QUANTITATIVE DETERMINATION OF KETO-ACIDS BY PAPER PARTITION CHROMATOGRAPHY

by

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From a biological point of view keto-acids are not a homogeneous group of compounds, and each one of them has different characters and functions. This is the reason why their determination *in toto* is of a limited use, and why biochemists have always looked for new methods which would make them able to detect separately every single one of these compounds. An attempt at solving this problem was carried out by CAVALLINI, FRONTALI AND TOSCHI[1,2], by using partition chromatography on filter paper of the 2,4-dinitrophenylhydrazones of these compounds.

The first results were satisfactory and the method, with minor modifications in some cases, was adopted by various workers[3–6]. In this paper a simplified method for the determination of keto-acids is reported; special attention was paid to the sensitivity and accuracy of the quantitative determination of pyruvic and α-keto-glutaric acid in biological material.

QUALITATIVE ANALYSIS

Fig. 1 gives the different positions of the yellow spots of the 2,4-dinitrophenylhydrazones of various keto-acids. The paper used was Schleicher-Schüll 2043b and the set of solvents was: *n*-butanol/ethanol/water (40/10/50 by volume). The synthetic phenylhydrazones were dissolved in phosphate buffer (0.01 *M*, pH 7.2) or in 1 *N* NH_3. Descending chromatography was used.

We want to discuss here some questions about the identification of these compounds on the basis of their chromatographic position, which, also in the most recent works on this subject, are not yet completely clear. When the synthetic phenylhydrazones are dissolved and put directly on the filter paper,

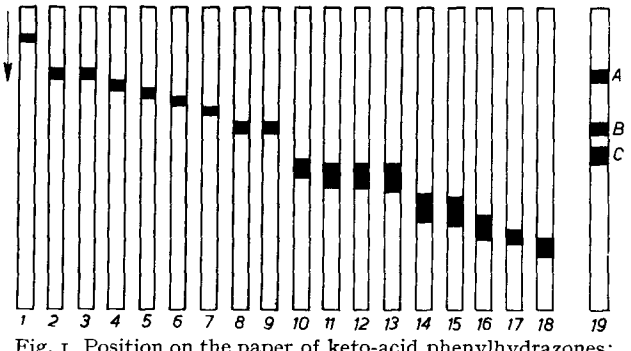

Fig. 1. Position on the paper of keto-acid phenylhydrazones: 1. oxalosuccinic; 2. α-ketoglutaric; 3. β-ketoadipic; 4. oxaloacetic; 5. glyoxylic; 6. α-ketoadipic; 7. mesoxalic; 8. pyruvic; 9. α-keto-γ-carboxyvalerolactone; 10. dimethylpyruvic; 11. α-keto*iso*valeric; 12. acetacetic; 13. α-ketobutyric; 14. α-keto-γ-methiobutyric; 15. succinic semialdehyde; 16. levulinic; 17. phenylpyruvic; 18. *p*-oxyphenylpyruvic; 19. A chromatogram from rat blood: A α-ketoglutaric, B pyruvic, C pyruvic (isomer).

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each one of them gives one single spot as is shown in the figure. But if we start from the keto-acids themselves instead of from their phenylhydrazones, and we submit them to the procedures for their transformation in phenylhydrazones and for the extraction of the latter (with the method described in the present work or with the method previously described[2]), the results are not completely the same as before: some compounds give rise to more than one spot on the paper.

