PAPER CHROMATOGRAPHIC DETERMINATION OF AROMATIC α-KETO ACIDS

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INTRODUCTION

Several methods concerning paper chromatography of α-keto acids have been published[1], but most of these methods have been worked out only for aliphatic α-keto acids.

Mixtures of free aromatic α-keto acids are inadequately separated by paper chromatography, each acid showing several spots on the chromatogram due to keto–enol tautomerism[2,3] and destructive oxidation by atmospheric oxygen. Paper chromatography of 2,4-dinitrophenylhydrazones of α-keto acids was introduced by CAVALLINI, FRONTALI AND TOSCHI[4], and several modifications of this method for the determination of aliphatic α-keto acids have been published. Multiple spots are, however, often obtained on paper chromatograms of α-keto acid 2,4-dinitrophenylhydrazones owing to the separation of *cis trans* isomers[5,6], and so a quantitative determination of aromatic α-keto acids by these methods was not possible.

3-Alkylquinoxalinols formed by condensation of α-keto acids with *o*-phenylenediamine are stable compounds easy to separate by paper chromatography in alkaline solvent systems, as shown by HOCKENHULL AND FLOODGATE[7]. This principle was adapted by TAYLOR AND SMITH[8] for the determination of aliphatic α-keto acids in blood, using 1,2-diamino-4-nitrobenzene as a 3-alkylquinoxalinol-forming reagent. Owing to the instability of the free aromatic α-keto acids in solution, and to the inertness of these acids in forming quinoxalinols as compared with the aliphatic α-keto acids, the method of TAYLOR AND SMITH is not generally applicable to aromatic α-keto acids.

In the present paper a method for the quantitative determination of phenylpyruvic acid, *o*-hydroxyphenylpyruvic acid, *p*-hydroxyphenylpyruvic acid and (3-indolyl)-pyruvic acid is described. Decomposition of the free acids by oxidation is avoided by using NaHS as an antioxidant, and the free acids are converted to quinoxalinols by condensation with *o*-phenylenediamine. After paper chromatography the amount of each quinoxalinol is determined by U.V.-spectrophotometry. The validity of the procedure, which may be of importance for the determination of aromatic α-keto acids in urine from patients with abnormal metabolism of phenylalanine, tyrosine and tryptophan[13–16], is indicated by tests with solutions of different α-keto acids of known concentration.

TABLE I

DATA FOR 3-ALKYLQUINOXALINOLS SYNTHETIZED FROM α-KETO ACIDS AND *o*-PHENYLENEDIAMINE

α-Keto acids	*3-Alkylquinoxalinols*	*M.p. (°C)*	*Analysis of the quinoxalinols*					
			% H		*% C*		*% N*	
			Exptl.	*Theor.*	*Exptl.*	*Theor.*	*Exptl.*	*Theor.*
PPA	3-Benzylquinoxalinol (BQ)	202.5	5.26	5.14	76.7	76.8	12.1	11.9
*o*HPPA	3-(*o*-Hydroxybenzyl)-quinoxalinol (*o*HBQ)	230.0	4.60	4.80	71.9	71.5	11.2	11.2
*p*HPPA	3-(*p*-Hydroxybenzyl)-quinoxalinol (*p*HBQ)	246.0	5.06	4.80	71.3	71.5	11.1	11.2
2,5DHPPA	3-(2,5-Dihydroxybenzyl)-quinoxalinol (2,5 DHBQ)	259–260	4.63	4.52	65.2	67.2	9.5	10.5
IPA	3-((3-Indolyl)methyl)-quinoxalinol (IMQ)	232	4.62	4.78	73.4	74.3	15.1	15.3
α-Ketoglutaric acid	3-(β-Propionic acid)-quinoxalinol (βPQ)	273.5						
Pyruvic acid	3-Methylquinoxalinol (MQ)	246.5						

EXPERIMENTAL*

Preparation of reference substances

Aromatic α-keto acids. Phenylpyruvic acid was prepared by hydrolysis of α-acetaminocinnamic acid in 1 *N* HCl[9]; *o*-hydroxyphenylpyruvic acid, *p*-hydroxyphenylpyruvic acid and 2,5-dihydroxyphenylpyruvic acid were prepared by alkaline hydrolysis of the corresponding azlactone-derivatives[10,11], and (3-indolyl)-pyruvic acid was prepared from tryptophan by the method of SHAW *et al.*[12].

