THE DETECTION OF KETO ACIDS IN BIOLOGICAL FLUIDS BY TWO DIMENSIONAL PAPER CHROMATOGRAPHY OF THEIR 2,4-DINITROPHENYLHYDRAZONES

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SUMMARY

After conversion to 2,4-dinitrophenylhydrazones, keto acids in biological fluids were extracted into isopropyl ether followed by $NaHCO_3$ and finally into ethyl acetate. The derivatives were chromatographed two-dimensionally on Whatman No. SG 81 paper. Limitations of the extraction and chromatographic procedures are discussed but it is considered that the method is superior to orthodox one-dimensional paper chromatography.

Reaction with 2,4-dinitrophenylhydrazine (DNPH) to form coloured 2,4-dinitrophenylhydrazones (DNPs) has been much used in connection with studies on biological keto acids. Paper chromatography of the derivatives is difficult however, and only one-dimensional techniques are possible by ordinary methods.

Recently useful results were obtained with authentic compounds using silica gel loaded paper (Whatman No. SG 81)[1] and the possibility of two-dimensional chromatography was mentioned. Application to the study of biological fluids is described in this paper.

EXPERIMENTAL

Reagents

All reagents were of the best commercially available quality. Diisopropyl ether was stored over 2 N H_2SO_4 saturated with $FeSO_4$. The DNPH reagent contained DNPH (0.2 %, w/v) in 2 N HCl.

Extraction and recovery of DNPs from aqueous solution

Absorption spectra of a number of authentic DNPs were determined in 0.1 M $NaHCO_3$. A solution of each derivative in 0.1 M $NaHCO_3$ containing sufficient material to give a suitable optical density at the wave-length of maximal absorption was then prepared. Each solution (10 ml) was acidified with 2 N HCl (10 ml) and extracted with isopropyl ether (5 ml). After centrifugation the upper layer was removed as completely as possible with a teat pipette. The lower layer was re-extracted with isopropyl ether (5 ml). After centrifugation and removal of the bulk of the upper layer the

aqueous layer was washed by careful addition and removal of several successive small portions of isopropyl ether. The combined extracts and washings (total volume *ca.* 12 ml) were shaken with 0.1 M $NaHCO_3$ (3 ml). The mixture was centrifuged, the organic layer removed and the aqueous layer washed several times with a little isopropyl ether before acidification with 0.2 N HCl (3 ml). The derivatives were finally extracted into ethyl acetate (6 ml); washings with the same solvent were used to adjust the final volume of extract to 10 ml.

For determination of recoveries 3 ml aliquots were evaporated in a stream of air at room temperature and redissolved in 0.1 M $NaHCO_3$ (3 ml). Optical densities were determined against a blank prepared by processing 0.1 M $NaHCO_3$ alone. Comparison with optical densities of the original solutions gave recoveries for derivatives of the following acids: ketomalonic, 37 %; oxaloacetic, 92 %; α-ketoglutaric, 96 %; pyruvic, 95 %; α-ketoisocaproic, 100 %; phenylpyruvic, 100 %; *p*-hydroxyphenylpyruvic, 94 %.

For extraction of biological fluids after treatment with DNPH, solvents and reagents were used in the proportions described above. With certain abnormal urines, however, modification of the procedure may be desirable (see Discussion).

Preparation and chromatography of extracts of biological fluids

Urine and sweat were treated with an equal volume of DNPH reagent. Saliva, whole blood or plasma (from heparinised blood) were deproteinised with 4 vol. of 5 %, w/v metaphosphoric acid and the centrifugate treated with a volume of DNPH equal to that of the original biological fluid. An extract of elder leaves was prepared using the boiling methanol technique of ISHERWOOD AND NIAVIS[2]; our facilities did not permit adoption of the superior techniques recommended by these authors. The extract was treated with half its volume of DNPH reagent.

All DNPH treated solutions were allowed to stand overnight at 4° before extraction by the procedure described above. Sometimes emulsion formation necessitated prolonged centrifugation although this trouble was not encountered frequently with urine. Aliquots of the final ethyl acetate extracts were evaporated in a stream of air at room temperature and stored dry (usually overnight) at —15° until chromatographed. Quantities equivalent to 2 mg urinary creatinine, 2 ml plasma, 4 ml whole blood, 5 ml saliva, 0.2 ml sweat or 0.25 g elder leaves proved suitable for chromatography.

