ELSEVIER

Journal of Chromatography A, 725 (1996) 287–294

JOURNAL OF CHROMATOGRAPHY A

Identification of the thermal degradation products of L-triiodothyronine sodium (liothyronine sodium) by reversed-phase high-performance liquid chromatography with photodiode-array UV and mass spectrometric detection

M. Andre[a,*], R. Domanig[a], E. Riemer[a], H. Moser[b], A. Groeppelin[b]

[a]*Biochemie GmbH, A-6250 Kundl, Austria*

[b]*Sandoz Pharma AG, Analytical Research and Development, CH-4002 Basle, Switzerland*

First received 8 March 1995; revised manuscript received 11 September 1995; accepted 13 September 1995

Abstract

The degradation products of the thermally stressed amino acid L-triiodothyronine sodium (liothyronine sodium) were investigated by high-performance liquid chromatography (HPLC) with photodiode-array UV detection and with mass spectrometric detection. Samples stressed at 80°C show a reproducible degradation pattern. Usually, amino acids are degraded to the corresponding aldehyde with one less carbon atom by oxidative deamination (Strecker degradation). In contrast, the unusual degradation of the amino acid triiodothyronine sodium results in the formation of the corresponding carboxylic acid, amide, amine, alcohol and alkane with one less carbon atom. The main degradation products of triiodothyronine sodium are triiodothyroacetic acid, triiodothyroacetic acid amide and triiodothyroethane; trace amounts of triiodothyroethylamine, triiodothyroethyl alcohol and the analogous degradation products of L-tetraiodothyronine sodium (levothyroxine sodium), a by-product of triiodothyronine sodium, were observed. Additionally, these results were compared with the degradation pattern of a differently stressed sample of L-tetraiodothyronine sodium.

Keywords: Liquid chromatography–mass spectrometry; Triiodothyronine sodium; Amino acids; Hormones; Liothyronine; Levothyroxine; Tetraiodothyronine sodium; Thyronines

1. Introduction

The pituitary gland hormones triiodothyronine (liothyronine) and tetraiodothyronine (levothyroxine) are biological compounds highly important as essential indicators of thyroid gland diseases in clinical diagnosis. The structure of the thyronines derived from the amino acid tyrosine is shown in Fig. 1.

The sodium salts are used as therapeutic agents. High-performance liquid chromatography (HPLC) has proved a reliable method for determining thyronines and related compounds in biological matrices and in pharmaceutical preparations [1–31]. HPLC has been established as the official testing method for quality control in the

* Corresponding author.

0021-9673/96/$15.00
SSDI 0021-9673(95)01007-6

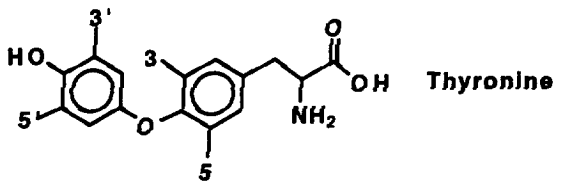

Fig. 1. Structure of thyronines with iodination sites 3, 5, 3′ and 5′.

US Pharmacopeia and the European Pharmacopoeia [1,2]. One of the most important aspects of quality assurance of drugs is a substantial knowledge of their thermal stability for establishing expiration dates. The main objective of this study was the elucidation of the thermal degradation pathway of L-triiodothyronine sodium by identification of unknown degradation products using HPLC and mass spectrometry (MS).

2. Experimental

2.1. Materials and reagents

A Nucleosil 5-μm particle size C_{18} column (Macherey–Nagel, Düren, Germany), 100 × 4.6 mm I.D., and a Nucleosil 5-μm particle size C_8 column (FZ Seibersdorf, Seibersdorf, Austria), 125 × 4 mm I.D., were used for HPLC–UV and HPLC–MS analyses, respectively. HPLC-grade solvents, phosphoric acid and acetic acid (analytical-reagent grade) were obtained from Merck (Darmstadt, Germany). Triethylamine (analytical-reagent grade) was purchased from Fluka (Buchs, Switzerland).