3-Alkylquinoxalinols. A mixture of 10 mmoles α-keto acid dissolved in 20 ml 96% ethanol and 1.2 g *o*-phenylenediamine dissolved in 20 ml 50% acetic acid in a stoppered tube is placed in a boiling water bath for 1 h. *o*-Hydroxyphenylpyruvic acid and 2,5-dihydroxyphenylpyruvic acid in their lactone form do not react with *o*-phenylenediamine by this procedure. Opening of the lactone-ring is accomplished by boiling the ethanolic solutions with 15 mmoles NaOH for 5 min under N_2 and neutralizing with 15 mmoles HCl. The precipitated quinoxalinols are filtered off after cooling, washed with 50% ethanol and crystallized once from 70% ethanol. The melting points and elementary composition of the 3-alkylquinoxalinols thus prepared are given in Table I.

Reagents for the paper chromatographic analysis

o-Phenylenediamine solution, 5% (w/v), in water, is prepared just before use (Merck, p.a.).

Sodium hydrogen sulphide solution is prepared by mixing equal parts of 1 *M* aqueous Na_2S solution and 1 *N* HCl (Merck, p.a.). Aromatic α-keto acids in solution

TABLE II

SEPARATION AND DETECTION OF 3-ALKYLQUINOXALINOLS BY PAPER CHROMATOGRAPHY

Solvent systems: S_1 = Methyl ethyl ketone–aq. 0.1 *N* NaOH (20:1, v/v). Used in an atmosphere equilibrated with 5 *N* aqueous NH_3.
S_2 = Methanol–chloroform–aq. 0.1 *N* NaOH (5:5:1, v/v).
S_3 = Ethanol–aq. 0.1 *N* NaOH (10:1, v/v).
S_4 = Ethanol–chloroform–aq. 0.1 *N* NaOH (5:5:1, v/v).
Paper: Whatman No. 20, sprayed with 0.1 *N* NaOH and dried immediately before use (descending technique).
Detection: By U.V. light (main emission about 350 mμ).

Quinoxalinols[a]	$R_F \times 100$				Colour (fluorescence)
	S_1	S_2	S_3	S_4	
BQ	78	78	72	80	light blue
*o*HBQ	60	67	70	62	grey-blue
*p*HBQ	63	44	62	52	dark blue
IMQ	80	69	68	75	yellow
MQ	38	55	61	50	light blue
βPQ	0	8	42	4	light blue

[a] For abbreviations see Table I.

* Abbreviations used: PPA = phenylpyruvic acid; *o*HPPA = *o*-hydroxyphenylpyruvic acid; *p*HPPA = *p*-hydroxyphenylpyruvic acid; 2,5DHPPA = 2,5-dihydroxyphenylpyruvic acid; IPA = (3-indolyl)-pyruvic acid. The abbreviations BQ, *o*HBQ, *p*HBQ, 2,5DHBQ, IMQ, MQ, and βPQ are used for the quinoxalinols, see Table I.

are protected against oxidation by addition of about 1 part of this reagent to 4 parts of the α-keto acid solution.

The solvent systems suitable for one- or two-dimensional paper chromatography of six 3-alkylquinoxalinols are given in Table II.

The chromatography paper, Whatman No. 20 (with a very slow flow rate) is sprayed immediately before use with 0.1 *N* NaOH and dried in a current of air.

Method

The paper chromatographic separation and quantitative determination of PPA, *o*HPPA, *p*HPPA and IPA* as their corresponding 3-alkylquinoxalinol derivatives is carried out as follows:

(1) 10 ml of an aqueous solution or urine specimen containing 0.1–2.0 μmoles of each aromatic α-keto acid and about 1 mmole NaHS is used for each determination.

(2) Immediately before the addition of *o*-phenylenediamine, 6 *N* HCl is added, bringing the H^+ concentration to about 0.15 *M*. 0.5 ml *o*-phenylenediamine solution is now added, and the mixture is heated to 90° for 45 min on a water bath. The reaction should be carried out in a hood because of the formation of H_2S.

(3) After cooling, the precipitated sulphur and impurities are removed by centrifugating, and the supernatant transferred to a glass-stoppered tube for extraction. The precipitate is washed twice with 1 ml methanol–ethyl acetate mixture (1:1) and the washings are added to the supernatant.

