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THE CHARACTERISATION OF IODOAMINO ACIDS AND THEIR DERIVATIVES BY THIN LAYER CHROMATOGRAPHY

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SUMMARY

A method of characterising iodoamino acids, *e.g.* thyroxine, iodothyronines and iodotyrosines and some of their commonly occurring derivatives, *e.g.* thyrolactic, -pyruvic and -acetic acids is described. This procedure, which is based on thin layer chromatography of the parent acids and on the preparation and chromatography of suitable derivatives, prepared in sequence, is exemplified by the characterisation of 3,3′,5′-triiodothyronine, a trace metabolite of thyroxine occurring in the plasma of plaice.

INTRODUCTION

Although naturally occurring iodoamino acids are commonly separated by paper[1,2], thin layer[3,4] and gas–liquid chromatography[5,6], chromatographic behaviour of the free acids does not, in itself, constitute an adequate criterion of chemical identification.

In the course of our work on thyroid hormones in marine fauna, several species of which had previously been examined by rather inadequate techniques, it became clear that there was a need for some general procedure by which small quantities of iodoamino acids could be more rigorously identified.

The present communication describes a system of characterisation based on chromatography of the free acids and the preparation and chromatography of suitable derivatives, prepared in sequence and involving different reactive sites on the parent iodoamino acid.

EXPERIMENTAL

Chromatography

TLC plates were spread at 250 μ thickness from a slurry of silica gel (Machery Nagel, N/HR UV^{254}; 30 g) and freshly prepared rice starch solution (11.5%; 65 ml) and were air dried before use.

Samples were applied from a solution in ethanol–2 *N* ammonia (1:1) containing methyl mercaptoimidazole (0.01 *M*) as antioxidant, the spots were dried under nitrogen.

Chromatograms were irrigated, without previous equilibration, in the following solvent systems (proportions quoted are volume:volume).

(a) Chloroform–methanol–ammonia, sp.gr. 0.880 (50:25:2.5) (ref. 7).
(b) Acetic acid–methanol–ammonia, sp.gr. 0.880 (40:20:3) (ref. 7).
(c) Formic acid–methanol–chloroform, (5:15:80) (ref. 8).
(d) Ethyl acetate–methanol–ammonia, sp.gr. 0.880 (diluted 1:5) (50:20:10).
(e) Ethyl acetate–methanol–ammonia, sp.gr. 0.880 (diluted 1:5) (50:13:10).
(f) Formic acid–methanol–chloroform, (15:15:70) (ref. 8).

Spots were normally located by viewing under U.V. light; on occasions, chromatograms were sprayed with specific locating reagents for phenolic, amino[7] and ketol groupings[1,9]. Radiochromatograms were scanned by the method of OSBORN AND SIMPSON[10]. Elutions of material from the layers, prior to rechromatography, was effected with ethanol–2 *N* ammonia (1:1) containing methyl mercaptoimidazole in a conventional sinter-disc thimble.

Preparation of derivatives

Acylations were performed by dissolving the dried sample (≯ 20 μg) in aqueous sodium hydroxide (0.1 *N*; 10 μl) and adding at 0.5 h intervals two 10 μl portions of reagent (0.4% (v/v) acetic anhydride in acetone or 0.4% (v/v) benzyl chloroformate in tetrahydrofuran). After the addition of further portions of base and reagent (10 μl of each) and standing for a further period of 0.5 h the product was isolated by evaporation in nitrogen.

Hydrolyses were effected by dissolving the dried sample on aqueous sodium hydroxide (0.5 *N*; 20 μl) and allowing to stand at 0° overnight.

Carboxylic acids were converted to their methyl esters by reaction with methanol (0.5 ml) and hydrochloric acid (sp.gr. 1.18; 2 drops) overnight at 40°; the products were isolated, as before, by evaporation in nitrogen.

Methyl ethers were prepared from the parent phenols by reaction with excess diazomethane in anhydrous methanol at 0° overnight[11]. Under these reaction conditions, phenolic iodoamino acids yielded the methyl esters of the phenol ethers from which the free carboxylic acids were obtained by hydrolysis, according to the method described, acidification and extraction with ether.

Authentic compounds

3,3′-Diiodothyronine was synthesised from 3,5,3′-triiodothyronine by the method of ROCHE *et al.*[12]. The pyruvic acid analogues of 3,5,3′-triiodothyronine and thyroxine were prepared by the method of NAKANO[9]; lactic acid derivatives were obtained from them by reduction with sodium borohydride in aqueous ethanol[13]. Iodothyroacetic acids, other iodothyronines, and iodotyrosines, were obtained from normal commercial sources.