2.2. Samples and reference compounds

Samples of L-triiodothyronine sodium were stressed by exposing them to air at 80°C for 3 weeks. A particular sample of L-tetraiodothyronine sodium was stressed at 60°C for 7 days. The reference substances used were either purchased from commercial sources or synthesized according to literature procedures. Triiodothyronine and tetraiodothyronine were USP Official Reference Substances (Rockville, MD, USA); diiodotyrosine was purchased from Senn Chemicals (Dielsdorf, Switzerland). Diiotothyronine, diiodothyroacetic acid, triiodothyroacetic acid and tetraiodothyroacetic acid were synthesized according to literature procedures [32–34]. Triiodothyroacetic acid amid was prepared by ammonolysis of the corresponding methyl ester.

2.3. Chromatographic equipment

All HPLC runs were carried out with an HP 1090 liquid chromatograph (Hewlett-Packard, Waldbronn, Germany), equipped with a binary solvent-delivery system and an HP 1040 photodiode-array UV–visible detector. The peaks were integrated by an HP 3357 Lab-Data-System. For on-line HPLC–MS analysis, an HP 1090 liquid chromatograph was connected to an HP 5987 mass spectrometer (Hewlett-Packard, Palo Alto, CA, USA) by a standard Vestec (Houston, TX, USA) thermospray interface. The tip temperature was adjusted to 250°C. Negative ionization with a discharge current or with a filament emission of 300 μA (thermospray with filament-on mode) was used. The range m/z 200–900 was scanned with an integration time of 250 μs and eight measurements per mass unit. A discharge or a filament current was added for better sensitivity and for the formation of fragment ions. During the analysis the source was kept at 320°C. The mass spectrometer was equipped with a cryogenic pump cooled by liquid nitrogen. The chromatographic separation was also controlled by an HP 1040 photodiode-array UV detector connected in-line between the HPLC system and the mass spectrometer. MS data were processed by an HP 1000 computer system.

2.4. Chromatographic conditions

(A) HPLC–UV detection

The mobile phase consisted of solvent A (0.05 *M* triethylammonium phosphate, pH 3.0) and solvent B (acetonitrile). A linear gradient from 5% to 70% B within 70 min with a flow-rate of 1.0 ml/min at ambient temperature was used. The column effluent was monitored at 231 nm. The sample solution was prepared by dissolving

40 mg of L-triiodothyronine sodium in 1 ml of 0.1 *M* NaOH and diluting with methanol–water (1:1) to a final volume of 10 ml. The reference solution, containing 1 mg of each reference substance in 10 ml, was prepared in the same way as the sample solution. Volumes of 10 μl of each solution were injected.

(B) HPLC–mass spectrometry

The mobile phase consisted of solvent A [water–acetonitrile (95:5) containing 0.05% acetic acid] and solvent B [water–acetonitrile (20:80) containing 0.05% acetic acid]. A step gradient from $t = 0$ min/0% B, $t = 12$ min/30% B, $t = 47$ min/55% B to $t = 70$ min/100% B with a flow-rate of 1.0 ml/min at ambient temperature was used. Additionally, the column effluent was monitored at 231 nm. The sample and reference solution were prepared as described under chromatographic conditions A.

3. Results and discussion

3.1. HPLC–photodiode-array UV detection

In a first study, HPLC separation with photodiode-array UV detection was optimized by gradient elution to study the degradation pathway as far as possible. The results obtained were used to establish a suitable routine procedure for studying product degradation according to official guidelines [1]. Triethylammonium phosphate proved to be the best mobile phase buffer with respect to peak shape and selectivity. A typical elution pattern of a stressed triiodothyronine sodium sample (A), the blank gradient (B) and reference compounds (C) is shown in Fig. 2.

In addition to the known by-products diiodothyronine (peak 2) and tetraiodothyronine (peak 4), there are numerous degradation products in low or trace concentrations. Two of the predominant degradation products (peaks 6 and 7) were identified by comparing the retention times and UV spectra of the sample peaks and the reference compounds. The UV spectra of the reference peak and the corresponding sample peak of triiodothyroacetic acid amide (peak 6)

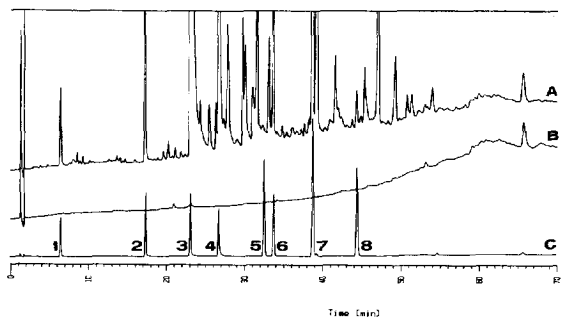

Fig. 2. Chromatograms (chromatographic conditions A) of (A) a stressed L-triiodothyronine sodium sample, (B) blank gradient and (C) reference substances. Peaks of reference substances: 1 = diiodotyrosine; 2 = diiodothyronine; 3 = triiodothyronine; 4 = tetraiodothyronine; 5 = diiodothyroacetic acid; 6 = triiodothyroacetic acid amide; 7 = triiodothyroacetic acid; 8 = tetraiodothyroacetic acid.

and triiodothyroacetic acid (peak 7) were congruent.