This happens in particular in the case of pyruvic acid: the first spot has the same R_F as the synthetic phenylhydrazone, the second one has a higher R_F. We tried to find out the reason of such a different behaviour, and we first thought of a separation of the pyruvic acid into two isomers. We therefore tried a fractionated distillation of pyruvic acid *in vacuo*, but each fraction gave us the same two spots. We also prepared some isomers and polymers of pyruvic acid, as β-pyruvic acid and α-keto-γ-carboxy-valerolactone, but they both showed a different R_F from that of the two spots of pyruvic acid. We then sought for some evidence of the hypothesis, which is suggested also by SELIGSON AND SHAPIRO[4], of an isomerisation of the molecule of the phenylhydrazone itself, which has a double bond between C and N and which therefore may undergo a *sin-anti* isomerisation. A certain amount of the synthetic phenylhydrazone of pyruvic acid was dissolved in phosphate buffer and divided in two portions. The first portion, put directly on the paper, gave one single spot. The second portion was submitted to the extracting procedures and chromatographed: it gave two spots. It is clear from these results that the isomerisation takes place during the process of extraction of the phenylhydrazone. The spectrophotometric analysis of the two forms, dissolved in 1 *N* NaOH, showed that while the first spot has the same curve as the synthetic phenylhydrazone of pyruvic acid, the second spot gives a quite different curve.

Another keto-acid which gives more than one spot is oxaloacetic acid, while its phenylhydrazone gives only one. Two new spots appear when the keto-acid is extracted, one with a lower and one with a higher R_F, but the process is probably due to another reason, probably to the decomposition of the instable oxaloacetic acid. Of the other keto-acids tried, only glyoxylic acid gave also two spots.

For the quantitative analysis of pyruvic acid, we have put together the two spots, not taking into account the small error due to a different absorption curve of the two compounds. This is the reason why our present results give a higher content of pyruvic acid in biological materials than with the previously described method.

QUANTITATIVE ANALYSIS OF KETO-ACIDS IN BIOLOGICAL MATERIALS

Deproteinization. The most suitable deproteinizer for our purposes was found to be tungstic acid, as it reduced to a minimum the disturbing formation of emulsions during the ether extraction. The tungstic acid solution is prepared just before use by mixing: water, 10% Na tungstate, 2/3 *N* sulphuric acid (60/20/20 by volume). The proportion between biological material and deproteinizing solution is 1:5 (w/v for solid tissues and v/v for blood). The organs are homogenized by a Waring blendor with the calculated amount of deproteinizing solution, or ground in a mortar with quartz sand. After 10' the liquid is filtered through filter paper and a portion of the filtrate is used for the analysis.

Reaction with dinitrophenylhydrazine. 20–30 ml of the filtrate are put in a 50 ml test tube of 2.5 cm diameter, provided with a well fitting ground stopper. 1 ml of 0.2%

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2-4-dinitrophenylhydrazine in 2 *N* HCl is added, and the solution left for 20′ at room temperature (FRIEDEMAN AND HAUGEN[7]).

Extraction with ether. We replaced the ethyl acetate used in the previous works with ethyl ether, because the phenylhydrazones are better extractable in this solvent[7], and it can be evaporated at a lower temperature. In the same test tube where the reaction with phenylhydrazine occurred, 5 ml of ethyl ether are added: the tube is closed and shaken gently by hand. If attention is paid not to open the tube until the liquid wetting the stopper has run down, the loss of substance is immaterial. After separation of the two layers, the supernatant is removed by using a pipette with a rubber teat, and put in a similar tube. This operation is repeated 4 or 5 times, with fractions of 3–5 ml of ether, till no more yellow substances are extracted. Sometimes the separation of the two layers is not perfect, perhaps owing to a not complete deproteinization; it is then necessary to centrifuge the mixture.

Removal of ether and drying of the residue. The ether solution is brought to dryness on a warm water bath, at reduced pressure (water pump). The last traces of water are removed with a warm air stream or in a vacuum desiccator over $CaCl_2$ and KOH.

Partition between chloroform and ammonia. To the dry residue an exactly measured amount of 1 *N* ammonia is added, for instance 1 ml. The walls of the test tube are accurately washed with the liquid, so as to bring in solution the highest quantity of the dried substance. Then equal amount of chloroform is added; the test tube containing the mixture is thoroughly shaken and its walls once again accurately washed. The mixture is transferred then in a small centrifuge tube and spun: the supernatant ammonia solution contains the phenylhydrazones of keto-acids and is used for chromatography, whereas the chloroform contains phenylhydrazine and almost all the neutral phenylhydrazones.

