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SEPARATION OF ENANTIOMERIC IODINATED THYRONINES BY LIQUID CHROMATOGRAPHY OF DIASTEREOMERS

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SUMMARY

A method for the separation of D,L-triiodothyronine and D,L-tetraiodothyronine optical isomers is described. The iodinated thyronines are coupled with L-leucine and the resulting diastereomers are separated by reversed-phase liquid chromatography. The derivatives are detected in the UV region at 230 nm. The technique can be used for the determination of the optical purity of thyroid hormones. It is possible to determine 0.2% of the L-isomer in D-tetraiodothyronine with a relative standard deviation of 8%. One complete analysis takes about 2 h. The results are in good agreement with those of an enzymatic method.

INTRODUCTION

The hormones triiodothyronine (T_3) and tetraiodothyronine (T_4) exist as enantiomers that have very different pharmacological effects. The chromatographic techniques used so far permit only a separation of the different thyronines without differentiation between the optical isomers. Such separations are possible by means of gas chromatography following derivatization[1–3], gel chromatography[4,5], ion-exchange chromatography[6,7] and high-performance liquid chromatography (HPLC) on reversed phases[8,9]. The HPLC determinations are fast and simple and applicable to routine determinations. For this reason, we tried to use the same technique for the separation of the optical isomers.

With chiral stationary phases separations by direct injection of the test substances can be performed. Most chiral stationary phases are polymers with proline or hydroxyproline as active centres. After complexation with Cu(II) ions a high stereo selectivity is attained[10–13]. High-speed separations have not proved possible with this technique. Recently, the synthesis of a similar chiral stationary phase based on silica was described[14]. For all of these separations Cu(II) ions must be present as complexing agent in the mobile phase. Therefore, it can be expected that problems

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will arise with iodinated compounds. Another possibility is the use of chiral eluents[15–17]. In all instances metal complexes are necessary for separation, and problems similar to those described for the chiral stationary phases can be expected. A review of these direct resolution techniques was published by Audebert[18].

An alternative approach is the synthesis of diastereomers and their separation by HPLC. The most important methods were reviewed recently[19]. *tert.*-Butyloxy-L-leucine-N-hydroxysuccinimide ester (BOC-L-Leu-SU) has proved to be well suited as a reagent for preparing diastereomeric peptides from racemic amino acids[20,21]. Racemization under the mild conditions of this reaction is below 0.1%[20,22]. The L-leucine derivatives prepared can be easily separated by reversed-phase liquid chromatography, as shown by Nachtmann[21]. In this paper the application of this technique to the determination of D,L-triiodothyronine (T_3) and D,L-tetraiodothyronine (T_4) enantiomers is demonstrated.

EXPERIMENTAL

Materials

The solvents used were of analytical-reagent grade (E. Merck, Darmstadt, G.F.R.). The iodinated thyronines were synthetized by Sanabo (Vienna/Schaftenau, Austria) and BOC-L-Leu-SU (puriss CHR) was provided by Fluka (Buchs, Switzerland). For the HPLC separations LiChrosorb RP-8 and RP-18 (Merck), particle size 7 μm, were used, packed in stainless-steel columns (150 × 3.2 or 250 × 3.2 mm I.D.) by a slurry technique[23].

Apparatus

A Waters 1000 A pump combined with a U6K injection system was used for the chromatographic separations. Detection was performed at 230 nm using a Schoeffel SF 770 spectrophotometric detector. Gradient elution and thermostating were not necessary, and the separations were performed isocratically at room temperature (20–22°C). For integration of the peak areas a Hewlett-Packard 3380 integrator and a Hewlett-Packard Laboratory Data System 3353 were used.