Comparison of the spectra of sample peaks at lower concentrations with the spectra of reference peaks failed owing to increased noise of the registered spectra. The elution patterns of the three stressed samples are identical regarding the number of peaks and the relative concentration of each degradation product. Triiodothyroacetic acid was identified as the main degradation product in a concentration range of up to 4%.

3.2. HPLC–mass spectrometry

The replacement of the mobile phase triethylammonium phosphate buffer system (chromatographic conditions A) by the volatile acetic acid buffer system (chromatographic conditions B) caused no change of the elution order of the reference substances but a considerable loss of peak symmetry and resolution. Typical elution patterns of a stressed triiodothyronine sodium sample monitored by UV detection at 231 nm and the extracted ion current (EIC) of m/z 254 are shown in Fig. 3. The EIC of m/z 254 was chosen, because all of the iodinated compounds bearing more than two iodine atoms showed a fragment ion represented by the peak at m/z 254. Most probably it corresponds to the molecu-

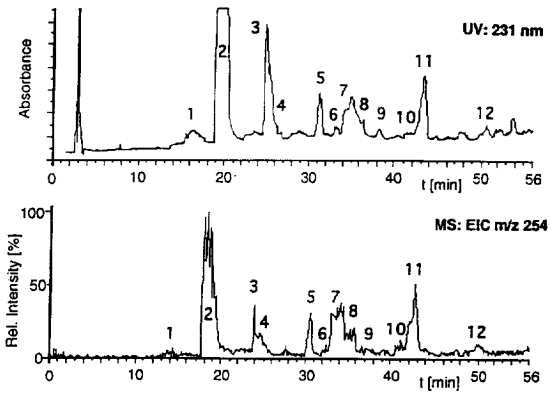

Fig. 3. Gradient elution (chromatographic conditions B) of stressed L-triiodothyronine sodium sample with UV detection at 231 nm and extracted ion chromatogram (EIC) of the ion at m/z 254.

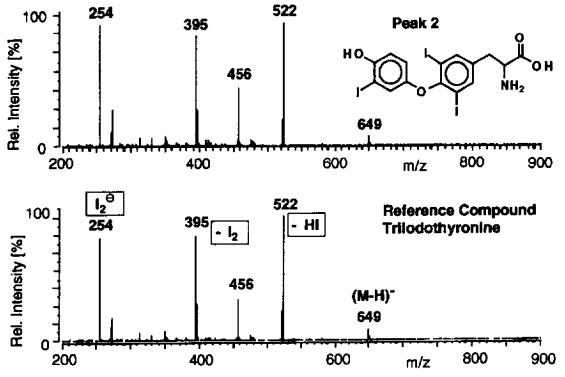

Fig. 4. Mass spectra of peak 2 in the chromatogram in Fig. 3 and reference compound triiodothyronine.

lar iodine ion. Both chromatograms show very similar elution patterns.

The order of the eluted peaks in the chromatogram (Fig. 3), the corresponding peaks of molecular ions, fragment ions and the compounds resulting from the interpretation of the mass spectra are summarized in Table 1.

Mass spectrometry

The mass spectra of 12 peaks were analysed and compared with those of the corresponding reference substances, whenever these were available. Most of the mass spectra exhibit peaks of molecular ions and fragment ions to characterize the degradation products. The fragmentation of all compounds represented by peaks 1, 2, 3, 5, 7 and 10 in the chromatogram shown in Fig. 3 is in exact agreement with the fragmentation of the corresponding reference compounds. The mass spectrum of diiodothyronine (peak 1, Fig. 3) exhibits only one ion at m/z 524. Obviously, the formation of the iodine fragment ion is not a dominant fragmentation with diiodo compounds.

