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N,O-Dipivalyl methyl esters of the thyroid hormones and related compounds: Synthesis, purification and gas chromatography

The classical methods for the detection and separation of non-radioactive iodoamino acids involve paper or thin-layer chromatography prior to their final quantitative determination by the ceric sulfate–arsenite color reaction. Only in recent years has gas chromatography been applied here. For this purpose heat-stable volatile derivatives would be needed. For instance, the N,O-bistrifluoroacetyl methyl esters derivatives were prepared[1], but no details on either yield or minimum detectable limit were given. With the availability of BSA, a powerful silylating agent, the TMS derivatives of the thyroid hormones and other iodoamino acids were made in macro quantities and separated by gas chromatography[2–4]. These derivatives can be easily prepared, but are not stable in air, and, therefore, would be difficult to use as precise standards. The N,O-dipivalyl methyl esters have recently been prepared in small quantities, tested by GC[5,6] and found to be particularly well suited for analyzing hormones in serum[7]. Pure derivatives in macro quantities were not isolated, however. These derivatives are more difficult to prepare than the TMS derivatives. On the other hand, however, they are quite stable under atmospheric conditions.

In this work we will describe the synthesis and purification of the N,O-dipivalyl methyl esters of MIT, DIT, T_2, T_3, T_4 and DBrT in weighable quantities.

Halogenated amino acids were esterified with methanolic hydrochloric acid in the usual way and dipivalyl derivatives were made from the crude methyl esters following essentially the published method[8]. Pure substances were isolated by adsorption chromatography on Florisil. The purity of each derivative was checked by melting point, elemental analysis, TLC and GC.

Experimental

Synthesis and purification. The halogenated amino acid (500 mg) was esterified by heating at 70° for 1 h with dry methanolic hydrochloric acid (25%, 20 ml) in a tightly closed screw cap vial. (All substances dissolved completely except T_4.) The crude methyl ester was checked by TLC (Silica Gel GF_{254}) using solvent system I, chloroform–methanol–formic acid (80 : 15 : 5)[5].

The dry crude ester was taken up in dry tetrahydrofuran (20 ml). Triethylamine (1 ml) and pivalic anhydride (1 ml) were added. The mixture was refluxed under nitrogen for 2 h. After cooling, the precipitated amine hydrochloride was removed by filtration and the filtrate evaporated to dryness under nitrogen, yielding a thick brown oil which did not solidify in the cold.

This oil was dissolved in a minimum quantity of benzene and chromatographed on a Florisil column, mesh 60–100, prepared in hexane. Elution was started with hexane–benzene (1 : 1) (100 ml), followed by benzene (100 ml) and 2% absolute ethanol in benzene (100 ml). 10 ml fractions were collected and tested on TLC (Silica Gel GF_{254} using solvent system II, isooctane–chloroform–formic acid (10:20:1)[5]). The fractions containing the largest amount of material as viewed under UV light on the TLC plates were further tested by GC to determine the degree of purity.

The derivatives were usually eluted with 2% ethanol in benzene. The relevant fractions were pooled and evaporated under a nitrogen stream. A light colored oil was obtained in all cases. This oil was triturated with light petroleum ether in the cold until it solidified. Recrystallization was performed by dissolving in dry benzene and adding light petroleum ether. In some cases charcoal was employed for decolorization. Preparative GC for purification was not successful.

Gas chromatography. F & M Model 609 with 400 oven and flame ionization detector was used.

Column: 6 ft. × 4 mm I.D. Pyrex column packed with 1% OV-1 coated on 80–100 mesh acid washed dimethylchlorosilane treated Chromosorb G. Column temperature: 200° for iodotyrosines, 250° for iodothyronines.

Helium was used as carrier gas with a flow rate of 80 ml/min. Gases used for the detector were: air and hydrogen at flow rates of 450 ml/min and 45 ml/min, respectively.

TABLE I

ELEMENTAL ANALYSIS OF N,O-DIPIVALYL METHYL ESTER OF HALOGENATED AMINO ACIDS

Amino acid	*Empirical formula*	*M.P.*	*Mol.wt.*		*Analysis (%)*		
					C	*H*	*Halogen*
MIT	$C_{20}H_{28}O_5NI$	80	489.33	Calc.	49.09	5.77	25.94
				Found	49.49	5.91	26.01
DIT	$C_{20}H_{27}O_5IN_2$	58–60	615.23	Calc.	39.04	4.42	41.25
				Found	38.88	4.47	41.22
T_2	$C_{26}H_{31}O_6NI_2$	93–95	707.32	Calc.	44.14	4.42	35.88
				Found	44.40	4.38	35.72
T_3	$C_{26}H_{30}O_6NI_3$	86–87	833.21	Calc.	37.48	3.63	45.69
				Found	37.83	3.75	45.66
T_4	$C_{26}H_{29}O_6NI_4$	103–106	959.10	Calc.	32.56	3.05	52.92
				Found	33.05	3.12	52.68
DBrT	$C_{20}H_{27}O_5NBr_2$	101–103	521.26	Calc.	46.08	5.22	30.66
				Found	46.36	5.50	30.59

