Notes

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Some easily prepared urinary extracts suitable for gas chromatography

With the development of silylation procedures and of liquid phases such as OV-1 and OV-17 which may be used at temperatures well in excess of 300° many urinary constituents have now become amenable to analysis by gas chromatography which will surely play an increasingly important part in physiological and clinical chemistry. Preliminary experiments in these laboratories indicated the complexity of chromatograms which may be obtained from both pathological and normal urines and emphasised the problems to be encountered in connection with the identity and homogeneity of peaks. Economic factors alone severely limit the use of ancilliary techniques such as mass spectrometry in solving such problems and most workers in clinical laboratories will, like ourselves, have to resort to analytical schemes based on the shift or disappearance of peaks resulting from the application of variations to a basic technique. Such schemes must invariably involve the tedious repetition of extraction procedures and it is therefore desirable that the latter should be as convenient as possible.

Four simple techniques giving extracts found to yield excellent gas chromatograms are described below and the types of compound for which they may prove suitable are indicated.

Experimental

Pyridine was stored over KOH pellets and ether over $FeSO_4$ in 2 N H_2SO_4. Urines were adjusted (HCl) to pH 1–2 before application of any of the procedures described below. Pure substances were extracted from solution in 0.1 N HCl. All evaporations were carried out *in vacuo* at 30°.

Evaporated urine extracts[1]. Urine (0.25 ml) was evaporated after addition of ethanol (5 ml).

Acetonitrile extracts. Urine (0.5 ml) was added to acetonitrile (5 ml). After gradual addition of Na_2SO_4 (1 g) the mixture was placed in the refrigerator for 1 h, with frequent shaking. The extract (2.5 ml) was evaporated to dryness.

This extraction procedure was tested on a number of pure compounds, the efficiency being judged roughly by paper chromatography. Good recoveries were obtained with the monosaccharides arabinose, fructose, glucose and galactose, the amino acids glycine, alanine, valine, leucine, cystine, methionine, phenylalanine, tyrosine, tryptophan, serine, proline and glutamic acid, the amines tyramine, metanephrine and normetanephrine and with thymine, adenine, uracil and hypoxanthine. Recoveries of glucosamine, sucrose and lactose, histidine and lysine and guanine were poor.

Ethyl acetate extracts[2]. Urine (2 ml) was treated as above with ethyl acetate

(10 ml) and Na_2SO_4 (4 g) and filtered. The filtrate and washings were evaporated.

Previous experience had indicated this method to effect highly efficient extraction of most aromatic substances from urine (although orthodox extraction procedures may be preferable in the case of acid-sensitive compounds such as 5-hydroxyindoleacetic acid) and to effect substantial recoveries of highly polar aliphatic compounds such as urea, citric acid and β-hydroxybutyric acid. Compounds such as monosaccharides and tyramine, although very poorly extracted, may sometimes be present in urine in quantities sufficient for detection.

Ether extracts. After addition of $(NH_4)_2SO_4$ (3 g) the urine (5 ml) was extracted with ether (3 × 10 ml). The combined ether layers, separated by centrifugation, were dried (Na_2SO_4) before evaporation.

The partition of several phenolic acids was determined. Each compound in moist ether (10 ml) was shaken with 0.1 *N* HCl (5 ml) and $(NH_4)_2SO_4$ (3 g). Phenols were determined in aliquots of the upper layers and original solutions using the Folin–Ciocalteu reagent. Percentages remaining in ethereal solution were as follows: *p*-hydroxyphenyllactic acid, 75; *p*-hydroxyphenylpyruvic acid, 94; 4-hydroxy-3-methoxymandelic acid, 66; 4-hydroxy-3-methoxyphenyllactic acid, 83; homogentisic acid, 60; 3,4-dihydroxyphenylacetic acid, 74; 3,4-dihydroxymandelic acid, 10. Indolelactic acid, estimated fluorimetrically, was retained quantitatively in the ether layer.

Gas chromatography. All extracts were silanised by leaving overnight with a mixture of bis-(trimethylsilyl)-acetamide (BSA) and pyridine (1:1; 0.5 ml). Aliquots were chromatographed on 1.5 m × 4 mm I.D. columns of OV-1 and OV-17 (each 10 % on Diatoport S) with temperature programming at 2°/min from 100° to 250°. Argon (50 ml/min) was used as carrier gas and compounds were detected with a hydrogen flame-ionisation detector. The ionisation amplifier attenuator was set at 2×10^3.