The fragmentation pattern of triiodothyronine (peak 2, Fig. 3) is shown in Fig. 4 in comparison with the reference compound. The molecular ion $(M-H)^-$ is represented by the peak at m/z 649. The fragment ions exhibit a higher intensity than

Table 1
Detected peaks in the chromatogram shown in Fig. 3, ions monitored (m/z) and the corresponding compounds derived

Peak No.	Molecular ion (m/z)	Fragment ions (m/z)	Compound
1	524	–	Diiodothyronine
2	649	254, 395, 456, 522	Triiodothyronine
3	776	254, 395, 522, 649	Tetraiodothyronine
4	–	254, 381, 508, 635	Structure not assignable
5	–	254, 365, 426, 492, 619	Triiodothyroacetic acid amide
6	605	254, 351, 412, 479	Triiodothyroethylamine
7	–	254, 322, 366, 449, 493, 576	Triiodothyroacetic acid
8	606	254, 352, 479	Triiodothyroethyl alcohol
9	745	254, 365, 491, 618	Tetraiodothyroacetic acid amide
10	–	254, 366, 448, 492, 574, 619	Tetraiodothyroacetic acid
11	591	254, 336, 464	Triiodothyroethane
12	716	254, 336, 463, 590	Tetraiodothyroethane

the molecular ion. A consistent explanation of the dominant fragmentation represented by the peaks at m/z 522 is the loss of hydrogen iodide and the loss of molecular iodine in the case of the fragment peak at m/z 395.

The same fragment ions were detected for tetraiodothyronine (peak 3, Fig. 3). The molecular ion $(M-H)^-$ at m/z 776 is not detectable. Peak 4 (Fig. 3) elutes as a shoulder on peak 3. The mass spectrum shows peaks at m/z 508, 381 and 254. The molecular ion is expected to exhibit a peak at m/z 635, because all of the fragment peaks show a mass difference of −14 as compared with the corresponding fragment peaks of the parent compound triiodothyronine. From the information obtained, no definite structure can be derived. The mass spectrum of the compound represented by peak 6 (Fig. 3) shows a fragment peak at m/z 605 which probably represents the molecular ion. The mass difference from the main compound triiodothyronine is −44, indicating a decarboxylation to the structure triiodothyroethylamine. The decarboxylation of amino acids to amines at elevated temperatures is known from the literature [35]. Triiodothyroacetic acid (peak 7, Fig. 3) is the main degradation product. The fragmentation pattern is shown in Fig. 5. In addition to the expected fragment ions generated by the loss of hydrogen iodide and molecular iodine, decarboxylation is observed as a third fragmentation reaction.

The mass spectrum of the compound represented by peak 8 (Fig. 3) shows a fragment peak at m/z 606 which probably represents the molecular ion $(M-H)^-$. The mass difference of all of these ions from the corresponding ions of the main compound is −43. The uneven number indicates the loss of the amino group yielding triiodothyroethyl alcohol. The compound represented by peak 9 (Fig. 3) has also been analysed by thermospray ionization with a filament emission resulting in a slightly milder ionization. The fragment peak at m/z 745 represents the molecular ion $(M-H)^-$. The combination of both mass spectra leads to the proposal of the structure tetraiodothyroacetic acid amide for peak 9. The structure proposed is in accord with the mass difference of +96 from triiodothyronine. Peak 11 (Fig. 3) represents a dominant degradation product of triiodothyronine. The ion at m/z 591 has been ratified as the molecular ion using thermospray ionization with filament emission. The mass difference from the main compound triiodothyronine is −59 and is consistent with the structure of triiodothyroethane. The non-polar structure is in agreement with the long retention time. The analogous interpretation is applicable to the compound represented by peak 12 (Fig. 3), again by using thermospray ionization with filament emission. The structure proposed is tetraiodothyroethane.

Additionally, for a preliminary comparison a tetraiodothyronine sodium sample stressed at a lower temperature (60°C) for a shorter period has been analysed by the same HPLC–UV–MS method (chromatographic conditions B). Only trace amounts of nine degradation products were detected with a low signal-to-noise ratio. A typical chromatogram is shown in Fig. 6.

