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DETERMINATION OF URINARY MONOHYDRIC AND DIHYDRIC PHENOLS BY GAS CHROMATOGRAPHY OF THE ACETATE AND TMS DERIVATIVES

4-DIMETHYLAMINOPYRIDINE AS A CATALYST FOR ACETYLATION REACTIONS

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SUMMARY

A procedure is described for the quantitative extraction of phenols from human urine. The compounds were chromatographed as the trimethylsilyl derivatives on wall-coated glass capillary columns.

A more specific method of extraction involving the formation of acetate derivatives is also described. The acetates were prepared by treating dilute solutions of phenols at room temperature with acetic anhydride in the presence of 4-dimethylaminopyridine, which is a far more powerful catalyst than pyridine. Under these conditions N-acylamino acids are converted to azlactones.

INTRODUCTION

There are many methods for determining monohydric phenols in urine, but at present no quantitative method exists for the dihydric compounds. For semi-quantitative measurements by paper chromatography, the phenols are usually extracted from neutralized urine with organic solvents. Using more sensitive and specific gas chromatographic (GC) methods for quantitation, Bakke and Scheline[1] found this method of extraction to be inefficient and irreproducible; they extracted acidified urine with ether, then back-extracted the ether layer with aqueous sodium bicarbonate. This procedure gives poor recoveries of the dihydric phenols, but, nevertheless, theirs has been the most satisfactory method available. We have re-investigated the extraction of phenols from aqueous solution, and a quantitative procedure is given in this paper.

GC of underivatised dihydric phenols[2] is somewhat difficult, but many suitable derivatives have been described in the literature. For many purposes the trimethyl-silyl (TMS) derivatives are satisfactory, but the acetates, being more resistant to hydrolysis, allow a further stage of purification after the derivatisation reaction. We

have developed a new method for preparing the acetates using acetic anhydride and 4-dimethylaminopyridine (DMAP), which is a far more powerful catalyst than pyridine[3]. The derivatives are formed rapidly and quantitatively, even in very dilute solutions.

EXPERIMENTAL

Standard phenols and reagents were purchased from BDH, Aldrich, Sigma and Koch-Light. Juice of *Helix pomatia* containing a mixture of glucuronidases and sulphatases was obtained from l'Industrie Biologique Française through Micro-Bio Labs. Diethyl ether was purified immediately before use by passage through activated alumina, as traces of peroxide led to selective losses of the phenols. Ethyl acetate was redistilled. Reference samples of the acetate derivatives were prepared by the method given by Vogel[4]. *p*-Hydroquinone diacetate was recrystallised from ethanol–water giving a product of m.p. 123–124° (literature 124°).

GC was performed on Pye Model 104 instruments. Packed columns (4 mm × 2.7 m) contained various OV phases (3 %) coated on Gas-Chrom Q by the filtration method[5]. Wall-coated glass capillary columns (50 m) of OV-101[6] were used with a packed pre-column (3 % OV-101 on Gas-Chrom Q) and inlet splitter, as described by German and Horning[7]. Argon or nitrogen was used as the purge gas in order to conserve helium. After injections columns were held at 70° for 10 min then programmed at 2 or 3°/min to 250°. GC–mass spectrometry (MS) was carried out on a Perkin-Elmer Model 270 instrument.

Extraction from aqueous solution

A solution containing exactly 50 μg/ml each of 13 standard phenols in ethyl acetate was prepared. Two 1-ml aliquots were evaporated just to dryness at 35° in a stream of nitrogen, then dissolved in 1 ml bis(trimethylsilyl)trifluoroacetamide containing 1 % chlorotrimethylsilane (BSTFA–CTMS). Aliquots of 1 μl were injected onto the capillary column, which was programmed from 60 to 250° at 2°/min.

Aliquots (10 ml) of an aqueous solution containing exactly 5 μg/ml of each of the 13 phenols were treated with 2 ml phosphate buffer (pH 7, 0.5 M) or acidified with 5 N HCl.

Acidic and neutral solutions were extracted in duplicate by the following methods: (1) 3 times with ether, or (2) 3 times with ethyl acetate, or (3) 3 times with ether then 3 times with ethyl acetate.

The extractions were repeated on aliquots which had been saturated with sodium chloride. The extracts were dried over sodium sulphate, and evaporated to a few millilitres on the rotary evaporator with a water-bath at 35°. The solutions were transferred to stoppered tubes, taken just to dryness at 35° in a stream of nitrogen, and dissolved in 1 ml BSTFA–CTMS. Aliquots of 1 μl were injected onto the capillary column as before.