Chromatograms obtained from a mixed urine from seven normal subjects are illustrated in Figs. 1 and 2. Evaporated urine extracts and ether extracts were also prepared in which the silylating mixture was replaced by an equal volume of hexamethyl disilazane–pyridine, trimethylsilyldiethylamine–pyridine or trimethylsilyldiethylamine–diethylamine (all 1:1). Chromatograms appeared generally to be very similar to those obtained after silylation with bis-(trimethylsilyl)-acetamide but a few

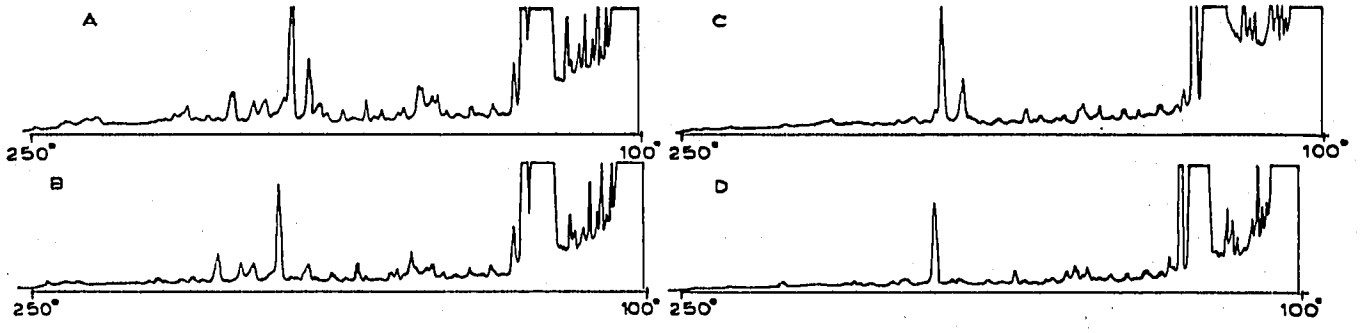

Fig. 1. Chromatography of major urinary constituents on OV-1 column. (A) Evaporated urine extract (10 μl of BSA–pyridine extract was chromatographed). (B) As A but extract was prepared from charcoal treated urine. (C) Acetonitrile extract (10 μl). (D) Acetonitrile extract (10 μl) of charcoaled urine. Comparison of these chromatograms illustrates the high proportion of aliphatic material in the extracts and the elimination of some peaks achieved through acetonitrile extraction.

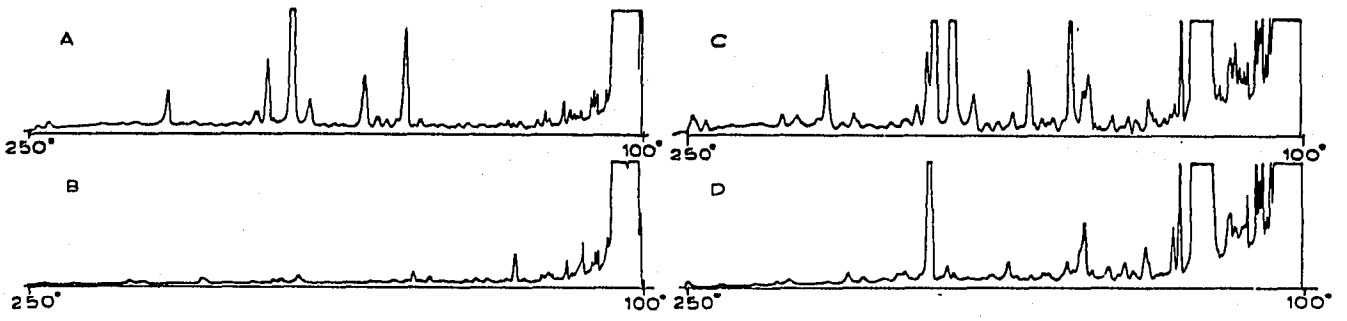

Fig. 2. Chromatography of minor urinary constituents on OV-1 column. (A) Ether extract (4 μl of BSA–pyridine extract was chromatographed). (B) Ether extract (4 μl) from charcoaled urine. (C) Ethyl acetate extract (10 μl). (D) Ethyl acetate extract (10 μl) from charcoaled urine. The essentially aromatic nature of A is illustrated by comparison with B where even the small amount of aliphatic material revealed is exaggerated owing to the presence of traces of aromatic material not completely removed by charcoal. Note an obvious artifact peak emerging at about 125° in B. By contrast, comparison of C and D illustrates the extraction of substantial amounts of aliphatic material by ethyl acetate in the presence of excess Na_2SO_4.

obvious differences were noted. Extracts were also prepared from urines treated at pH 1–2 with activated charcoal (40 mg/ml).

Discussion

Major constituents of urine may be detected after simple evaporation; we have found this procedure to be very useful for preliminary screening purposes[1]. Some selectivity with regard to compounds detected may be achieved by extraction with acetonitrile according to the procedure described above. The use of charcoal demonstrates that both types of extract contain much aliphatic material. Minor constituents of urine, including highly polar and amphoteric compounds demanding extraction techniques outside the scope of this paper, must be concentrated before gas chromatography may be attempted. In this respect solvent extraction is often effective.

In recent years interest in minor urinary constituents has centred particularly upon acidic and neutral aromatic compounds, representatives of which have proved to be of clinical importance in such conditions as phenylketonuria, phaeochromocytoma, neuroblastoma, melanoma, argentaffinoma, alkaptonuria and tyrosinosis. Such compounds may be extracted with ether or ethyl acetate, but most workers using paper chromatography have used the latter solvent which is usually the more effective for highly polar urinary constituents. However ether is equally efficient for the extraction of less polar aromatic compounds and has been stated to be superior to ethyl acetate for some indolic acids[3], *p*-hydroxymandelic acid[4], phenyllactic acid[4] and homogentisic acid[4]. Our experiments indicate conditions for the convenient extraction of clinically important aromatic compounds less polar than 3,4-dihydroxymandelic acid; under such conditions charcoal treatment indicates extraction of interfering aliphatic compounds to be normally almost negligible.