Derivatization

As a result of the optimization studies the following procedure can be recommended: 1.5 mg of T_3 or T_4 is weighed into a 25-ml flask and 200 μl of a 0.1 *M* solution of sodium hydrogen carbonate and 400 μl of methanolic reagent solution (7 mg/ml BOC-L-Leu-SU in methanol) are added. The reagent solution must be prepared immediately before use. The reaction mixture is placed in an ice-bath for 30 min and stirred well, then the solution is evaporated to dryness (the temperature must not exceed 30°C). Trifluoroacetic acid (100 μl) is added to the dry residue and, after standing for 30 min at room temperature it is neutralized by the addition of 2 ml of 1 *M* sodium hydrogen carbonate solution. The precipitate is centrifuged, the supernatant liquid removed and the precipitate dissolved in 600 μl of methanol–0.02 *M* sodium hydroxide solution (1:1). The solution obtained is injected into the chromatograph.

RESULTS AND DISCUSSION

Derivatization

The D,L-T_3 and D,L-T_4 enantiomers react with BOC-L-Leu-SU and give diastereomeric peptides. For the detection of small amounts of one isomer in a large excess of the other, pure derivatives must be obtained. As the iodinated thyronines are relatively unstable, the reaction was optimized with respect to mild reaction conditions. The formation of the peptides is not only possible in tetrahydrofuran–sodium hydrogen carbonate solution, as described by Mitchell et al.[20], as methanolic solutions have also proved to be suitable. The basicity of the reaction solution is of great importance. Solutions of sodium hydrogen carbonate, sodium hydroxide and sodium carbonate of different molarity were tested. The best results were obtained with 0.1 *M* sodium hydrogen carbonate solution. In methanol–0.1 *M* sodium hydrogen carbonate solution (1:1) the derivatization is completed within 10 min at 0°C. For quantitative determinations the best reproducibility was achieved with a reaction time of 30 min.

Recently, Nachtmann[21] found that the quantitative determination of small amounts of L-penicillamine in the D-form was not possible using the BOC-protected L-Leu derivatives, and the same is true for the iodinated thyronines. The deprotection step must be carried out under very mild conditions in order to avoid the formation of degradation products. Mixtures of trifluoroacetic acid with solvents previously used[20,21] could not be used owing to the formation of by-products.

With 100 μl trifluoroacetic acid, added to the evaporated dry residue of the derivatization reaction, deprotection of the peptides succeeds within a few minutes. Owing to the low solubility of the residue, a reaction time of 30 min is recommended. The evaporation of the acid is not possible without the formation of by-products. An alternative is the addition of 2 ml of 1 *M* sodium hydrogen carbonate solution, which causes precipitation of the peptides. After centrifugation the precipitate can be dissolved in methanol–0.02 *M* sodium hydroxide solution (1:1) and injected into the chromatograph.

Using the optimal conditions, the excess of reagent for quantitative derivatization of D,L-T_3 and D,L-T_4 was investigated (Table I). To 200 μl of the substrate solutions in 0.1 *M* sodium hydrogen carbonate solution (7,5 mg/ml) various amounts of BOC-L-Leu-SU (7 mg/ml in methanol) were added. The chromatographic determination was performed as shown in Fig. 3. For L-T_3 and L-T_4 200 μl of BOC-L-Leu-SU

TABLE I

OPTIMIZATION OF THE REACTION OF BOC-L-LEU-SU WITH D,L-T_3 AND D,L-T_4

Reaction conditions: see Experimental. Chromatographic conditions: see Fig. 3.

Volume of BOC-L-Leu-SU (µl)	*Relative peak height of L-Leu-derivatives (mm)*			
	L-T_3	L-T_4	D-T_3	D-T_4
50	16	12	7	7
100	45	32	22	23
200	65	36	27	44
400	62	38	45	64
600	62	38	43	65

solution are sufficient for derivatization, whereas for the D-forms a larger excess of reagent is necessary.

