erfolgt und die Chromatogramme über einen 2 mV Servogor Potentiometerschreiber der Firma Goerz Elektro aufgezeichnet worden.

Institut für Organische und Pharmazeutische Chemie der Universität Graz, Universitätsplatz 1, A-8010 Graz (Österreich)

HEINRICH BINDER
KARL GROKE

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A general screening method for urine constituents utilising gas–liquid chromatography

Few of the many constituents of urine are amenable to GLC without prior conversion to suitably volatile derivatives. For this purpose conversion to trimethylsilyl derivatives is often very suitable. Various silylation procedures have proved more or less successful according to the types of compound examined but a recent important advance has been achieved through the introduction of bis(trimethylsilyl) acetamide (BSA)[1,2]. This reagent rapidly and quantitatively silylates alcohols, enols, phenols, carboxylic acids, amines, amides, ureas, heterocyclic compounds such as purines and pyrimidines and has been shown to yield derivatives suitable for GLC with such complicated molecules as steroid glucuronides[3] and sugar phosphates[4]. It may thus be considered as a general reagent for GLC purposes.

Similarly, although a bewildering variety of liquid phases are still in use, interest seems gradually to be focussing on a few siloxane polymers as being most generally useful. These give excellent results with most trimethylsilyl derivatives and are not easily overloaded; relatively enormous quantities of urea, for instance, interfere only to the extent of obscuring immediately adjacent compounds on chromatograms.

It seemed to us that it should now be technically possible to develop a GLC method capable of screening urines for many types of compound in one operation. Such a method would have obvious practical limitations owing to overlaps and to wide quantitative differences between various compounds excreted, but should suffice to detect moderate pathological increases in relatively major urine constituents and larger increases in minor ones. Within these limitations such a general technique would be of obvious value in many pathological conditions and would doubtless further extend the range of diseases in which chemical analysis is of diagnostic value.

Experimental

Gas chromatography. The instrument used was a Pye 104 series dual-column model 64 with 1.5 m × 4 mm I.D. coiled glass columns. A hydrogen flame-ionisation

detector was used with a hydrogen flow-rate of 50 ml/min. The detectors were maintained at 280°. Injection heaters were used to give an initial temperature at the point of injection about 80° above the temperature at which programming (2°/min from 100° to 250°) was started. Argon at a flow rate of 50 ml/min was used as carrier gas.

Various columns were assessed roughly for performance in terms of the ratios of peak heights to base-widths and on the ease with which the excess reagent and solvent were eliminated to give a low, stable base-line. Columns investigated included QF-1 (3% on Gas Chrom Q, 85–100 mesh), SE-30 (10% on siliconised Celite, 100–120 mesh), polyphenylether (6 ring), XE-60, W-98, SE-52, OV-1 and OV-17 (all 10% on Diatoport S, 80–100 mesh). The column containing W-98 was selected as being of average performance and more likely to reveal potential technical difficulties at an early stage than would the better columns (SE-52, OV-1 and OV-17). W-98 (Hewlett-Packard Ltd., now listed as W-982) is described as a silicone gum rubber containing methyl and vinyl groups.

Chromatograms were recorded on a Leeds and Northrup Speedomax W recorder using a chart speed of 0.5 in./min. The ionisation amplifier attenuator was set at 1×10^3 to give full scale deflection at 10^{-9} A.

Preliminary experiments. All silylations were carried out with a 2:1 mixture of solvent and BSA. An ampoule of the reagent (Sigma; usually a very generous 1 ml) was opened, the contents mixed with 2 ml solvent and the mixture used at once.

Comparison of chromatograms carried out after varying amounts of evaporated urine (see below) had been treated with 0.5 ml of pyridine–BSA (2:1) indicated that, as the amount of reagent became increasingly insufficient to effect complete silylation, a few peaks disappeared from chromatograms at a stage when the majority were not noticeably affected. Among such peaks hippuric acid was recognised; the amide grouping in this compound is silylated with relative difficulty. Hence a suitable higher homologue, benzoyl-DL-phenylalanine (BPA), which is not naturally present in urine

