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Identification and quantitation of sodium-thyroxine and its degradation products by LC using electrochemical and MS detection

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Abstract

High performance liquid chromatography (HPLC) was used in combination with an amperometric and mass spectrometric detection to elucidate and quantitate the degradation products and contaminants of the photo-sensitive Na-thyroxine. Using HPLC with amperometric detection, seven decomposition compounds were separated. These products, which occur mostly as contaminants, were then identified by a developed liquid chromatography-mass spectrometry technique. The same HPLC method was also employed to analyze Na-thyroxine and its degradation products in three commercially available brands of Na-thyroxine tablets. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Sodium-thyroxine; Degradation products; High-performance liquid chromatography; Amperometric detection; HPLC-mass spectrometry

1. Introduction

Sodium-thyroxine, sodium *o*-(4-hydroxy-3,5-di-iodophenyl)-3,5-di-iodo-L-tyrosinate, is mainly used in tablet and oral powder forms as a replacement therapy in hypothyroidism. The drug is not stable against the environmental factors such as high temperature and light. It decomposes photochemically by both normal daylight and under irradiation to form several degradation products [1,2]. There is no detailed information in the literature regarding the degradation pathway and quantification of these photo degradation products. Liothyronine is the most frequently studied contaminant and may be found in bulk as row materials or pharmaceutical formulations [3–5].

Several LC methods have been employed for separation and quantitation of Na-thyroxine in biological samples as well as pharmaceutical formulations [3–12]. The United States Pharmacopoeia [4] and BP 1998 [5] does not permit the presence of liothyronine in sodium-thyroxine at

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levels in excess of 2%, without mentioning other contaminants caused mostly by light. Such a high level of impurities accepted by these pharmacopoeia may be linked to the fact that the LC-UV methods suggested by these sources can only detect an impurity such as liothyronine at some limited and selected levels.

Electrochemical detection has been successfully applied to determine a wide range of electroactive drugs and their degradation products or metabolites in pharmaceutical and biological samples [13,14] and the LC-MS has successfully shown to be a very useful interface for the structural elucidation of impurities and degradation products of pharmaceuticals [15,16].

The purpose of this work is to identify and quantitate Na-thyroxine along with its degradation products by LC-EC and LC-MS with a view to detect the degradation products caused by light and high temperature as well as increasing the sensitivity and specificity of the present HPLC methods employing amperometric detection mode.

2. Experimental

2.1. Materials

Sodium-thyroxine and liothyronine standards were obtained from Sigma (Sydney, Australia). Formulated Na-thyroxine samples were obtained from Glaxo-Wellcome, Sydney, Australia (Oroxine, 50 mcg tablets) and Iran Hormone, Tehran, Iran (Levothyroxine 50 and 100 mcg tablets) as examples of high-quality preparation. The acetonitrile used (Mallinckrodt) was Chrom AR, HPLC grade. All other substances used were of analytical grade.

2.2. Instrumentation

The HPLC separation system incorporated a modular system consisting of an LKB Model 2105 pump (Bromma, Sweden) equipped with a Rheodyne Model 7125 injector with 20-μl loop, an ESA Coulochem Model 5100 A detector (Bradford, MA, USA) coupled in series with a Spectra-Physic variable wavelength detector Model Spectra 100 (Santa Clara, CA, USA). The mobile phase I consisted of 0.5% formic acid in 40% acetonitrile (adjusted to pH 3.12 with ammonium hydroxide solution). Mobile phase system II was 0.3% acetic acid in 40% acetonitrile and had a pH of 3.27. The mobile phases were filtered through a 0.2-μm Millipore filter and degassed in an ultrasonic bath immediately before use.

Chromatography was carried out on a Merck LiChrospher 60 RP-select B Column (5-μm packing, 125 × 4.6 mm i.d.). The operation conditions were flow rate 0.7 ml min^{-1}; injection volume 20 μl; detection mode electrochemical at 0.8 V.

The aqueous sample solutions to be irradiated, were contained in stoperred cylindrical quartz vessels of 10 mm pathlength and irradiated with a Cermax 500 W xenon arc source through an applied Photophysics f 3.4 monochromator set to 320 nm with a bandwidth of 5 nm.

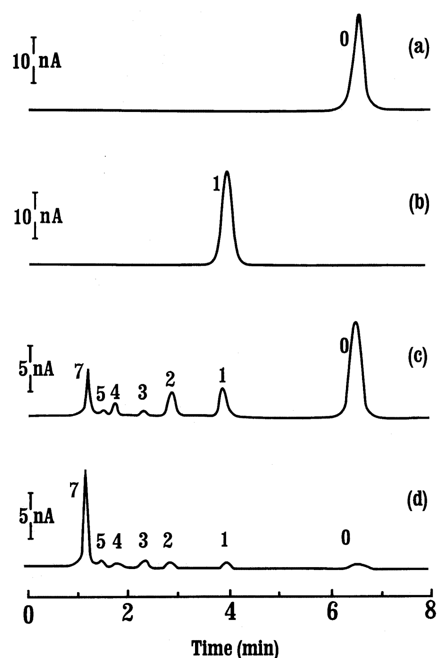


Fig. 1. Representative LC-EC chromatogram of (a) 10 ng μl^{-1} Na-thyroxine immediately after preparation of solution; (b) 10 ng μl^{-1} liothyronine immediately after preparation of solution; (c) solution a after 1 h irradiation and (d) solution a after 90 min irradiation; peaks 1, liothyronine; 2, diiodothyronine; 3, iodothyronine; 4, diiodotyrosine; 5, iodotyrosine and 7, tyrosine.

