{-12} A for f.s.d.) necessary to detect the small amounts of urinary phenol normally present. A difficulty was caused by urinary hippuric acid which on hydrolysis gave a peak for benzoic acid. Reference to this cause of interference was not found in the literature, although "back-flushing" was suggested for the removal of high boiling compounds introduced on to the column[32]. Fig. 2 shows the chromatogram obtained after injecting a phosphoric acid–urine mixture on a column with the mixed stationary phase. Raising the inlet temperature resulted in artifact peaks. Lowering the temperature decreased the extent of hippuric acid hydrolysis, but with concomitant increase in peak tailing. Subsequent injections could only be made at 45-min intervals, although the retention time for *p*-

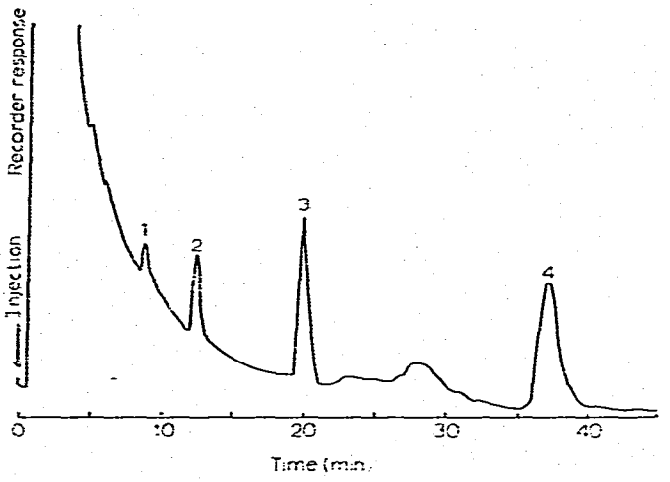

Fig. 2. GLC of urine with NPGS–H_3PO_4 stationary phase. Conditions: Microtek MT-220, dual FID. Glass pre-column, 10 cm × 2.5 mm I.D., packed with borosilicate glass powder 30–50 mesh. Glass column, 4 m × 2.5 mm I.D., packed with Chromosorb W 70–80 mesh coated with NPGS (3% w/w) and H_3PO_4 (1% w/w). Inlet temperature, 140°. Oven temperature, 116°. Nitrogen flow-rate, 20 ml/min. Attenuation, 8×10^{-13} A for f.s.d. Sample: urine with bibenzyl internal standard mixed with phosphoric acid (1:1) and 4 μl injected. 1 = Phenol; 2 = *p*-cresol; 3 = bibenzyl; 4 = benzoic acid.

cresol was 12 min. Glass bead pre-columns soon became blocked due to accumulation of phosphoric acid and non-volatile substances. Gently crushed borosilicate glass helices (4-mm diameter) were more satisfactory. When Porapak was used in the pre-column, this led to a threefold increase in the retention time of phenol. Quite sudden deterioration of polyester columns was observed after a period of repeated urine injections and replacement of the injection end of the column with fresh packing material did not give much improvement in the separations.

Using a GLC column with neopentyl glycol adipate and H_3PO_4 Duran *et al.*[18] showed the presence of benzoic and phenylacetic acids in urines which were pretreated with acid but did not comment on their origin. It was shown that phenyl conjugates were hydrolysed by acid retained at the top of the column. A single injection of an acidic sample was sufficient to cause this effect. Urine was extracted with ether and estimations made on a column with NPGS stationary phase. The values for free phenol and *p*-cresol were 0.05 mg/l and 1.82 mg/l, respectively (Table I). When an aliquot of the untreated urine was injected on to a pre-column previously used once for the injection of an acid mixture, the values were increased to 23 mg phenol/l and 88 mg *p*-cresol/l. When the urine was mixed with phosphoric acid in the ratio 1:1 and injected immediately or following a period of pre-heating at 100° for 15 min there was no observable difference. The results in Table I confirm the lability of some conjugated phenols and might explain the high average values reported for free phenol (8.5 mg/l) and free *p*-cresol (27.2 mg/l) in urine[23], particularly if acid samples had been previously injected on the column.

Urine samples did not give satisfactory phenol peaks with butanediol succinate coated on Gas-Chrom Q (ref. 28) or low loadings of diethylene glycol adipate (0.3% w/w) on glass beads[32].