(4) The supernatant is extracted 3 times with ethyl acetate (5 ml, 2 ml and 2 ml), and the combined extracts are washed first with 5 ml 1 *M* aqueous sodium hydrogen carbonate solution and then with 3 ml water.

(5) The ethyl acetate phase, containing the quinoxalinols, is evaporated to dryness under reduced pressure in a tube (100 mm × 30 mm ø), the bottom of which is shaped as a reservoir (15 mm × 7 mm ø). The whole of the remainder is transferred to the reservoir by repeated washing of the inner wall of the tube with ethyl acetate, followed by evaporation. The dry residue in the reservoir is dissolved in 200 μl methanol, containing about 3 % ammonia, and an aliquot is transferred to the chromatography paper.

(6) The quinoxalinol mixture is separated by descending paper chromatography (see Table II).

(7) The chromatograms are dried in an air current at room temperature, and the quinoxalinols are made visible in ultra-violet light. The quinoxalinol spots are marked in pencil, cut out, weighed and eluted by rotating gently for 20–30 min in a tube with 5 ml 0.1 *N* NaOH. A paper blank is treated in the same way as the samples. The optical densities of the eluates are measured in a Beckman spectrophotometer at the following wavelengths, corresponding to the light absorption maxima of the quinoxalinols: BQ* and *p*HBQ: 350 mμ; *o*HBQ: 358 mμ; IMQ: 348 mμ.

RESULTS AND DISCUSSION

Paper chromatography of 3-alkylquinoxalinols

Alkaline solvent systems are the most suitable for paper chromatography of the 3-

* See footnote on p. 465.

alkylquinoxalinols, because of the poor solubility of these compounds in acid and neutral aqueous solutions. Several solvent systems were tested, but the best separations of the four quinoxalinols BQ, *o*HBQ, *p*HBQ and IMQ were obtained with the solvent systems given in Table II. The R_F values of MQ and βPQ are also given in Table II, because pyruvic acid and α-keto-glutaric acid are commonly found in blood and urine. Two-dimensional chromatography must be carried out if these six quinoxalinols are to be separated simultaneously.

Chromatography paper with a slow flow rate has proved to be most suitable for the solvent systems given in Table II. The spots tend to streak on chromatograms developed on papers having a faster flow rate. It appears that the treatment of the chromatography paper by spraying with 0.1 *N* NaOH and drying in an air current immediately before use gives the best separations and the most compact spots.

The smallest quantity of a quinoxalinol that can be detected on the paper by U.V.-light after two-dimensional chromatography is about 0.02 μmole when pure quinoxalinols are separated. Fluorescing impurities on the chromatograms may raise the detection limit to about 0.05 μmole, if the quinoxalinols are isolated from urine before chromatography.

Pure 3-alkylquinoxalinols eluted from chromatography paper with 5 ml 0.1 *N* NaOH after one- or two-dimensional chromatography obey Beer's law in the concentration range 0.05–1.00 μmole quinoxalinol per 5.0 ml eluate (E/μmole, see Table III experiment B). The recoveries of the quinoxalinols by chromatography and elution from the paper are also set out in Table III (*cf.* experiments A and B).

Extraction of the 3-alkylquinoxalinols from aqueous solutions

Ethyl acetate is commonly used when extracting aromatic compounds from aqueous solutions. Extraction from urine is often lengthy owing to the formation of a relatively stable emulsion. This is easily avoided by adding 1 ml 10 *N* NaOH to the urine specimen after the quinoxalinol formation (described in point (2) of the method) and heating on a boiling water bath for 5 min. After this alkaline treatment the mixture is neutralized and the quinoxalinols are isolated by extraction and chromatographed as described below.

The quinoxalinol yield by the extraction method is reproducible and independent of the quinoxalinol concentration in the range 0.1–5.0 μmoles per 10 ml aqueous solution (E/μmole, see Table III experiment C).

Quinoxalinol formation

Attempts to use 1,2-diamino-4-nitro-benzene for the formation of 3-alkyl-nitroquinoxalinols with aromatic α-keto acids present at these low concentrations were not successful. On the other hand, the nitroquinoxalinols could be synthetized as reference substances from PPA and *p*HPPA, but not from IPA.

1,2-Diamino-4-methyl-benzene and *o*-phenylenediamine react so readily with the aromatic α-keto acids present at low concentrations, that these compounds could be used as the quinoxalinol-forming reagents in the method here described. Preliminary investigations into the quinoxalinol yield per μmole α-keto acid did not show that either of these reagents offered any advantage over the other and therefore only *o*-phenylenediamine was used in the further development of this method.