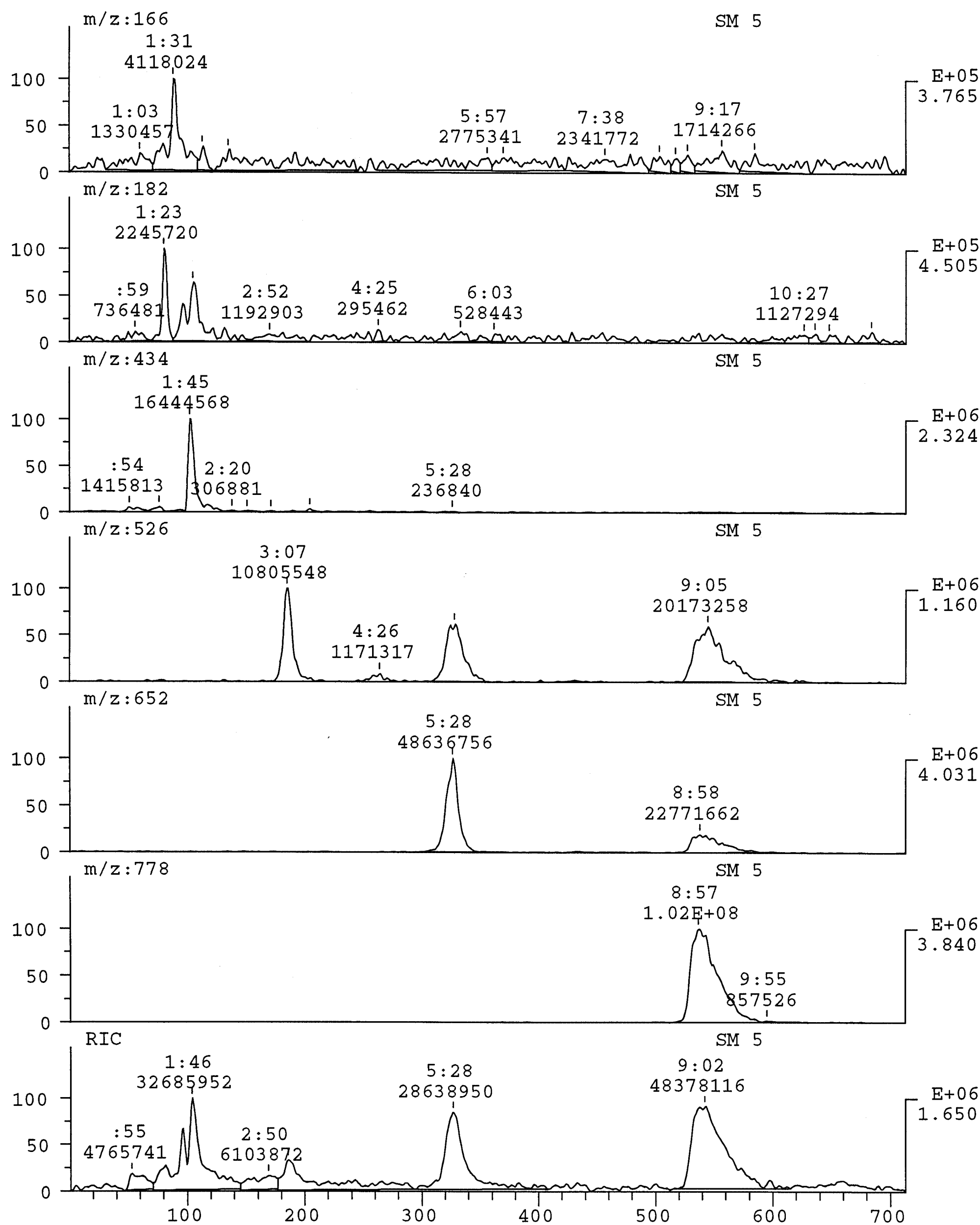


Fig. 2. LC-MS chromatogram obtained from a 0.5 mg ml^{-1} solution of Na-thyroxine after 1-h irradiation. Conditions are as described in text.

The LC-MS analysis was performed on Finnigan/Mat TSQ 7000 LC/MS/MS (San Jose, CA, USA) operating in APCI mode. The system was linked to a Hewlett Packard HP 1090 liquid chromatograph, which is controlled by the software of the TSQ 7000. The mass spectrometer was operated in full scan mode from mass 150–850 amu in 2 s. Vaporizer of the APCI and the heated capillary were operated at 420 and 180°C, respectively.

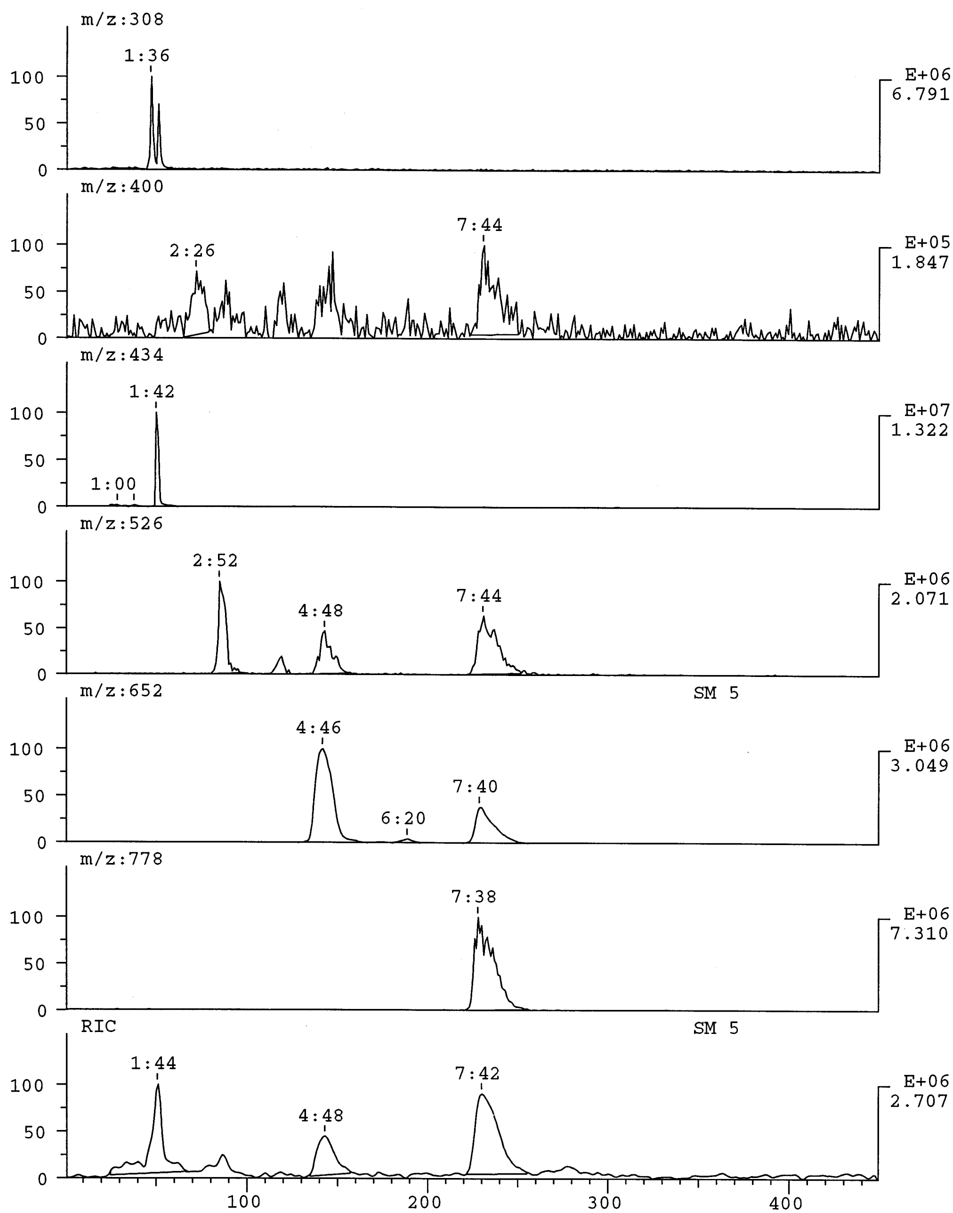


Fig. 2. (*Continued*)

The system was tuned previously in electrospray (ESI) mode, then when in APCI mode, it was optimized by tuning the capillary and the tube lens. In addition, a tuning solution of irradiated thyroxine infused into the LC from the APCI vaporizer was also optimized in this manner. It was found that optimal sensitivity and solvent dedustening was obtained by using a CID (source) voltage of 12 eV.