TABLE I

ON-COLUMN HYDROLYSIS OF URINARY PHENOLS DURING GLC

Microtek MT-220, dual FID. Glass pre-column, 10 cm × 2.5 mm I.D., packed with borosilicate powder 30–50 mesh. Glass column, 4 m × 2.5 mm I.D., packed with Chromosorb W 70–80 mesh coated with NPGS (3% w/w). Inlet temperature, 140°. Oven temperature, 116°. Nitrogen flow-rate, 20 ml/min. Attenuation, 1×10^{-11} A to 8×10^{-13} A for f.s.d. Benzyl alcohol was used as internal standard. Means of duplicate results (mg/l urine, uncorrected).

Sample treatment	*Phenol (mg/l)*	*p-Cresol (mg/l)*
Untreated urine; ether extract injected	0.05	1.82
Untreated urine injected directly	23	88
Urine–H_3PO_4 (1:1): mixed and injected directly	26	86
Urine–H_3PO_4 (1:1): heated at 100° for 15 min before injection	28	75

Polyethyleneglycol phases. PEG 6000 on Chromosorb W (5% w/w)[22] in well matched columns gave high efficiencies with simple phenols (HETP 0.7–0.9 mm) but noisy baselines could not be avoided with a column temperature (160°) close to the recommended upper limit. "Ghosting" and solvent peak tailing developed with urine analyses. Frequent replacement of the upper section of the column packing and that of the glass powder of the pre-column gave some improved results. These problems were less than with polyester phases. The causes of ghosting were studied. A 1-μl aliquot of an aqueous solution of phenyl glucuronide partially hydrolyzed with β-glucuronidase (*Helix pomatia*) and containing *p*-cresol as internal standard was injected on to a freshly prepared and conditioned column packed with Chromosorb W

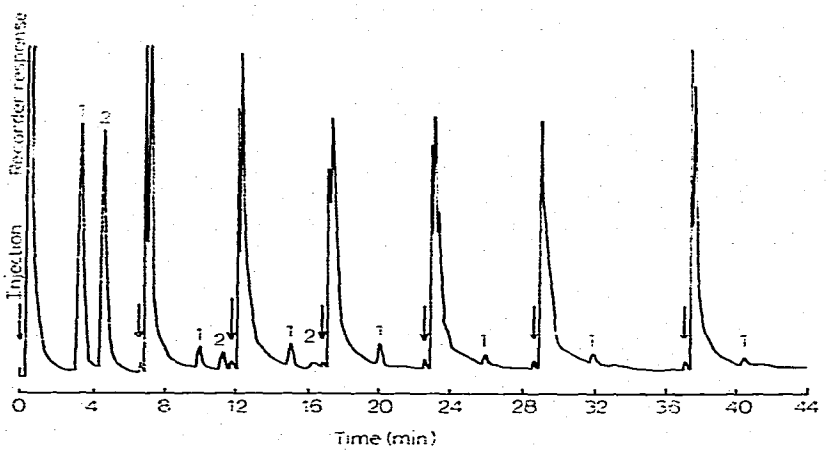

Fig. 3. "Ghost" peaks obtained by repeated injection of water on to a GLC column. Conditions: Microtek MT-220, dual FID. Pre-column as in Fig. 2. Glass column, 2 m × 2.5 mm I.D., packed with HP Chromosorb W 70–80 mesh coated with PEG 6000 (10% w/w). Inlet temperature, 200°. Oven temperature, 165°. Nitrogen flow-rate, 14 ml/min. Attenuation 2×10^{-11} A for f.s.d. Test solution: phenyl glucuronide partially hydrolysed with β-glucuronidase (*Helix pomatia*) with *p*-cresol added as internal standard. 1 = Phenol; 2 = *p*-cresol. Samples injected: test solution 4 μl, followed by six 4-μl injections of water.

TABLE II

RELATIVE RETENTION TIMES AND GLC DATA FOR SIMPLE PHENOLS

The retention times of the cresols are given relative to that of phenol taken as 1.0. The retention time in min for phenol is given in brackets.

