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Note

A fast derivatization method for the gas chromatographic analysis of thyroid hormonal compounds

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In the 1980s there is an apparent trend towards the use of gas chromatography (GC) for the analysis of thyroid hormones[1–4] as the detection principles generally used in liquid chromatography (LC) are in this respect not applicable to low biological levels[5–9]. The continuing interest in GC is undoubtedly connected with improvements in the associated technology which have resulted in the availability of instruments suitable for work with capillary columns and selective detectors, *i.e.*, high resolution combined with high sensitivity.

As with the protein amino acids[10], the thyroid hormones were converted into their acylated alkyl esters or persilylated forms, the latter being less stable[11,12]. Stable derivatives were obtained upon treatment of the iodothyronine methyl esters with pivalic (trimethylacetic) anhydride[13–16], but more volatile and highly electron-capture-sensitive derivatives were formed when heptafluorobutyric anhydride was used[1–4,17,18]. The drawback of these two step esterification–acylation approaches is the long derivatization time of about 2 h and the necessity of a fresh 25% gaseous HCl–methanol solution.

We describe here a mild and rapid procedure, based on a cooperative action of two reagents, dichlorotetrafluoroacetone and a perhalogenated anhydride, on the hydrogen-containing groups in the iodo compounds. The method is also suitable for derivatization of the deamination and decarboxylation products of the iodothyronines, *i.e.*, for triac and tetrac.

EXPERIMENTAL

Material

3,5-Diiodo-L-thyronine (T_2), 3,3′,5-triiodo-L-thyronine (T_3) and L-thyroxine (T_4) were obtained from Koch-Light (Colnbrook, U.K.), 3,3′,5′-triiodothyronine (rT_3) from Calbiochem (Lucerne, Switzerland) and 3,3′,5-triiodothyroacetic acid (triac, T_3Ac) and 3,3′,5,5′-tetraiodothyroacetic acid (tetrac, T_4Ac) from Sigma (St. Louis, MO, U.S.A.). An equimolar stock solution containing 1 nmol of each compound in 1 μl methanol–ammonia (19:1) solvent was prepared and stored at 4°C.

The internal standard (I.S.), 2-methylnaphthalene-bis(hexachlorocyclopentadiene) adduct, was obtained from Aldrich Europe (Janssen Pharmaceutica, Beerse, Belgium) and dissolved in heptane to form a stock solution of the same concentration as above.

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The anhydride reagents, heptafluorobutyric (HFBA), pentafluoropropionic (PFPA) and trifluoroacetic anhydride (TFAA), were purchased from Pierce Eurochemie (Rotterdam, The Netherlands) and 1,3-dichlorotetrafluoroacetone (DCTFA), originally from Fluka (Buchs, Switzerland), but from 1983 no longer available, can be purchased from Riedel de Haën (Seelze, F.R.G.). The organic solvents, acetonitrile, pyridine, hexane and heptane, were from Lachema (Brno, Czechoslovakia) as the best available grade.

Procedure

The dry residues of the compounds of interest including the I.S. were treated in a 2-ml glass reaction vial (50 × 12 mm O.D. with a ground joint 12.5/15 mm[19]) as follows.

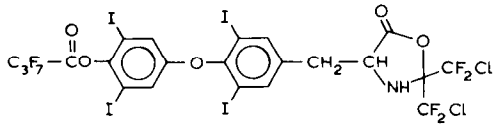
A 120-μl volume of acetonitrile–pyridine (40:1, v/v) and 15 μl of DCTFA were left to stand for 1–2 min and then 6 μl of HFBA or 5 μl of PFPA were added. After a further 1–2 min the derivatized forms were extracted into hexane (400 μl) by shaking with water (300 μl) for about 10 sec. The lower phase was sucked off and the organic solvent evaporated at 50–60°C under a stream of nitrogen in the same or a second reaction vial. After addition of heptane (20–100 μl) the sample was subjected to GC.

Analysis

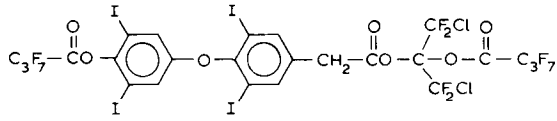
A Hewlett-Packard 5736A gas chromatograph equipped with two flame ionization detectors, a capillary inlet system 18740B and a computing integrator 3380 A was used for the GC analysis. Two microlitres of the sample in heptane were injected via the splitless mode (delay 25 sec) in a 2.5 mm I.D. glass insert heated to 250°C, to which a 5 m × 0.31 mm I.D. fused-silica capillary column containing OV-1 cross-linked methylsilicone phase (layer thickness 0.17 μm; Hewlett-Packard, Avondale, PA, U.S.A.) was connected. The column was operated in the range of 190°C (hold for 2 min) to 240°C with a linear temperature increase of 4°C/min and a hydrogen flow-rate of 4.7 ml/min (*ca.* 100 cm/sec, measured at the initial column temperature). The nitrogen make-up flow-rate was equal to that of the additional hydrogen, *i.e.*, 30 ml/min. The detector temperature was maintained at 300°C.