It appears from point (2) of the method that the quantity of *o*-phenylenediamine

TABLE III

RECOVERIES OF QUINOXALINOLS AT THE SUCCESSIVE STEPS OF THE PAPER CHROMATOGRAPHIC PROCEDURE

E indicates the extinctions of the quinoxalinols in 5 ml 0.1 *N* NaOH solution (1 cm light path). μmole indicates the quantity of the quinoxalinols or the corresponding α-keto acids. The numbers in parentheses indicate the recoveries in relation to the pure quinoxalinol solution.

Expt.	*Quinoxalinols*	*E*/μmole			
		BQ	*oHBQ*	*pHBQ*	*IMQ*
A	Pure quinoxalinols	1.80 (100%)	1.60 (100%)	2.06 (100%)	1.86 (100%)
B	Pure quinoxalinols separated by two-dimensional paper chromatography and eluted from the paper (points (6) and (7) in the method)	1.75 (97%)	1.55 (97%)	1.87 (91%)	1.80 (97%)
C	Pure quinoxalinols extracted from water with ethyl acetate, separated by two-dimensional paper chromatography and eluted (points (4), (5), (6) and (7) in the method)	1.54 (86%)	1.38 (86%)	1.69 (82%)	1.49 (80%)
D	Quinoxalinols formed from α-keto acids in aqueous solutions and determined by the method (points (1)–(7)) (results from Table IV)	1.03 (57%)	1.12 (70%)	1.18 (57%)	0.86 (46%)
E	Quinoxalinols formed from α-keto acids in urine and determined by method (points (1)–(7)) (results from Table IV)	1.03 (57%)	1.16 (72%)	0.99 (48%)	0.57 (31%)

that must be added to the α-keto acid solutions is 10^2 to 10^3 times greater than is stoichiometrically required for the formation of quinoxalinol. Fig. 2 shows the results of an experiment demonstrating how the quinoxalinol yield per μmole of the four aromatic α-keto acids depends on the quantity of the *o*-phenylenediamine added.

The quinoxalinol yield per μmole α-keto acid depends on the concentration of H^+ during condensation. The optimal concentration range is 0.1 to 0.2 *M* H^+ for quinoxalinol formation with *o*HPPA and IPA and 0.1 to 0.5 *M* H^+ with PPA and *p*HPPA.

The reaction temperature of 90° during the formation of the quinoxalinols was arbitrarily chosen in order to maintain the temperature at a constant high level. Increasing the reaction time to more than the recommended 45 min will not increase the quinoxalinol yield per μmole α-keto acid.

A comparison between the results of experiments C, D and E in Table III gives the yield of the quinoxalinols formed from the corresponding α-keto acids, using the method described in the concentration range 0.167 to 2.0 μmoles α-keto acid per 10ml solution. In spite of their low values the yields are reproducible with reasonable accuracy (see Table IV).

Protection of the aromatic α-keto acids against oxidation

Various inorganic reducing compounds (I^-, HSO_3^-, HS^- and HSe^-) were added to the aqueous solutions of the aromatic α-keto acids to prevent their destruction by atmospheric oxygen, but only hydrogen sulphide was found suitable for this purpose.

To demonstrate the stability of PPA, *o*HPPA, *p*HPPA and IPA in aqueous hydrogen sulphide solutions, 0.25 μmole and 1.00 μmole of these acids were added to