Dried aliquots were transferred with ethyl acetate to sheets of Whatman No. SG 81 paper (23 × 23 cm) and developed with 2-nitropropane containing formic acid (1 %, v/v) followed, after brief drying in a current of warm air, by benzene containing acetic acid (5 %, v/v). The whole procedure occupied *ca.* 6 h. Spots were detected by their (usually) yellow colour. The papers were also examined under U.V. light and after exposure to vapours from aqueous NH_3 or, better, methylamine.

Typical results obtained are illustrated in Fig. 1.

In preliminary experiments it was noticed that DNP derivatives left overnight between the two solvent runs tended to undergo decomposition or to become irreversibly adsorbed. The various stages in the chromatography should therefore be carried out with the minimum of delay.

Best results were obtained when chromatograms were run at 20–25°. A recent deterioration in quality following cutting off of central heating in these laboratories

appears to have been successfully counteracted by performing the first run in an incubator at 35° and increasing the acetic acid content of the benzene for the second run to 7 %, v/v.

The sheets of paper were cut from standard sheets 46 × 57 cm. The residual paper, cut into squares 11 × 11 cm, has been found very useful for running preliminary microchromatograms (*e.g.* on urine extracts equivalent to 0.1–0.5 mg creatinine) which may be completed two-dimensionally in little more than 60 min.

