(8) D. J. Macey and W. B. Gilboy, *Nucl. Instrum. Methods*, **92**, 501 (1971).
(9) G. E. Coote, N. E. Whitehead, and G. J. McCallum, *J. Radioanal. Chem.*, **12**, 491 (1972).
(10) L. Shabason and B. L. Cohen, *Anal. Chem.*, **45**, 284 (1973).
(11) J. F. Janni, Tech. Report AFWL-TR-65-150, Air Force Weapons Lab., New Mexico, U.S.A.F. (1966).
(12) R. B. Boulton, Ph.D. Thesis, Department of Physics, Queen's University, Kingston, Ontario, Canada, 1975.
(13) R. B. Boulton and G. T. Ewan, unpublished work.
(14) R. B. Boulton and G. T. Ewan, unpublished work.
(15) P. H. Ribbe and J. V. Smith, *J. Geol.*, **74**, 217 (1966).
(16) R. F. Martin, *Am. Mineral.*, **56**, 281 (1971).
(17) S. J. Horska, Department of Geology, McGill University, Montreal, Canada, private communication, 1975.
(18) A. F. Clarke, Department of Biochemistry, Queen's University, Kingston, Ontario, Canada, private communication, 1974.

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Analysis of Thyroid Hormones as Their Heptafluorobutyryl Methyl Ester Derivatives by Gas Chromatography–Mass Spectrometry

B. A. Petersen[1] and Paul Vouros*

Institute of Chemical Analysis, Applications and Forensic Science, Northeastern University, Boston, Massachusetts 02115

The mass spectra of the heptafluorobutyryl methyl ester derivatives of several thyroid hormones and their analogues have been examined, and their fragmentation patterns are discussed. Ions of significant relative intensity dominate the high mass ($m/e > 700$ amu) region of the spectra of these derivatives. In view of the decreased interference of background peaks at that high mass region, this feature has been effectively utilized in the GC-MS analysis of these compounds by selective ion monitoring. Detection limits of 0.5 pg have been attained.

The two thyroid hormones which are found in significant quantities in human serum are thyroxine (T_4) and 3,5,3′-triiodothyronine (T_3). Although their total concentrations are approximately 72 ng/mL and 2 ng/mL, respectively, only about 0.03% of T_4 and 0.03% of T_3 are free, that is, not protein bound, in normal serum. Nevertheless, these forms are of considerable interest because it is only the free hormone (i.e., FT_3 and FT_4) which is believed to penetrate cells, stimulate metabolism and exert feedback control to the pituitary (*1*). In addition, reverse T_3 (3,3′,5′-triiodothyronine; RT_3), although it is generally regarded as physiologically inactive, has recently become of interest partly because it occurs at different levels in the serum of euthyroid and pathological states (*2, 3*). For example, RT_3 is markedly elevated in the serum of the newborn and in amniotic fluid at 15 weeks pregnancy (*4, 5*).

Many difficulties are associated with the quantitation of the thyroid hormones both due to their presence in trace levels and the inexact methods of analysis currently employed. A variety of methods based on radiochemical techniques have been selected for estimating and monitoring FT_3 and FT_4 (*6–9*). Although currently utilized, these methods suffer from certain disadvantages such as the need for accompanying assays of the total amounts of T_3 and T_4 and the necessity to work with short half-lived radioisotopes and their degradation products.

A number of investigators have used gas chromatographic analysis with electron capture detection (GC-ECD) of volatile derivatives of the thyroid hormones as a direct means of quantitation (*10–13*). These methods required a minimum quantity of 1 mL of serum for the analysis, because of their relatively high detection limits and the low concentrations of thyroid hormones involved. Recently, however, it was shown that direct analysis of the free levels of these hormones can be achieved by formation of their *N,O*-diheptafluorobutyryl (HFB) methyl ester (ME) derivatives using GC-ECD (*14*).

In a logical extension of this earlier study we have now looked into the mass spectrometric properties of these derivatives and, in the process, examined the general utility of combined gas chromatography–mass spectrometry for their analysis by selective ion monitoring (SIM). For the purposes of structure elucidation from the mass spectral fragmentation patterns, several related compounds containing different halogen substituents on the phenyl system were synthesized and subsequently derivatized. In the following section we report on the fragmentation patterns of T_3 and RT_3 with particular emphasis on the sample requirements for quantitation of the latter compounds using selective ion monitoring.

EXPERIMENTAL

A. Reagents and Chemicals. Diiodothyronine (T_2), triiodothyronine (T_3), and tetraiodothyronine (T_4) were obtained as the free acids from Sigma (St. Louis, Mo.). Reverse T_3 (RT_3) and tetrachlorothyronine (Cl_4To) were kindly provided by R. I. Meltzer of Warner-Lambert Research Laboratories. The esterifying reagent was 25% (w/w) gaseous HCl (Matheson, Gloucester, Mass.) in methanol (Burdick and Jackson, Muskeegon, Mich.). The methanol was redistilled and dried over molecular sieve Type 5A. The acetylating reagent was heptafluorobutyric anhydride (K & K, Plainview, N.Y.) in acetonitrile (Burdick and Jackson). A deuteriomethyl ester derivative of several of the thyroid hormones was prepared by reacting the free acid with CD_3OD (99.999% isotopic purity; Stohler Isotopes, Billerica, Mass.).

B. Synthesis of 3′,5′-Dibromo-3,5-diiodothyronine (Br_2T_2) and 3′,5′-Dichloro-3,5-diiodothyronine (Cl_2T_2). (*1*) *Br_2T_2.* Five hundred mg of T_2 was placed in a 50-mL round bottom flask and dissolved in 10 mL of glacial acetic acid. Three hundred mg of Br_2 were then added and the solution was allowed to stand at room temperature for 72 h. The solution was then removed under vacuum with a rotary evaporator at 60 °C. The product (Br_2T_2) was recrystallized from ethanol/2N HCl (95:5,V/V) in 90% yield. (mp 136–138) (*15*).

(*2*) *Cl_2T_2.* Five hundred mg of T_2 was placed in a 50-mL round bottom flask and dissolved in 10 mL of glacial acetic acid. Cl_2 was allowed to bubble through the solution for 15 min at room

[1]Present address, Battelle Columbus Laboratories, 505 King Avenue, Columbus, Ohio 43201.