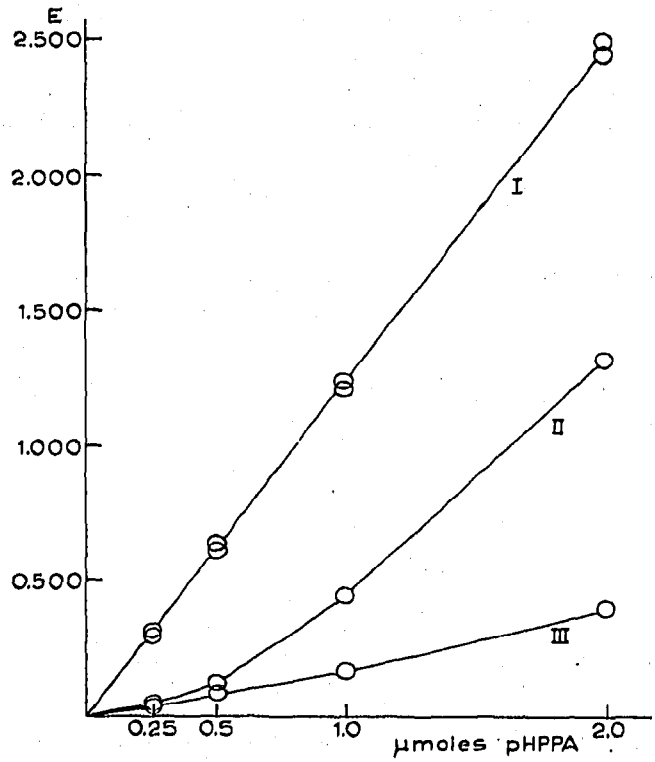

Fig. 1. Calibration curves of *p*HPPA determined as *p*HBQ by paper chromatography. *E* indicates the extinction of *p*HBQ eluted from the chromatography paper by 5 ml 0.1 *N* NaOH. μmole refers to the quantity of *p*HPPA. Curves I, II and III correspond to solutions I, II and III, see text.

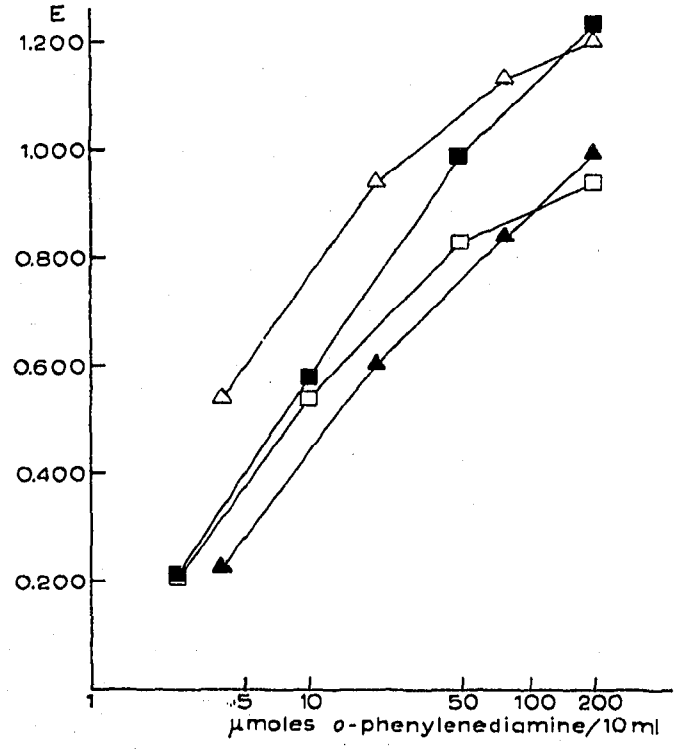

Fig. 2. Relationship between the quantity of quinoxalinol formed and the concentration of *o*-phenylenediamine. *E* indicates the extinction of the quinoxalinols derived from 1 μmole of the corresponding α-keto acids in solutions with different concentrations of *o*-phenylenediamine. ▲—▲, PPA; △—△, oHPPA; ■—■, *p*HPPA; □—□, IPA.

10 ml 0.1 *M* NaHS solutions and stored at 0° in a loosely corked tube. The quantities of the α-keto acids were determined by the procedure described in specimens taken during 7 days. The loss of α-keto acid, which gradually increased with time, was about 10% for PPA, 0% for *o*HPPA, about 12% for *p*HPPA and about 16% for IPA after 7 days.

The antioxidative effect of H_2S during formation of the quinoxalinol is demonstrated by the experiment illustrated in Fig. 1. Different amounts of *p*HPPA were added to three series of aqueous solutions I, II and III. I consisted of 0.1 *M* NaHS solution, II of water almost saturated with N_2 and III of water equilibrated with the atmosphere. The concentration of H^+ was brought to 0.15 *N*, *o*-phenylenediamine was added and the formation and determination of the quinoxalinols was carreid out as described in the method by one-dimensional paper chromatography.

TABLE IV

REPRODUCIBILITY OF THE DETERMINATION OF FOUR AROMATIC α-KETO ACIDS BY ONE- OR TWO-DIMENSIONAL PAPER CHROMATOGRAPHY

Determinations with the same group number are carried out simultaneously. In the determinations numbered 1, 2, 3, 4, 5 and 6 the α-keto acids are added to water, and in determinations numbered 7 and 8 to urine. In determinations 1, 2, 3 and 4 one-dimensional chromatography with solvent system S_1 is used, and in determinations 5, 6, 7 and 8 two-dimensional chromatography with solvent systems S_1 and S_2.