Support material[*]	*Stationary phase*	*% (w/w)*	*Mesh size*	*Column temperature (°C)*	*Carrier gas flow (ml/min)*	*HETP (mm)*	*Relative retention time*			
							Phenol	*o-Cresol*	*m-Cresol*	*p-Cresol*
Anakrom (ABS)	Apiezon L	5.0	80–90	127	20	2.1	(5.0)	1.80	1.85	1.85
Ballotini glass beads (AS)	DEGA	0.3	80	150	24	4.0	(4.0)	1.00	1.35	1.35
Anakrom (ABS)	JXR	5.0	80–90	96	20	0.8	(5.0)	1.44	1.67	1.67
Anakrom (ABS)	NPGS	1.0	80–90	106	14	1.5	(6.0)	1.16	1.52	1.47
Chromosorb W (AS)	NPGS	5.0	100–120	160	24	1.3	(7.5)	1.00	1.40	1.38
Silocel C22 (AS)	NPGS	5.0	60–90	112	24	0.9	(10.0)	1.10	1.60	1.50
Anakrom (ABS)	OV-17	5.0	80–90	130	24	1.5	(10.0)	1.40	1.40	1.40
Chromosorb W (AS)	PEG 6000	5.0	70–80	160	14	0.9	(18.0)	1.00	1.70	1.70
Chromosorb T	PEG 20M	5.0	40–60	160	20	1.5	(4.0)	1.00	1.34	1.34
Anakrom (ABS)	QF-1	5.0	80–90	120	24	1.2	(10.0)		1.70	1.70
Anakrom (ABS)	SE-30	3.8	80–90	110	20	1.1	(7.0)	1.70	1.70	1.70
Chromosorb W (AS)	NPGS H_3PO_4	3.0 1.0	70–80	120	20	0.9	(15.8)	1.07	1.47	1.47

[*] A = Acid washed; B = base washed; S = silanized.

coated with PEG 6000 (10% w/v). The initial injection showed peaks for phenol (1) and *p*-cresol (2) as seen in Fig. 3. This was followed by six consecutive injections of 1 μl of water. True ghost peaks as described by Geddes and Gilmour[33] were observed with *p*-cresol, with each of two water injections. A second cause of ghosting not traced in the literature was due to the retention of phenyl glucuronide at the injection end of the column. On-column hydrolysis occurred with each subsequent injection of water. Even after six injections of water a phenol peak with area equivalent to 3–4% of the original peak was seen in Fig. 3. The presence of acid greatly increased the extent of ghosting. Lowering the inlet temperature from 200° reduced the amount of hydrolysis occurring *in situ*, but caused problems with tailing peaks of benzoic acid deriving from urinary hippuric acid. Phenyl sulphates, present in larger amounts in urines and more acid-labile than the glucuronides accentuated this form of ghosting.

PEG 20M, PEG 6000 and FFAP were coated on to diatomaceous earth and PTFE support materials but urine samples led to solvent tailing and ghosting. FFAP on PTFE was recommended for aqueous phenolic solutions[34], but artifact peaks were always obtained. Table II summarizes retention time data for simple phenols on polar and non-polar stationary phases obtained by injecting solutions of these in organic solvents. In no case was it possible to effect a separation between the *o*-, *m*- and *p*-cresol isomers.

Phosphate ester phases. Separations of phenolic compounds reported on phosphate ester stationary phases could not be repeated with aqueous samples on either TCP[35] or TXP[36]. The aim of estimating phenols by direct aqueous injection was aban-

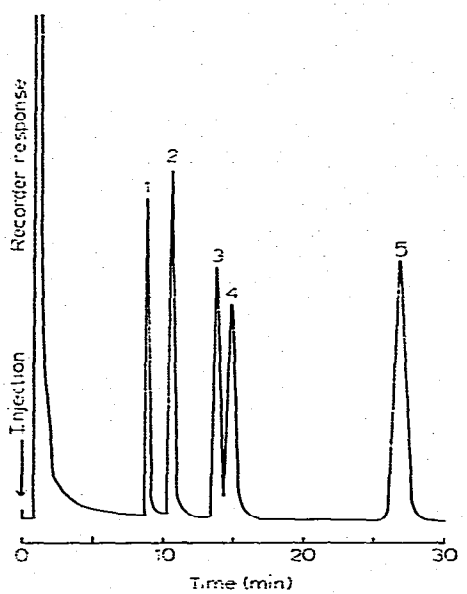

Fig. 4. Separation of phenols with TXP (0.5% w/w) stationary phase. Conditions: Pye 104, Model 24, dual FID. Glass column, 2 m × 3.0 mm I.D., packed with HP Chromosorb W 80–100 mesh coated with TXP (0.5% w/w). Inlet temperature, 130°. Oven temperature, 105°. Nitrogen flow-rate, 20 ml/min. Attenuation, 5 × 10^{-10} A for f.s.d. Sample injected (1 μl) contained 10 nmoles of each compound. Acenaphthene was the internal standard. 1 = Phenol; 2 = *o*-cresol; 3 = *p*-cresol; 4 = *m*-cresol; 5 = acenaphthene.

doned in favour of the extraction of phenols into organic solvents for GLC. The same conclusion was reached by Lechner *et al.*[37] for chlorophenols.