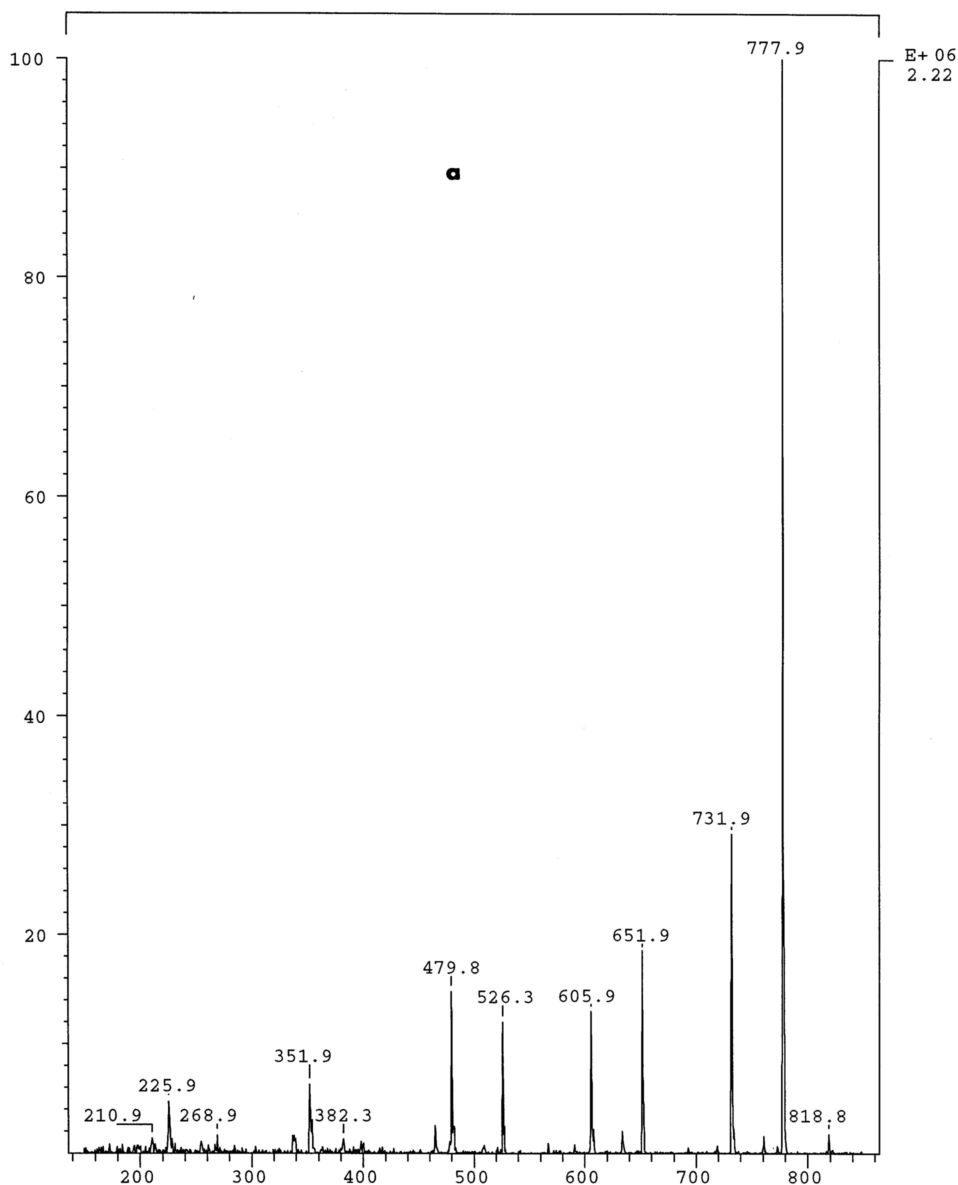


Fig. 3. Ion mass spectrums from degraded solution of 0.5 mg ml^{-1} Na-thyroxine after 1 h irradiation; (a) thyroxine; (b) liothyronine; (c) diiodothyronine; (d) diiodotyrosine; (e) iodothyronine; (f) iodotyrosine; (g) acetyltyrosine; (h) acetylphenylalanine.

3. Results and discussion

In order to achieve precise quantitation of Na-thyroxine and sufficient separation of degradation products, a number of HPLC mobile phase systems and columns were examined. Na-thyroxine and its decomposition products required an acidic mobile phase for good separation by liquid chromatography. Four different acidic mobile phase were used and finally the mobile phase system I for LC-EC was utilized. This mobile phase consisting of acetonitrile–water–formic acid (40:60:0.5) containing 0.075% ammonium hydroxide (30%) with a pH 3.12 demonstrated good