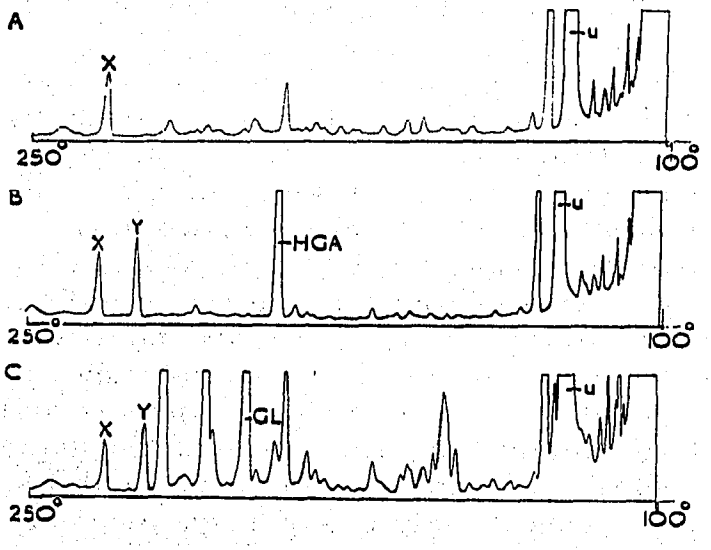

Fig. 1. Typical chromatograms of evaporated urine extracts. In each case extract equivalent to 2 μg urinary creatinine was chromatographed. Peaks U, X and Y represent urea, BPA standard and docosane standard, respectively. (A) Normal healthy male subject. (B) Case of alcaptonuria showing a large peak due to homogentisic acid (HGA). (C) Case of Lignac's disease with multiple abnormalities, known to include aminoaciduria and glucosuria. Peak GL corresponds to glucose.

and which appears on chromatograms at a point where no significant peaks normally appear, was subsequently added to urine extracts in order to indicate the efficiency of silylation. Docosane was added as a standard to correct for the intrinsic errors of GLC.

All chromatograms were dominated by a large early peak due to urea (Fig. 1). Peaks appearing before this included reagent peaks and probably some derived from compounds of appreciable volatility which may be erratically lost in the evaporation process. When chromatograms were compared variations in these early peaks were ignored and only those emerging after urea were considered.

Aliquots of one normal urine were processed as described below, using as silylating reagent a 1:2 mixture of BSA and one of the following solvents: pyridine, ethyl acetate, triethylamine, hexamethyldisilazane, acetonitrile and dioxan. Almost identical chromatograms resulted, dioxan giving rise to most differences. The least amount of undissolved material was obtained when pyridine was used. Addition of trimethylchlorosilane (0.125 ml) to the extract obtained using pyridine did not alter the chromatographic pattern.

Chromatography of urines. Urine was adjusted to pH 1–2. An aliquot containing 0.25 mg creatinine was placed in a 100 ml round-bottom flask, diluted with 10 vols (or more if convenient) ethanol and evaporated to dryness *in vacuo* at not greater than 30°. Standards of BPA (250 μg) in ethyl acetate (0.5 ml) and docosane (200 μg) in ethanol (2 ml) were added, together with a few millilitres benzene, and evaporation was repeated. The dry residue was treated with BSA–pyridine (1:2, 0.5 ml) and the flask tightly stoppered with a polypropylene stopper. After a few minutes, with occasional rotation of the flask, the crust of solids disintegrated and a solution containing usually only a little undissolved material was obtained. The mixture was allowed to stand overnight before aliquots (4 μl) were chromatographed as described above; no attempt was made to remove solids. Occasionally crystals appeared in the mixture: these could be readily redissolved by gentle warming and appeared to be a useful indication of incomplete silylation.

Ten adult urines examined by the above procedure were found to be silylated satisfactorily as judged by the ratio of the peak heights of the BPA and docosane standards. When additional BSA (0.125 ml) was added to the extracts and chromatography was repeated, using 5 μl aliquots, chromatograms were virtually identical with the originals. Infant urines, containing relatively much more urea, provided a much more stringent test of conditions and in a high proportion (14 of 20) of those examined silylation proved to be incomplete before addition of extra BSA; usually the BPA peak was completely absent. It is probably not worth examining such urines without increasing the amount of BSA relative to that of urine.

Discussion

The work described was undertaken with two objectives in mind, to determine whether it was technically possible to obtain good chromatograms from urines subjected to the minimum treatment, and to investigate conditions necessary to ensure consistent silylation of reactive constituents.