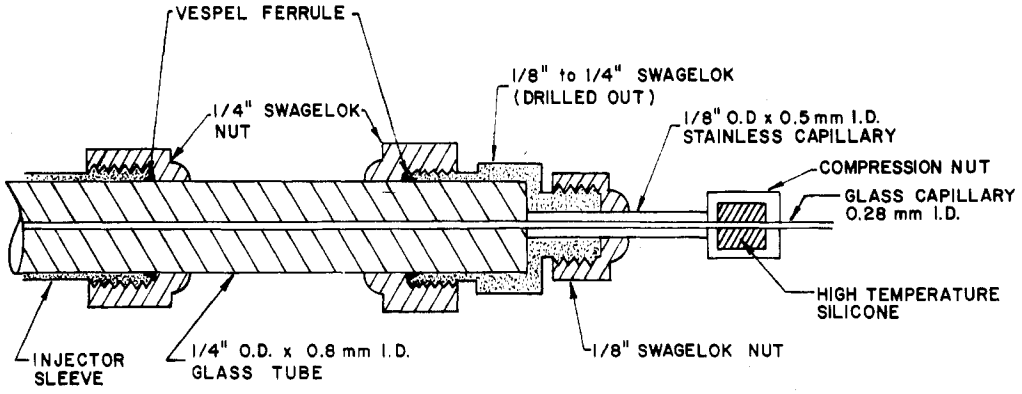


Figure 1. Schematic of capillary column connection to injection port of GC-MS system

Table I. Relative Intensity Values of Significant Ions of the N,O-diHFB-Me Derivatives of the Thyroid Hormones

	1 (X = H)	2 (X = I)	3 (X = I)	4 (X = I)
$(M)^{+\cdot}$	62[a] (931)[b]	67 (1057)	63 (1057)	60 (1183)
$(M - 213)^{+\cdot}$	100 (718)	100 (844)	70 (844)	100 (970)
$(M - 284)^{+}$	22 (647)	67 (773)	100 (773)	48 (899)
$(M - 410)^{+}$	36 (521)	25 (647)	15 (647)	28 (773)
$(M - 213 - I, X)^{+\cdot}$	14 (590)	22 (590)	14 (590)	36 (716)
$(M - 284 - I, X)^{+\cdot}$	32 (519)	35 (519)	38 (519)	30 (645)
$(M - 410 - I, X)^{+}$	17 (393)	6 (393)	5 (393)	8 (519)
$(M - 284 - I, X - 197)^{+\cdot}$	33 (322)	21 (322)	32 (322)	21 (448)

[a] % relative intensity. [b] *m/e* value.

temperature. The excess chlorine was then removed by bubbling N_2 through the solution. The solvent was again removed by use of the rotary evaporator, and the product recrystallized as before in 80% yield (mp 123–126) °C (*15*).

C. Derivative Formation. Esterification of the thyroid hormone standards and related compounds was accomplished separately by placing 1.0 mg of each into a 3-mL microreaction vial (Pierce, Rockford, Ill.) adding 1.0 mL of the 25% HCl–alcohol solution, sealing with a Teflon coated septum and heating to 60 °C for 1 h. The solution was subsequently evaporated to dryness under a stream of dry N_2. A freshly prepared solution of 400 μL of heptafluorobutyric anhydride in 500 μL of acetonitrile was then added to acetylate the amino nitrogen and phenolic oxygen of the thronines. The tube was kept at 60 °C for 1 h, cooled to room temperature, and the solution blown to dryness with dry N_2.

D. Preparation of the Physiological RT_3 Sample. Twenty mL of euthyroid sera were placed in a clean dialysis membrane (Visking Nojax, Union Carbide, Chicago, Ill.). This membrane was then inserted into a clean, silanized 100-mL Erlenmeyer flask and 30 mL of 0.15 M phosphate buffer (19.70 g K_2HPO_4–4.92 KH_2PO_4/L H_2O, pH 7.40) added. Dialysis was allowed to continue for 16 h at 37.0 ± 0.5 °C with gentle stirring. The dialysate was purified using cation-exchange chromatography (AG 50W-X2, 100-200 mesh, Bio-Rad, Richmond, Calif.) by placing on a 10 cm × 1 cm i.d. column, washing with 50 mL of 0.15 M ammonium acetate (pH 8.5), and 6 mL of methanol. The free thyroid hormone fraction was collected with 10 mL of methanol–12 N NH_4OH (97:3,v/v). After evaporation of this solution, the derivatization was conducted in the identical fashion as above. A detailed description of all procedures used in the preparation of the physiological sample has appeared in a recent publication (*14*).

E. Mass Spectra of the Thyroid Hormone Derivatives. Low resolution mass spectra were obtained with a Nuclide 12-90-G mass spectrometer by direct insertion probe. The accelerating potential was 4 kV, electron energy was 70 eV, the ionization current 50 μA, and the temperature of the ion source maintained at 300 °C.

High resolution mass spectra of the thyroid hormone derivatives were kindly provided by Catherine Costello of the Massachusetts Institute of Technology, Cambridge, Mass.

F. Combined Gas Chromatographic–Mass Spectrometric (GC-MS) Analysis. A Varian 2700 gas chromatograph was coupled to the mass spectrometer via a direct transfer line. Since the instrument was equipped with high capacity diffusion pumps and the ion source was differentially pumped, no molecular separator was used. A high temperature valve system (Valco, Chicago, Ill.) was used in the GC detector cavity to direct the effluent from the GC to the MS or to the atmosphere. The direct transfer line was a stainless steel capillary 12 inches long with an inside diameter of 0.010 inch. This transfer line could be resistance heated to 350 °C.

The GC column used to separate the derivatives was a high resolution wall-coated glass capillary (20 m × 0.15 mm i.d.) coated with 1% OV-101. (Gas Chromatographic Supplies, Tokyo, Japan). The injector system of the GC was modified for direct capillary injection without splitting by inserting a glass sleeve ($^1/_4$-inch o.d. × 0.80 mm i.d.) into the injection port and maintained at 350 °C. A $^1/_4$-inch to $^1/_8$-inch reducing union was drilled out so that the capillary column connector could be attached to the glass sleeve without excessive dead volume. The glass capillary was connected to the system with high temperature silicone septums. A schematic diagram of this capillary column connection is shown in Figure 1. The carrier gas was ultra high purity helium (99.999%; Matheson) and the flow rate was 2 mL/min. The column was operated at 290 °C to separate all the thyroid hormone derivatives and at 275 °C during SIM of the T_3 and RT_3 derivatives. The effluent from the GC was monitored using the total ion current detector while the output from the electron multiplier was monitored using both a strip chart and the oscillographic recorder. The valve system, transfer line and ion source were maintained at 300 °C during GC-MS. Ionization conditions were identical to those in Section E.

RESULTS AND DISCUSSION

A. Interpretation of the Mass Spectra of the Thyroid Hormone Derivatives. The compounds examined in this study were the *N,O*-di-HFB-ME derivatives of 3,5-diiodothyronine (T_2; **1**), 3,3′,5-triiodothyronine (T_3; **2**) 3,3′,5′-triiodothyronine (RT_3; **3**), 3,3′,5,5′-tetraiodothyronine (T_4; **4**), 3′,5′-dibromo-3,5-diiodothyronine (Br_2T_2; **5**), 3′,5′-dichloro-3,5-diiodothyronine (Cl_2T_2; **6**), and 3,3′,5,5′-tetrachlorothyronine (Cl_4T_0; **7**). The spectra of these derivatives are summarized in Table I (Compounds **1–4**) and Table II (Compounds **5–7**). Complete mass spectra are shown in Figures 2–8. To assist in the interpretation of the spectra, the fragmentation of the d_3-methyl ester of the thyroxine derivative T_4 (**4**) was also examined. The mass shifts of the

Table II. Relative Intensity Values of Significant Ions of the N,O-diHFB-Me Derivatives of Compounds Related to the Thyroid Hormones