Chromatographic separation. A measured amount of the ammonia solution (usually 0.2 ml) is put on a strip of paper two or more cm wide. Best results were obtained with the paper Schleicher-Schüll 2043b and the solvent: *n*-butanol, ethanol and water (40/10/50 by volume).

Quantitative determination. The paper containing the spot is cut to pieces with the scissors and put in a centrifuge tube. A measured amount of 1 *N* NaOH is added (8 ml if Coleman, 4 ml if Beckmann spectrophotometer is used for the reading) and the paper is completely homogenized by stirring it with a glass rod. After 10′ the mixture is centrifuged; the clear supernatant solution is decanted and its light absorption determined.

Wavelength. Phenylhydrazones dissolved in NaOH have characteristic spectrophotometric curves which can be used for their identification[8]. The maximum absorption lies usually between 400 and 450 mμ, however, we prefer to read at 510 mμ, firstly because any possible traces of phenylhydrazine do not absorb at this wavelength, and secondly because near the spots of pyruvic acid traces of another phenylhydrazone, which absorbs at a lower wavelength, sometimes appear.

The filter paper, treated similarly with NaOH, gave a very slight absorption under 400 mμ, above this wavelength the absorption was zero.

Standard curve. A standard curve is drawn of the phenylhydrazones dissolved in 1 *N* NaOH, and, as the curves are straight between 10 and 50 μg, that is with the quantities most commonly used, a conversion factor can be calculated. Instead of using the synthetic phenylhydrazones, it is preferable to start from known amounts of keto-

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acids in acid solution, to extract them, to run them chromatographically, and to draw the standard curve of the phenylhydrazone extracted from the paper. In this way the curve includes the loss of substance which inevitably happens during the extraction from aqueous solutions, and the recovery of the determined keto-acids is some 6% higher. The values given below are calculated on the basis of this second curve.

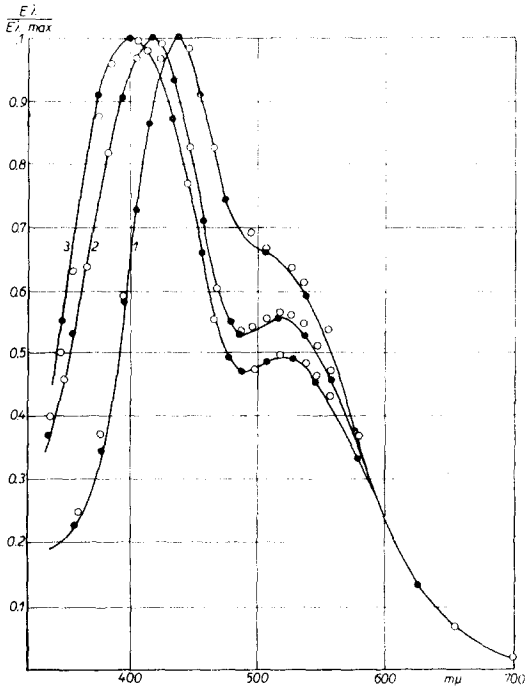

Fig. 2. 1. Black circles: spectrophotometric curve of the first of the two spots of pyruvic acid hydrazone, obtained by extraction of the synthetic keto-acid from an aqueous solution. White circles: spectrophotometric curve of the corresponding spot obtained from blood. 2. Black circles: spectrophotometric curve of the second of the two spots obtained from synthetic pyruvic acid. White circles: spectrophotometric curve of the corresponding spot obtained from blood. 3. Black circles: spectrophotometric curve of the spot obtained by extraction of synthetic α-ketoglutaric acid from an aqueous solution. White circles: spectrophotometric curve of the corresponding spot obtained from blood.

Recovery starting from a known solution of pyruvic and α-ketoglutaric acid. The substances used were: Na pyruvate and α-ketoglutaric acid, and their purity, estimated by the method of LONG[9], was respectively 97.3 and 98.9%. The results are reported in Fig. 3: the optical density of known amounts of the two keto-acids extracted from aqueous solutions using the described method are compared with the optical density of corresponding amounts of synthetic phenylhydrazones. The recovery averages 94%.