Optimization of the chromatographic conditions

For the separation of iodinated thyronines reversed-phase materials with

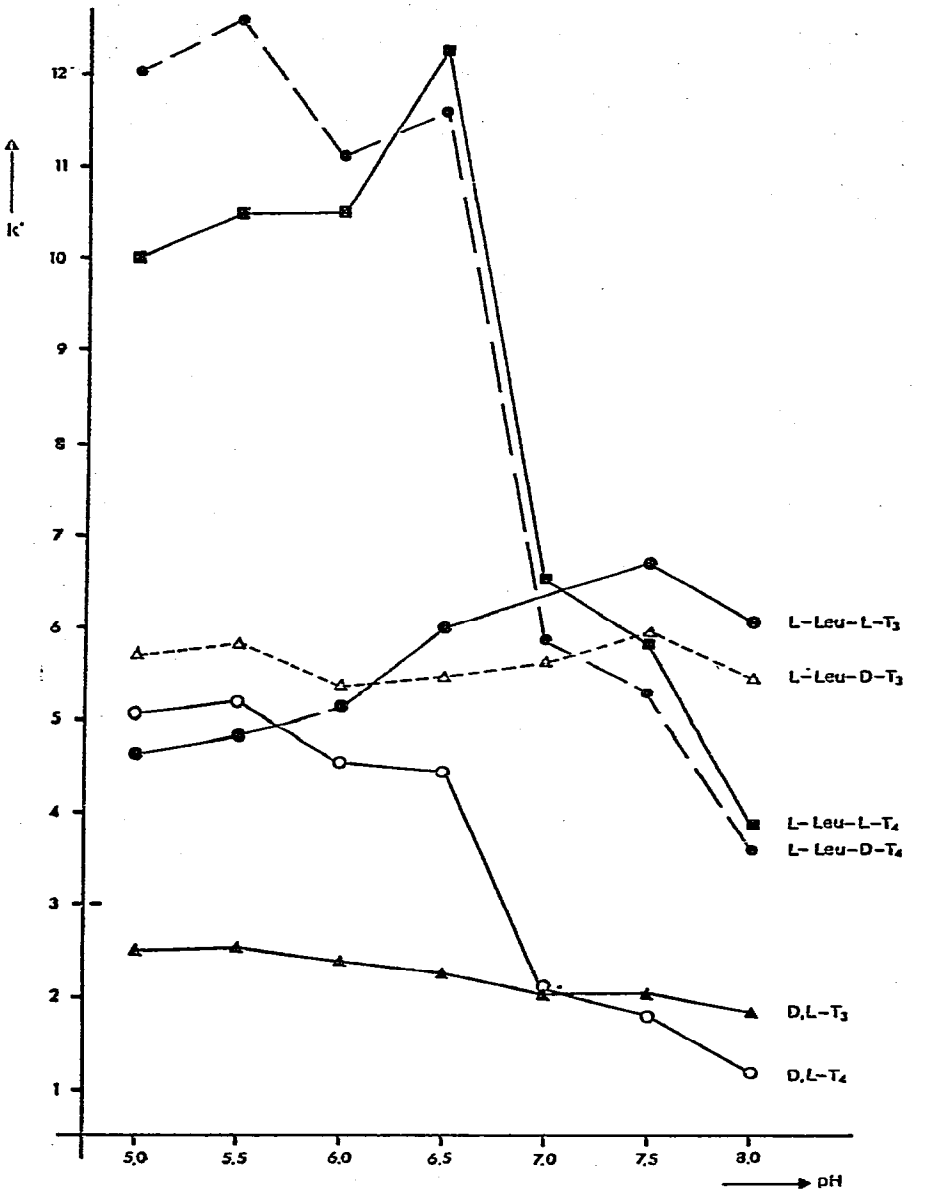

Fig. 1. Chromatographic characteristics of D,L-T_3, D,L-T_4 and their L-Leu derivatives. Column: LiChrosorb RP-18 (7 μm), 150 × 3.2 mm I.D. Mobile phase: methanol–phosphate buffer (0.033 *M*) of various pH (60:40). Flow-rate: 1 ml/min.

mixtures of phosphate buffer and methanol have been described[8]. Systems of this type were studied for the separation of the diastereomeric L-Leu derivatives of D,L-T_3 and D,L-T_4 (Fig. 1). At pH below 6.0 the L-form of the derivatives of T_3 and T_4 elute before the D-form. At higher pH the order of elution is reversed. For quantitative determinations of small amounts of the L-isomer in the D-form, pH values lower than