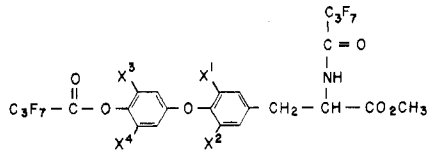
	Compound		
	5 (X_1 = I, X_3 = Br)[a]	6 (X_1 = I, X_3 = Cl)[b]	7 (X_1 = Cl, X_3 = Cl)[b]
$(M)^{+\cdot}$	24[c] (1087)[d]	75 (999)	7 (815)
$(M - 213)^{+\cdot}$	100 (874)	100 (786)	82 (602)
$(M - 284)^{+}$	47 (803)	42 (715)	69 (531)
$(M - 410)^{+}$	23 (677)	20 (589)	100 (405)
$(M - 213 - X_1, X_3)^{+\cdot}$	29 (668)	8 (624)	8 (532)[e]
$(M - 284 - X_1, X_3)^{+}$	26 (597)	9 (553)	6 (461)
$(M - 410 - X_1, X_3)^{+}$	6 (471)	5 (427)	7 (335)
$(M - 284 - X_1, X_3 - 197)^{+\cdot}$	12 (400)	7 (356)	9 (264)

[a] *m/e* values based on ^{79}Br in ion peak cluster. [b] *m/e* values based on ^{35}Cl in ion peak cluster. [c] % relative intensity. [d] *m/e* value. [e] % relative intensity corrected for isotopic composition from *m/e* 531 (i.e. $[M - 284]^{+}$).

Table III. Mass Shifts of the Principal Ions in the Mass Spectrum of the *N,O*-Diheptafluorobutyryl-d_3-methyl Ester Derivative of T_4

	HFB-ME-T_4	HFB-d_3-ME-T_4	ΔM
$(M)^{+\cdot}$	1183	1186	3
$(M - 213)^{+}$.	970	973	3
$(M - 284)^{+}$	899	899	0
$(M - 410)^{+}$	773	776	3
$(M - 213 - 2I)^{+\cdot}$	716	719	3
$(M - 254\ 2I)^{+}$	645	645	0
$(M - 410 - 2I)^{+}$	519	522	3
$(M - 254 - 2I - 197)^{+\cdot}$	448	448	0

principal fragment ions are indicated in Table III.



1 (T_2) : $X^1 = X^2 = I$; $X^3 = X^4 = H$

2 (T_3) : $X^1 = X^2 = X^3 = I$; $X^4 = H$

3 (RT_3) : $X^1 = X^3 = X^4 = I$; $X^2 = H$

4 (T_4) : $X^1 = X^2 = X^3 = X^4 = I$

5 (Br_2T_2) : $X^1 = X^2 = I$; $X^3 = X^4 = Br$

6 (Cl_2T_2) : $X^1 = X^2 = I$; $X^3 = X^4 = Cl$

7 (Cl_4T_0) : $X^1 = X^2 = X^3 = X^4 = Cl$

The following noteworthy features are common to the mass spectra of compounds 1–7:

(a) The molecular ion peaks of the *N,O*-di-HFB methyl ester derivatives of the thyroid hormones and related compounds investigated were of generally high relative intensity. This intensity seemed to vary with halogen substitution on the phenyl system in the order Cl < Br < I.

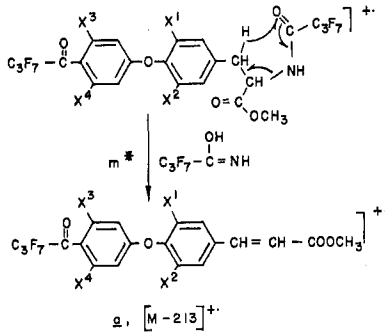
(b) Noteworthy is the high intensity of the ion peaks appearing at the high mass region (*m/e* > 700) of the spectra.

(c) A major fragment ion corresponding to $[M - 213]^{+\cdot}$ was observed in all of the spectra. It is conceivably formed via a McLafferty rearrangement as indicated in Scheme I. The three mass unit shift of the $[M - 213]^{+\cdot}$ ion in the spectrum of the d_3-methyl ester of T_4 (Table III) and high resolution data support the structure *a* given in Scheme I.

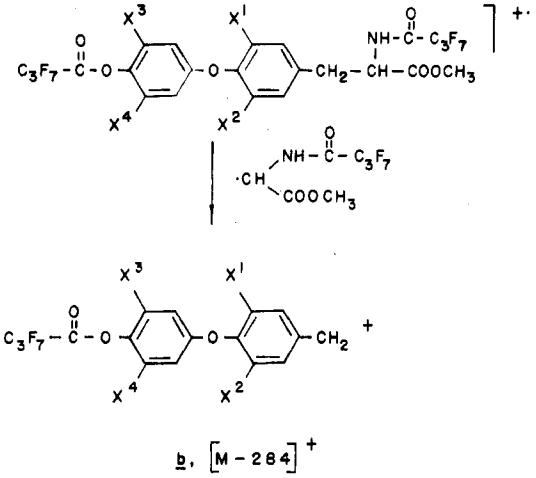
(d) The abundant ion corresponding to $[M - 284]^{+}$ is formed by cleavage β to the aromatic ring in a manner analogous to the fragmentation of related compounds such as tyrosine (*15, 16*), *N*-trifluoroacetyl-*O*-butyl aromatic amino acids (*17*), and trimethylsilyl (TMS) derivatives of thyroxine (*18*). Scheme II.

(e) An ion corresponding to $[M - 410]^{+}$ is formed by elimination of C_3F_7–CO· from $[M - 213]^{+\cdot}$, as supported by metastable transitions (Scheme III). This ion can be stabilized by resonance through the π-system as indicated by

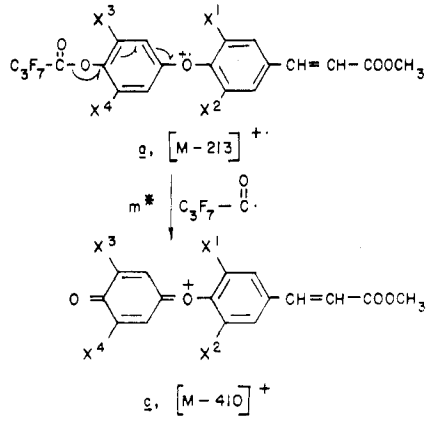
SCHEME I



SCHEME II



SCHEME III



structure *c* (Scheme III). Noteworthy is the increased abundance of this ion in the spectrum of the tetrachloro derivative (7) where it constitutes the base peak. This may

