An Improved Method for Separation of Radioactive Thyroid Hormone Metabolites by Thin-Layer Chromatography.* (30835)

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The separation of iodine compounds occurring in the body has been carried out to a great extent using paper chromatography. This type of separation has certain inherent disadvantages which, in principle, could be overcome by using thin layer chromatography (TLC). Massaglia and Rosa(1) have shown that monoiodotyrosine (MIT), diiodotyrosine (DIT), tyrosine and iodide can be readily separated by TLC on silica gel. Patterson and Clements(2) analysed animal feed by TLC on starch bound cellulose and separated thyroxine (T_4) triiodothyronine, 3,5 diiodothyronine (3,5 T_2) DIT and iodide. The latter technique is an improvement on paper chromatography but is still affected by the disadvantages of using cellulose as a support for chromatography. We have developed a solvent system for TLC on silica gel, which efficiently separates a larger number of iodocompounds than hitherto reported. The details of this separation are given below.

Methods and results. Preparation of plates. 30 g of Kieselgel G (Merck-Germany) are shaken vigorously with 60 ml of doubly distilled water. The suspension is then spread on glass plates (20 × 20 cm) with a spreader (Desaga). The approximate thickness of the layer is 250 μ. Thirty grams of powder are sufficient for 5 plates. The plates are dried at 37°C, overnight, and stored at the same temperature until used. Desiccation at higher temperatures is not necessary and may affect the efficiency of separation.

Chromatography. The sample is applied 1.5 cm from the lower edge of the plate, with a micropipette. The plate is chromatographed twice in the same direction. The first run is carried out in tertiary-amyl alcohol, acetone, concentrated NH_4OH (25:8:7 v/v) and takes about 16-18 hours (overnight). The time of this run influences the separation. Shorter periods do not give satisfactory results. In this run it is possible to separate the iodide from other iodocompounds.

To separate the iodotyrosines and iodothyronines, a second chromatographic run is required. The plate is dried after the first run and placed in a developing system consisting of tertiary-amyl alcohol, acetone, 2 N NH_4OH containing 0.1% sodium thiosulfate (25:8:7 v/v). The time required for completion of the run is about 4 hours (*i.e.*, until the solvent front reaches the upper edge of the plate). The plate is then dried.

For a successful separation is it necessary to prepare new developing mixtures before each chromatography.

Carriers of the iodo-amino compounds are detected by spraying with a mixture of 0.2% ninhydrin in a solvent consisting of 5% acetic acid solution in n-butanol. The iodide ion is detected by spraying with a solution of 0.1% $PdCl_2$, Triac and Tetrac are detected by spraying with a mixture of cold 0.05 M sulfanilic acid in 9% HCl in an equal volume of 4.5% $NaNO_2$ (freshly prepared).

Under these conditions the chromatographic behaviour was examined of iodide (I), monoiodotyrosine (MIT), diiodotyrosine (DIT), the O-sulfate ester of triiodothyronine (ST_3), 3′,5′-3 triiodothyronine (Rev T_3), thyroxine (T_4), triiodothyronine (T_3), 3,5 diiodothyronine (3,5 T_2), tetraiodothyroacetic acid (Tetrac) and triiodothyroacetic acid (Triac). 50 μg of each compound was present in 5 μl of the mixtures. The results of representative runs are shown in Table I. While there was some variability in Rf values in any individual run all the compounds were separable one from another (Fig. 1).

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TABLE I. Rfs for Mixtures of Various Iodocompounds in a Number of Representative Runs.

Run	DIT	MIT	ST_3	$RevT_3$	T_4	T_3	$_{35}T_2$	Tetrac	Triac	I
1	.22	.27	—	—	.44	.55	—	—	—	.77
2	.27	.32	.42	—	.50	.61	—	—	—	.80
3	.23	.29	—	.36	.41	.51	—	—	—	.76
4	.24	.29	—	—	.38	.46	.50	—	—	.70
5	.24	.29	—	—	.39	.48	—	—	.69	.72
6	.24	.30	—	—	.42	.51	—	.69	—	.74

TABLE II. Effect of Various Constituents on the Area of Spots After TLC Chromatography.

	Area of spots in cm^2				
	I	MIT	DIT	T_3	T_4
A. Amount of each carrier in mixture applied					
10 μg	1.30	1.30	1.30	.47	.57
25 μg	2.09	1.53	1.50	.69	.73
50 μg	2.43	1.87	1.94	.97	1.15
100 μg	2.91	2.18	2.21	1.06	1.41
B. Amount of plasma containing 50 μg of each carrier iodocompound					
10 μl	2.37	.99	.70	1.06	.99
25 μl	2.56	.92	.80	.70	.94
50 μl	1.80	1.02	.87	.70	.99
100 μl	3.79	1.30	1.30	.95	1.63
C. Amount of 6% thyroid hydrolysate containing 50 μg of each carrier iodocompound					
10 μl	2.22	.83	.87	1.15	1.35
25 μl	2.75	1.04	.87	.92	1.50
50 μl	3.34	1.46	1.34	.94	2.30
100 μl	2.97	2.61	2.43	1.72	2.97

The effect of loading was then investigated. Four mixtures of I, T_3, T_4, DIT and MIT were run on the same plate. These contained 10, 25, 50 and 100 μg of each substance in 10 μl of mixture, respectively. Separation of all 5 substances was obtained up to a load of 50 μg of each. With the 100 μg mixtures, overlapping of the I and T_3 spots and the DIT and MIT spots occurred. The effect on the area of the spots is shown in Table IIA.