RESULTS AND DISCUSSION

The derivatized thyroid iodo compounds are really perhalogenated substances of high molecular weights (range 1000–1200), unusual for solutes analysed by GC. The formation of a 2,2-bis(chlorodifluoromethyl)-1,3-oxazolidinone-5 ring after condensation of α-amino acids with DCTFA was confirmed mass spectrometrically in our previous study[20]. Thus, the structure of, *e.g.*, the derivative of T_4 formed upon treatment with DCTFA and HFBA is as shown:



For triac and tetrac we assume coupling of the DCTFA reagent to the carboxyl group first occurs followed by esterification of the resulting secondary hydroxyl group by HFBA, *e.g.*, the structure of the tetrac derivative would be as follows:



II

The formation of the cyclic oxazolidinone ring was monitored radiometrically in one of our previous studies[21] with the iodothyronines. Under conditions similar to those given in Experimental and at a slightly higher temperature (37°C), a total time of 15 min was required to complete the cyclization. Similar results were obtained with iodotyrosines[22] treated with DCTFA, where evaporation of the first condensation medium, *i.e.*, removal of DCTFA, prolonged the time required for the subsequent esterification of the phenolic group by HFBA to 30 min at a higher temperature.

In contrast, we have now found that under the stated conditions of the condensation medium (acetonitrile as the solvent with traces of pyridine as the catalyst) the ring closure need not be complete as the subsequent addition of the reactive anhydride results in a cooperative effect. The same is true for esterification of the phenolic group, where excess of DCTFA promotes the dehydration process, which is then immediate. Thus, the cooperative action of both strong dehydrating agents enables the whole procedure to be shortened to a few minutes. The molar ratio between the particular anhydride and pyridine is important concerning the yields for triac and tetrac. The molar amount of the anhydride should be slightly higher than half that of pyridine, in order to create an aqueous acidic phase upon liberation of the corresponding organic acid after addition of water. During the extraction with hexane and acidic water, pyridine is removed from the organic phase without any distortion of the derivatives. If greater amounts of the anhydride are added, the yields for triac and tetrac are reduced. On the contrary, the extraction yields are lower for all derivatized compounds if the water phase is neutral, *i.e.*, the amount of anhydride employed is lower than that recommended. The reasons for these phenomena are not known.

The employment of TFAA led to worse results with triac and tetrac and also with T_2 and T_3, in which the esterified hydroxyl group is not protected by the presence of neighbouring bulky iodine atoms against a possible hydrolytic cleavage. This was not the case with the HFB- or PFP-acylated forms, where no losses caused by the possible hydrolytic cleavage were observed although others[23] have reported on the relative instability of the N,O-diHFB methyl esters of T_3 in a packed chromatographic column. However, in this case we assume that the chromatographic support together with a relatively high column operating temperature (275°C) are responsible for the losses as even with a capillary column operated above 265°C a decreased recovery of the derivatized iodothyronines was found[1].

The variations in molar responses obtained upon derivatization of ten individually prepared samples and repeated injection of one sample are given in Table

TABLE I

VARIATIONS IN FLAME IONIZATION DETECTOR MOLAR RESPONSES (RELATED TO INTERNAL STANDARD)

The values were obtained through derivatization of ten individual standard samples (a) and GC analyses of one sample injected ten times (b), and represent means ± standard deviations.

Method	*T_3Ac*	*T_4Ac*	*T_2*	*T_3*	*rT_3*	*T_4*
a	0.514 ± 0.011	0.447 ± 0.017	0.656 ± 0.017	0.573 ± 0.014	0.532 ± 0.014	0.507 ± 0.020
b	0.518 ± 0.017	0.445 ± 0.021	0.637 ± 0.025	0.564 ± 0.022	0.523 ± 0.013	0.509 ± 0.018

I. There are no significant differences among the substances. The GC analysis of the compounds after the DCTFA–HFBA treatment is illustrated in Fig. 1. Similar results were achieved with PFPA instead of HFBA, but the retention times for the PFP-treated forms were slightly lower. The internal standard (III) undergoes no changes during the chemical treatment and its retention time suits perfectly for the required