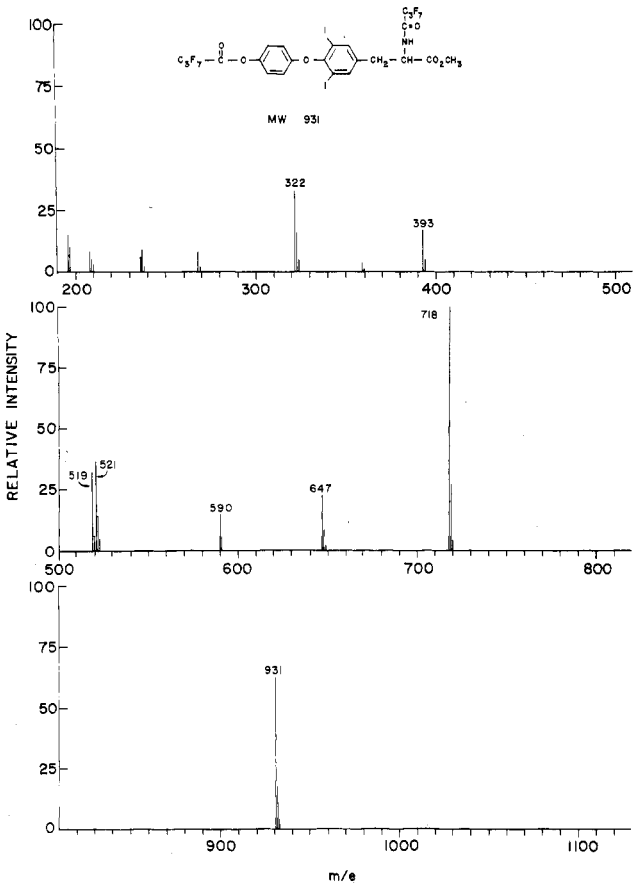


Figure 2. Mass spectrum of the HFB-ME derivative of 3,5-diiodothyronine (T_2; **1**)

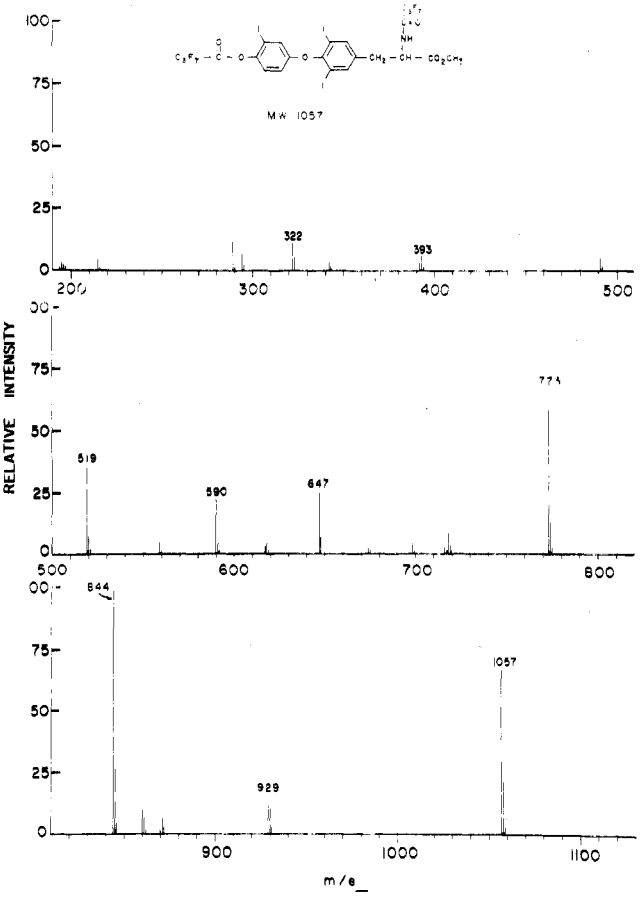
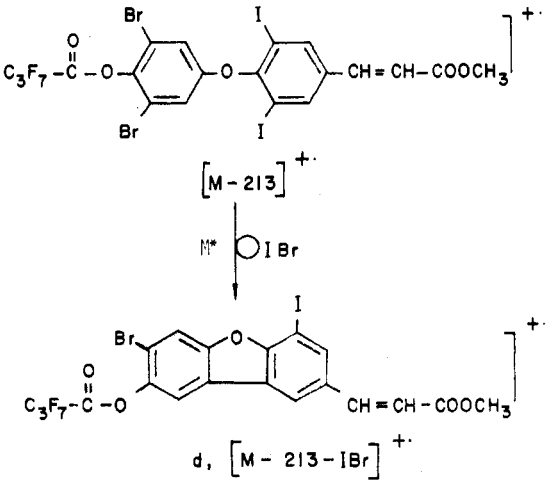
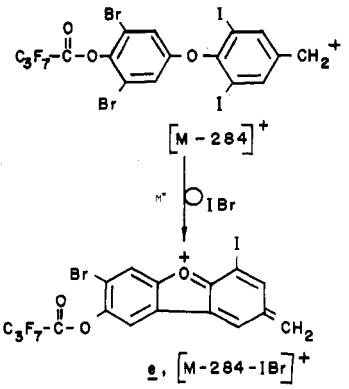


Figure 3. Mass spectrum of the HFB-ME derivative of 3,3′,5-triiodothyronine (T_3; **2**)

SCHEME IV



SCHEME V



be attributed to the decreased steric inhibition to resonance of the smaller chlorine atoms in 7 compared to the larger bromine and iodine atoms in 1–6. This is consistent with earlier studies by Bursey and co-workers on the effect of ortho substituents on the resonance stabilization of fragment ions (*19*, *20*).

(f) In all of the spectra, appear ions of significant intensity corresponding to the loss of two halogens from the various fragment ions. To ascertain from which phenyl ring the halogens were eliminated, the mass spectra of the related derivatives Br_2T_2 (**5**) and Cl_2T_2 (**6**), which contain different halogens on the two phenyl rings, were examined. In the spectrum of Br_2T_2 there is no evidence for the simultaneous loss of either two iodines or bromines from the molecular ion or the major fragment ions. Instead an apparently concerted loss of BrI occurs from $[M - 213]^+\cdot$ (*m/e* 874) and $[M - 284]^+$ (*m/e* 803) as shown in Schemes IV and V, respectively. It is speculated that these eliminations are accompanied by an intramolecular hydrogen shift to yield the resonance stabilized cyclic structures *d* and *e*. Fragment ions corresponding to the above, and originating from $[M - 213]^+$ (*m/e* 786) and $[M - 284]^+$ (*m/e* 715), also appear in the spectrum of Cl_2T_2 (**6**). They occur at *m/e* 624 and *m/e* 553 (i.e. loss of ClI from each). These mechanisms and structures are also supported by metastable transitions and high resolution data.

An additional fragment ion in the spectra of these derivatives, corresponding to the loss of two halogens and presumably ring condensation, also appears after cleavage of the heptafluoro-butyryl group attached to the phenolic oxygen of $[M - 284 - 2X]^+$. The general structure for this fragment ion (*f*) is shown in Scheme VI.