A column packed with HP Chromosorb W 80–100 mesh coated with TXP (0.5% w/w) stationary phase gave the separation of phenol, *o*-, *p*- and *m*-cresol shown in Fig. 4, with acenaphthene as internal standard. Because of the long retention times on this column with dihydric phenols their trifluoroacetyl (TFA) derivatives were studied. They gave symmetrical GLC peaks but quantitative results were unsatisfactory, due to breakdown of the O–TFA bond caused by traces of water[38] present in ethereal extracts prepared from urines. In addition, after the solvent peak, there was often a long tailing peak due to trifluoroacetic acid. Attempts to eliminate this by concentrating the sample led to losses of volatile phenols. Reacting the trifluoroacetic acid with diazomethane in ethereal solution to convert it to the more volatile methyl ester was not practicable owing to further dilution of the sample and subsequent need for its concentration.

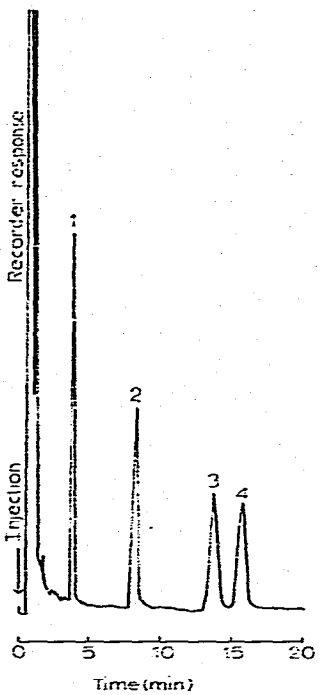

Fig. 5. Separation of TMS phenols with TXP stationary phase. Conditions: Pye 104, Model 24, dual FID. Glass column, 2 m × 3.0 mm I.D., packed with HP Chromosorb W 80–100 mesh coated with TXP (5.0% w/w). Inlet temperature, 100°. Oven temperature, 75°. Nitrogen flow-rate, 20 ml/min. Attenuation 10^{-9} – 10^{-10} A for f.s.d. Sample size (1 μl) contained 10 nmoles of each compound. 1 = TMS *p*-cresol; 2 = TMS catechol; 3 = TMS resorcinol; 4 = TMS quinol.

TMS derivatives were easily prepared and they all gave single peaks as shown in Fig. 5 for *p*-cresol, catechol, resorcinol and quinol. Because of their high volatility the separation was made on a column with TXP 5% (w/w). In Table III the retention time data are given for phenols on a column with TXP 0.5% (w/w) and for TMS

TABLE III

RELATIVE RETENTION TIMES OF PHENOLS AND THEIR TRIMETHYLSILYLATED DERIVATIVES

Microtek MT-220, dual FID, glass columns, 3.25 m × 2.5 mm I.D., packed with HP Chromosorb W 80–100 mesh coated with TXP. Nitrogen gas flow-rate, 24 ml/min. Attenuation between 0.5×10^{-11} and 2.0×10^{-11} A for f.s.d. Sample size, 1 µl. Conditions for non-derivatized phenols: TXP (0.5% w/w). Oven temperature, 110°. Figures given are retention times relative to that of phenol taken as 1.0. The retention time (min) is given for phenol in brackets. Conditions for TMS phenols: TXP (5.0% w/w). Oven temperature, 75°. Figures given are retention times relative to that of TMS phenol. The retention time (min) is given for TMS phenol in brackets. Benzoic and phenylacetic acids were not derivatized.

Compound	*Non-derivatized*	*TMS phenols*
Phenol	(10.00)	(10.00)
o-Cresol	1.20	1.56
m-Cresol	1.68	1.71
p-Cresol	1.56	1.90
2-Ethylphenol	1.72	2.18
3-Ethylphenol	2.60	2.68
4-Ethylphenol	2.46	3.16
2,3-Xylenol	2.24	3.38
2,5-Xylenol	1.81	2.36
2,6-Xylenol	0.91	2.94
3,4-Xylenol	2.98	3.82
3,5-Xylenol	2.62	2.79
2-Methoxyphenol	0.46	3.54
3-Methoxyphenol	5.00	5.31
4-Methoxyphenol	5.80	5.75
2,6-Dimethoxyphenol	3.49	13.40
o-Chlorophenol	0.55	2.94
m-Chlorophenol	7.00	2.90
p-Chlorophenol	6.50	3.54
Catechol	—	3.60
Resorcinol	—	5.72
Quinol	—	6.48
Acenaphthene	2.93	—
Benzoic acid	4.50	4.62
Phenylacetic acid	7.80	4.25

phenols on a similar column with TXP 5% (w/w). The *ortho* effect[30] was not so pronounced and was sometimes lost completely when the reactive hydroxyl groups were protected by TMS groups. Non-derivatized *p*-cresol had a shorter retention time than *m*-cresol, but for the TMS derivatives the reverse occurred. This reversal of elution times was seen with other *m*- and *p*-isomeric pairs in Table III. A comparison between the two sets of relative retention times shows that even with the concentration of stationary phase increased tenfold and the oven temperature 35° lower, the TMS derivatives showed comparable retention times with the non-derivatized phenols.