Evaluation of acetylation reaction

A solution of 4-chlororesorcinol diacetate (1 mg) in ether (30 ml) and ethyl acetate (30 ml) was placed in each of six separating funnels. Hydroquinone (1 mg) was added to three of the solutions, and hydroquinone diacetate (1.76 mg, equivalent to

1 mg hydroquinone) to the other three. Each solution was then treated with acetic anhydride (0.5 ml) and DMAP (10 mg in ethyl acetate). After 1 h the solutions were extracted with 3 × 20 ml saturated aqueous sodium bicarbonate, taking care to vent the carbon dioxide frequently. Carbon dioxide evolution ceased after they had been shaken intermittently for about 10 min. The organic layers were dried over sodium sulphate and reduced in volume to about 1 ml. Aliquots were injected onto a column of OV-7.

Extraction of urine

An aliquot of a 24-h urine sample corresponding to 5–10 min excretion was adjusted to pH 5 with acetic acid, then incubated at 37° for 16 h with 100 μl *Helix pomatia* juice. 100 μl aqueous 4-chlororesorcinol solution (1 mg/ml) and 2 ml phosphate buffer (pH 7, 0.5 *M*) were added. The mixture was saturated with sodium chloride and extracted with 3 × 10 ml ether followed by 3 × 10 ml ethyl acetate. Aqueous standards were treated similarly, but the hydrolysis step was omitted.

The subsequent steps depended on the derivatives required: TMS ethers were prepared as described above; for acetates, after each stage in the extraction the organic layer was passed through a column (10 × 100 mm) of sodium sulphate into a separating funnel (100 ml) fitted with a PTFE stopcock. The column was rinsed with 3 × 10 ml ethyl acetate and the combined extract treated as described above for hydroquinone.

RESULTS AND DISCUSSION

Extraction

The phenols were extracted with equal efficiencies from neutral or acidic solutions. This result is in disagreement with the work of Bakke and Scheline[1] who obtained poor recoveries unless the solutions were acidified. A likely explanation for this discrepancy is that Bakke and Scheline did not buffer their standard solutions, so that traces of alkali in the distilled water, or on the glassware, could react with the weakly acidic phenols.

Extracting three times with either ether or ethyl acetate gave somewhat low recoveries (70–90%) of some of the compounds. When both solvents were used, making a total of six extractions, the relative peak heights were within 10% of the values given by the original mixture, except for pyrogallol. The poor recovery of this compound could be due to oxidation. The addition of salt did not significantly improve recoveries, but it considerably reduced the amount of water taken up by the solvents.

Recently, Cooper and Wheatstone have found that phenols are efficiently extracted from coal carbonisation effluent by isobutyl methyl ketone[8]. The only disadvantage of this solvent is its high boiling point (118°), but, unlike ether, it does not form peroxides.

Absolute recoveries were not determined, but comparison of the GC responses produced by the extract and the original mixture suggests that these are essentially quantitative.

Derivative formation

Addition of BSTFA–CTMS to a phenol extract gave a clear solution within

an hour at room temperature. The reaction was much faster if a few microlitres of trifluoroacetic acid or pyridine were added. The silylation reaction may be assumed to be quantitative.

Hydroquinone was used to evaluate the acetylation reaction because pure samples of the phenol and its derivative were readily obtainable. The extracts prepared from free hydroquinone gave peak height ratios hydroquinone: 4-chlororesorcinol of 1.20, 1.12 and 1.16 (average 1.16). The pure diacetate gave 1.12, 1.25 and 1.14 (average 1.17), so the reaction is quantitative, at least for this compound. When the acetylation was carried out in the absence of DMAP, each of the dihydric phenols gave more than one GC peak.

Gas chromatography

The OV-101 capillary column resolved the TMS derivatives of all the phenols tried (Fig. 1). Unfortunately, the acetate derivatives did not give satisfactory chromatograms on this column. A similar column fitted with a solids inlet gave broad tailing peaks, and tailing was also observed on several packed columns of OV-101. However, Higginbottom *et al.*[9] have obtained excellent separations of phenol acetates on a copper capillary column of SE-30, which had first been coated with an inert silicone. Good results were obtained on packed columns of several other OV phases. It seems likely that a slightly polar liquid phase is required to mask active sites on the support and the column wall. None of the packed columns resolved the 13 standard phenol acetates, but OV-210 separated all the major normal urinary compounds.