It is interesting that ions representing cleavage of the ethereal bond and retention of the charge with either part of the molecule were not observed. This is unfortunate with

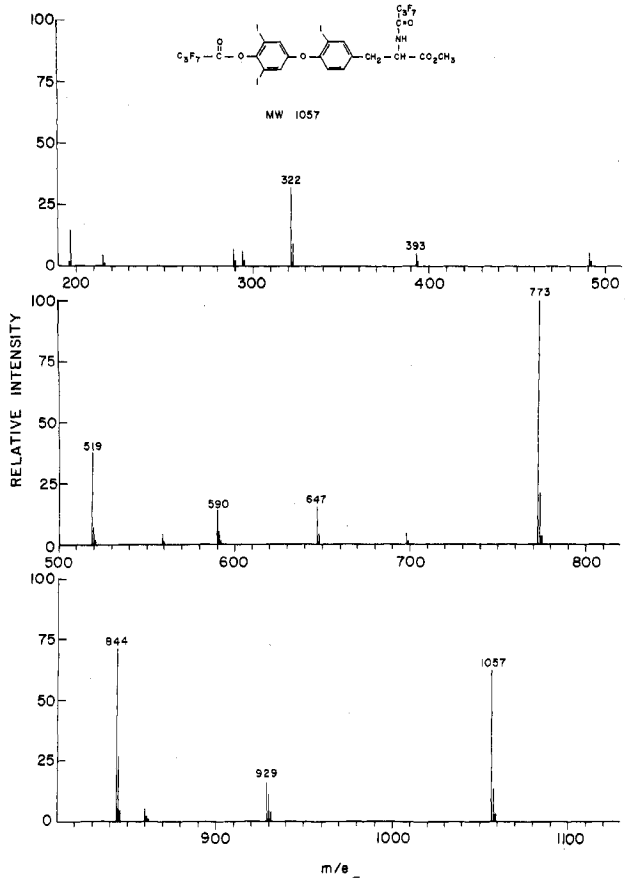
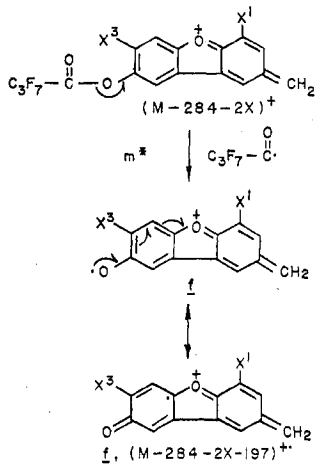


Figure 4. Mass spectrum of the HFB-ME derivative of 3,3′,5′-triiodothyronine (RT_3; **3**)

SCHEME VI



regard to mass spectral identification based on discriminating between the positional isomers T_3 and RT_3. The fragmentation patterns of these isomers are identical, differing only on relative peak intensities. However, in view of the significant difference in chromatographic retention time of these two compounds, a study of their GC-MS properties was conducted using SIM for their detection and quantitation.

GC-MS Studies. As stated earlier, analysis of the free thyroid hormones in serum presents a major challenge due to their occurrence in trace levels. This is further compounded by the fact that the serum sample itself is often in short supply and of such complexity that it requires considerable cleanup and pretreatment to achieve isolation of the hormones in a medium and form suitable for final analysis. Previous investigations (*14*) have shown that, Dialysis–GC–ECD can be

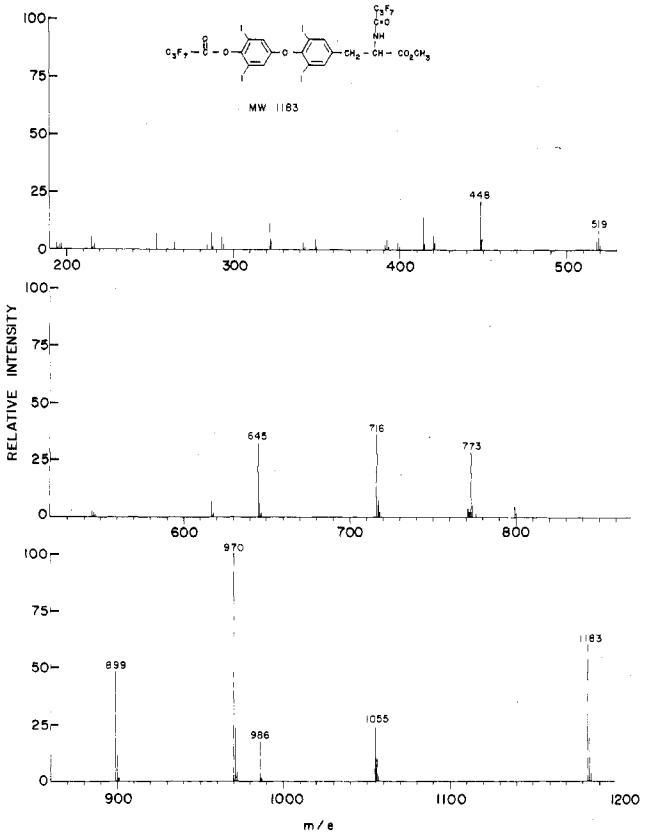


Figure 5. Mass spectrum of the HFB-ME derivative of 3,3′,5,5′-tetraiodothyronine (T_4; **4**)

effectively used for the concurrent analysis of FT_3 and FT_4 in both normal and pathological sera as their di-HFB-ME derivatives. However, due to the fact that free RT_3 (FRT_3) occurs in extremely low concentrations in normal serum (i.e., 0.5–2.0 pg/mL) severe demands are placed on its detection by GC-ECD. Furthermore, confirmation of the identity of the eluted components is also extremely difficult when present in such low concentrations. Because of the fact that formation of the HFB-ME derivatives of these hormones had been shown to be quantitative at physiological concentrations, their excellent mass spectrometric properties, and the high selectivity and specificity of GC-MS, we examined the utility of this technique for the analysis of FRT_3 in normal serum using selective ion monitoring (SIM) techniques.

Some of the requirements for effective utilization of SIM techniques in GC-MC include: (a) the presence of an ion(s) indicative of and specific to the compound(s) in question, (b) occurrence of this ion(s) at an m/e value(s) free of interferences from column bleed or background, (c) possession of a substantial fraction of the total ion current with the ion(s) used for SIM, and (d) the ready availability of an isotopically labeled analogue for use as an internal standard and carrier. Co-injection of this analogue can also minimize the losses arising from adsorption in the chromatographic system and interface, thereby improving the detection limits of the analysis.

The ion in the spectrum of the HFB-ME derivative of RT_3, which satisfied all of these requirements, was $[M - 213]^+$ (m/e 844). The high m/e value of this ion virtually eliminates the possibility of any interference due to column bleed, as such ion peaks usually occur at much lower mass numbers. Furthermore, this ion is common to the spectra of both RT_3 and T_3 comprising over 20% of the total ion current, thus permitting the concurrent analysis of these compounds in view of their significantly different elution times. This is illustrated in Figure 9 which shows the total ion current and selective

