IDENTIFICATION AND QUANTITATIVE DETERMINATION OF KETO ACIDS BY PAPER CHROMATOGRAPHY*

ERNEST KUN** AND MARIO GARCIA-HERNANDEZ

Institute for Enzyme Research and Department of Biochemistry, The University of Wisconsin, Madison, Wis. (U.S.A.)

Physical properties of 2,4-dinitrophenylhydrazine (DNPH) derivatives of keto acids render these compounds suitable for isolation and identification. Extraction of the DNPH derivatives with organic solvents from their aqueous solutions has been used for quantitative determination of certain keto acids of biological importance[1]. Identification of mono-DNPH derivatives, as carried out in strong alkaline solutions by means of spectrophotometry, is generally unreliable because the colors produced by alkali (in N NaOH) are unstable. An additional complication arises from the fact that the DNPH derivative of a single pure keto acid often yields a mixture of *cis* and *trans* isomers, each of them exhibiting different absorption spectra (in N NaOH, between 350 and 600 mμ). Since the ratio and rates of formation of isomers are unpredictable, the measured visible spectrum, which is the sum of the two components, may vary considerably.

Much work has already been published, dealing with chromatographic separation of DNPH derivatives of pyruvic, oxalacetic and α-ketoglutaric acids[2–12]***. The identification of these keto acid derivatives was based in almost all instances on the position of each compound on paper chromatograms, and only few workers[11, 13, 14] used more reliable chemical criteria.

The diversity of papers dealing with the chromatographic separation of DNPH derivatives appears to indicate that it is desirable to develop a more uniform procedure which permits a systematic analysis of mixtures of carbonyl compounds.

EXPERIMENTAL AND RESULTS

Preparation of DNPH derivatives from biological material

Deproteinization is best accomplished by addition of 0.05 to 0.1 volume of ice-cold 60% $HClO_4$, while the incubation mixture (homogenate etc.) is kept in an ice bath. Ten minutes after agitation, the mixture is filtered (Whatman No. 1 paper) directly into an excess (50 to 200 ml) of freshly prepared solution of 2,4-dinitrophenylhydrazine (1 g dissolved in 1 l of 2 N HCl). This reaction mixture is kept for 2 hours at room temperature and then overnight at 4° C. With the exception of β-mercaptopyruvate, which requires several days of incubation, all other keto acids so far investigated are

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*** References quoted are examples, not a complete list of bibliography.

quantitatively converted to the DNPH derivatives under these conditions. With the exception of acetoacetate, which yields significant amounts of acetone, none of the commonly occurring keto acids tested were measurably decomposed by dilute perchloric acid. Diketosuccinic and oxaloglycolic acids, the most unstable keto acids so far encountered, are decomposed by traces of bivalent ions in neutral aqueous solutions[19]. For preparation of DNPH derivatives these keto acids should be dissolved directly in the DNPH reagent. Their decomposition in biological material is diminished in the presence of 0.02 M ethylenediamine-tetra-acetate[19].

Chromatographic separation of DNPH derivatives of keto acids from other carbonyl compounds

When it is desirable to analyze for certain carbonyl compounds (formaldehyde, acetaldehyde, acetone) besides keto acids, a preliminary separation of the two groups of substances is carried out. The total amount of DNPH derivatives is quantitatively extracted in separatory funnels with ethyl acetate (2 successive extractions with 10 ml portions). The combined extract is filtered through anhydrous Na_2SO_4, and then evaporated to dryness under a stream of N_2 at 30° to 40° C. The dried DNPH derivatives are dissolved in the smallest volume of dry ethyl acetate and quantitatively applied on the starting line of an impregnated (see below) Whatman No. 1 filter paper strip by means of a capillary pipette. Descending chromatography is carried out in a solvent system, described by SYKORA AND PROHÁZKA[15], consisting of 20 volumes of petroleum ether and 80 volumes of 95 % ethanol. Sufficient separation is achieved in a period of 4 to 6 hours at room temperature (20° C). Non-acidic bis-2,4-dinitrophenylhydrazones of glyoxal and methylglyoxal remain on the starting line, the DNPH derivatives of keto acids move together in one line closer to the start, while DNPH derivatives of non-acidic carbonyl compounds aggregate below the front of keto acids. Each group of compounds is separated by re-extraction of the cuts of the chromatograms as described below.