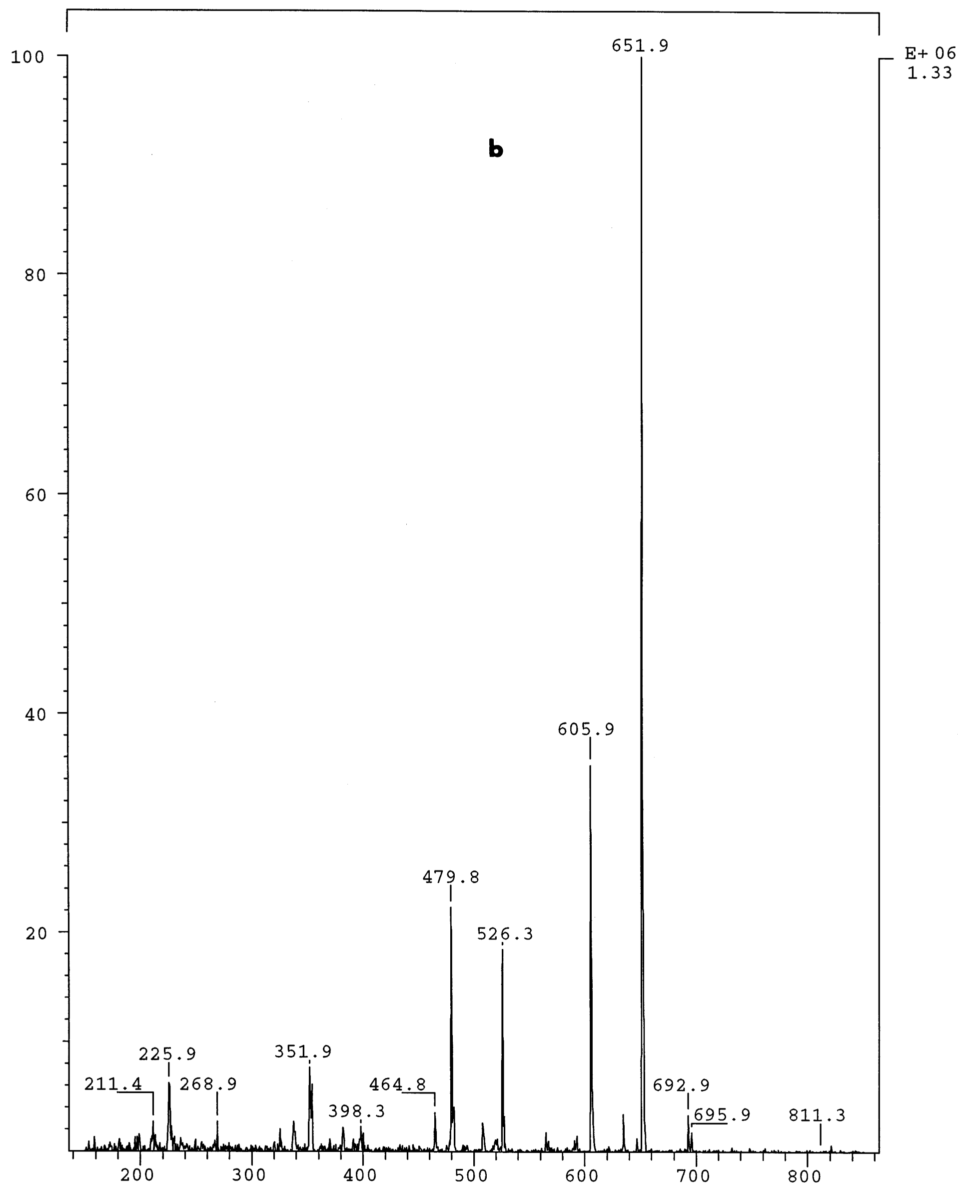
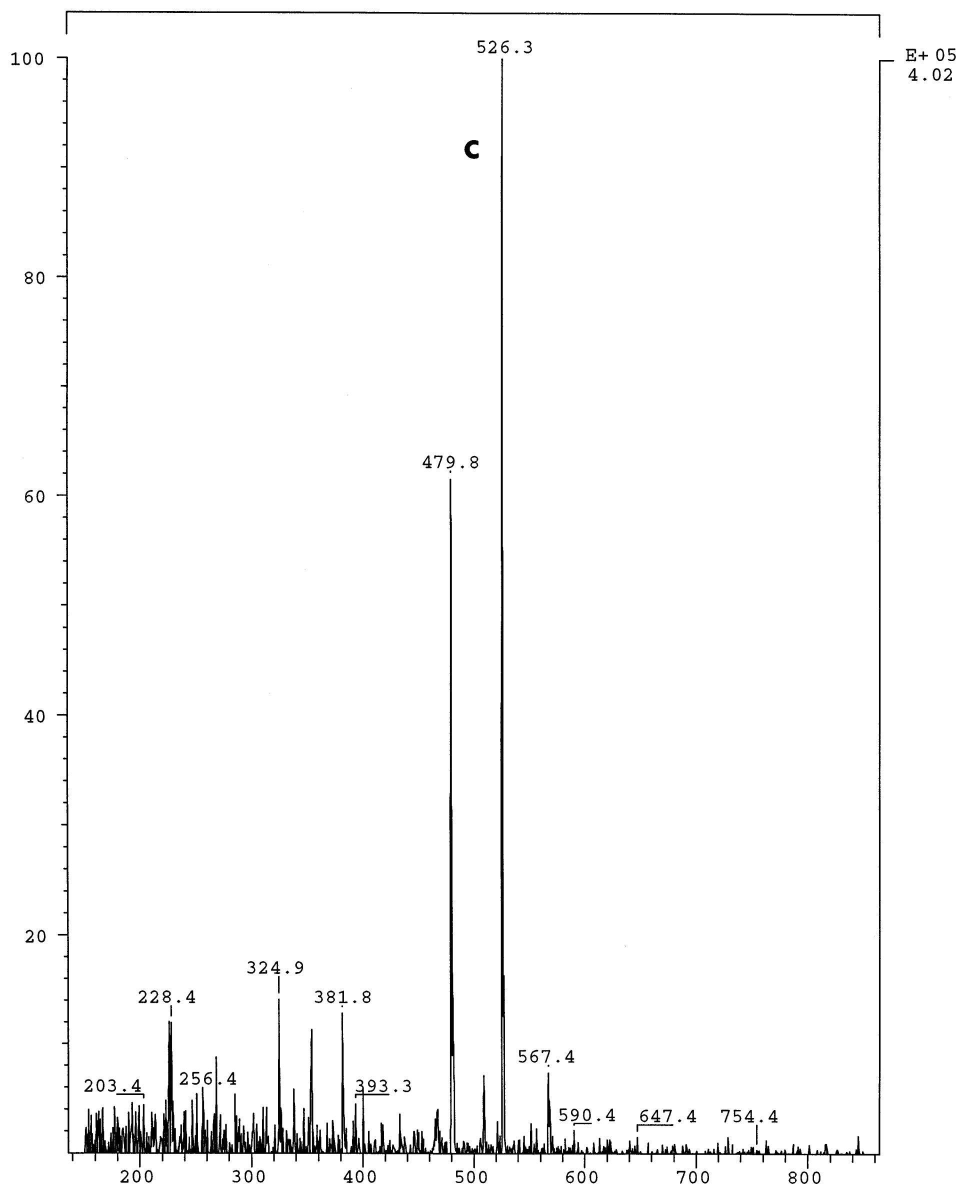


Fig. 3. (*Continued*)

resolution between Na-thyroxine and all its degradation products and gave excellent separation and sensitivity for LC-EC studies. A pH of 3.12 was used in order to ensure that Na-thyroxine and its degradation products are kept in free form. Owing to the low solubility of the compound and some degradation products, the study was carried out in a mixture of acetonitrile–water. Using the mobile phase II for LC-EC, the separation and quantitation of Na-thyroxine and its degradation products was not satisfactory.

The degradation products were expected to be more polar than Na-thyroxine. Due to the polar nature of thyroxine and degradation products, no



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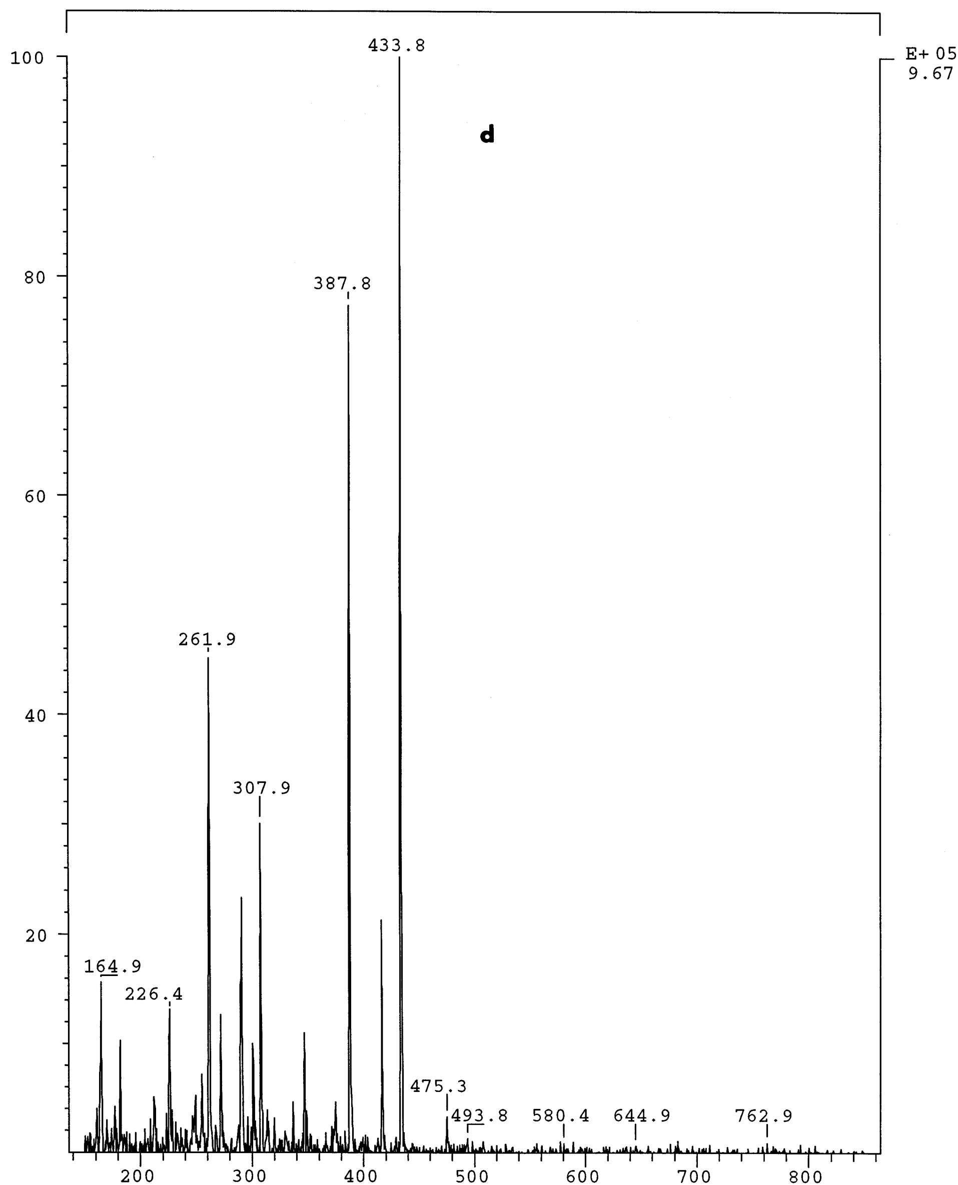
chromatographic difficulties were encountered for separation and quantitation, using commercially available reversed phase silica-bonded C_8 column.