Conditions for achieving maximal trimethylsilylation of phenol, methylphenols, dimethylphenols, ethylphenols, catechol, resorcinol, and quinol were examined. Pyridine, which is often used as a solvent for the reaction, gave long tailing peaks in the chromatogram and methylene chloride or chloroform was substituted. Monohydric phenols rapidly formed derivatives at room temperature using a mixture of

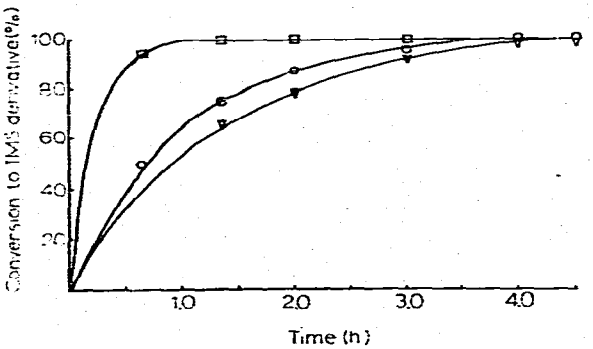

Fig. 6. Conversion of dihydric phenols to their TMS derivatives. The phenols were reacted at room temperature in stoppered tubes with a mixture of chloroform–HMDS–TMCS in the proportion 9:3:1. Aliquots were taken for GLC analysis. □, Resorcinol; ○, catechol; ▽, quinol.

chloroform–HMDS–TMCS in the ratio 9:3:1. In practice the reaction was allowed to proceed for about 10 min. Fig. 6 presents the time course for the trimethylsilylation of resorcinol, catechol and quinol at room temperature. Resorcinol was exceptional in reaching maximal derivatization in 1 h. Attempts to speed up the reaction by using higher temperatures resulted in losses of the more volatile phenol derivatives, despite the use of stoppered tubes. No better results were obtained with BSA, Silyl-8 and TMSIM. With mixtures of dihydric phenols trimethylsilylation was allowed to proceed for 4.5 h at room temperature. The TMS derivatives gave reproducible results. Traces of water did not create a problem.

Linear relationships were established with the FID between weight and peak area ratios of each of the pairs of compounds phenol and acenaphthene, *p*-cresol and acenaphthene, TMS phenol and anisole, TMS *p*-cresol and anisole. The results are given in Table IV. Molar response values (Table V) were used for quantitative studies.

TABLE IV

LINEARITY OF FID RESPONSE BETWEEN WEIGHT RATIOS AND PEAK AREA RATIOS OF PHENOLS AND STANDARD COMPOUNDS

The number of determinations is shown in brackets.

Weight ratio	*Peak area ratio (mean ± S.D.)*	*Weight ratio*	*Peak area ratio (mean ± S.D.)*
Phenol		*p-Cresol*	
Acenaphthene		*Acenaphthene*	
0.505	0.370 ± 0.025 (4)	0.511	0.353 ± 0.030 (4)
1.009	0.739 ± 0.005 (4)	1.023	0.726 ± 0.014 (4)
2.018	1.640 ± 0.040 (4)	2.046	1.640 ± 0.020 (4)
4.036	3.350 ± 0.100 (4)	4.092	3.380 ± 0.120 (4)
TMS phenol		*TMS p-cresol*	
Anisole		*Anisole*	
0.445	0.485 ± 0.001 (3)	0.406	0.453 ± 0.004 (3)
0.886	0.968 ± 0.004 (3)	0.812	0.912 ± 0.004 (3)
1.777	1.940 ± 0.010 (3)	1.625	1.800 ± 0.030 (3)
3.550	3.940 ± 0.001 (3)	3.250	3.540 ± 0.010 (3)

TABLE V

MOLAR RESPONSES OF PHENOLS

Pye 104, Model 24, dual FID. Conditions for non-derivatized phenols: Column, 2 m × 3.0 mm I.D., packed with HP Chromosorb W 80–100 mesh coated with TXP (0.5% w/w). Molar responses of non-derivatized phenols relative to that of acenaphthene taken as 1.0. Conditions for TMS phenols: Column as above with TXP (5.0% w/w). Molar responses of other compounds relative to that of TMS phenol taken as 1.0. Means of five determinations.