Behaviour of acetoacetic, pyruvic and α-ketoglutaric acid DNPs

Although the results indicated in Fig. 1 suggest otherwise, these three keto

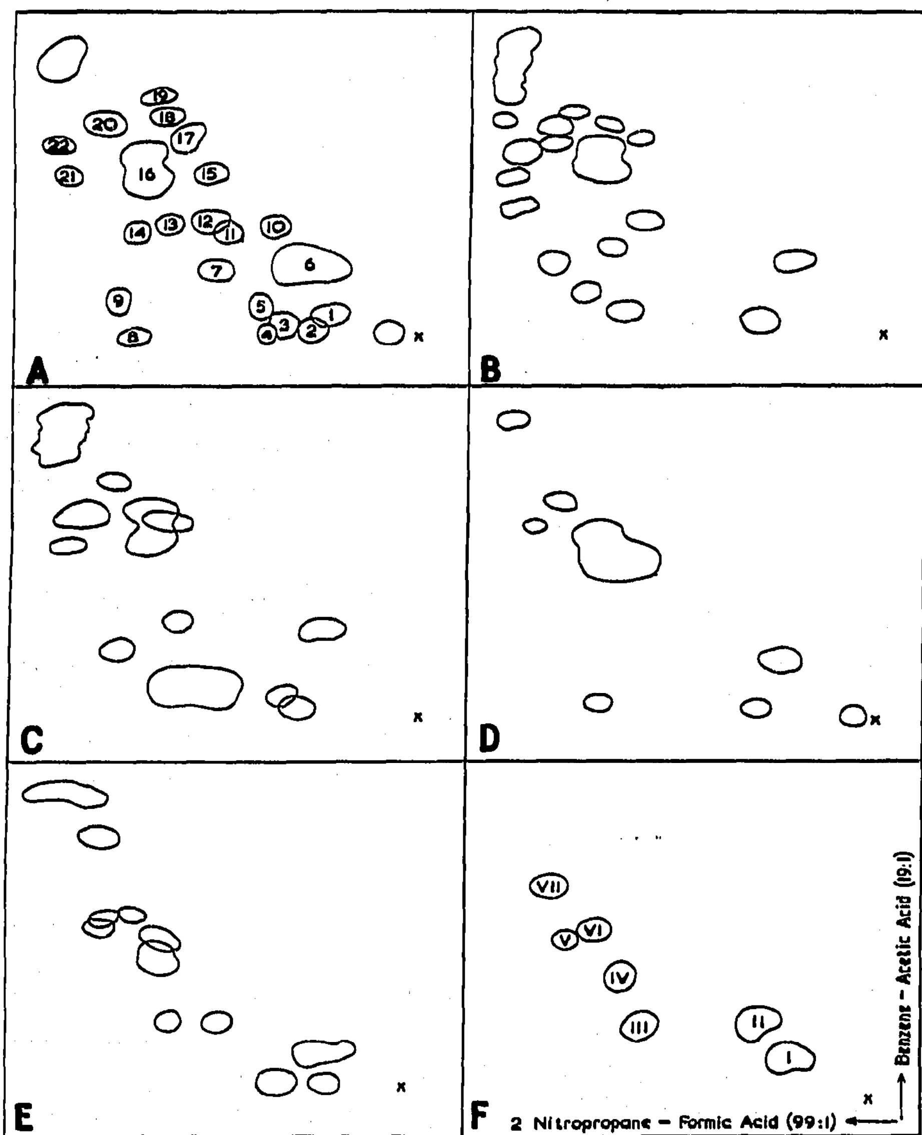

Fig. 1. Typical chromatograms from biological fluids. (A) Normal human urine. The spots are numbered for reference in this and future publications. (B) Normal human plasma. (C) Normal human saliva. (D) Sweat collected from a normal male exposed to high ambient temperature. (E) Extract of elder leaves. (F) Some authentic DNPs of biological interest: (I) oxaloacetic acid; (II) α-ketoglutaric acid; (III) *p*-hydroxyphenylpyruvic acid; (IV) 4-hydroxy-3-methoxyphenylpyruvic acid; (V) 3-indolylpyruvic acid; (VI) pyruvic acid; (VII) α-ketoisovaleric acid (not separated from other higher homologues of pyruvic acid or from phenylpyruvic acid).

acids have been commonly believed to be the only ones occurring to any extent in normal human fluids. They are certainly major constituents and the behaviour of their DNPs deserves mention.

Acetoacetic acid DNP decomposes under the conditions of chromatography to give acetone DNP, one constituent of an undoubtedly heterogeneous spot of high R_F value in both solvents.

The behaviour of pyruvic acid DNP is variable. It is depicted as a dumb-bell shaped spot (No. 16) in *e.g.* Fig. 1A but sometimes appears as a normal single spot whilst at other times the two halves of the dumb-bell are clearly separated in benzene–acetic acid. In one such case confirmation of the identity of each half was achieved by the increase in its size observed when another sample of the urine was processed after addition of sodium pyruvate. When pure sodium pyruvate was processed the spot of higher R_F value in benzene–acetic acid was only observed in traces and pure pyruvic acid DNP gave only one spot. Definite conclusions would clearly require repetition of these experiments on numerous occasions but they suggest the danger of misinterpreting chromatograms owing to variable behaviour of compounds not indicated by previous data[1].

Only one spot has ever been observed in the case of α-ketoglutaric acid DNP. Several replicates of the chromatogram illustrated in Fig. 1A were prepared and the appropriate spots (No. 6) eluted with 0.1 M $NaHCO_3$ and extracted, after acidification, into ethyl acetate. Chromatography in *sec.*-butanol–water (4:1), *sec.*-butanol–2 N NH_4OH (4:1) or 5 % w/v aqueous $NaHCO_3$ on Whatman No. 20 paper indicated spot 6 to be homogeneous and identical in behaviour with the α-ketoglutaric acid derivative. Sufficient α-ketoglutaric acid may be present in urine for papers to be somewhat overloaded with respect to its DNP if aliquots equivalent to 2 mg urinary creatinine are used; some streaking of the compound may then occur, particularly in the first direction.

DISCUSSION

Successful two-dimensional chromatography requires solvent systems of significantly different properties. Guided by results obtained with authentic keto acid DNPs we selected the combination described above as being the best of a limited choice. Application of variations to biological extracts has never revealed any combination of solvents giving generally superior results although specific pairs of spots may be slightly better resolved.

Treatment of biological fluids demands a method for separating keto acid derivatives from other DNPs and from excess reagent. Because of the wide variety of compounds present, sometimes in large amounts, no method can be perfect for every purpose. For the concentration of acidic derivatives we examined only extraction from organic solvent into aqueous alkali although ion-exchange presents a possible alternative. Since it is more specific for the carboxyl group we preferred the use of $NaHCO_3$ to that of Na_2CO_3 or NH_4OH; nevertheless the appearance of a yellow compound in the appropriate fraction is no absolute guarantee that it is the derivative of a ketocarboxylic acid (or even that it is a DNP at all). Because some derivatives are easily salted out, the use of very dilute $NaHCO_3$ was found to be preferable.