E/μmole is the ratio of the extinction of the 3-alkylquinoxalinols extracted with 5 ml 0.1 *N* NaOH and the quantity of the corresponding α-keto acid added. Each value in columns A represents a duplicate determination. Columns B show the arithmetical mean values of determinations from the same group.

Determination group No.	*Quantity of α-keto acid (μmoles)*	*E*/μmole							
		PPA		*oHPPA*		*pHPPA*		*IPA*	
		A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
1	0.250	1.09	1.05	1.28	1.24	1.27	1.24	0.91	0.91
	1.00	1.01		1.20		1.21		0.92	
2	0.250	1.07	1.06	1.24	1.20	1.27	1.29	0.89	0.89
	1.00	1.05		1.15		1.30		0.90	
3	0.250	1.00		1.16		1.18		0.92	
	0.500	1.00	1.01	1.16	1.15	1.18	1.18	0.95	0.94
	1.00	1.03		1.14		1.18		0.96	
4	0.125	1.03		1.15		1.27		1.08	
	0.250	1.02		1.22		1.24		0.92	
	0.500	0.97	1.01	1.22	1.21	1.27	1.24	0.93	0.95
	1.00	1.02		1.25		1.22		0.91	
	2.00	1.01		1.22		1.24		0.91	
5	0.167	1.04	1.03	1.09	1.12	1.17	1.17	0.89	0.87
	1.667	1.02		1.14		1.16		0.84	
6	0.167	0.99	1.03	1.10	1.12	1.18	1.19	0.86	0.85
	1.667	1.07		1.15		1.19		0.84	
7	0.167	1.07	1.04	1.16	1.15	0.98	0.96	0.57	0.55
	1.667	1.02		1.13		0.93		0.53	
8	0.167	1.08	1.03	1.22	1.16	1.03	1.02	0.61	0.58
	1.667	0.97		1.09		1.01		0.55	

Accuracy of the method

In order to test the accuracy of the method described, aliquots from methanolic stock solutions of PPA, *o*HPPA, *p*HPPA and IPA (10 μmoles/ml) were added to 10 ml samples of water or urine, each containing NaHS in a concentration of 0.1 *M*. The method was carried out on these solutions as described, and the extinction of the eluates was determined. The results expressed as E/μmole are given in Table IV, each value in column A representing the average of a duplicate determination. It appears that the E/μmole values from determinations carried out simultaneously (same group number in Table IV) are independent of the amount of α-keto acid, and that the average values of E/μmole for such experiments can be reproduced with reasonable accuracy, when the paper chromatographic technique used and the solutions to which the α-keto acids are added are identical.

The difference between two single determinations of E/μmole carried out on the same quantity of different α-keto acids does not vary significantly from one keto acid to another or from determination to determination carried out in the same manner. A common standard error on single determinations of E/μmole is therefore calculated for determinations of the four α-keto acids added in the same quantity. The standard errors on single determinations of E/μmole in experiment 1,2, 3 and 4 (Table IV) are 0.04/μmole when the quantity of α-keto acid is 0.25 μmole and 0.02/μmole when the quantity is 1.00 μmole. In experiments No. 5 and 6 the standard errors on single determinations of E/μmole are 0.04/μmole and 0.02/μmole and in experiments No. 7 and 8 they are 0.06/μmole and 0.03/μmole, when the quantities of α-keto acids are 0.167 μmole and 1.667 μmoles respectively.

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SUMMARY

A paper chromatographic method is described for the quantitative determination of phenylpyruvic acid, *o*-hydroxyphenylpyruvic acid, *p*-hydroxyphenylpyruvic acid and (3-indolyl)-pyruvic acid in aqueous solutions or urine. The α-keto acids, protected against oxidation by sodium hydrogen sulphide, react with *o*-phenylenediamine to form 3-alkylquinoxalinols. The 3-alkylquinoxalinols are separated by paper chromatography, eluted from the paper, and the quantities determined by U.V.-spectrophotometry. The successive steps of the method are discussed, and the accuracy of the method is demonstrated by tests with solutions of α-keto acids of known concentration.

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