Recovery of known amounts of keto-acids from biological materials. The recovery of pyruvic and α-ketoglutaric acids from deproteinized rat blood and liver homogenates were tried. 5–10 ml of rat blood were added to 5 volumes of the deproteinizing solution; 10–20 g of rat liver were homogenized in a Waring blendor (semimicro) with the same proportion of the deproteinizing solution. The filtrate was divided in two equal portions; to one of these a known amount of keto-acid was added, while the second was used

TABLE I

Experiment	*No. of experiments*	*μg of keto-acid added*	*Mean recovery %*	*Standard error*
Recovery of added pyruvic acid from rat blood	8	15–20*	90.8	± 2.1
Recovery of added pyruvic acid from rat liver	7	13–20	92.4	± 2.52
Recovery of added α-ketoglutaric acid from rat blood	7	22–27	96.0	± 1.09
Recovery of added α-ketoglutaric acid from rat liver	7	22–27	97.4	± 0.86

* Minimum — maximum amount added.

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to determine the amount of the same keto-acids already present in the filtrate. All the determinations were carried out in duplicate: the differences observed on the average were within 1%. The means of the recoveries are given in Table I.

PYRUVIC AND α-KETOGLUTARIC ACID CONTENT OF RAT BLOOD, MUSCLE, LIVER AND BRAIN

Pyruvic and α-ketoglutaric acids are certainly the two most abundant keto-acids in the biological material that we have studied (however, sometimes other spots appear, which have spectrophotometric curves characteristic of phenylhydrazones; these spots sometimes are difficult to separate from those of pyruvic and α-ketoglutaric acid). We

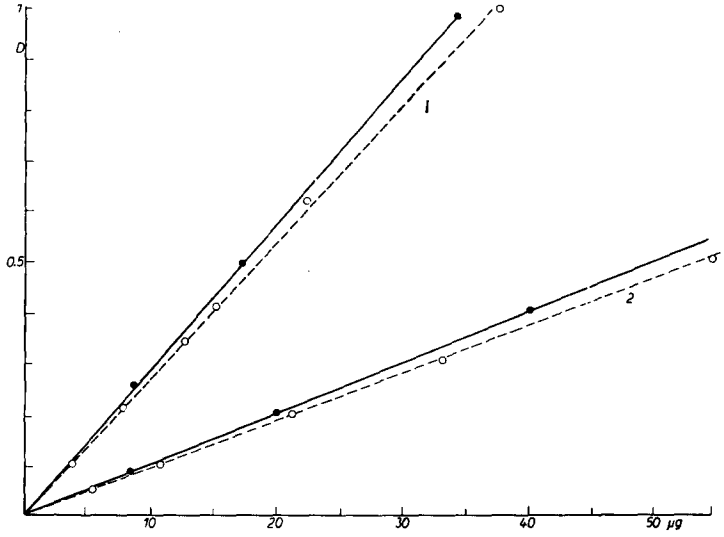

Fig. 3. ——— Standard curves from known solutions of pyruvic (1) and α-ketoglutaric (2) acids dinitrophenylhydrazones in *N* NaOH; – – – – Standard curves from known amounts of pyruvic (1) and α-ketoglutaric (2) acids extracted, chromatographied and eluted from paper.

investigated whether the three spots which appear in chromatograms from biological material, as for instance rat blood, have the same spectrophotometric curves as a mixture of pyruvic and α-ketoglutaric acids submitted to the same procedures. As is shown in Fig. 2 the curves are perfectly identical.

The quantitative analysis was carried out as follows. For each determination two rats of about 100 g were used; they were killed by stunning, and the blood was collected from the jugular veins in a beaker containing the deproteinizing solution, and weighed. The brain, the liver and part of the hind legs were quickly removed and immediately frozen between two blocks of solid CO_2. The brain and liver were then weighed and homogenized in a Waring blendor with 5 volumes of deproteinizing solution. After 10' the four samples were filtered and a known amount of the filtrate was treated with phenylhydrazine, extracted and run chromatographically with the above described method. The results of seven of these determinations are given in Table II. The last

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two determinations were obtained from rats anesthetized with ether and bled: the values are not much different from those from rats killed by stunning, and therefore we have included them in the same table.