Na-thyroxine and most of its degradation products each contains an electrochemically oxidizable phenolic substituent. In amperometric detection, oxidation of these compounds occurs from this moiety. The optimum working potential for amperometric detection was chosen at 800 mV. This potential assures the oxidation of Na-thyroxine and its major degradation products.

The linearity of the assay was determined by injecting 20 μl aliquots of a series of Na-thyroxine standard solutions ranging from 0.1 to 100 ng ml^{-1} into the LC-EC system. The linearity over this range was excellent with the linear regression



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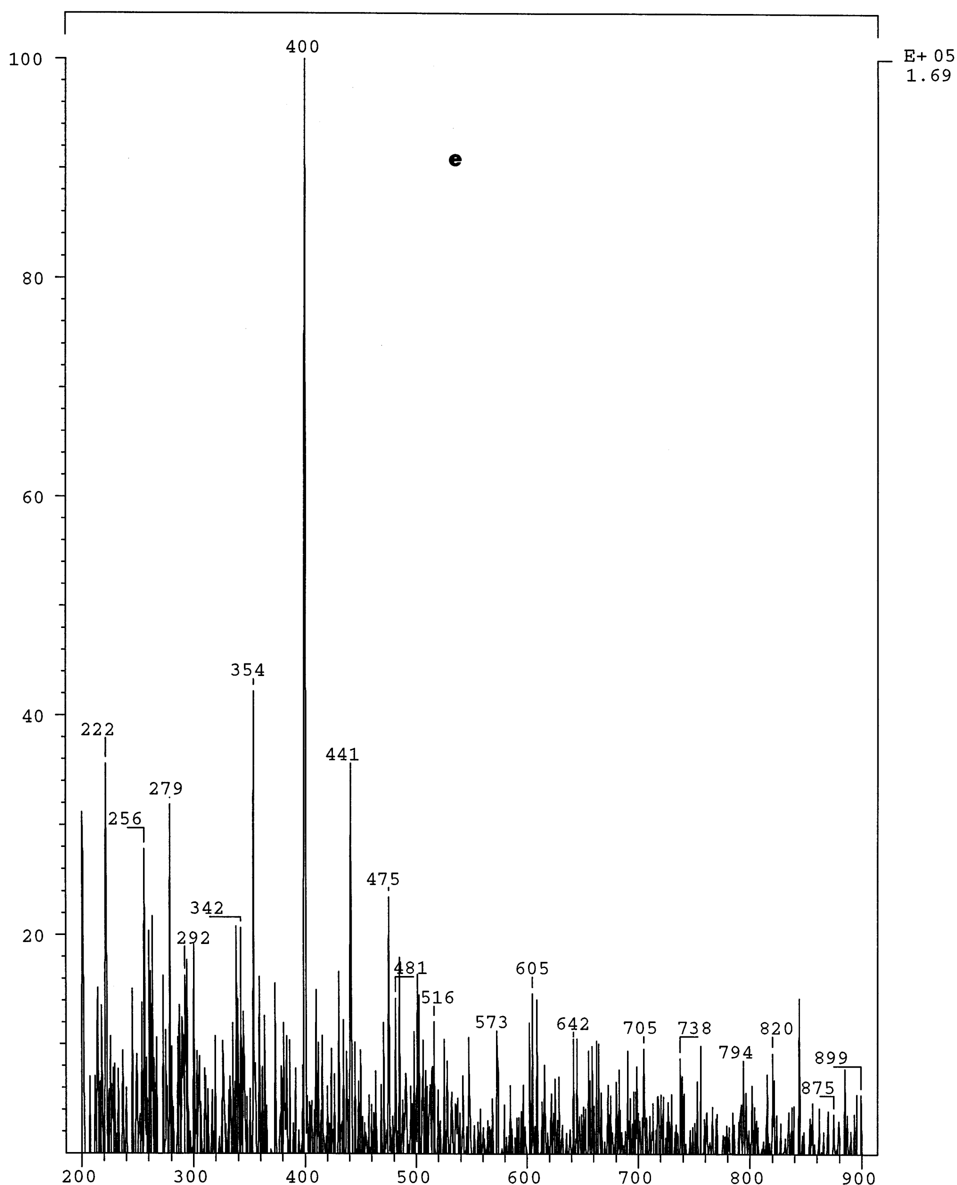
coefficient of $r = 0.9990$. The detection limit of the assay, based on signal to noise ratio of 3:1, was calculated to be less than 0.1 ng μl^{-1}. Reproducibility and recovery values for the assay were determined by analyzing six replicates of sample solutions ranging from 10 to 20 ng μl^{-1} on each of 3 days. The overall values for recovery and relative standard deviation (R.S.D.) were 99 and 1.1% in both the cases.

In order to predict the stability of Na-thyroxine in different environments, the effect of high temperature and direct light have been studied. The results showed that solid Na-thyroxine is slightly affected by both high temperature and irradiation.