Application of characterisation procedure to biological materials

Whole blood was collected from freshly killed plaice *(Pleuronectes platessa)* which had been injected intraperitoneally with ^{125}I labelled thyroxine 24 h before

sacrifice. The plasma fraction (5 ml) was acidified (pH 2), extracted twice with *n*-butanol and the combined extracts immediately made alkaline to reduce ester formation. After the addition of methyl mercaptoimidazole (0.01 *M*; 10 μl) and appropriate inactive carriers (3,5,3′-triiodothyronine, thyroxine, 3,5,3′-triiodothyroacetic acid, 3,5,3′,5′-tetraiodothyroacetic acid; 20 μg of each) the extract was evaporated at 37° *in vacuo*, redissolved in ethanol–2 *N* ammonia (1:1, v/v), applied to a TLC plate and chromatographed in system (a). Scanning revealed the presence of several radioactive peaks, the peak of particular interest being located just behind the thyroxine carrier. This material was eluted, supplemented with authentic 3,3′,5′-triiodothyronine (T_3') (20 μg) and chromatographed successively in systems (b), (a) and (d). In all chromatograms, exact coincidence of the radio peak and authentic T_3', visualised under U.V. light, was observed. The sample was then eluted, esterified with methanol–hydrochloric acid and chromatographed successively in systems (a) and (c); exact correspondence of radiopeaks and authentic T_3' methyl ester was again observed. The esters were next eluted, saponified, acetylated and chromatographed in system (a). Two radiopeaks were observed, on scanning, coincident with O,N-diacetyl- and N-acetyl-T_3'; after elution and rechromatography in system (d), the same coincidence was observed. Elution of the diacetate, followed by hydrolysis and chromatography in system (a) furnished the N-acetyl compound coincident with authentic carrier. The monoacetates were then combined, methylated with diazomethane, saponified to release the free acid and chromatographed successively in systems (a) and (d). Exact coincidence of the radio-peak with authentic N-acetyl-O-methyl-T_3' was observed on both chromatograms.

RESULTS AND DISCUSSION

The uncertainty which is properly attached to assignments of chemical identity to compounds on the basis of their chromatographic behaviour, even in a number of

TABLE I

R_F VALUES OF SOME IODO COMPOUNDS

Compound	*System*					
	a	*b*	*c*	*d*	*e*	*f*
Thyroxine	0.13	0.45	0.27	0.38	0.29	0.50
3,5,3′-Triiodothyronine	0.22	0.37	0.19	0.48	0.33	0.41
3,3′,5′-Triiodothyronine	0.09	0.49	0.31	0.35	—	—
3,5-Diiodothyronine	0.19	0.34	0.15	0.43	—	0.34
3,3′-Diiodothyronine	0.16	0.29	0.21	0.40	—	—
3-Monoiodotyrosine	0.06	0.25	0.10	0.16	0.13	0.25
3,5-Diiodotyrosine	0.03	0.35	0.13	0.10	0.07	0.37
3-Monoiodohistidine	0.05	0.07	0.02	0.09	0.09	0.05
3,5,3′,5′-Tetraiodothyroacetic acid	0.24	0.89	0.73	0.47	0.34	0.87
3,5,3′-Triiodothyroacetic acid	0.33	0.85	0.63	0.57	0.39	0.81
3,5-Diiodothyroacetic acid	0.28	0.87	0.64	0.58	—	0.77
3,5,3′,5′-Tetraiodothyropyruvic acid	0.21	—	0.67	0.49	—	—
3,5,3′-Triiodothyropyruvic acid	0.30	—	0.59	0.58	—	—
3,5,3′,5′-Tetraiodothyrolactic acid	0.19	—	0.61	0.43	—	—
3,5,3′-Triiodothyrolactic acid	0.27	—	0.55	0.53	—	—
3,5,3′-Triiodothyronamine	0.58	—	0.23	0.65	—	—
Iodide	0.33	0.26	0.10	0.55	0.37	3.0

