A STUDY OF THE SEPARATION OF UROPORPHYRIN OCTAMETHYL ESTERS I AND III BY THIN-LAYER AND PAPER CHROMATOGRAPHY

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Several methods are available for the identification of the isomeric uroporphyrin esters; these include (i) chromatography in a dioxan system as described by FALK AND BENSON[1] and CORNFORD AND BENSON[2]; (ii) decarboxylation[3] followed by chromatography in lutidine of the resulting free coproporphyrins according to ERIKSEN[4] or by chromatography of their esters[5]; and (iii) determination of melting point and mixed melting point as was done by NICHOLAS AND RIMINGTON[6]. In this laboratory, work has been confined mainly to the dioxan method and to confirmation by decarboxylation followed by chromatography in lutidine. Within the known limitations of these methods, the results obtained by these two techniques have agreed well, but doubts have remained as to their absolute reliability. It has been clearly demonstrated by several workers[2,3,6,7] that some form of molecular association occurs between the I and III series isomers. Subsequent work in this laboratory (unpublished), showing that the dioxan method is very misleading for mixtures of uroporphyrins I and III when the amount of uroporphyrin III exceeds 65% of the total, has led us to investigate the method further and at the same time to try thin-layer chromatography[8], followed by elution and spectrophotometry of the separated porphyrins. JENSEN[9] separated coproporphyrins I and III by thin-layer chromatography with lutidine–ammonia as the solvent system but there has been no attempt to adapt this useful method to the esters or, more particularly, to the uroporphyrin esters.

This paper describes such an attempt, using a mixture of kerosene and dioxan as the solvent system, and also reports a further investigation of a chromatographic separation on paper.

MATERIALS

Solvents

The solvents were purified and used as described by CORNFORD AND BENSON[2].

Standard reference substances

Uroporphyrin I octamethyl ester (uro I ester), m.p. 293°, was fraction A_1 shown in Fig. 1 of the paper of RIMINGTON AND MILES[10]; further data concerning it are given by RIMINGTON AND SVEINSSON[11]. Uroporphyrin III octamethyl esters

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(uro III ester), m.p. 253–260° and m.p. 264°, were prepared from turacin; decarboxylation and chromatography by the lutidine method showed coproporphyrin III only. Pseudouroporphyrin ester (ψ-uro ester), m.p. 211–216°, was fraction O_4 shown in Fig. 1 of the paper of CANIVET AND RIMINGTON[12] which, on dioxan chromatography, ran to a position in advance of uro III ester and on decarboxylation gave coproporphyrin III only. Uroporphyrin III (SFM 200), uroporphyrin III ex benzene/heptane and uroporphyrin III (SFM 100, 11/81) were synthetic esters prepared by Dr. S. F. MACDONALD and kindly made available by him.

Cellulose powder. Three types of cellulose powder were used. They were (1) cellulose MN300, a standard cellulose powder manufactured by Macherey, Nagel & Co., Germany, and obtained from CamLab (Glass) Ltd., Cambridge; $CaSO_4 \cdot \frac{1}{2}H_2O$ content nil; average particle size $< 10\ \mu$. (2) Cellulose MN300G, a standard cellulose powder, also manufactured by Macherey, Nagel & Co., and containing gypsum, $CaSO_4 \cdot \frac{1}{2}H_2O$, approx. 10%; average particle size $< 10\ \mu$. (3) Cellulose powder, Whatman standard grade.

METHODS

The dioxan method for paper chromatography, including elution, was used exactly as described by CORNFORD AND BENSON[2]. The method for thin-layer chromatography was essentially the same as for paper chromatography with only slight changes in the volumes of the developing solvents. The plates (10 × 20 cm and between 1 and 3 mm thick) were prepared as follows: 15 g cellulose powder was suspended in 100 ml glass-distilled water and mixed using an ultrasonic disintegrator operating at 18,000–20,000 c.p.s., maximum output 6 watts, for 8 min. The resulting slurry was then used for preparing about six plates using a Camag Thin Layer Chromatography Apparatus as designed by MUTTER AND HOFSTETTER. The plates were dried at 100–105° for 1 h and stored in a container over $CaCl_2$ and H_2SO_4 until used. Before use the cellulose was reactivated by heating at 105° for 10 min.