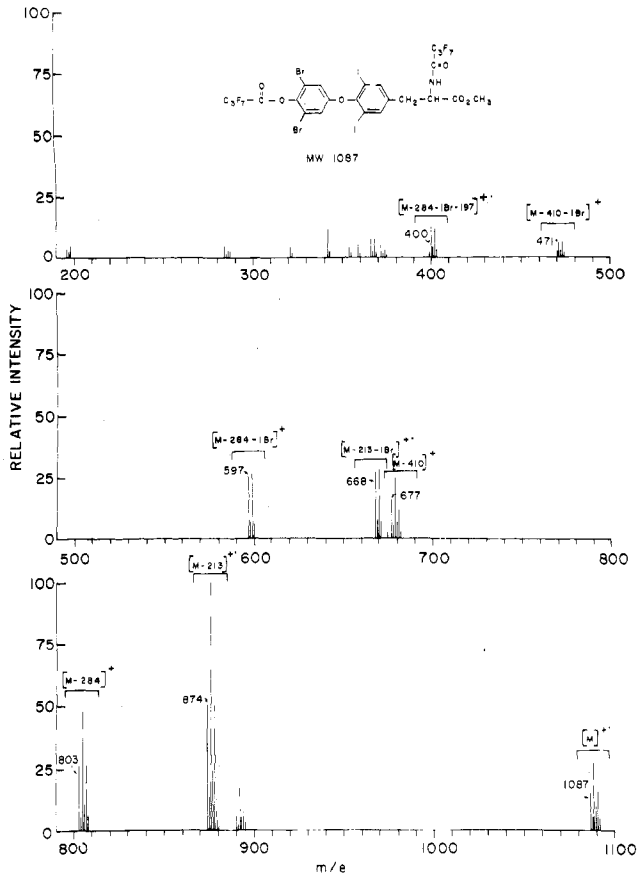


Figure 6. Mass spectrum of the HFB-ME derivative of 3′,5′-dibromo-3,5-diiodothyronine (Br_2T_2; **5**)

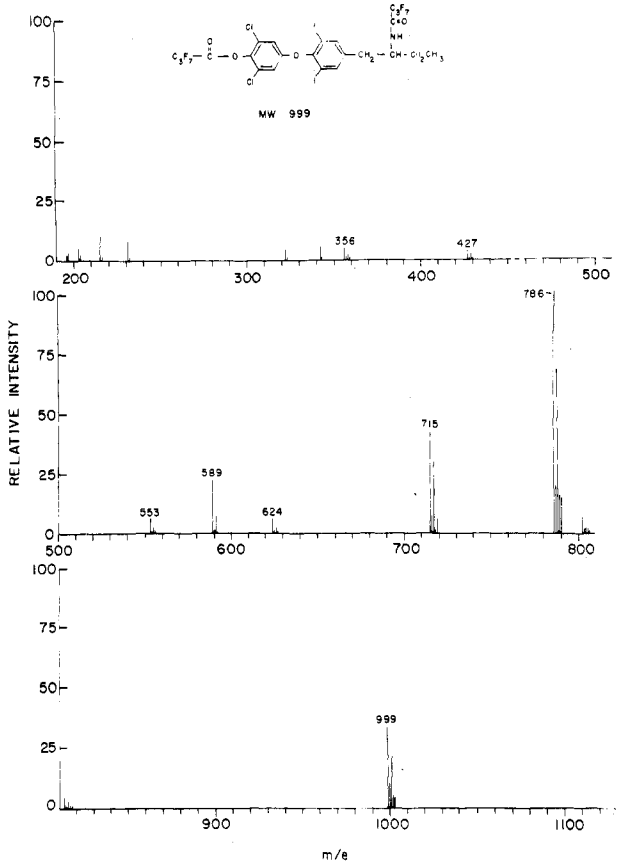


Figure 7. Mass spectrum of the HFB-ME derivative of 3′,5′-dichloro-3,5-diiodothyronine (Cl_2T_2; **6**)

ion chromatograms of a standard mixture containing 100 ng each of T_3 and RT_3. Finally, a deuteriomethyl ester derivative of RT_3 can be easily synthesized and the $[M - 213]^+$ ion contains the CD_3 group (Scheme I), thus shifting its value from m/e 844 to m/e 847. This feature should permit the deuteriated compound to act as an internal carrier to minimize loss of the physiological compound in the chromatographic system.

To establish the conditions for GC-MS analysis and quantitation of T_3 and RT_3, the mass spectrometric sensitivity of their HFB-ME derivatives was determined by SIM of the m/e 844 ion. As the particular instrument employed in this study (Nuclide 12-90-G) was equipped with a retractable Faraday cup in the collector housing, absolute ion current measurements under SIM conditions could be obtained. Therefore, by eliminating the variable of electron multiplier gain, quantitative analysis could be performed in terms of coulombs/microgram ($C/\mu g$) of sample introduced into the system. Details of this technique have appeared in an earlier publication (*21*). Standard sensitivity values were obtained by introducing known quantities of the respective derivatives into the ion source via the direct inlet probe and monitoring the ion current over the lifetime of the sample. Values of 9.6×10^{-10} $C/\mu g$ and 6.5×10^{-10} $C/\mu g$ for T_3 and RT_3, respectively, were obtained. Based on these measurements the transfer efficiency of the GC-MS system for these derivatives was found to be 97% at sample levels of 0.1 μg. A calibration curve of RT_3 (Figure 10) for quantitative analysis was obtained by injection of standard sample aliquots into the GC-MS system.

The sensitivity values for T_3 and RT_3 were used to evaluate quantitatively the extent to which the isotopically labeled internal carrier can improve the trace level analysis of a compound during GC-MS. To a solution containing known amounts of HFB-ME derivatives of T_3 and RT_3, a 1000-fold excess of the HFB-d_3-ME derivative of RT_3 was added, and varying amounts of sample over a range exceeding 10^5 (100 ng–0.5 pg) were injected into the GC-MS. The value of using a deuteriated internal carrier is demonstrated from the graph

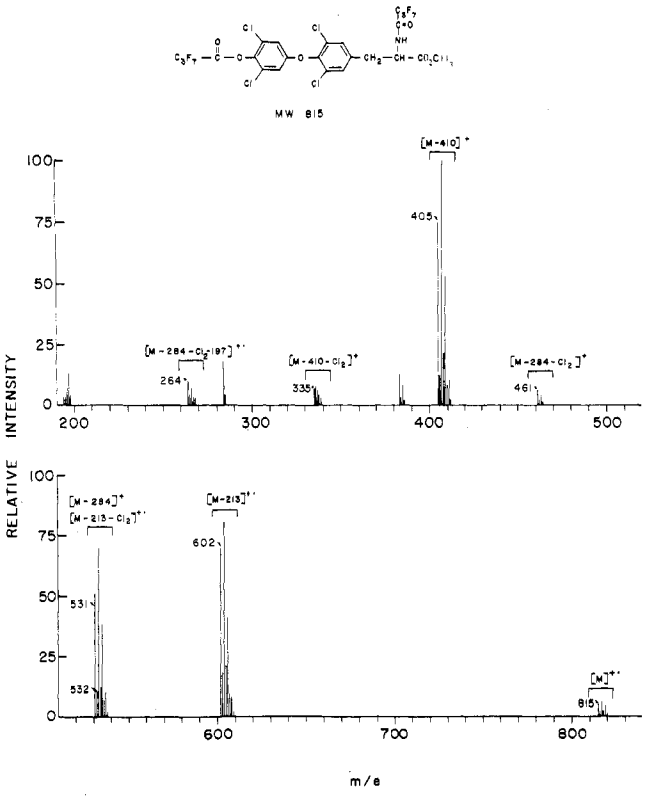


Figure 8. Mass spectrum of the HFB-ME derivative of 3,3′,5,5′-tetrachlorothyronine (Cl_4T_0)