TABLE II

R_F VALUES OF ACYL DERIVATIVES

Compound	*System*				
	a	*b*	*c*	*d*	*e*
N-Acetylthyroxine	0.21	0.82	0.52	0.45	—
N-Acetyl-3,5,3′-triiodothyronine	0.32	0.77	0.48	0.55	—
N-Acetyl-3,3′,5′-triiodothyronine	0.14	0.85	0.55	0.41	—
N-Acetyl-3,5-diiodothyronine	0.31	0.77	0.45	0.53	—
N-Acetyl-3,3′-diiodothyronine	0.27	0.79	0.43	0.47	—
N-Acetyl-3-monoiodotyrosine	0.13	0.72	—	—	0.21
N-Acetyl-3,5-diiodotyrosine	0.08	0.77	—	—	0.13
N-Acetyl-3-monoiodohistidine	0.11	0.33	—	—	0.15
O,N-Diacetylthyroxine	0.54	0.85	0.63	0.77	—
O,N-Diacetyl-3,5,3′-triiodothyronine	0.70	0.82	0.59	0.89	—
O,N-Diacetyl-3,3′,5′-triiodothyronine	0.40	0.86	0.63	0.67	—
O,N-Diacetyl-3,5-diiodothyronine	0.68	0.83	0.57	0.85	—
O,N-Diacetyl-3,3′-diiodothyronine	0.63	0.82	0.58	0.80	—
N-Carbobenzoxythyroxine	0.35	0.92	0.72	0.60	—
N-Carbobenzoxy-3,5,3′-triiodothyronine	0.49	0.88	0.63	0.67	—
N-Carbobenzoxy-3,3′,5′-triiodothyronine	0.32	0.92	0.75	0.57	—
N-Carbobenzoxy-3,5-diiodothyronine	0.45	0.87	0.61	0.66	—
N-Carbobenzoxy-3,3′-diiodothyronine	0.43	0.89	0.66	0.64	—
N-Carbobenzoxy-3-monoiodotyrosine	0.33	0.83	—	—	0.38
N-Carbobenzoxy-3,5-diiodotyrosine	0.26	0.89	—	—	0.30
N-Carbobenzoxy-3-monoiodohistidine	0.30	0.57	—	—	0.32
O,N-Dicarbobenzoxy-3,5-diiodotyrosine	0.66	—	—	—	0.56
O,N-Dicarbobenzoxy-thyroxine	0.65	—	0.80	0.82	—
O,N-Dicarbobenzoxy-3,5,3′-triiodothyronine	0.68	—	0.75	0.83	—
O,N-Dicarbobenzoxy-3,3′,5′-triiodothyronine	0.63	—	0.80	0.81	—
O,N-Dicarbobenzoxy-3,5-diiodothyronine	0.67	—	0.75	0.83	—
O,N-Dicarbobenzoxy-3,3′-diiodothyronine	0.67	—	0.76	0.83	—
N-Acetyl-O-methyl-thyroxine	0.33	—	—	0.57	—
N-Acetyl-O-methyl-3,5,3′-triiodothyronine	0.40	—	—	0.63	—
N-Acetyl-O-methyl-3,3′,5′-triiodothyronine	0.30	—	—	0.54	—
N-Acetyl-O-methyl-3,5-diiodothyronine	0.37	—	—	0.60	—
N-Acetyl-O-methyl-3,3′-diiodothyronine	0.35	—	—	0.58	—
O-Acetyl-tetraiodothyropyruvic acid	0.49	—	0.77	0.62	—
O-Acetyl-3,5,3′-triiodothyropyruvic acid	0.50	—	0.69	0.69	—
O-Acetyl-tetraiodothyrolactic acid	0.25	—	0.75	0.45	—
O-Acetyl-3,5,3′-triiodothyrolactic acid	0.32	—	0.32	0.54	—
O,O-Diacetyl-tetraiodothyrolactic acid	0.50	—	0.81	0.60	—
O,O-Diacetyl-3,5,3′-triiodothyrolactic acid	0.51	—	0.51	0.66	—
N-Acetyl-3,5,3′-triiodothyronamine	0.85	—	0.63	—	—
O,N-Diacetylthyroxamine	0.90	—	0.78	—	—

different solvent systems, may be reduced to negligible proportions if these criteria are supplemented by evidence of the formation and chromatographic mobility of suitably chosen derivatives. The requirements which determine the suitability of derivatives for identification purposes may be summarised thus: they should chromatograph as compact spots and be well separated from likely contaminants, they should constitute modifications of different reactive centres on the parent molecule, should be capable of formation in near quantitative yields and, where the available amounts of material are low, should be capable of being prepared in sequence. A procedure of characterising steroids by chromatography of the free compounds and of derivatives, prepared in sequence has already been described[14].