Calibration

Satisfactory linear calibration curves were obtained for aqueous standards
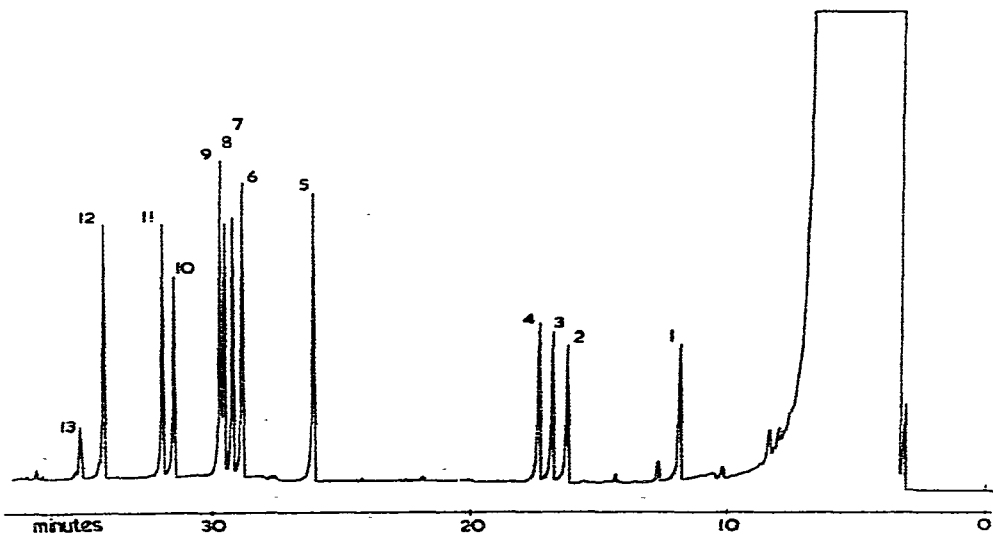

Fig. 1. Separation on OV-101 capillary column of TMS derivatives of phenols extracted from aqueous phosphate buffer (pH 7). For conditions see Experimental. 1 = Phenol; 2 = *o*-cresol; 3 = *m*-cresol; 4 = *p*-cresol; 5 = catechol; 6 = resorcinol; 7 = 4-methylcatechol; 8 = 3-methylcatechol; 9 = quinol; 10 = orcinol; 11 = 2-methylresorcinol; 12 = 4-chlororesorcinol; 13 = pyrogallol.
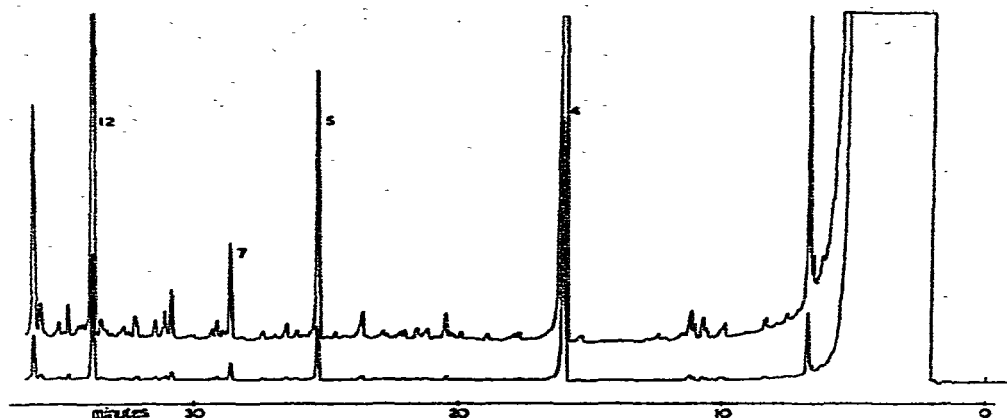
Fig. 2. Phenols extracted from a 24-h urine sample from a normal adult were chromatographed as the TMS derivatives on the OV-101 capillary column. The upper trace was recorded at five times the sensitivity of the lower. For conditions see Experimental. For identification of peaks see Fig. 1. Peak 12 (4-chlororesorcinol) was the internal standard.

(20–1000 μg) of *p*-cresol, catechol, 4-methylcatechol and resorcinol, using either type of derivative. 4-Chlororesorcinol (1000 μg) was used as internal standard. Recoveries of standards added to urine were 100 ± 10%.