Since most analyses are made on tissues or body fluids, the effect of the load of adventitious materials on the separation was studied. Varying amounts (10-100 μl) of rat plasma were added to a 5 μl mixture containing 50 μg of I, T_3, T_4, DIT and MIT. There was some increase in size of spot with increasing load of plasma (Table IIB). However, the 5 substances were separated clearly and no overlapping occurred. The same experiment was repeated but the plasma was replaced by increasing amounts (10-100 μl) of a hydrolysate of a 6% rat thyroid suspension. There was greater enlargement of spots (Table IIC) than in the previous experiment, but with the highest load separation was obtained and there was no overlapping.

From these results the following standard conditions were adopted. Mixtures of carriers (of 10-25 μg each) were present on all plates

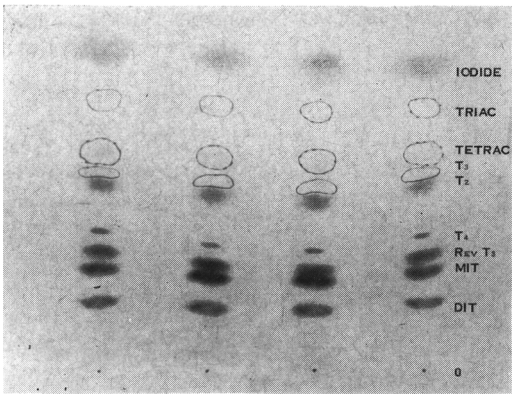

FIG. 1. A separation of iodocompounds on TLC (as described in *Methods*). The spots of Triac and Tetrac are yellowish and do not reproduce well in the photograph. The T3 spot is not obvious because the diazotized sulfanilic acid spray (used for visualization of the acetic acid analogues) causes fading of the ninhydrin colour. The T3 spot is therefore outlined before the second spray is used.

to compensate for any variation in Rf from run to run. The amount of carrier Triac or Tetrac necessary was 50 μg. The quantity of protein or other organic material present in any sample for separation was less than 6 mg, and if possible less than 3 mg.

Detection and recovery of radioactive compounds. The method has been applied to analyses of mixtures of radioiodine containing compounds. The isotope most frequently used has been I^{125}. Because of the low energy of the radiation of this isotope, there was the possibility of difficulty in obtaining autoradiographs of the chromatograms. The following technique was evolved. The dry chromatogram was wrapped in cellophane and tightly pressed against a sheet of X-ray film (Kodak No-screen) in an X-ray cassette. The sandwich was exposed for periods up to a month, depending on the activity estimated in the separation. With this maximum exposure time a spot containing 1×10^{-3} μc was easily detectable.

Using the autoradiograph as a guide, the radioactive spots were located, scraped off the plate into test tubes and counted. The recovery of known amounts of MIT, DIT, T_3, and T_4 and I was 98%. The efficiency of elution of the spots was tested using spots of I^{125}-labelled T_3 and T_4. The spots were eluted 4 times with a double volume of a mixture of 95% ethanol and concentrated NH_4OH (1:1 v/v). The combined elutions gave a recovery in excess of 98% of the original activity.

The possible deiodination of T_3, T_4, DIT and MIT during chromatography was tested by rechromatographing in the second dimension on the same plate. In all cases less than 3% of the radioactivity was found as iodide and about 86% of each of the original compounds was recovered in its corresponding spot. Thus there is moderate damage due to the technique. This is in contrast to our experience with chromatography on paper using butanol-dioxane-ammonia. Here the procedure may result in over 30% breakdown of these compounds with the formation of a variety of breakdown products. In view of the above results, the TLC technique described readily lends itself to separation of mixtures of labelled compounds resulting either from the metabolism of iodide or from *in vitro* synthesis of these compounds. In both cases our experience with the method has been satisfactory.

Summary. 1. A technique for TLC separation of iodide, monoiodotyrosine, diiodotyrosine, triiodothyronine, thyroxine, 3,5-diiodothyronine, 3′,5′-3 triiodothyronine and O-sulfate ester of triiodothyronine, triiodothyroacetic acid (Triac) and tetraiodothyroacetic acid (Tetrac) is described. 2. The effect of the amount of iodocompound or adventitious organic materials on efficiency of separation was tested. The best conditions were an iodocompound content of not more than 25 μg (50 μg for Triac and Tetrac) of each and not more than 6 mg, and preferably less than 3 mg, of organic material. 3. The detection and method of estimation of I^{125}-labelled compounds is described. Recoveries in the spots are about 98%. Elution from spots is similarly about 98%. The chromatographic procedure resulted in not more than 3% deiodination of triiodothyronine or thyroxine.

1. Massaglia, A., Rosa, U., J. Chromatography, 1964, v14, 516.

2. Patterson, Stella J., Clements, R. L., The Analyst, 1964, v89, 328.

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