Standard solutions of uro I and uro III esters were prepared as described by CORNFORD AND BENSON[2] at a concentration of 1 μg/10 μl in washed chloroform. Varying ratios of uro I and uro III esters were prepared by mixing appropriate volumes of these standards and all solutions were stored in stoppered tubes at —10°.

After reactivating the cellulose, the plate was allowed to cool and the porphyrin esters applied from a micro-pipette along a base line 1.5 cm from the short edge of the plate, the spots being about 1 cm apart.

Development was carried out by the ascending method at 22–26° in the glass cylinders described by CORNFORD AND BENSON[2].

Chromatography

First run. Atmosphere 10 ml ethanolic chloroform, developing mixture 8 ml kerosene + 12 ml ethanolic chloroform. Development was continued until the porphyrins had moved approximately 2 cm from the base line. Time 3–5 min. The plate was then dried for 5 min under a warm air blast and the position of the spots marked with a 4H pencil (a hard lead was used to avoid contamination when eluting).

Second run. Atmosphere 7 ml dioxan, developing mixture 24 ml kerosene + 9 ml dioxan. Development was continued until the solvent front had moved about 14 cm

from the base line. Time 1.5 h. The plate was dried for 20 min by a warm air blast and the final position of the spots marked.

Elution of the porphyrin. The fluorescent spots were scraped off the plate with a small spatula on to a small piece of extra-smooth tracing paper and transferred to stoppered tubes. Freshly washed chloroform (3 ml) was added and, after mixing thoroughly, the tubes were allowed to stand for 30 min. Each sample was then filtered through Whatman No. 1 paper into a 5 ml measuring cylinder, the precipitate being washed with washed chloroform, and the volume adjusted to 3 ml. The solutions were stored at —10°, and the extinctions of the chloroform solutions measured in a Unicam S.P. 500 photoelectric spectrophotometer in a 1-cm light path on the following day. The ester content of the eluates was calculated from the following expression[2]:

$$\mu g \text{ uro ester} = [2D_{405-6} - (D_{380} + D_{430})] \times 2.721 \times V$$

where

V = volume of solution

D = extinction at wavelength indicated by the subscript.

To check for possible loss by retention on the cellulose, 1.0 μg of the standard uro III ester was added to each of four tubes containing 3 ml washed chloroform. Into two of the tubes cellulose MN300 or cellulose MN300G was added equal in amount to that which would be scraped off the plate for elution. Each tube was filtered as described above and the ester content in the eluate calculated. Recoveries of 87–96% were found.

A preliminary survey using all three cellulose powders showed cellulose MN300G to be the most suitable and all results reported here were obtained with this type of cellulose powder.

RESULTS

Thin-layer chromatography

Preliminary thin-layer chromatograms using 100% uro I, 100% uro III and a 50:50 mixture of these two esters, established that a kerosene–dioxan ratio of 4:1.5 was the most suitable for achieving a good separation based on uro I:uro III recoveries by elution. Table I shows the results obtained by running a series of mixtures

TABLE I

SEPARATION OF VARYING AMOUNTS OF A RANGE OF RATIOS OF URO I AND III ESTERS BY THIN-LAYER CHROMATOGRAPHY

Ratio uro I:uro III applied	*Ratio uro I:uro III found by elution*					
	0.3 μg	0.5 μg	0.6 μg	0.7 μg	0.8 μg	0.9 μg
0:100	0:100	0:100	2:98	35:65	10:90	49:51
10:90	0:100	4:96	0:100	35:65	18:82	40:60
20:80	0:100	0:100	3:97	35:65	9:91	41:59
25:75	0:100	0:100	3:97	38:62	18:82	34:66
50:50	4:96	16:84	22:78	50:50	45:55	53:47
75:25	16:84	38:62	54:46	69:31	59:41	71:29
90:10	29:71	66:34	78:22	80:20	80:20	84:16
100:0	55:45	76:24	75:25	84:16	84:16	92:8

of uro I and uro III esters in known proportions and using kerosene–dioxan (4:1.5), for varying amounts of total porphyrin applied.

As with the paper technique, an interdependence was found between mobility of uro III ester and the amount applied; the greater the quantity of pure uro III ester placed initially upon the spot, the greater the amount which occupied the uro I position after development. This is illustrated in Table I. A 50:50 mixture of I and III esters could be satisfactorily separated (*i.e.* equal distribution of the porphyrin between the I and III position) by making the initial total application 0.7 μg. When, however, 0.7 μg of uro III ester was run under these same conditions, approximately 35% of the total moved no further than the uro I ester position. This is strikingly similar to our experience with paper chromatography.