TABLE III

R_F VALUES OF METHYL ESTERS AND METHYL ETHERS

Compound	*System*		
	a	*c*	*d*
Thyroxine methyl ester	0.77	0.40	—
3,5,3′-Triiodothyronine methyl ester	0.86	0.30	—
3,3′,5′-Triiodothyronine methyl ester	0.70	0.46	—
3,5-Diiodothyronine methyl ester	0.82	0.26	—
3,3′-Diiodothyronine methyl ester	0.79	0.36	—
3-Monoiodotyrosine methyl ester	0.75	0.24	—
3,5-Diiodotyrosine methyl ester	0.57	0.27	—
3-Monoiodohistidine methyl ester	0.60	0.07	—
Tetraiodothyroacetic acid methyl ester	0.88	0.85	—
3,5,3′-Triiodothyroacetic acid methyl ester	0.91	0.79	—
3,5-Diiodothyroacetic acid methyl ester	0.90	0.78	—
Tetraiodothyropyruvic acid methyl ester	0.90	0.82	—
3,5,3′-Triiodothyropyruvic acid methyl ester	0.93	0.75	—
Tetraiodothyrolactic acid methyl ester	0.85	0.77	—
3,5,3′-Triiodothyrolactic acid methyl ester	0.90	0.72	—
O-Methyl-thyroxine	0.32	0.32	0.47
O-Methyl-3,5,3′-triiodothyronine	0.33	0.27	0.50
O-Methyl-3,5-diiodothyronine	0.31	0.28	0.49
O-Methyl-tetraiodothyroacetic acid	0.51	0.87	0.58
O-Methyl-3,5,3′-triiodothyroacetic acid	0.48	0.85	0.63
O-Methyl-3,5-diiodothyroacetic acid	0.49	0.85	0.63

Suitable derivatives for the characterisation of iodothyronines, iodotyrosines and their metabolites have been found to be methyl esters, N-acetates and O,N-diacetates, or the corresponding carbobenzoxy derivatives, and the O-methyl ethers; R_F values of these compounds in a number of different solvent systems are listed in Tables I, II and III. These derivatives are readily prepared in near quantitative yield. Characterisation of small quantities of iodothyronines and iodotyrosines, as exemplified by the identification of 3,3′,5′-triiodothyronine, a trace metabolite of thyroxine occurring in the blood plasma of plaice, is based on chromatography of the parent amino acid and on the preparation and chromatography, in sequence, of the methyl ester, O,N-diacetates, N-acetates and N-acetyl-O-methyl derivatives. Characterisation of iodothyrolactic acids is based on chromatography of the parent acids and on the preparation and chromatography of the methyl esters, O,O-diacetates and of O-acetyl derivatives having an unesterified phenolic hydroxyl group. The derivatives recommended for characterising iodothyro-pyruvic and -acetic acids are, respectively, methyl esters, O-acetates, O-methyl ethers and methyl esters, O-methyl methyl esters and O-methyl acids.

The procedures described in this communication have been in routine use in these laboratories for the last twelve months and have been applied to the analysis of iodoamino acids in a variety of biological materials including thyroid tissue, blood, urine and bile. Chromatography of the parent compounds and preparation of two derivatives have commonly proved to provide adequate criteria for identification purposes; however, when sufficient material is available, preparation of the third derivative is recommended.

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REFERENCES

1 L. G. PLASKETT, in M. LEDERER (Editor), *Chromatographic Reviews*, Vol. 6, Elsevier, Amsterdam, 1964, p. 91.
2 J. ROCHE, R. MICHEL AND S. LISSITZKY, in D. GLICK (Editor), *Methods of Biochemical Analysis*, Vol. 12, Interscience, New York, 1964, p. 143.
3 A. FAVINO, D. EMRICH AND A. MÜHLEN, *Acta Endocrinol.*, 54 (1967) 362.
4 T. SOFIANIDES, C. R. MELONI, E. ALGER AND J. J. CANARY, *Proc. Soc. Exptl. Biol. Med.*, 123 (1966) 646.
5 P. JAAKONMÄKI AND J. E. STOUFFER, *J. Gas Chromatog.*, (1967) 303.
6 A. H. RICHARDS AND W. B. MASON, *Anal. Chem.*, 38 (1966) 1751.
7 J. G. HEIDER AND J. R. BRONK, *Biochim. Biophys. Acta*, 95 (1965) 353.
8 J. E. STOUFFER, P. I. JAAKONMÄKI AND T. J. WENGER, *Biochim. Biophys. Acta*, 127 (1966) 261.
9 M. NAKANO, *Endocrinology*, 67 (1959) 880.
10 R. H. OSBORN AND T. H. SIMPSON, *J. Chromatog.*, 35 (1968) 436.
11 K. TOMITA, H. A. LARDY, D. JOHNSON AND A. KENT, *J. Biol. Chem.*, 236 (1961) 2981.
12 J. ROCHE, R. MICHEL AND W. WOLF, *Compt. Rend.*, 239 (1954) 597.
13 R. PITT-RIVERS, *Endocrinology*, 66 (1960) 628.
14 T. H. SIMPSON AND R. S. WRIGHT, *Anal. Biochem.*, 5 (1963) 313.