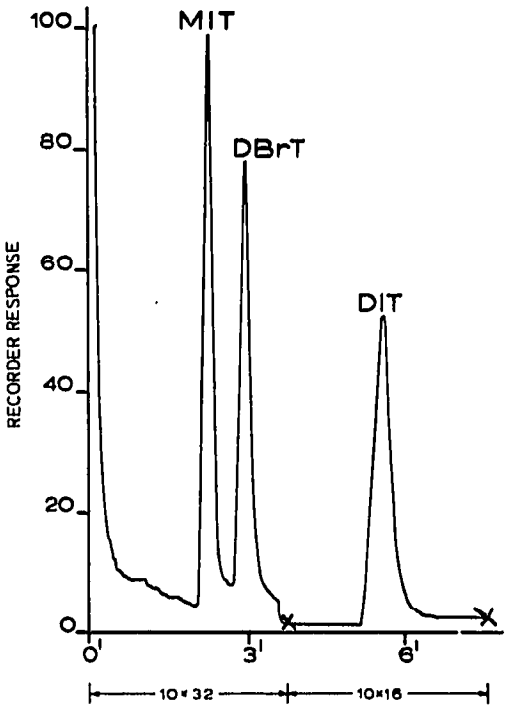
Results

Table I indicates melting points and elemental analysis of derivatives. Figs. 1 and 2 show the type of separation obtained by GC, isothermally at two different temperatures, of mixtures of the purified derivatives of the three halogenated tyrosines and of the three iodothyronines.

Table II summarizes GC data for all six compounds.

Discussion

A quantitative estimation of thyroid hormones and their precursors and metabolites from biological systems such as thyroid, and serum, has so far been difficult due to the similarity of many of their chromatographic properties as well as to their presence in extremely small quantities. In gas chromatography one of the difficulties has been lack of suitable, thermally stable, volatile derivatives in the pure state to be used directly as standards. The N,O-dipivalyl methyl ester derivatives have been employed for measuring thyroid hormones in serum by GC with the help



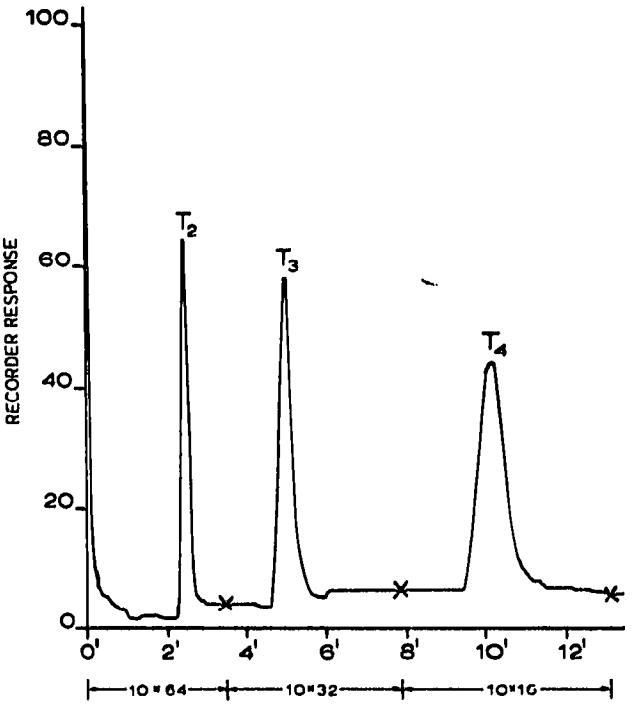


Fig. 1. Gas chromatogram of the N,O-dipivalyl methyl esters of MIT, DBrT and DIT, isothermal at 200°. About 10 μg of each compound injected.

Fig. 2. Gas chromatogram of the N,O-dipivalyl methyl esters of T_2, T_3, and T_4, isothermal at 250°. About 10 μg of each compound injected.

of the electron capture detector[7]. Very small amounts could be detected, but precise answers were difficult to obtain due to the non-availability of the pure derivatives. We have been successful now in isolating these derivatives in macro quantities and in pure form. These substances are solid at room temperature and can be weighed without special precautions. With these pure standards, the analysis of biological samples by GC and the ^{63}Ni-electron capture detector will be possible. The DBrT derivative, which would not be found in biological samples, has a retention time between that of MIT and DIT and would probably be a suitable internal standard for the estimation of these two iodoamino acids. The T_2-derivative, presumably also not found in biological samples, may serve to quantitate T_3 and T_4. Studies along those various lines are in progress.

TABLE II

GAS CHROMATOGRAPHIC DATA FOR HALOGENATED AMINO ACIDS

N,O-Dipivalyl methyl ester	*Column temperature (°C)*	*Retention time*
MIT	200	2.3
DBrT	200	3.0
DIT	200	5.8
T_2	250	2.4
T_3	250	5.0
T_4	250	10.2

Abbreviations

BSA	N,O-bis(trimethylsilyl) acetamide
TMS	Trimethylsilyl
MIT	L-Monoiodotyrosine
DIT	L-3,5-Diiodotyrosine
T_2	L-3,5-Diiodothyronine
T_3	L-3,5,3'-Triiodothyronine
T_4	L-Thyroxine
DBrT	L-3,5-Dibromotyrosine
TLC	Thin-layer chromatography
GC	Gas chromatography

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Rapid gas chromatographic separation of amino acid enantiomers using N-perfluoroacyl esters

Amino acid enantiomers can be resolved by gas chromatography using optically active stationary phases[1–5]. Stationary phases most commonly used are N-trifluoroacetyl (TFA)-L-valyl-L-valine cyclohexyl ester[3,5] and N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester[4]. N-TFA amino acid isopropyl esters have been utilized thus far as the most notable derivatives. However, the retention times were long, resulting in extended gas chromatographic runs. It was of interest to investigate the influence of perfluoroacyl derivatives of amino acid esters other than the trifluoroacetyl group with respect to retention times and resolution factors.

We wish to report the preparation of different N-pentafluoropropionyl (PFP), N-heptafluorobutyryl (HFB), and N-pentadecafluorooctanoyl (PDFO)-D,L-leucine