Extraction of the DNPH derivatives of keto acids

When it is intended to analyze only for keto acids the following procedure may be followed. The combined ethyl acetate extract containing all DNPH derivatives is re-extracted once with 20 ml of 10 % Na_2CO_3. Repetition of extraction with carbonate is unnecessary, and only increases the amount of alkaline decomposition products of DNPH. The carbonate extract is immediately chilled in an ice bath and acidified in the cold with HCl to pH 1.0, then re-extracted twice with ethyl acetate (2 × 5 to 10 ml). This extract is dried by filtration through anhydrous Na_2SO_4 and the ethyl acetate evaporated as described above.

Chromatography of the DNPH derivatives of keto acids

All keto acid derivatives so far tested could be quantitatively separated in one solvent system, by the use of Whatman No. 1 filter paper which was impregnated with 0.1 M potassium phosphate buffer of pH 7.3. Filter paper strips of the size of 10 × 45 cm are immersed in a tray containing the buffer and suspended lengthwise for drying for 5 to 8 hours at room temperature. It should be emphasized that only freshly prepared (impregnated and dried) filter paper strips should be used for chromatography. The samples are applied on the starting line which is drawn at a distance of 10 cm from the upper end of the dried paper. The amount of DNPH derivatives which can be separated on this strip is about 1 micromole. Proportionally larger strips should be used when more keto acid derivatives are expected to be present. The developing solvent, finally adapted, was 83 vol. of abs. ethanol + 17 vol. of H_2O. In all instances descending chromatography was used, and the chambers were equilibrated with 95 % ethanol. It was found that both the concentration and pH of the potassium phosphate buffer as well as the H_2O ethanol ratio of the solvent has a decisive influence on both separation and size of separated lines of DNPH derivatives. Temperature optimum is between 17 to 20° C.

Extraction of DNPH derivatives from paper chromatograms

Quantitative extraction of the two separated groups of DNPH derivatives, (*i.e.* keto acids and non-acidic carbonyl compounds) as obtained in the petroleum ether–ethanol solvent system is best done with ethyl acetate. Traces of filter paper are removed by filtration of the ethyl acetate extracts through a sintered glass filter. The combined DNPH derivatives of keto acids are subsequently rechromatographed in the ethanol–water solvent system, while the other carbonyl compounds analyzed as amines after catalytic reduction (see below). For quantitative analysis of separated mono-2,4-dinitrophenylhydrazones of keto acids 5 or 10 ml of 0.1 *M* phosphate buffer of pH 7.3 is used as extracting solvent. These DNPH derivatives are easily soluble in this buffer and are stable for several days in solution. The bis-DNPH derivatives (osazones) are extracted with 1 *N* NaOH for quantitative analysis. They form an intensive violet colored solution which is stable for at least 2 hours.

Quantitative analysis and identification of DNPH derivatives of keto acids

Observance of the following conditions make it possible to perform qualitative and quantitative analysis of keto acids in mixtures: (1) The chromatographic separation of DNPH derivatives must be carried on to the point when each component is clearly separated. This occurs generally in 14–18 hours at 20°. However, when a number of DNPH derivatives travel close together (*e.g.* oxaloglycolic, diketosuccinic, mesoxalic, oxalacetic, and α-ketoglutaric acids in one slowly moving group, and hydroxypyruvic, pyruvic, glyoxylic, acetoacetic and α-ketobutyric in a more rapidly migrating group) it may become necessary to divide the first paper chromatogram into several horizontal sections and after quantitative extraction of the DNPH derivatives repeat their chromatographic separation on individual strips of filter papers. Ethyl acetate is used for re-extraction. (2) Each component is eluted separately for quantitative analysis. Absorbance of mono-DNPH derivatives in phosphate buffer is measured between 365 to 370 mμ (Beckman spectrophotometer) while the violet-colored solution of bis-DNPH derivatives in NaOH is read at 560 mμ. (3) A measured aliquot of each of the mono-DNPH derivatives (in phosphate buffer) is acidified with HCl and extracted with ethyl acetate which is then evaporated. A known amount of the ethyl acetate eluate of the bis-DNPH derivatives is also evaporated to dryness. Each of these samples is subjected to repeated paper chromatography separately and in mixtures with synthetic pure DNPH derivatives. When the unknown DNPH derivative does not separate in mixed chromatograms from a pure synthetic DNPH compound, their identity is probable. (4) A more definitive proof is obtained by reduction of both (*i.e.* unknown and known) DNPH derivatives to the corresponding amino acids. This is done as follows. Each of the isolated components is extracted from the chromatogram with ethyl acetate, the solvent is evaporated and the DNPH derivatives redissolved in 3 to 5 ml of glacial acetic acid. Reduction to amino acids is carried out by shaking the solutions with 1 to 2 mg of platinum oxide catalyst in pure H_2 atmosphere. Complete reduction occurred within 2 to 5 hours (the amounts of DNPH derivatives should not exceed 1 to 3 micromoles) with all DNPH derivatives so far tested. It is safest to determine H_2 absorption manometrically and extend the time of catalytic reduction until H_2 absorption stops. The volume of glacial acetic acid is reduced *in vacuo* to 0.1 ml and samples of the glacial acetic acid solution are directly used for chromatographic amino acid analyses. The following solvent systems were used for amino acid analyses: *n*-butanol–acetic acid–water[16], phenol–water, and methyl ethyl ketone–propionic acid–water[17].