Compound	*Molar response ± S.D.*
Acenaphthene	1.00
Phenol	0.44 ± 0.009
o-Cresol	0.55 ± 0.011
m-Cresol	0.54 ± 0.014
p-Cresol	0.50 ± 0.009
TMS phenol	1.00
TMS *o*-cresol	1.14 ± 0.018
TMS *m*-cresol	1.16 ± 0.046
TMS *p*-cresol	1.13 ± 0.020
TMS catechol	1.42 ± 0.028
TMS resorcinol	1.37 ± 0.012
TMS quinol	1.30 ± 0.015
Anisole	0.60 ± 0.008

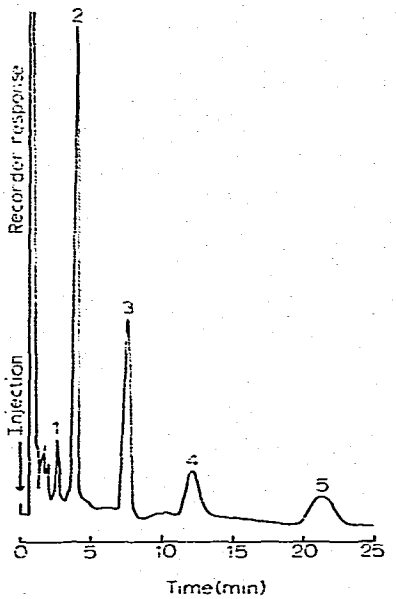

Fig. 7. GLC of ether extract prepared from urine after hydrolysis of sulphate-conjugated phenols. Apparatus and column as in Fig. 4. Inlet temperature, 140°. Oven temperature, 115°. Attenuation, 4 × 10^{-10} A for f.s.d. Sample injected, 1 μl. The urine sample was hydrolysed at pH 1.0 and ether extracted as described in Materials and methods. 1 = Phenol; 2 = *p*-cresol; 3 = acenaphthene; 4 = benzoic acid; 5 = phenylacetic acid.

Analysis of urinary extracts

The chromatogram of an ether extract from urine after acid treatment to hydrolyse sulphate-conjugated phenols is shown in Fig. 7. Free phenol was always present in much smaller quantities than *p*-cresol. *o*-Cresol and *m*-cresol were rarely detected. Benzoic and phenylacetic acids usually appeared after treatment of the urine with acid or enzymes. These acids could not be removed by washing the ether extracts with sodium bicarbonate solution because losses of dihydric phenols occurred during this procedure[39]. Columns used for the analysis of urinary extracts lasted a few months provided that temperatures did not exceed 115–120° and the column packing did not come into contact with the heated inlet zone. Contamination of the inlet zone with non-volatile constituents caused a gradual reduction in peak efficiencies but regular replacement of the glass thread and cleaning of the injection end of the column generally restored efficiency.

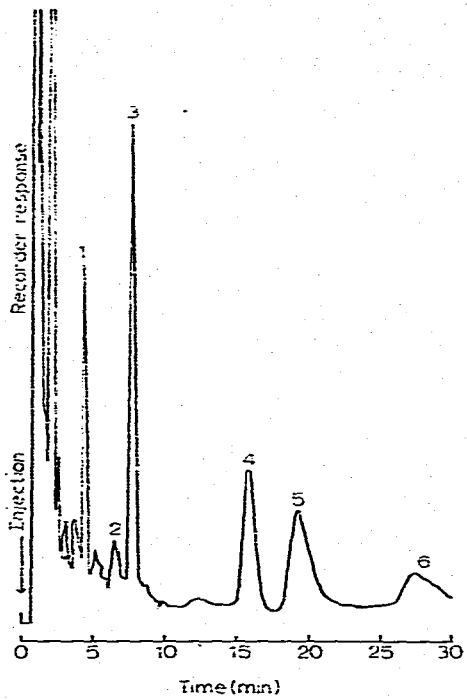

Fig. 8. Chromatogram of trimethylsilylated ether extract prepared from urine after hydrolysis of sulphate-conjugated phenols. Conditions: Pye 104, Model 24, dual FID. Glass column, 2 m × 3.0 mm I.D., packed with HP Chromosorb W 80–100 mesh coated with TXP (5%, w/w). Inlet temperature, 90°. Oven temperature, 60°. Attenuation 4 × 10^{-10} A for f.s.d. Sample injected 1 μl. After hydrolysis at pH 1.0 the ether extract was trimethylsilylated for GLC as described in Materials and methods. 1 = TMS phenol; 2 = TMS *o*-cresol; 3 = TMS *p*-cresol; 4 = TMS catechol; 5,6 = ?.