If desired the aqueous residues from which ether extracts have been found to be of interest may be further extracted with ethyl acetate: additional quantities of highly polar aromatic and aliphatic material are then recovered. However, the ability of ethyl acetate to extract polar compounds may most advantageously be exploited if extrac-

tion be performed in the presence of excess Na_2SO_4 as described above; this procedure should be most useful for aliphatic compounds present in charcoaled urine.

Many authentic compounds have been found to behave satisfactorily under the conditions of silylation and chromatography described above. However owing to multiple peak formation the behaviour of sugars is very complex. Similarly although good results were obtained with aromatic α-keto acids (including 4-hydroxy-3-methoxyphenylpyruvic acid, a sample of which yielded multiple peaks using an alternative procedure[3]), α-ketoglutaric and α-ketocaproic acids gave multiple peaks. Such compounds are best silylated with hexamethyldisilazane[5]. Examination of urine extracts silylated under various conditions indicated that other compounds may behave differently with different procedures; occasionally this may perhaps be exploited for purposes of characterisation or identification of peaks.

We are finding the above techniques invaluable in current work on methods suitable for the estimation of aromatic constituents of urine. When investigating the chemical nature or homogeneity of a peak we prefer if possible to use peak disappearance techniques in which chromatographic conditions are kept constant whilst the nature of extracts is varied. Then, for example, the presence of such groups as carboxyl, keto, amino or hydroxyl in compounds can be deduced if peaks found on chromatography of silylated extracts disappear when the extracts are first treated with appropriate reagents before silylation. Charcoal treatment and conditions of solvent extraction (including variations in both solvent and pH) represent almost perfect examples of such techniques in that peaks tend to vanish completely from chromatograms rather than merely from their original positions. However occasional ambiguities due to artifacts may arise (*e.g.* Fig. 2). The use of charcoal for detecting aliphatic contaminants of aromatic peaks is obvious enough. The conditions under which a compound may be extracted may provide valuable clues as to its chemical nature and in some cases as to the homogeneity of peaks. Thus the finding that peak X is larger in an ethyl acetate extract than in an ether one, despite the fact that compound X is known to be quantitatively extracted by ether, not only proves its heterogenity in the former case but suggests it in the latter also, since impurities obviously extracted by ethyl acetate may well be extracted in smaller quantity by ether.

The use of two different types of column may provide valuable information in favorable cases when peaks are easily identified because of their exceptional size but may otherwise prove ambiguous when, as in the cases of OV-1 and OV-17, the columns give substantially different chromatographic patterns. More easily interpreted results may be often obtained by taking advantage of the more subtle differences between chromatograms resulting from the use of columns containing the same liquid phase but at differing concentrations[5]. However, our own present preference lies towards the use of columns of different lengths, all containing similar packings: peaks unresolved with one column length may often be resolved with another. Using columns of OV-1 (10%) 1.5, 2.7 and 5.4 m long we have examined ether extracts from a series of normal urines. Results indicate that the number of compounds possibly present far exceeds the number of peaks observed in any one urine using any one column. It seems quite evident that one chromatogram is unlikely to provide reliable quantitative data concerning the normal excretion of more than a few, if any, compounds: optimum conditions must be investigated for each substance.

Nevertheless quantitative estimations of reasonable to high accuracy may be made in many pathological cases. Almost any conditions of chromatography will permit the detection and estimation of compounds such as homogentisic acid in alkaptonuria, phenyllactic acid in phenylketonuria and *p*-hydroxyphenyllactic acid in gross tyrosyluria. However it is always desirable to check results with a second column and may be essential when only moderately abnormal excretions, such as that of 4-hydroxy-3-methoxymandelic acid in some cases of phaeochromocytoma, are encountered.

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The determination of acetonitrile and other trace impurities in acrylonitrile by gas chromatography

In a recent communication[1], a column packed with 10% Bentone 34–10% PEG on Chromosorb P was used to estimate acetonitrile in acrylonitrile. The method was not suitable at low concentration (<100 p.p.m.) because the acetonitrile was eluted on the tail of the acrylonitrile peak.

We experienced similar difficulties using oxydipropionitrile as stationary phase. With the introduction of Porapak (Waters Associates), however, the analysis became much more satisfactory because acetonitrile was eluted before acrylonitrile.

This packing has been used for over two years for this particular analysis under the gas chromatographic conditions shown below.

Experimental

Gas chromatograph: Perkin Elmer F-11.
Detector: flame ionisation.
Column dimensions: length 2.5 m, diameter 2.5 mm I.D.
Column temperature: 160°.
Injection temperature: nominal 150°.
Carrier gas: helium, flow rate about 70 ml/min.
Sample size: 1 μl.

The retention data are given in Table I.