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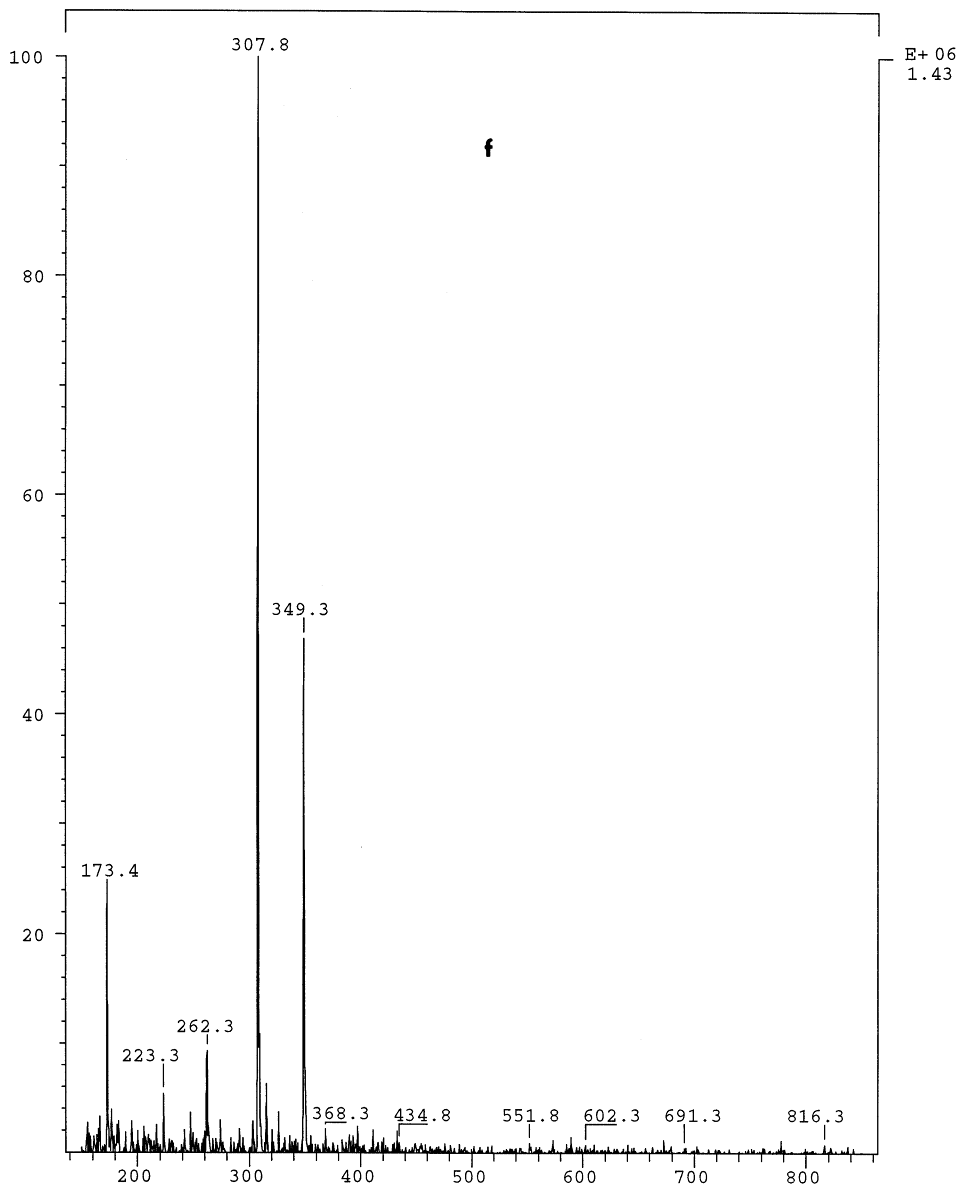
When the solid form of the drug is kept for 100 h at 75°C, up to 10% will be degraded to equal amount of compounds **1** and **2** (later identified as liothyronine ad diiodothyronine), whereas 1-h irradiation resulted in 20% degradation to compound **1**. The degradation studies performed on Na-thyroxine solution Fig. 1a, indicated that a considerable decomposition (up to 65%) can be observed in this solution, when irradiated for 1 h, and the rate of photodegradation of Na-thyroxine in aqueous solution yielded nearly total degradation on irradiation when increased to 90 min (Fig. 1d).

The chromatogram of irradiated solution revealed a number of products (six compounds detected by LC-EC) being eluted at very shorter



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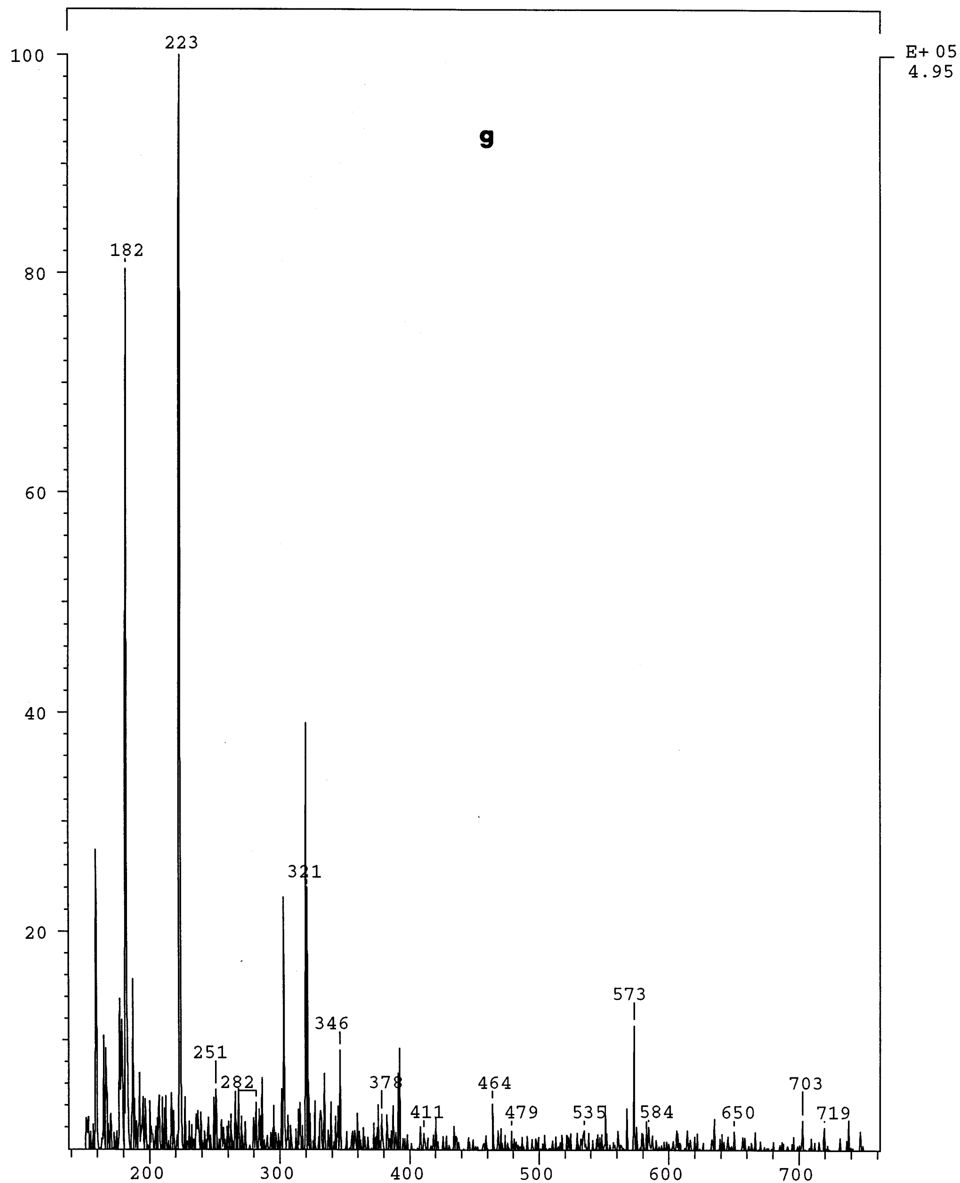
retention times compared with Na-thyroxine (Fig. 1c). Peak 1 was broad and constituted the immediate degradation product. This product was later identified as liothyronine by LC-MS and by its LC-EC behavior using the authentic substance (Fig. 1b). Liothyronine had similar sensitivity as thyroxine to irradiation and degraded to the same products. Peak 7 constituted the major end product of the degradation process, judging by its peak area. The chromatogram of irradiated solution showed further small amount of components 2, 3, 4, and 5, which had to be identified by LC-MS. Degradation product 6 was not electroactive and not detectable by LC-EC. In a



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separate experiment, we detected this by LC-UV using photodiode array, after it was identified as phenylalanine by LC-MS.