For the compounds represented by peaks 2, 3 and 9 in the chromatogram shown in Fig. 6 an unambiguous interpretation of the mass spectra is possible. In addition to the main compound tetraiodothyronine (peak 3), the expected by-product triiodothyronine (peak 3) has been identified. Studies of the thermal inactivation of the free acid of tetraiodothyronine have established the degradation to triiodothyronine as the only degradation product [36]. The mass spectrum and the retention time of the compound repre-

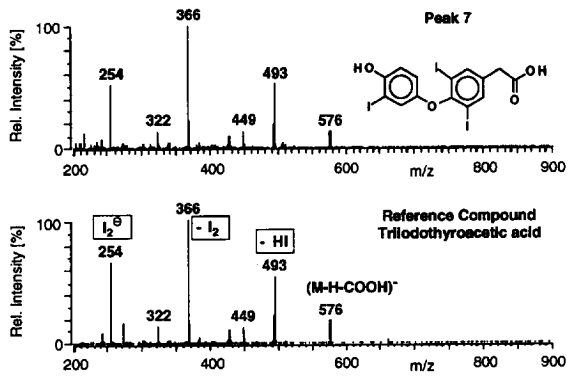

Fig. 5. Mass spectra of peak 7 in the chromatogram in Fig. 3 and reference compound triiodothyroacetic acid.

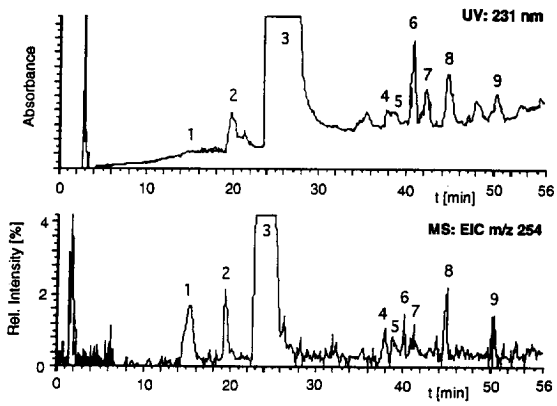

Fig. 6. Gradient elution (chromatographic conditions B) of stressed L-tetraiodothyronine sodium sample with UV detection at 231 nm and extracted ion chromatogram (EIC) of the ion at m/z 254.

sented by peak 9 are identical with the corresponding data for peak 12 of the stressed triiodothyronine sample (see Fig. 3 and Table 1). The derived structure is tetraiodothyroethane, indicating a degradation pathway common to triiodothyronine. The mass spectrum of the compound represented by peak 4 shows fragment peaks identical with those observed for tetraiodothyroacetic acid. The structure of tetraiodothyroacetic acid is in agreement with the mass spectrum but not with the retention time. A possible explanation is an iodine shift as a side-reaction during the synthesis in very low concentration. An aromatic halogen shift is a well known phenomenon; especially the iodine shift is catalysed by hydrogen iodide which is generated in the iodination step of thyronines [37].

The compounds represented by peaks 5 and 6 exhibit the same fragment peaks at m/z 254, 352, 478, 479 *and* 604. The mass difference from the main compound tetraiodothyronine is −44. Tetraiodothyroethylamine is a structure in agreement with the mass spectrum. Again an iodine shift reaction is an acceptable explanation for the different retention times. From the information obtained no definite structure can be derived for peaks 1, 7 and 8 in the chromatogram shown in Fig. 6. Further investigations of samples of tetraiodothyronine stressed under harsher conditions will provide exact identification.

3.3. Degradation pathways

Upon thermal stress, the sodium salt of the amino acid triiodothyronine show unusual degradation compared with regular amino acids: the main degradation pathway consists in the formation of the carboxylic acid with one less carbon atom and its subsequent reaction to the amide. Usually, amino acids degrade to the aldehyde with one less carbon atom according to the Strecker degradation, i.e., oxidative deamination and subsequent decarboxylation of the oxocarboxylic acid [38–42].

In the case of the less stressed tetraiodothyronine sodium sample, only trace amounts of degradation products were observed. Therefore, the interpretation of the resulting mass spectra is ambiguous for some peaks. The analogous tetraiodinated thyronine exhibits degradation products mostly with the same chemical functionalities, indicating a common degradation pathway of thyronines. These preliminary results will be confirmed by further investigations of severely stressed tetraiodothyronine sodium samples. In both cases no aldehyde with one less carbon atom (Strecker degradation) was detected.