Urine extracts

Profiles of extracts of normal urines as the TMS and acetate derivatives are shown in Figs. 2 and 3, respectively. The acetate profiles are much less complex than
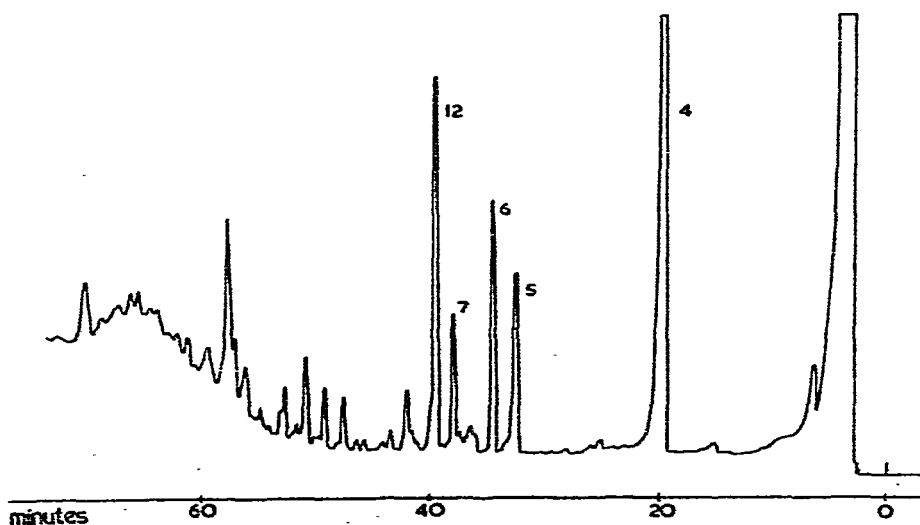

Fig. 3. Phenols extracted from a 24-h urine sample from a normal adult were chromatographed as acetate derivatives on a packed column of OV-210. The urine was not from the same individual as the one shown in Fig. 2. For conditions see Experimental. For identification of peaks see Fig. 1. Peak 12 (4-chlororesorcinol) was the internal standard.

TABLE I

24-h URINARY EXCRETION OF SOME MAJOR PHENOLS BY NORMAL ADULTS IN mg PER 24 h

Subject	p-Cresol	4-Methylcatechol	Resorcinol	Catechol
1	17	2.9	0	15
2	46	5.0	12.6	24
3	33	2.5	0	10
4	43	1.2	0.3	2.8
5	42	1.8	1.6	22
6	21	3.2	1.7	6.7
7	52	7.3	0	12
8	26	1.2	7.9	9.2
9	18	1.7	2.6	9.9
10	>100	3.7	3.2	16
11	45	2.4	0.5	11

the TMS ones; this would be expected as the method of extraction is more specific. However, it is rather more tedious, and for the major phenols the TMS method is adequate because of the good resolution given by the capillary column.

Of particular interest is the absence of urea from the TMS profile. Large amounts of urea were extracted from the urine but the compound did not react with BSTFA unless a base (such as pyridine) was added. The suspended solid caused no trouble. When urinary acids are extracted with organic solvents and converted to the TMS derivatives, the urea nearly always dissolves and produces a broad peak on the chromatogram. Possibly, one of the acids catalyses the silylation of urea. If the acetate derivatives are used, urea is removed by the aqueous bicarbonate.

Concentrations of some of the major phenols in normal urines, determined by the TMS method are listed in Table I. The excretion of phenols varies quantitatively and qualitatively both between individuals and from day to day in the same individual[10,11]. This is to be expected since the compounds are mostly derived from the metabolism of certain constituents of food by the gut bacteria. The normal urines contained only small amounts (1 or 2 mg per 24 h) of unconjugated phenols, but a patient with periodic catatonia produced varying amounts of free resorcinol whose origin is unexplained[12].

Effect of acetic anhydride and DMAP on N-acyl amino acids

Our original motive for using acetate derivatives was to avoid the loss of dihydric phenols when an extract from acidified urine was shaken with aqueous sodium bicarbonate as in the method of Bakke and Scheline[1]. The idea was to reduce the polarity of the phenols by acetylating them, so that only the organic acids would be removed by the bicarbonate. This approach gave interesting profiles, but some of the major peaks were not due to phenols .The largest component was identified by GC–MS as the azlactone derived from hippuric acid. Azlactones are usually prepared by heating an N-acylamino acid with acetic anhydride[13]; presumably DMAP catalyses the reaction at room temperature. Good GC peaks were obtained by the same method from N-acetyl norleucine and N-acetyl glycine; possibly this reaction could form the basis of a specific extraction procedure for this type of compound. The method is un-

likely to be quantitative as it stands, as the azlactones are easily hydrolysed. GC separations of some azlactones have been described[14].

CONCLUSIONS

The two methods presented here enable the sensitive, selective and accurate determination of the major mono- and dihydric phenols in normal urine.

The use of DMAP as a catalyst in acylation reactions[3] could have wider application, both for the derivatisation of different types of compound, and for the attachment of different acyl groups.

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