In order to further examine the nature of the degradant peaks, LC-MS was employed to generate spectrograms of the light degraded samples. For mass spectrometry, a volatile mobile phase is necessary because solvents and buffer components have to evaporate before the sample ions are introduced into the vacuum system of the mass spectrometer. Mobile phase system I was not acceptable for LC-MS, because in general, the addition of a buffer salt at a high concentration will strongly reduce the sensitivity in MS for ionic compounds. In contrast to that, the mobile phase system II containing 0.3% acetic acid was the

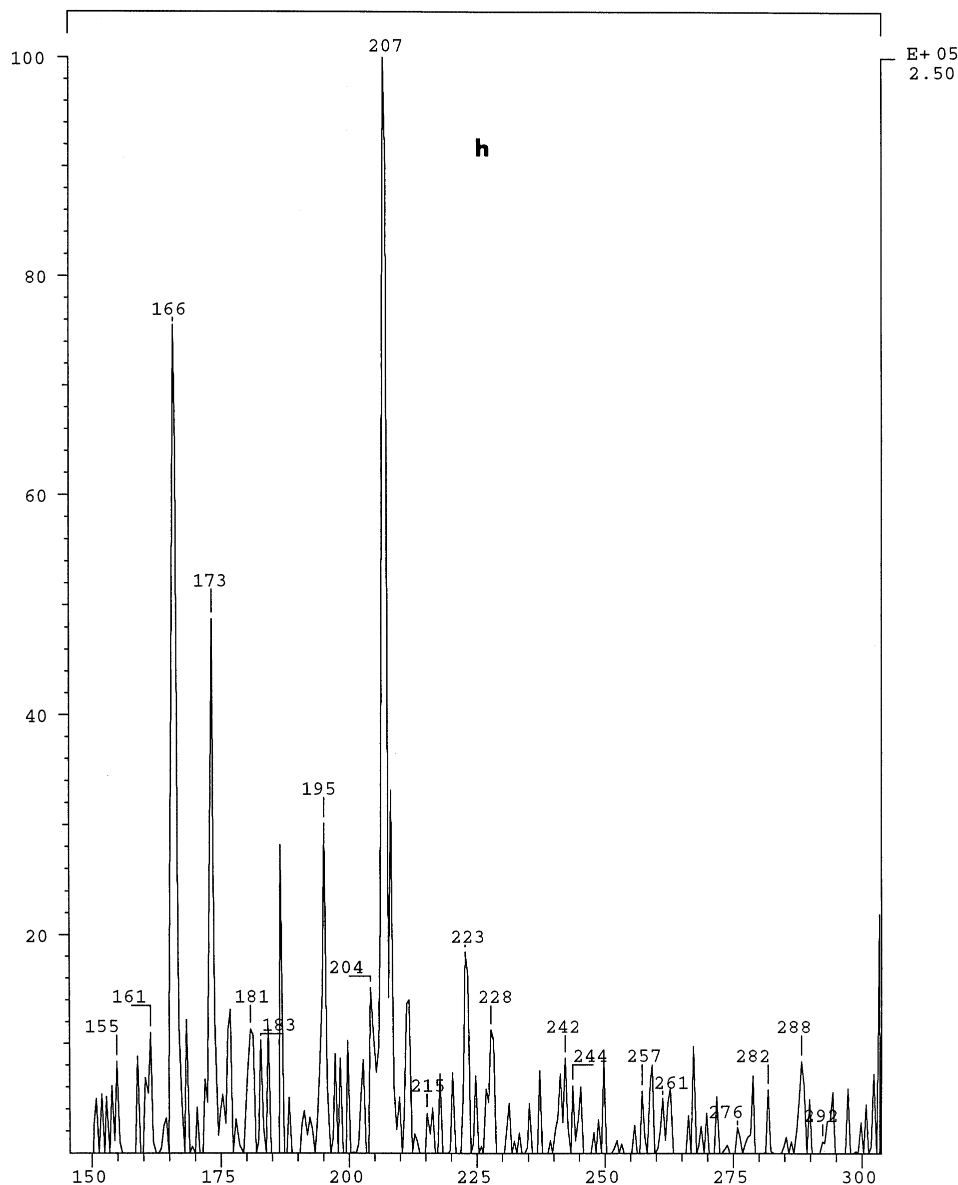

Fig. 3. (*Continued*)

most convenient system for LC-MS studies. The online LC-MS techniques offered a powerful tool for the analysis of these polar degradation products as it has been shown in total ion chromatogram and spectra for the LC-MS run after injecting an aliquot (20 μl) of 1 h irradiated sample of 0.5 mg ml^{-1} Na-thyroxine standard solution.

Fig. 2 shows an example of a LC-MS chromatogram, corresponding to the light degraded Na-thyroxine solution. The well-separated thyroxine, its immediate and major degradation product liothyronine, and six other identified degradation products with different m/z and retention times have been presented in this figure. Fig. 3a shows the structure of Na-thyroxine and its ion mass