The chromatogram in Fig. 8 shows peaks for the TMS derivatives of phenol, *o*-cresol, *p*-cresol and catechol. These were obtained from an ether-extracted urine sample which was hydrolyzed at pH 1.0. Other peaks could only be identified in certain cases (see Table III).

Reproducibility of the method

From a single urine sample, 5-ml aliquots were extracted with ether for analysis of free phenol and *p*-cresol on a GLC column with TXP (0.5% w/w). Further 5-ml aliquots adjusted to pH 1.0 were heated on a boiling water-bath for 1 h to hydrolyse sulphate-conjugated phenols before ether extraction. The results are given in Table VI. The average value for free phenol (0.26 mg/l urine) represented about 2.6% of the amount of sulphate-conjugated phenol (9.8 mg/l). The free *p*-cresol (4.26 mg/l) was 7.8% of the sulphate-conjugated *p*-cresol (54.6 mg/l). When concentrating ether extracts losses of volatile phenols may occur if they are taken to complete dryness[40].

TABLE VI

ANALYSIS OF A SINGLE URINE SAMPLE FOR FREE PHENOL AND *p*-CRESOL AND THEIR SULPHATE-CONJUGATED FORMS

Results are given as mg/l urine ± S.D. for four determinations in each case.

Extract No.	*Free*		*Extract No.*	*Free + sulphate-conjugated*	
	Phenol	*p-Cresol*		*Phenol*	*p-Cresol*
1	0.28 ± 0.038	4.21 ± 0.09	4	10.25 ± 0.65	58.2 ± 6.0
2	0.27 ± 0.044	4.46 ± 0.11	5	10.30 ± 1.14	57.2 ± 4.8
3	0.22 ± 0.023	4.12 ± 0.08	6	9.65 ± 0.58	61.1 ± 2.8

The analyses obtained by GLC from six urine samples from healthy adults are presented in Table VII. The summation of the values for free, sulphate- and glucuronide-conjugated phenol gave total phenol values which agreed well (except for sample 1, 5.48 mg/l) with the total phenol value obtained with the independent method using *Helix pomatia* juice. Although sulphate-conjugated phenol represented between 60 and 95% of total phenol, the glucuronide-conjugated fraction varied between 1 and 27% of the total.

Thirteen urine samples from healthy adults were incubated with *Helix pomatia* juice for the determination of total phenol and ether extracted for GLC. A time-

TABLE VII

FREE AND CONJUGATED PHENOL IN NORMAL URINES

Free phenol: no urine treatment. Sulphate-conjugated phenol: incubation at pH 1.0 and corrected for free phenol. Glucuronide-conjugated phenol released with *E. coli* glucuronidase and corrected for free phenol. Total phenol: *Helix pomatia* juice incubation. Means of duplicate results (mg/l urine, uncorrected).

Sample No.	*Phenol*			*Total*
	Free	*Sulphate-conjugated*	*Glucuronide-conjugated*	
1	0.30	4.23	0.95	6.50
2	0.04	0.84	0.22	1.06
3	0.06	1.06	0.08	1.32
4	0.43	2.03	0.92	3.36
5	0.56	2.02	0.03	2.77
6	0.09	4.69	0.36	4.96

TABLE VIII

URINARY TOTAL PHENOL DETERMINED BY TWO GLC METHODS

Urines corrected to specific gravity 1.016. Methods of Van Haaften and Sie, using acid hydrolysis[22] and enzymic hydrolysis followed by GLC as described in Materials and Methods.

Sample No.	*Phenol (mg/l)*	
	Acid hydrolysis	*Enzyme hydrolysis*
1	30.0	3.2
2	12.3	11.2
3	44.5	13.2
4	10.0	10.6
5	16.7	9.5
6	7.8	5.8
7	8.9	4.1
8	11.1	3.9
9	3.0	2.2
10	6.6	4.2
11	13.1	7.2
12	25.0	14.7
13	19.3	8.6

TABLE IX

PHENOL IN URINE: COMPARISON BETWEEN GIBB'S COLORIMETRIC AND GLC METHODS

The GLC figures for free and sulphate-conjugated phenol are the means of two determinations.

Sample No.	*Phenol (mg/l)*		*Other phenolic compounds in urine identified by GLC**
	Colorimetric	*GLC*	
1	3.5	2.9	2,6-xylenol
2	1.5	0.5	*o*-cresol
3	7.8	5.4	*o*-cresol, g.
4	2.0	7.9	*o*-cresol, g.
5	7.5	3.5	g.
6	27.0	30.8	g. small amount
7	25.0	25.3	
8	11.0	25.2	2,6-xylenol, *o*-cresol, g.
9	6.2	4.5	g.
10	3.0	3.1	*o*-cresol
11	8.0	3.6	g.
12	1.0	1.2	g. small amount
13	8.8	3.2	2,6-xylenol, *o*-cresol
14	11.2	3.8	g. large amount
15	10.0	2.5	g. large amount
16	12.6	1.4	2,6-xylenol, g.
17	9.0	1.8	2,6-xylenol, *o*-cresol
18	4.0	1.5	2,6-xylenol
19	12.0	9.0	2,6-xylenol, *o*-cresol, g.
20	9.8	2.7	*o*-cresol, g.