When large amounts of colored by-products of the catalytic reduction of DNPH derivatives are present, the amino acid (after spotting of a sample with ninhydrin) should be re-extracted from the paper with glacial acetic acid and rechromatographed. The identity of each amino acid is established when in mixed chromatograms a pure known amino acid does not separate from the "unknown" in all three solvent systems. In general, 1 to 10 micrograms of amino acids are sufficient for identification.

An example of combined analyses of keto acid derivatives is given in Tables I and II. Some keto acid derivatives which are close to each other in the first chromatogram (Table I) separate into clear distinct bands upon rechromatography (Table II). During rechromatography the solvent is allowed to flow through the paper for 2 days.

TABLE I

DISTRIBUTION OF DNPH DERIVATIVES ON IMPREGNATED WHATMAN NO. 1 FILTER PAPER

No.	*Compound*	R_F	*% recovery*
1	Glyoxal[b]	0.00	
2	Methylglyoxal[b]	0.00	
3	Oxaloglycolic acid[a]	0.03	80
4	Mesoxalic acid[a]	0.04	80
5	Oxalacetic acid[a]	0.05	81
6	Diketosuccinic acid[b]	0.06	85
7	α-Ketoglutaric acid[a]	0.08	83
8	Hydroxypyruvic acid[b]	0.20	77
9	Glyoxylic acid I[a]	0.30*	88
10	Pyruvic acid[a]	0.30*	80
11	β-Thiopyruvic acid[a]	0.40**	—
12	Acetoacetic acid[a]	0.40	68
13	Glyoxylic acid II[a]	0.45	75
14	α-Ketobutyric acid[a]	0.50	76
	Acetone Acetaldehyde Formaldehyde [a]	0.90–0.95	—

a = mono-DNPH derivative.
b = bis-DNPH derivative.
% Recovery = the amount of DNPH derivative recovered after chromatography, elution, etc. A mixture containing one micromole of each keto acid was analyzed.

* The distinction between glyoxylic acid I and pyruvic acid is carried out by separation of the two amino acid components of the product of catalytic reduction (see text).

** β-Thiopyruvic acid can be identified by a color reaction given by –SH compounds, as described by BENESCH, BENESCH, GUTCHO AND LAUFER (*Science*, in press), which consists of dipping the paper into 0.05 *M* N-ethyl maleimide (in absolute *iso*propanol), drying, followed by treatment with 0.05 *M* KOH (also in *iso*propanol). Red color appears at the site of the –SH compound.

Although several operations are involved (elution, extraction, etc.), the loss in DNPH derivatives is not serious (Table I) and recoveries are between 78 to 90 %. The optimal range of spectrophotometric analysis of DNPH derivatives is shown in Figs. 1 and 2. Identity of the *cis* and *trans* isomers of glyoxylic acid was proven by the fact that both components yielded glycine upon catalytic reduction. Repeated chromato-