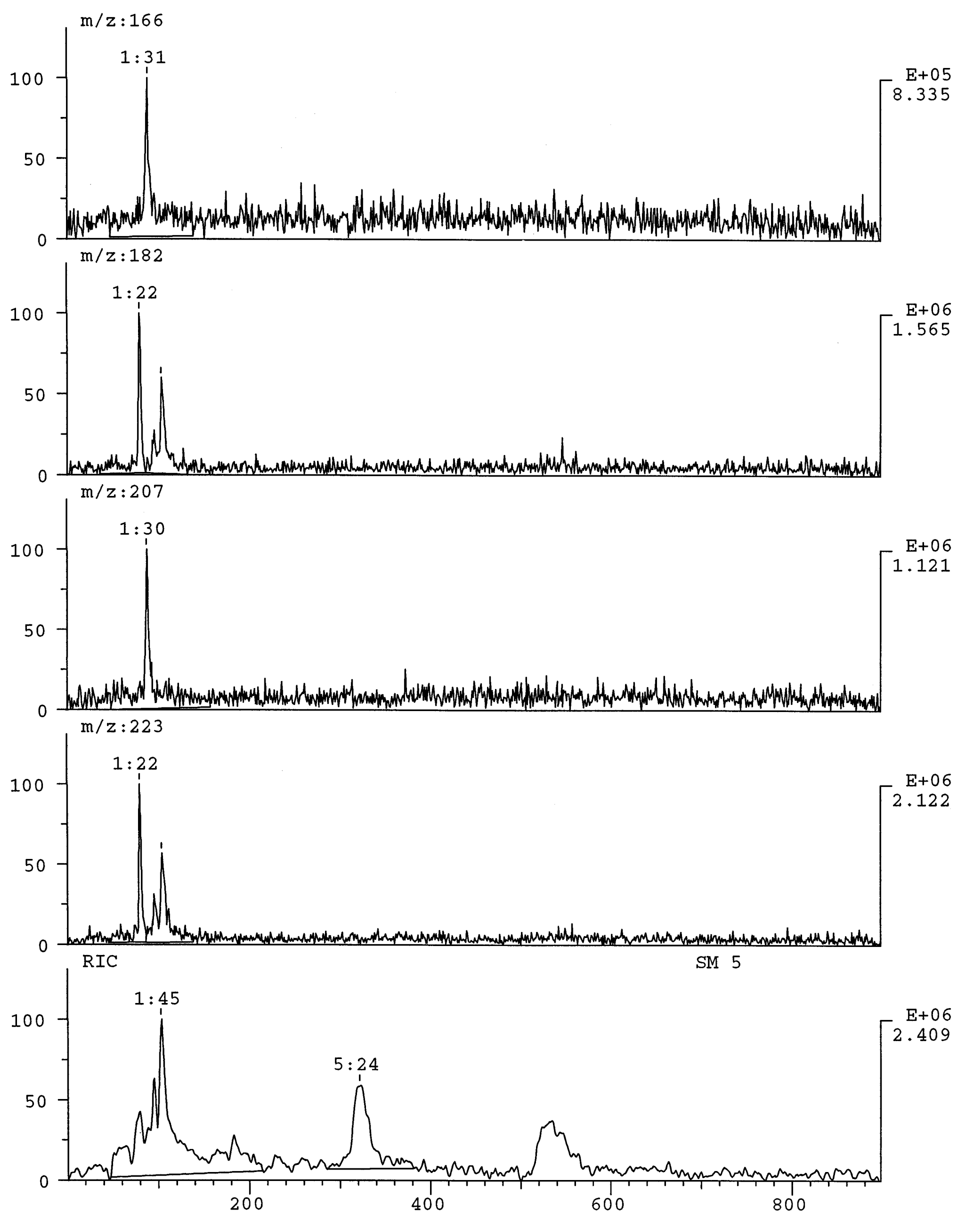

Fig. 4. LC-MS chromatogram from degraded and acetylated compounds **8** and **9** obtained after 1-h irradiation of 0.5 mg ml^{-1} Na-thyroxine. Conditions described in text.

spectrum. The most important ion is the thyroxine molecular ion at *m/z* 778. This is typical of ion LC-MS, with few fragment ions being produced. When this molecule was irradiated in solution, its immediate degradation product was liothyronine at *m/z* 652 (Fig. 3b), which was further degraded to the other products (Fig. 3c–h) listed below.

Compounds **8** and **9** were formed only in decomposed solution prepared in aqueous acetic acid or using mobile phase II for LC-MS studies. The molecular weights were, in each case, 41 amu

higher than that of phenylalanine or tyrosine, indicating compounds **8** and **9** to be acetylated derivatives of the named degradation products (Fig. 4). The apparent retention time for phenylalanine and tyrosine differs in Figs. 3 and 4, because of the different scan parameters.

The degradation profile of Na-thyroxine, considering the detected products, is shown in Fig. 5. Compound **1** (liothyronine) reached a maximum concentration after 20 min, and when this compound started to decrease, the other deiodinated compounds became more abundant. This proba-

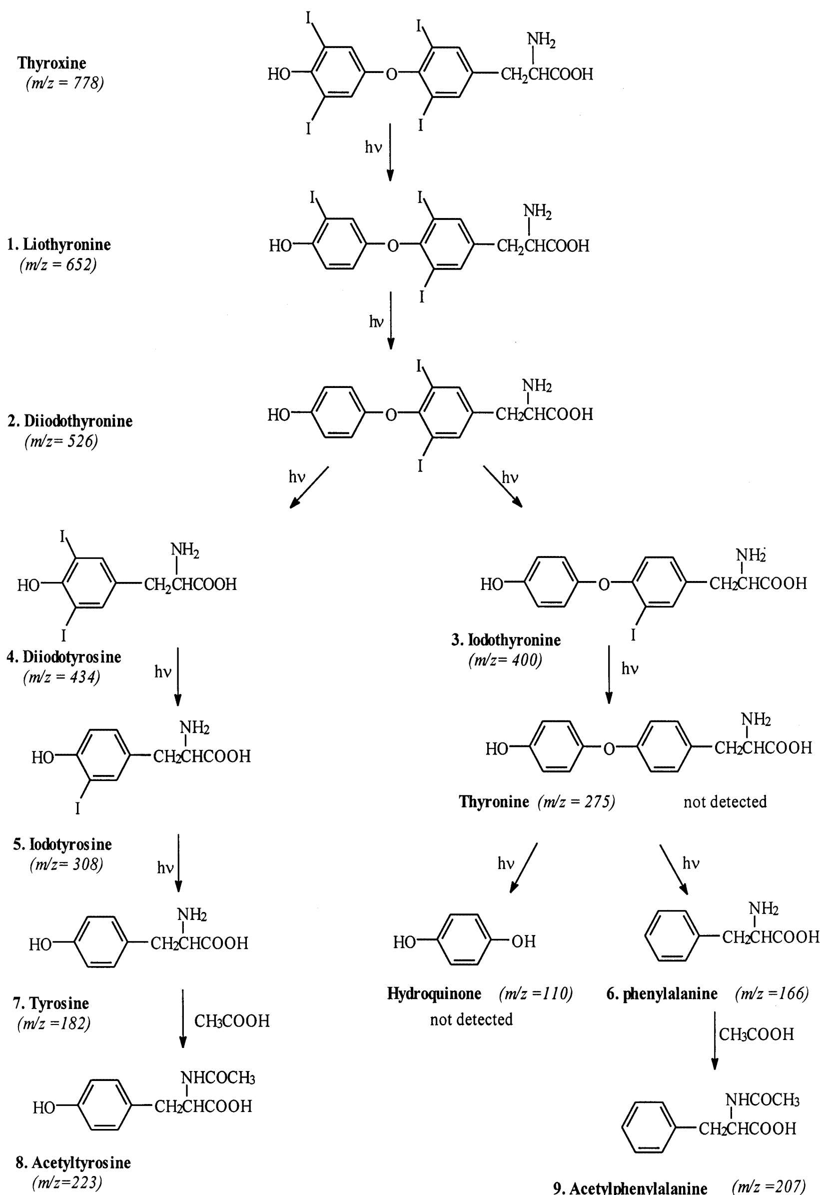

Fig. 5. Proposed structures for the degradation products formed from irradiated Na-thyroxine solution.

bly was caused by cleavage of C–O bond. The degradation profile of liothyronine is similar to that of Na-thyroxine. To make sure that the high decomposition is only produced by light action, one of the sample solutions was protected from light with aluminum foil. After 2 days, no degradation products were detected in the protected sample.

Online ionspray MS is a very useful technique for the structural characterization of small amount of polar decomposition products with a mixture of acetonitrile and acetic acid in the mobile phase of LC-MS. Thus, the degradation products of Na-thyroxine were easily characterized with this technique.

By way of illustrating the LC-EC method developed, tablets of three brands of Na-thyroxine were analyzed. This showed that one of the brands (100 mcg tablets) studied, contained a considerable amount of the major decomposition products detected. Therefore, these findings illustrate the need for careful control of storage condition for Na-thyroxine formulation. The LC-EC techniques described in the present study have provided the basis for the development of the assay for Na-thyroxine and it's degradation products both in the raw material state and in complex formulation. The developed method permits satisfactory control to be exercised, whilst at the same time allowing such degradation to be identified and quantified.

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