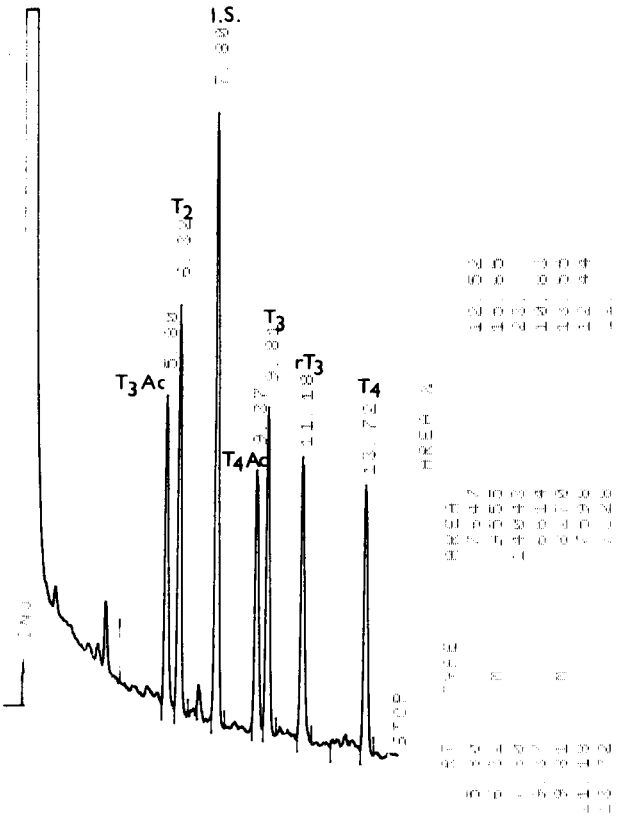
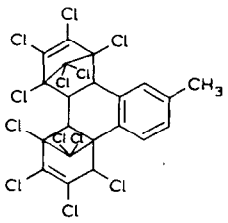


Fig. 1. GC analysis of a standard equimolar mixture (20 pmol of each compound injected) of the thyroid substances after treatment with DCTFA and HFBA as described. For the internal standard (I.S.), added in equimolar amount, and the chromatographic conditions see the text. Attenuation: 10^{-11} A.



III

analytic range. Its molar response is nearly twice that of the derivatized iodothyronines. This seems to confirm our earlier findings that the halogen atoms do not necessarily diminish the flame ionization detector response[24].

Whereas the N,O-diHFB methyl esters of iodothyronines can be chromatographed successfully also in packed columns[17,18,23], the GC analysis of the present perhalogenated substances fails completely in the presence of a chromatographic support. This is probably due to presence of the additional benzene ring separated from the first one by an ether linkage and the concomitant increase in the molecular weights caused by the additional iodine atoms, as the iodotyrosines, treated in the

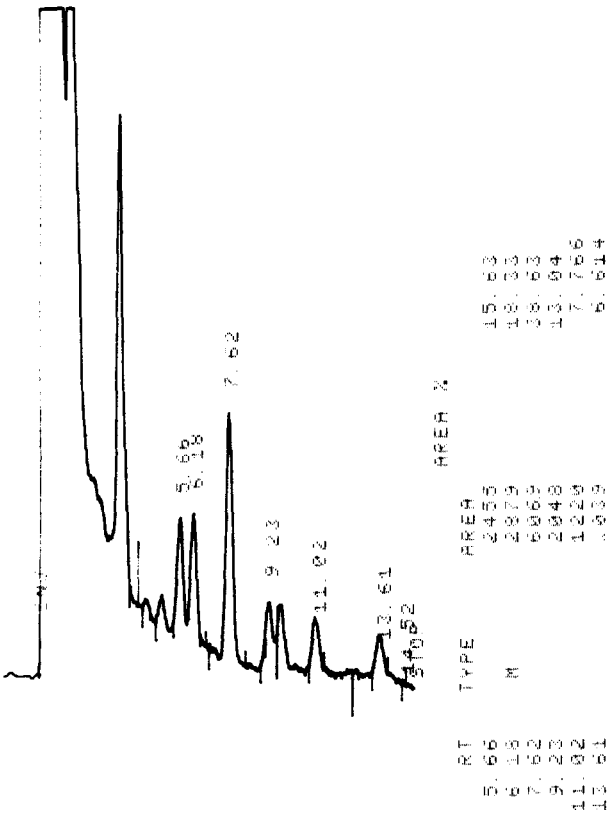


Fig. 2. GC analysis of the same sample as in Fig. 1, except that nitrogen (5 ml/min) instead of hydrogen was used as the carrier gas for the capillary column.

same way, can be eluted even from packed columns without difficulties[22,25]. The employment of a relatively short fused-silica capillary with a chemically bonded phase proved to be the best means for GC analysis of the compounds of interest while maintaining the benefit of the splitless injection mode. The employment of hydrogen as the carrier gas was confirmed to be of prime importance (Fig. 1), the results obtained with nitrogen (Fig. 2) being unsatisfactory especially for compounds of high molecular weight.

The behaviour of the derivatives in the subpicomole range of injected masses and with electron capture detection is currently under study. Difficulties in this respect have recently been comprehensively discussed[1–3].

CONCLUSIONS

A procedure for rapid conversion of thyroid hormonal substances (including triac and tetrac) into derivatives convenient for GC analysis is presented. The time required for the chemical treatment and the subsequent analysis is about 20 min. The combination of splitless injection with phase immobilization in a short fused-silica capillary enables one to analyse picomole amounts by use of a flame ionization detector. A convenient internal standard was found for this application.

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