No troubles were encountered in connection with the first objective and there remains considerable latitude for such variations in conditions as experience may indicate to be advisable. Evaporation of urine may be carried out in various ways,

with or without the addition of ethanol and samples may be buffered if this is thought to be desirable. The method described seemed to be quicker than possible alternatives and to offer conditions sufficiently mild to minimise possible decomposition or reaction of urine constituents. Nevertheless it has been noted, for instance, that phenylpyruvic acid does not yield the expected peak in phenylketonuria urine or when added to normal urine or to aqueous urea. Very good chromatograms were obtained using a column of average performance, suggesting that suitable columns should be immediately available in most laboratories equipped for GLC. However, attention should be drawn to the excellent results obtainable with OV-1 and OV-17, the thermal stabilities of which are such that they may be used at temperatures well in excess of 300° if desired.

Although BSA appears to be the best available silylating reagent, to be fully effective it must be present in excess and in this respect has two obvious defects. First, it is extremely sensitive to atmospheric moisture and must be handled appropriately. It did not seem advisable to risk the variable amounts of hydrolysis which might have occurred in extracts if manipulations such as centrifugation had been attempted and for this reason pyridine extracts containing relatively little solid were preferred to extracts obtained using solvents such as dioxan which may themselves be more readily dried. Secondly, the present high cost of BSA dictates its use in an economical manner. The experiments outlined above indicate conditions offering a good chance of complete silylation but it is hoped that the reagent will eventually become cheap enough to be used more liberally. But in any case it is desirable to include a standard to indicate the presence of excess BSA in a reaction mixture. In this connection BPA was found to possess suitable properties; it is of course theoretically possible for BPA to be just completely silylated whilst other less reactive compounds present in a mixture remain unreacted but no indication of this was observed in practice.

It was noted that BPA present in an adequately silylated urine extract always yielded a rather taller peak than did a similar quantity of the pure substance. In the latter case a small poorly shaped peak emerged from the column just before the major peak, suggesting silylation to be incomplete. It seems possible that other urinary constituents catalyse the reaction between BPA and BSA. In the steroid field it has been shown that reaction between sterically hindered hydroxyl groups and BSA may be catalysed by addition of hydrogen chloride or trimethylchlorosilane[3]. In GLC methods involving conversion of compounds to derivatives, allowance should be made for the possibility that a compound may behave in a different manner when pure than when in admixture with other compounds.

Under the conditions described above normal urines yielded chromatograms showing numerous, mostly rather small, peaks (Fig. 1A). It is not possible to define accurately the amount of substance necessary to give an 'abnormal' peak since this will depend on whether the compound emerges early or late, on the normal variation of the peak and of any overlapping peaks, and on the sensitivity of the detector towards the compound, as well as on the experience of the observer in examining chromatograms. However, it seems likely that most substances will be readily detectable if excretion is of the order of 200 μg per mg urinary creatinine (calculated if necessary as the trimethylsilyl derivative). Because of the complexity of urine we envisage the method primarily as a means of detecting abnormalities in the first place, prior to detailed investigation by other means, rather than as a research tool in its own right.

However, it seems likely that more specific applications may be possible through the use of selective detectors (*e.g.* electron capture or thermionic). The method can be adapted to form a very rapid screening procedure. Reaction of functional groups with BSA is rapid and limited largely by solubility of compounds[2]; hence preliminary chromatograms of extracts may be performed within a few minutes, particularly if the mixture is warmed. Given a urine of known creatinine content and a GLC apparatus ready for use, it is easily possible to screen a urine within 1 h if temperature programming at 4°/min is employed.

Extreme examples of pathological results obtained are illustrated. Fig. 1B shows a simple case in which enormous quantities of a single abnormal compound (homogentisic acid) were excreted. The method was here entirely adequate in itself, requiring only recognition of the peak and confirmation of its identity using alternative columns. In Fig. 1C numerous abnormal peaks were observed; clearly, detailed examination of a urine as complex as this would require more specific and sophisticated techniques. Nevertheless even in such cases, much provisional diagnostic information may be immediately available on inspection of the initial chromatograms when major peaks, although numerous, form a pattern characteristic of a particular condition.

RAF Institute of Aviation Medicine,
Farnborough, Hants. (Great Britain)

R. F. COWARD
P. SMITH

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