* g. = Guaiacol.

course reaction was followed in each case to ensure maximal release of bound phenol. The results in Table VIII may be compared with those obtained by the acid hydrolysis–GLC method of Van Haaften and Sie[22]. The higher acid hydrolysis values were due to artifact production and to the presence of compounds such as *o*-cresol and 2,6-xylenol which co-eluted with phenol under the GLC conditions used. Correlation between the two sets of results was not significant ($r = 0.533$, $t = 2.1$, $p = 5$–10%). Hunter[41] reported values of 1.3 to 41.7 mg/l uncorrected for 100 normal urines using the method of Van Haaften and Sie.

Twenty urine samples taken from men suspected to have been exposed to benzene were mixed with perchloric acid, steam distilled and the phenols determined with Gibb's reagent, using the standard method for screening urines[42]. Under these conditions, glucuronide-conjugated phenol was not released and it was appropriate to use for comparison our GLC method for determining free plus sulphate-conjugated phenol. The results are given in Table IX. A statistical correlation was established between the two methods ($r = 0.786$, $t = 5.4$, $p < 0.1\%$). In most samples the colorimetric method gave higher results, which could be attributed to the co-distillation of interfering compounds reported by Dacre[43], such as cresol and 2,6-xylenol which were shown to be present. Thus, in samples 6 and 7 no interfering substances were detected by GLC and the two methods gave comparable results. Using Gibb's reagent, Ikeda and Ohtsuji[44] quoted mean values of 26.1, 25.2 and 22.8 mg/l urine uncorrected for groups of non-exposed healthy subjects.

TABLE X

COLOUR REACTIONS OF 4-AMINOANTIPYRINE WITH PHENYL COMPOUNDS*

Numbers refer to the strength of the test, letters to the solubility of dyes in chloroform. 3 represents a reaction taken to be over 25% of the reaction of an equivalent concentration of phenol (w/v) measured at 460 nm in chloroform solution; 2 moderate reaction, 5–25% that of phenol; 1 reaction less than 5% that of phenol; 0 no absorbance. S = soluble; SIS = slightly soluble.

Compound	*Reaction*	*Compound*	*Reaction*
Phenol	3-S	Resorcinol	3-SIS
Phenyl glucuronide	0	Quinol	1-S
Phenyl sulphate	0	Catechol glucuronide	3-SIS
o-Cresol	3-S	Quinol glucuronide	3-S
m-Cresol	3-S	3-Methylcatechol	2-S
p-Cresol	1-S	4-Methylcatechol	1-SIS
2-Ethylphenol	3-S	Phloroglucinol	3-SIS
3-Ethylphenol	3-S	Anisole	0
4-Ethylphenol	1-S	Phenetole	2-S
2,3-Xylenol	3-S	Phenacetin	0
2,5-Xylenol	3-S	Benzyl alcohol	1-S
2,6-Xylenol	3-S	Benzoic acid	1-S
3,4-Xylenol	0	Salicylic acid	1-S
3,5-Xylenol	3-S	*m*-Hydroxybenzoic acid	3-SIS
2-Methoxyphenol	3-S	*p*-Hydroxybenzoic acid	3-S
3-Methoxyphenol	3-S	*m*-Coumaric acid	3-SIS
4-Methoxyphenol	3-S	Vanillic acid	3-S
2,6-Dimethoxyphenol	3-S	Protocatechuic acid	3-S
Catechol	3-SIS	Syringic acid	3-S

* After Emerson[45], who reported colour reactions judged visually.

The colour reaction with 4-aminoantipyrine[45] was used for urinary phenol following steam-distillation[46] and for total phenol by an automated method[47]. Our investigations with this reagent carried out with a spectrophotometer are reported in Table X in the same form used by Emerson[45]. Many compounds known to exist in urine reacted, and steam distillation of phenol could not entirely eliminate problems of non-specificity of reaction.

The methods described in this paper allowed the determination of simple phenols in both free and conjugated forms, with the elimination of errors due to interfering compounds which, in the past, have resulted in the reporting of higher values than those quoted here. The results of dietary and other studies are being presented elsewhere.

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