TABLE II

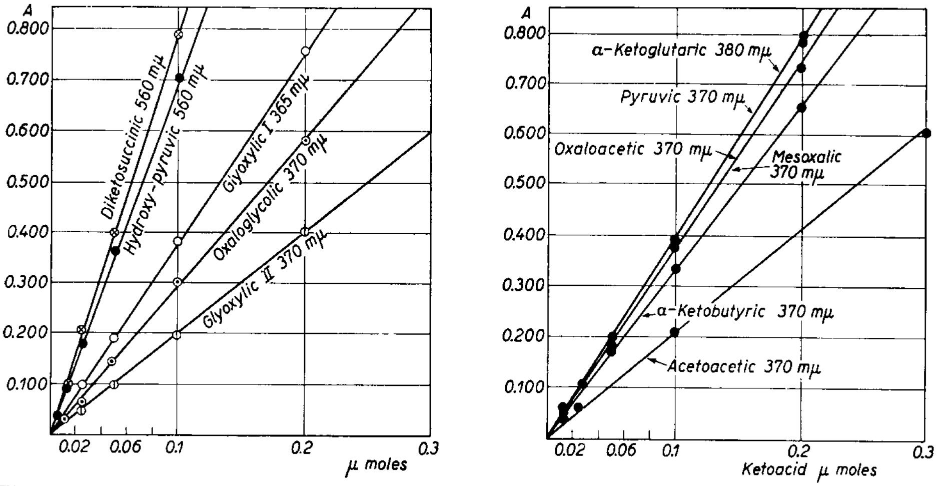
FURTHER SEPARATION OF SOME DNPH DERIVATIVES BY RECHROMATOGRAPHY

Duration of rechromatography = 60 hours.

Compound	R_P*
Oxaloglycolic acid	0.21
Mesoxalic acid	0.27
Oxalacetic acid	0.33
α-Ketoglutaric acid	0.44

* R_P = relative position, *i.e.* distance from the zero line divided by the length of the chromatogram.

graphy of closely related DNPH derivatives like one of the isomers of glyoxylic acid and of pyruvic acid may separate the two dinitrophenylhydrazones, but unquestionable identification is achieved by chromatography of the products of catalytic reduction, in which case glycine and alanine readily separate. Since the conversion of the DNPH derivatives of glyoxylic and pyruvic acids to amino acids is quantitative, their amount can be determined by spectrophotometric analysis of the colored product of amino acids, developed by the ninhydrin–$CdCl_2$ reagent of BARROLIER[18].



Figs. 1 and 2. Correlation between absorbancy (*A*) and the amount of DNPH derivatives, as measured in the Beckman spectrophotometer (1 cm light path) at a wave length of maximum light absorption. All mono-DNPH derivatives are dissolved in 5 ml of 0.1 *M* phosphate buffer of pH 7.3 while bis-DNPH derivatives are dissolved in 5 ml of 1 *N* NaOH.

Identification of the DNPH derivatives of formaldehyde, acetaldehyde and acetone

This was done by converting these compounds to the corresponding amines by catalytic reduction. Since the resulting amines are volatile, significant loss is encountered during evaporation of the solvent, *i.e.* glacial acetic acid. This can be prevented by the addition of 0.1 to 1 ml of concentrated HCl to the samples before evaporation. Good chromatographic separation of amines is readily achieved with *n*-butanol–acetic acid–water[16] as developing solvent. Ninhydrin dissolved in butanol reacts with the amines so far tested (methyl-, ethyl- and *iso*propylamine) but BARROLIER's reagent[18] does not.

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The combination of these procedures was extensively used in biochemical studies, dealing with the enzymic oxidation of certain hydroxy acids[19]. References to preparative methods of various keto acids are also listed in this paper.

SUMMARY

A systematic analysis of keto acids and other carbonyl compounds in biological material was based on the following procedures: (1) their conversion to DNPH derivatives under mild conditions, (2) preliminary chromatographic separation into acidic and non-acidic DNPH derivatives, (3) isolation of each component by paper chromatography and (4) identification by means of rechromatography and catalytic reduction to the corresponding amino derivative. The method is quantitative for keto acids and its accuracy is between 10–20%.

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THE QUANTITATIVE DETERMINATION OF AMINO ACIDS BY PAPER CHROMATOGRAPHY*

A SOLVENT TO REPLACE PHENOL

M. WOLFE

Botany Department, University College, London (England)

Water-saturated phenol, used in an ammoniacal atmosphere, has been found to be a satisfactory first solvent for two-dimensional qualitative separations of amino acids on paper chromatograms, for which *n*-butanol/glacial acetic acid/water (the organic layer of a mixture of 4:1:5 parts by volume) is used as the second solvent. To obtain

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