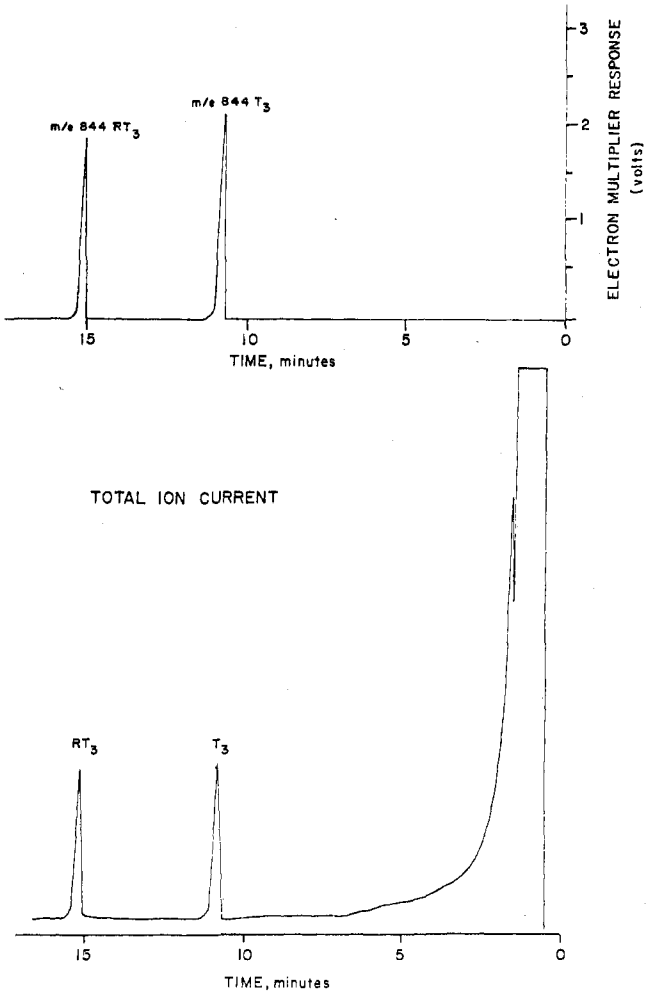


Figure 9. Gas chromatographic separation of 100 ng each of the HFB-ME derivatives of T_3 and RT_3. Simultaneous recording of the total ion current (bottom) and electron multiplier response (SIM of *m/e* 844; top). GC conditions: 20 m × 0.28 mm i.d. glass capillary coated with 1% OV-101, Gas flow 2 mL/min, temperature: 275 °C, isothermal

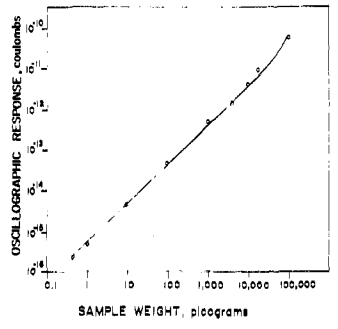


Figure 10. Calibration plot of the HFB-ME derivative of RT_3 as oscillographic peak area (coulombs) vs. weight of RT_3 injected (SIM of *m/e* 844)

in Figure 11. Under ideal conditions, the sensitivity vs. sample weight curve should have a straight line with a slope of zero, since the sensitivity is normalized with respect to the electron multiplier gain. In fact, the slope for the sensitivity curve of RT_3, which was co-eluted with the deuterium labeled carrier, closely approaches that ideal value while, on the other hand, the sensitivity curve for T_3 drops sharply at levels below 10 ng. Furthermore, the lowest detection limit for RT_3 which was co-eluted with its d_3-labeled counterpart was approximately 0.5 pg as compared to 100 pg for the T_3 derivative. It should be noted that a 0.1-μg sample of the HFB-d_3-ME derivative of RT_3 exhibited no detectable contribution to *m/e*

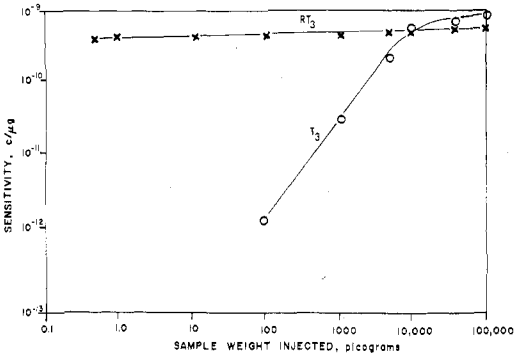


Figure 11. Normalized plot of mass spectrometric sensitivity vs. sample weight of RT_3 and T_3. Effect of labeled internal carrier on sensitivity and detection limits. Component ratio in mixture injected into GC-MS: HFB-ME-T_3:HFB-ME-RT_3:HFB-d_3-ME-RT_3 = 1:1:1000

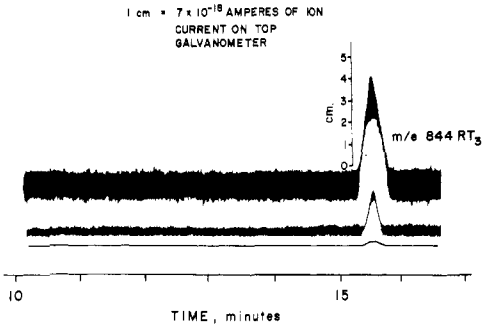


Figure 12. Oscillographic response to *m/e* 844 during GC-MS-SIM of a physiological sample containing RT_3 and T_3 as their HFB-ME derivatives. Galvanometer sensitivity 30 (top), 10 (middle), 1 (bottom). Electron multiplier gain, $G = 10^8$. Chromatographic conditions as in Figure 9

844 due to any d_0 impurity under SIM conditions.

To assess the general applicability of our results, we examined a physiological sample of known concentration of RT_3 (2 pg/mL). The ratio of free T_3 to free RT_3 in this sample had previously been established at 4:1 by Dialysis–GC–ECD (22). After dialysis, ion exchange chromatography, derivatization, and concentration of a 20-mL euthyroid serum sample, an aliquot was analyzed by GC-MS and monitoring of the *m/e* 844 ion. The oscillographic recording of this analysis is shown in Figure 12. Note that although the physiological concentration of T_3 is 4–5 times higher than that of RT_3, no signal was detected at its expected retention time, (i.e. ~11 min) whereas the response for the RT_3 derivative is clearly evident. Based on our calibration data (Figure 10), the signal obtained corresponds to approximately 1 pg injected, or a free concentration of 2 pg/mL of serum, i.e., well within the expected value. It should be noted that several blanks containing solvent and reagents exhibited no signal under the same conditions at *m/e* 844. In view of the extremely low concentrations involved, it is not entirely clear at this point whether the RT_3 detected represents the physiologically free forms or may result from mono de-iodination of T_4 during dialysis. However, our data clearly indicate its presence.

CONCLUSIONS

The data presented above point out the usefulness of combined gas chromatography–mass spectrometry and, in particular, the use of selective ion monitoring techniques for the analysis of the thyroid hormones. To perform quantitative analysis at the trace level, the use of an isotopically labeled internal carrier has been evaluated, and was shown to be essential for this purpose.