Alternatively, if the amount of porphyrin applied is decreased so that pure uro III ester moves completely to the III position, then the 50:50 I:III mixture also moves mainly to the III position. Correction by the nomogram of CORNFORD AND BENSON[2] did not significantly alter the result at those points where the percentage of uro I ester was below 50%, but it did appear to improve those where the amount of uro I ester present was 50% and over.

Paper chromatography

Experience with the paper chromatographic method of uro ester separation has shown considerable variation in the behaviour of uro III ester specimens, in part at least accountable for by the variation in the total amount applied as a spot. However, it has come to light recently in this laboratory that almost all samples of known uro III ester, at whatever concentration they are applied, will leave some porphyrin at the I position. This amount, although variable (Table II), rises to the region of 35–45% of the total when the quantity applied is such that a satisfactory separation is achieved of known mixtures, applied in this same total quantity and containing

TABLE II

SEPARATION OF URO III ESTERS BY PAPER CHROMATOGRAPHY
Kerosene–dioxan (4:1).

Uroporphyrin III	*Amount applied* (μg)	*CI:CIII or CIV:CII by decarboxylation*	*Ratio uro I:uro III by elution*
Turacin	1.0	0:100:0	28:72
original, m.p. 264°	0.75		46:54
Uro III Turacin	1.0	0:100:0	26:74
ex MgO 9	0.75		33:67
m.p. 253–260°	0.75		45:55
	0.5		36:64
S.F.M. 200	1.0	10:90:0	0:100
'IIIb'	1.0		4:96
m.p. 255–258°*	0.75		8:92
Synth. uro III ex benzene/heptane	1.0	2:96:2	38:62
S.F.M. 100, 11/81	1.0	1:99:0 0:100:0	41:59

* This specimen, previously used as a standard by CORNFORD AND BENSON[2] was subsequently found to contain other uro isomers (see text).

TABLE III

SEPARATION OF VARYING AMOUNTS OF A RANGE OF RATIOS OF URO I AND URO III* ESTERS BY PAPER CHROMATOGRAPHY

Kerosene–dioxan (4:1).

Ratio I:III applied	*Ratio I:III found by elution*				
	0.3 μg	0.5 μg	0.75 μg	1.0 μg	1.25 μg
0:100	5:95	30:70	34:66	59:41	72:28
	4:96	10:90	33:67	40:60	58:42
			46:54		
10:90	12:88	31:69	39:61	63:37	76:24
	4:96	22:78	26:74	38:62	51:49
			54:46		
20:80	20:80	30:70	38:62	53:47	70:30
	9:91	10:90	12:88	36:64	46:54
			34:66		
25:75	6:94	19:81	44:56	49:51	61:39
	0:100	5:95	26:74	40:60	51:49
			29:71		
35:65	17:83	33:67	50:50	57:43	68:32
	10:90	11:89	36:64	41:59	58:42
			42:58		
50:50	27:73	48:52	55:45	72:28	76:24
	15:85	31:69	43:57	62:38	62:38
			53:47		
75:25	57:43	66:34	72:28	77:23	84:16
	42:58	67:33	73:27	75:25	81:19
			75:25		
90:10	68:32	74:26	82:18	91:9	90:10
	79:21	65:35	92:8	92:8	89:11
			84:16		
100:0	79:21	84:16	84:16	88:12	93:7
	79:21	82:18	94:6	92:8	91:9
			93:7		

* Uro III ester from turacin.

35% or more of uro I ester (see Table III). The sample of uro III ester coming nearest to an ideal separation of I:III = 0:100 by elution is the uro III ester used as a standard by CORNFORD AND BENSON[2]. It has since been found that this material could contain all four possible isomers[13] and decarboxylation and subsequent lutidine chromatography shows it to contain, based on visual assessment, a ratio of CI:CIII or CIV:CII of 10:90:0. Whether the presence of some uroporphyrin IV could increase the mobility of this sample in dioxan is worth considering; earlier work in this laboratory, using the FALK AND BENSON[1] dioxan method and a sample of synthetic uro IV ester showed it to behave in a similar manner to uro III ester, whilst uro II ester behaved more like uro I ester.