For the initial extraction of derivatives, ethyl acetate is an excellent solvent

which has been widely used. However it extracts much material of negligible R_F value, particularly from urine, which limits the amounts which may be chromatographed without gross distortion. Moreover the extraction of some DNPs (*e.g.* of higher homologues of pyruvic acid) from ethyl acetate into alkali is incomplete in one operation. These difficulties are not encountered if a non-polar solvent such as benzene is employed, but then some important derivatives, such as that of α-ketoglutaric acid, are poorly extracted from aqueous solution.

As a compromise we selected isopropyl ether which in conjunction with 0.1 *M* $NaHCO_3$ gave good recoveries of authentic derivatives and appeared adequate for normal biological fluids. However, it is not essentially a good solvent for DNPs and in some cases (*e.g.* tyrosinosis urine containing much *p*-hydroxyphenylpyruvic acid) the quantities recommended above may not prove sufficient to dissolve a derivative completely. Such cases will usually be obvious owing to the presence of a visible precipitate which may, of course, be removed and examined separately. By contrast, some isopropyl ether extracts (*e.g.* from phenylketonuria urine) may contain enough acidic material to render the quantity of $NaHCO_3$ used insufficient to extract DNPs completely. As a precaution against this we have found it convenient to examine separately a second extract of the isopropyl ether, using 2 *M* aqueous methylamine. In addition to removing any strongly acidic DNPs incompletely extracted into $NaHCO_3$ this also extracts any weakly acidic compounds (*e.g.* *p*-hydroxybenzaldehyde DNP) which may be present.

Application of the technique to a variety of biological fluids yielded satisfactory chromatograms (Fig. 1) varying in complexity from the comparatively simple sweat, containing predominently pyruvic acid, to normal urine yielding a multitude of spots. We have studied particularly normal (and a few pathological) human urines and find the method much more informative than orthodox one-dimensional paper chromatography. Nevertheless it has obvious limitations. Some difficulties encountered, such as those mentioned above involving the extraction processes or the tendency of compounds present in large amount to streak, may be readily remedied as the occasion arises. Double spotting, presumably resulting from geometrical isomerism, should not prove unduly troublesome provided its possibility is anticipated, especially since data from pyruvic acid (this paper) and a few cases previously recorded[1] suggest that the spots should be close together. Perhaps the greatest difficulties lie in the identification of compounds by inspection of papers owing to the generally similar position and appearance of many spots. Thus distinction between phenylpyruvic acid (in phenylketonuria) and higher homologues of pyruvic acid (in maple syrup urine disease) is not possible and the method must then be used only as a preliminary stage to some alternative procedure such as reduction of DNPs to the corresponding amino acids.

The presence of a few compounds of distinctive colour is some aid to the interpretation of the chromatographic pattern. Thus in the urine depicted in Fig. 1A spots 11 and 14 appeared orange and spot 2 (from dehydroascorbic acid) red. Further distinction between compounds may be achieved by exposing papers to alkaline vapours. Most DNPs slowly acquire a brownish colour, particularly if papers have been thoroughly dried, but some give an immediate and characteristic colour. For examples, spots 4 and 5 (Fig. 1A) became orange-brown and spot 22 became violet when exposed to methylamine. Finally, advantage may sometimes be taken of sprays designed to reveal specific groups such as an indole or phenolic nucleus. Thus the

identity of spot 7 (Fig. 1 A) as *p*-hydroxyphenylpyruvic acid DNP, suggested by R_F values, was supported by the development of an orange-red colour with diazotised sulphanilic acid and of a blue-green colour with 2,6-dichloroquinone chloroimide–borax.

REFERENCES

1 P. SMITH, *J. Chromatog.*, 30 (1967) 273.
2 F. A. ISHERWOOD AND C. A. NIAVIS, *Biochem. J.*, 64 (1956) 549.