It should be emphasized that for quantitative analysis, the calibration data of Figure 12 cannot be used on a day-to-day

basis because of the possibility of variations in instrumental conditions as shown in an earlier study (*21*). For this purpose, multiple ion detection using an accelerating voltage alternator (AVA) (*23*) and simultaneous monitoring of several ions, including one or more of the isotopically labeled internal standard, is necessary. Ideally, the internal standard should be subjected to the identical sample preparation and derivatization conditions as the compound in question. This was not possible in this specific case, as the labeled analogue of RT_3 had to be prepared separately during esterification. This problem can be alleviated by using a compound containing deuterium atoms on the thyronine phenyl rings, which, of course, would be a major project in itself. Nevertheless, the methodology developed for the analysis of free RT_3 by Dialysis–GC–MS–SIM can be applied to the analysis of all the thyroid hormones and their precursors as their HFB-ME derivatives.

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LITERATURE CITED

(1) P. R. Larsen, *Med. Clin. North Am.*, **59**, 1063 (1975).
(2) P. Nicod, A. Burger, V. Stacheli, and M. V. Vallotton, *J. Clin. Endocrinol. Metab.*, **42**, 823 (1976).
(3) H. Meinhold, K. W. Wenzel, and P. Schürnbrand, *Z. Klin. Chem. Klin. Biochem.*, **13**, 571 (1975).
(4) I. J. Chropra and B. F. Crandell, *N. Engl. J. Med.*, **293**, 740 (1975).
(5) D. A. Fisher, *N. Engl. J. Med.*, **293**, 770 (1975).
(6) K. A. Sterling and A. Hegedus, *J. Clin. Invest.*, **41**, 1031 (1962).
(7) S. S. Levinson and S. U. Reider, *Clin. Chem. (Winston-Salem, N.C.)*, **20**, 1568 (1974).
(8) J. S. Annino and R. W. Giese, "Clinical Chemistry", Little, Brown & Co., Boston, Mass., 1976, p 306.
(9) K. A. Sterling and M. A. Brenner, *J. Clin. Invest.*, **45**, 153 (1966).
(10) M. Tajuddin and S. G. Elfbaum, *Clin. Chem., (Winston-Salem, N.C.)*, **19**, 109 (1972).
(11) N. N. Nihei, M. C. Gershengorn, T. Mitsuma, L. R. Stringham, A. Cordy, B. Kuchmy, and C. S. Hollander, *Anal. Biochem.*, **43**, 433 (1971).
(12) P. I. Jaakonmäki and J. E. Stouffer, *J. Gas Chromatogr.*, **5**, 303 (1967).
(13) J. E. Stouffer, P. I. Jaakonmäki, and T. J. Wenger, *Biochim. Biophys. Acta*, **127**, 261 (1966).
(14) B. A. Petersen, R. N. Hanson, R. W. Giese, and B. L. Karger, *J. Chromatogr.*, **126**, 503 (1976).
(15) A. Dibbo, J. C. P. Sly, L. Stephenson, T. Walker, W. K. Warburton, and K. D. E. Whiting, *J. Am. Chem. Soc.*, **90**, 2890 (1968).
(16) K. M. Baker, M. S. Shaw, and D. H. Williams, *Chem. Commun.*, 1108 (1969).
(17) E. Gelpi, W. A. Koenig, J. Bilbert, and J. Oro, *J. Chromatogr. Sci.*, **7**, 604 (1969).
(18) A. M. Lawson, D. B. Ramsden, P. J. Raw, and R. Hoffenberg, *Biomed. Mass Spectrom.*, **1**, 374 (1974).
(19) M. M. Bursey, *J. Am. Chem. Soc.*, **91**, 1861 (1969).
(20) M. M. Bursey and M. K. Hoffman, *J. Am. Chem. Soc.*, **91**, 5023 (1969).
(21) D. J. Marshall, B. A. Petersen and P. Vouros, submitted for publication.
(22) B. A. Petersen, R. W. Giese, B. L. Karger, and P. R. Larsen submitted for publication.
(23) J. F. Holland, C. C. Sweely, R. E. Thrush, R. E. Teets, and M. A. Bieber, *Anal. Chem.*, **45**, 308 (1973).

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Determination of Mefruside and Its Metabolites in Urine by High Performance Liquid Chromatography

C. J. Little,* A. D. Dale, D. A. Ord,[1] and T. R. Marten

Roche Products Ltd., P.O. Box 8, Welwyn Garden City, Hertfordshire, United Kingdom

A method is described for packing high performance liquid chromatography (HPLC) columns using isopropanol as the slurry medium. Highly reproducible columns can be made by this technique which applies equally well to reversed phase columns. Chromatographic conditions are described suitable for the analysis of a urine extract of the sulfonamide "Mefruside" and its metabolites. Prior to extraction with ethyl acetate, the urine should be acidified, to convert all of the major metabolite (5-oxo-Mefruside) into its lactone form. The extract is then quantitatively analyzed by an external standardization technique which gives essentially the same result as a radiochemical check method.

Sulfonamide drugs have been important for many years as antibacterial agents. Recently they have been used as antidiabetic and diuretic agents. Of the many and varied methods described for their analysis, chromatography is one of the more specific. Paper and thin-layer chromatography have been used extensively (*1*), as have chromatography on polyamide (*2*), zinc ferrocyanide (*3*), and paper impregnated with strong cation-exchange resins (*4*). Recently, high pressure anion exchange (*5*), reverse phase (*6*), and ion pair partition (*7*) methods have been published for specific sulfonamides.

This paper describes the application of high performance liquid chromatography (HPLC) on silica columns to the analysis of the sulfonamide Mefruside "I" (Ro-8-3725) and its metabolites "II" and "III" (Figure 1).

EXPERIMENTAL

Apparatus. Modular chromatographic equipment was used comprising the following items: (a) Constametric II pump, (b) Rheodyne 7010 injection valve, (c) Cecil CE 212 UV monitor (set at 248 nm). Column packing was performed with a high pressure pump (maximum ~15 000 psig) from Stansted Fluid Power, Stansted, Essex. "Apollo" stainless steel tubing, with a $^1/_2$-μm internal surface finish was used throughout (Accles and Pollock, Oldbury, Warley, Worcestershire, U.K.).

Reagents. All solvents were of analytical reagent (AR) grade and were used without further purification.

Microparticulate silica used in the course of this work was Partisil-5, and was used straight from the bottle.

TLC investigations were carried out on precoated plates (E.M. Merck, Darmstadt—BDH) which were used without activation.

Samples of Mefruside "I" and its metabolites "II" and "III" were obtained from Bayer.

Column Preparation. The balanced density slurry method of packing columns (*8–10*) and several variants of the method (*11, 12*) are now common practice. For several years we have slurried silica and bonded silicas in alcohol mixtures which rapidly wet the silica surface. Initially we used methanol but now use iso-

[1] Present address, Spectra-Physics Ltd., 17 Brick Knoll Park, St. Albans, Hertfordshire, U.K.