The formal thermal degradation pathway (Fig. 7) of triiodothyronine sodium is the normal oxidative deamination of the amino acid and the decarboxylation of the resulting intermediate oxocarboxylic acid with subsequent disproportionation of the aldehyde to the corresponding thyroacetic acid and thyroethyl alcohol, analogous to the Cannizzaro reaction. Up to now, the degradation of amino acids to the corresponding carboxylic acids with one less carbon atom has been observed only with anodic oxidation of amino acids [43]. The formation of thyroethane could be assisted by reduction of the thyroethyl alcohol by hydrogen iodide [44]. A second route is the decarboxylation of the parent compound triiodothyronine.

3.4. MS fragmentation

Regarding the mass spectrometry of iodinated thyronines and their degradation products, three intrinsic characteristics have been observed:

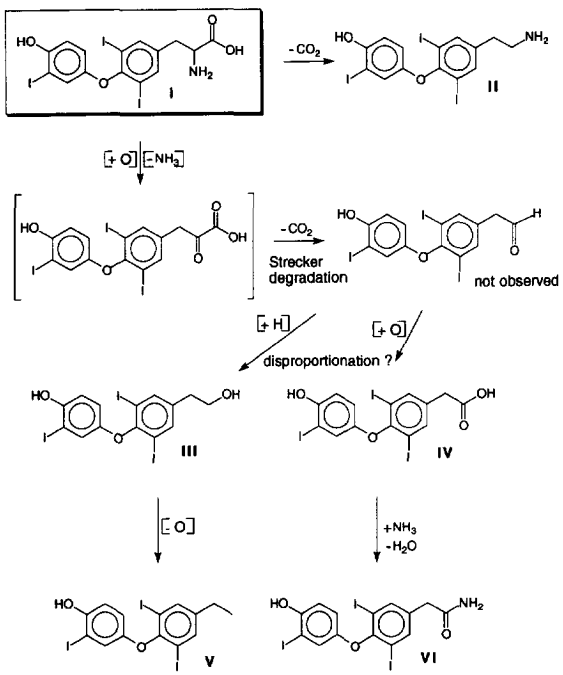

Fig. 7. Thermal degradation pathway of L-triiodothyronine sodium (**I**) to triiodothyroethylamine (**II**), triiodothyroethyl alcohol (**III**), triiodothyroacetic acid (**IV**), triiodothyroethane (**V**) and triiodothyroacetic acid amide (**VI**).

(1) The intensity of the molecular ion decreases as the content of iodine increases.

(2) The main type of fragmentation is a splitting off of hydrogen iodide and molecular iodine. The carboxylic acids show decarboxylation as a third minor fragmentation reaction. This type of fragmentation has been observed for all of the corresponding degradation products, even for those to which no definite structure was assignable.

(3) All of the tri- and tetraiodothyronine compounds showed an ion at m/z 254, probably representing the molecular iodine ion. Degradation products with more than two iodine atoms can easily be detected by the extracted ion chromatogram of the ion at m/z 254.