TABLE II

No.	*Liver*	*Muscle*	*Blood*	*Brain*
	Pyruvic acid content of rat organs (μg in 100 g)			
1	183	146	1465	585
2	92.5	126	2160	543
3	256.6	467	2086	1460
4	142.5	347.5	2480	790
5	417	—	3000	2330
6	304	538	1830	614
7	173	388	2170	364
mean	224	335	2170	955
	α-ketoglutaric acid content of rat organs (μg in 100 g)			
1	142.5	90.5	480	330
2	68.5	44.2	832	164
3	264	—	585	468
4	104	80.7	552	231
5	—	—	578	233
6	—	—	937	—
7	166	187	1090	281
mean	149	100.6	722	284.5

SUMMARY

A simplified method for the quantitative determination of keto-acids by means of paper chromatography of their 2,4-dinitrophenylhydrazones is reported.

1. The identification of these compounds on the filter paper is discussed, especially of those compounds which give more than one spot.

2. The method is described in detail, especially with regard to the pyruvic and α-ketoglutaric acid content in biological material. The recovery of these two keto-acids, starting from known aqueous solutions, averages 94 %; on the basis of these results a standard curve can be drawn. According to this curve, recovery from deproteinized extracts of liver and blood averages 91.6 % for pyruvic acid and 96.7 % for α-ketoglutaric acid.

3. Using this method the pyruvic and ketoglutaric acid content of rat blood, muscle, liver and brain is estimated.

RÉSUMÉ

Les auteurs exposent une méthode simplifiée pour la détermination des acides cétoniques au moyen de la chromatographie sur papier filtre de leurs dinitrophenylhydrazones.

1. On a trouvé que certains composés donnent lieu à plus d'une tache à cause de l'isomérisation du derivé phenylhydrazone. On a étudié en particulier le dédoublement du phenylhydrazone de l'acide pyruvique.

2. On expose en détail la technique de la détermination chromatographique des acides cétoniques dans les milieux biologiques, en particulier pour ce qui régarde l'acide pyruvique et α-cétoglutarique. Ces deux acides cétoniques sont recouvrés à partir de solutions aqueuses avec une moyenne de 94 %. Une courbe standard peut être établie de ces données. D'après cette courbe le rendement à partir du foie et du sang de rat déprotéinisés est en moyen 91.6 % pour l'acide pyruvique et 96.7 % pour l'acide α-cétoglutarique.

3. En utilisant cette méthode les auteurs ont déterminé le contenu en acides pyruvique et cétoglutarique du sang, muscle, foie et cerveau de rat.

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ZUSAMMENFASSUNG

Es wird über eine vereinfachte Bestimmungsmethode für Ketosäuren durch Papierchromatographie der bezüglichen Dinitrophenylhydrazonen berichtet.

1. Die Identifizierung dieser Stoffe auf Grund ihrer chromatographischen Lage wird unter besonderer Berücksichtigung jener Stoffe, die mehr als einen Fleck erzeugen erörtert.

2. Die technischen Einzelheiten der quantitativen chromatographischen Bestimmung von Ketosäuren in biologischen Stoffen werden unter besonderer Berücksichtigung von Brenztraubensäure und Ketoglutarsäure erläutert. Die Rückgewinnung dieser zwei Ketosäuren aus bekannten wässerigen Lösungen beträgt ungefähr 94 %; auf Grund dieser Daten kann man eine Standardkurve aufbauen. Nach dieser Kurve, ergibt sich die Rückgewinnung aus enteiweissten Extrakten von Leber und Blut durchschnittlich zu 91.6 % für die Brenztraubensäure, und zu 96.7 % für die Ketoglutarsäure.

3. Bei Verwendung dieser Methode wurde der Gehalt an Brenztraubensäure und Ketoglutarsäure von Blut, Muskel, Leber und Gehirn von Ratten bestimmt.

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