The variation in apparent uro I:uro III found by elution after chromatography shows itself in the artificial mixtures of authentic I:III esters up to the ratio of I:III = 35:65 at a concentration of 0.75 μg; the run being in kerosene–dioxan (4:1). When the relative amount of uro I ester is greater than this, the agreement between the ratio I:III applied and the ratio I:III found by elution remains good (Table III). With higher amounts of uro I, use of the nomogram improves these figures still further so that expected ratios result.

In the search for conditions which would bring about satisfactory separation of the individual isomers, it was thought that an alternative to varying the total quantity of porphyrin ester applied as a spot would be an alteration in the ratio of the developing solvents. However, if the kerosene–dioxan ratio is altered so as to move all the authentic uro III ester off the I position, then the separation of mixtures made in various ratios is altered and bad agreement results.

The effect of pseudouroporphyrin on the mobility of uro III esters was also investigated. Where large amounts of ψ-uro ester are formed, as in the human erythrocyte system of CORNFORD[14], the agreement between the isomers found by dioxan and by decarboxylation was very good and the possibility of the ψ-uro playing a role in the I : III separation was considered. The presence of ψ-uro may increase the mobility of the uro III ester thereby giving good agreement with the decarboxylation results, or in our case, with the ratio expected from the standard mixtures. A chromatogram was run applying mixtures of various ratios of uro III:ψ-uro esters, such that the amount of uro III ester present was in the region of 0.6–0.8 μg, whatever ratio of uro III:ψ-uro was applied. The spots at the I, III and ψ-uro positions were eluted but the results showed that the presence of ψ-uro had no effect whatsoever on the amount of porphyrin still remaining at the uro I position.

DISCUSSION

When the dioxan paper chromatographic method is adapted for thin-layer chromatography, it shows no apparent advantage over the former method. The same factor which limited the paper method, namely the interdependence of quantities of uro III ester applied and the amount of uro III found at the I position, was also the limiting factor for thin-layer chromatography. The main advantages of thin-layer chromatography, namely, speed of running with more compact spots, ease of elution and greater loading power, were barely noticeable. The running times were practically the same as for the paper method and there was the same degree of diffusion of the spots. The elution technique was more laborious and the concentration of material applied was limited by the method and not the media used.

Further investigation into the behaviour of several samples of uro III ester together with a sample whose isomer composition was doubtful has shown that the dioxan paper technique, whilst still the only available method for direct isomer determination, can give misleading results in the range where the percentage of uro III ester lies between 100 and 65%. At 100%, a constant amount of porphyrin, between 35 and 45%, was found at the I position when the concentrations of porphyrin applied were such that ratios of between 35 and 100% uro I ester separate ideally. This same figure, 35%, is given by the pure uro III ester in thin-layer chromatography (Table I) when a theoretical 50% separation is achieved from a 50:50 mixture of uro I and uro III. It may be noted that CORNFORD AND BENSON[2] found that when a 50:50 mixture separated under ideal conditions to give equal amounts of porphyrin at both positions, and these porphyrins were eluted separately and rechromatographed, they each again split into two fractions estimated with the help of the nomogram correction to be uro I:uro III = 65:35 and 35:65, respectively. That such behaviour is due to some form of molecular association between the two isomers seems beyond doubt. Notwithstanding its drawbacks, the method can still be of very great use in deter-

mining the ratio of uro I to uro III esters when the amount of uro III ester present is less than 65% of the total and it is used in conjunction with decarboxylation and lutidine chromatography. It is still of great value for the detection and determination of pseudouroporphyrin.

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SUMMARY

A quantitative study has been made of the separation of uroporphyrin octamethyl esters I and III by thin-layer chromatography using cellulose powder containing 10% gypsum (MN300G) and a kerosene–dioxan system. The separation by paper chromatography has been further studied.

Thin-layer chromatography offered no apparent advantage over paper chromatography. Results by both methods indicated some interaction between the two porphyrin isomers leading to inability to separate quantitatively uroporphyrin ester mixtures when the III isomer comprised 65% or over.

Paper chromatography is still of great value for separation of the I and III isomers when uroporphyrin III ester is less than 65% and also for the detection and determination of pseudouroporphyrin.

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