References

[1] United States Pharmacopeia, XXIII Revision, US Pharmacopeial Convention, Rockville, MD, 1995, pp. 883, 893 and 1957.
[2] European Pharmacopoeia, (1991) Maisonneuve, Saint-Ruffine, 2nd ed., 1991, Fifteenth Fascicule, Monograph 728.
[3] N.M. Alexander, CRC Handbook of HPLC for the Separation of Amino Acids, Peptides and Proteins, Vol. 1, CRC Press, Boca Raton, FL, 1984, pp. 291–301.
[4] F. Nachtmann and M. Andre, CRC Handbook of HPLC for the Separation of Amino Acids, Peptides and Proteins, Vol. 1, CRC Press, Boca Raton, FL, 1984, pp. 303–308.
[5] S.L. Richheimer and T.M. Amer, J. Pharm. Sci., 72 (1983) 1349.
[6] M.T.W. Hearn and B. Grego, J. Liq. Chromatogr., 7 (1984) 1079.
[7] J.F. Brower, T.Y. Toler and J.C. Reepmeyer, J. Pharm. Sci., 73 (1984) 1315.
[8] E. Besenfelder, J. High Resolut. Chromatogr. Chromatogr. Commun., 7 (1984) 566.
[9] S.L. Richheimer and C.B. Jensen, J. Pharm. Sci., 75 (1986) 215.
[10] P.R. Bedard and W.C. Purdy, J. Liq. Chromatogr., 9 (1986) 1971.
[11] A.S. Sidhu, J.M. Kennedy and S. Deeble, J. Chromatogr., 391 (1987) 233.
[12] C.M. Selavka and I.S. Krull, Anal. Chem., 59 (1987) 2699.
[13] P.R. Kootstra, H.H. Van de Broek, E.A. Hogendoorn, C.E. Goewie and J.J.M. De Vijlder, J. Chromatogr., 458 (1988) 175.
[14] L.V. Oberkotter, J. Chromatogr., 487 (1989) 445.
[15] L. Dalgaard, J.J. Hansen and J.L. Pedersen, J. Pharm. Biomed. Anal., 7 (1989) 361.
[16] T. Ohmori, O. Tarutani and T. Hosoya, J. Biochem., 265 (1990) 931.
[17] G. Lovell and P.H. Corran, J. Chromatogr., 525 (1990) 287.
[18] J. Escribano, M. Asuncion, J. Miguel, L. Lamas and E. Mendez, J. Chromatogr., 512 (1990) 255.
[19] G. Ducrotoy, L. Richert, V. De Sandro, D. Lurier and E. Pacaud, J. Chromatogr., 566 (1991) 415.
[20] E.H.J.M. Jansen, L. Doorn and F.X.R. Van Leeuwen, J. Chromatogr., 566 (1991) 471.
[21] L. Doorn, E.H.J.M. Jansen and F.X.R. Van Leeuwen, J. Chromatogr., 553 (1991) 135.
[22] R.M. Sweeting and J.G. Eales, Gen. Comp. Endocrinol., 85 (1992) 367.
[23] T.M. Hays and R.R. Cavalieri, Metab. Clin. Exp., 41 (1992) 494.
[24] C.E. Hendrich, J. Berdecia-Rodriguez, V.T. Wiedmeier and S.P. Porterfield, J. Chromatogr., 577 (1992) 19.
[25] B. Loun and D.S. Hage, J. Chromatogr., 579 (1992) 225.
[26] E. Abe, S. Murai, Y. Masuda and H. Saito, Naunyn-Schmiedebergs Arch. Pharmacol., 346 (1992) 238.
[27] C.E. Hendrich and S.P. Porterfield, Endocrinology (Baltimore), 131 (1992) 195.
[28] K. Takatera and T. Watanabe, Anal. Chem., 65 (1993) 759.
[29] A. Whitaker and J.G. Eales, Fish Physiol. Biochem., 10 (1993) 431.

[30] T.T. Nguyen, J.J.III. Di Stefano, H. Yamada and Y.M. Yen, Endocrinology, 133 (1993) 2973.
[31] Y.M. Yen, J.J.III Di Stefano, H. Yamada and T.T. Nguyen, Endocrinology, 134 (1994) 1700.
[32] F. Langer, Österreichisches Patentamt, Patentschrift Nr. 243246 (1965).
[33] H. Ziegler and C. Marr, J. Org. Chem., 27 (1962) 3336.
[34] J.H. Wilkinson, Biochem J. 63 (1956) 601.
[35] Ullmann's Encyclopedia of Industrial Chemistry, Vol. A2, VCH, Weinheim, 5th ed., 1985, p. 65.
[36] J. Wortsman, D.C. Papadimitriou, M. Borges and C.L. Defesche, Clin. Chem., 35 (1989) 90.
[37] J. March, Advanced Organic Chemistry, Wiley, New York, 1985, p. 510, and references cited therein.
[38] T.M. Reynolds, Adv. Food Res., 12 (1963) 1.
[39] T.M. Reynolds, Adv. Food Res., 14 (1965) 167.
[40] Comprehensive Organic Chemistry, The Synthesis and Reaction of Organic Compounds, Vol. 2, Pergamon Press, Oxford, 1979, pp. 824 and 825.
[41] Houben Weyl Methoden der Organischen Chemie, Vol. 11/2, Georg Thieme, Stuttgart, 1958, p. 332.
[42] Kirk-Othmer Encyclopedia of Chemical Technology, Third Edition, Vol. 2, Wiley, New York, 1978, p. 392.
[43] Houben Weyl Methoden der Organischen Chemie, Vol. 11/2, Georg Thieme, Stuttgart, 1958, p. 363.
[44] Houben Weyl Methoden der Organischen Chemie, Vol. 5/1a, Georg Thieme, Stuttgart, 1970, pp. 227 and 285.