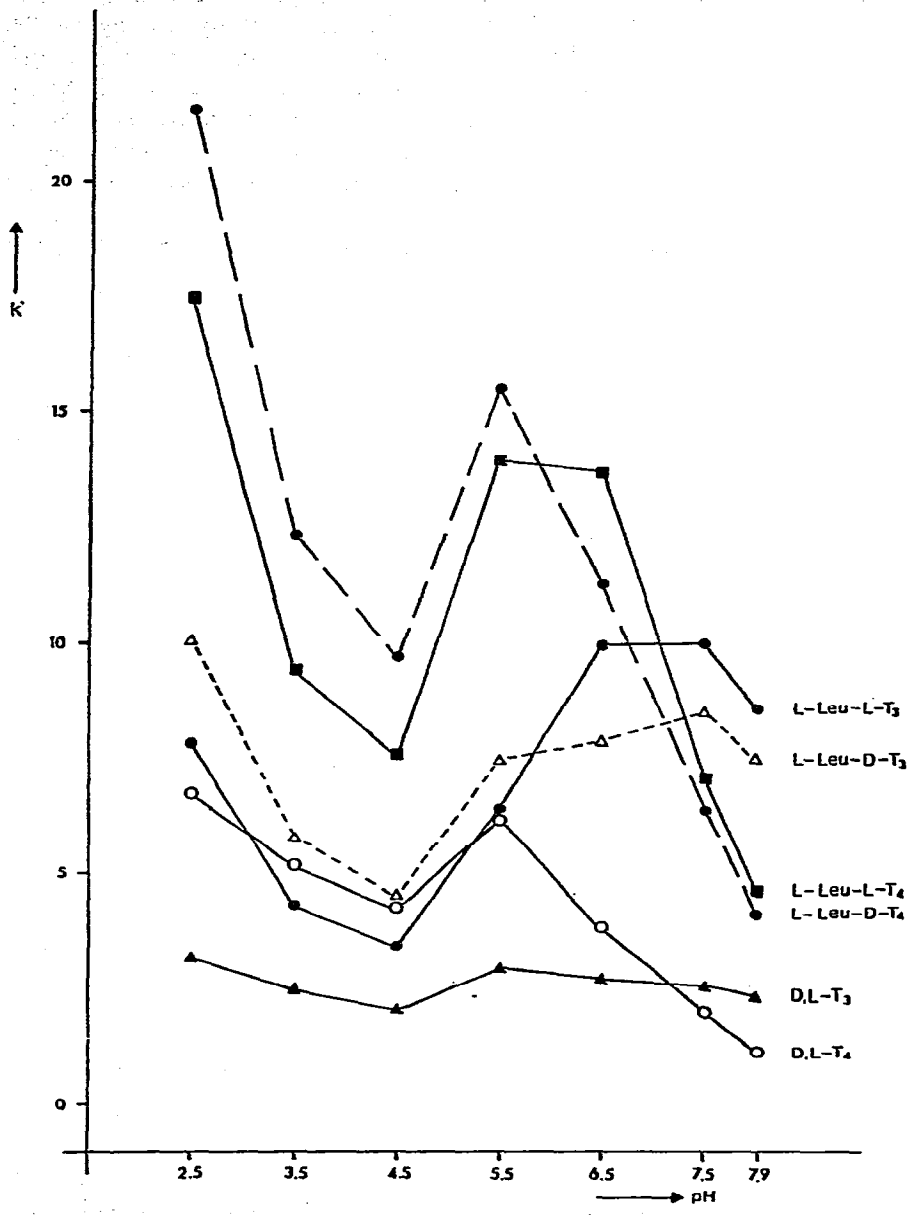

Fig. 2. Chromatographic characteristics of D,L-T_3, D,L-T_4 and their L-Leu derivatives. Column: LiChrosorb RP-18 (7 μm), 150 × 3.2 mm I.D. Mobile phase: methanol–phosphate–citrate buffer of various pH (60:40). Flow-rate: 1 ml/min.

6.0 should be chosen. It is also interesting that in the pH range 7.0–7.5 the order of elution of T_3 and T_4 is changed. In acetic media T_3 is always eluted before T_4, and in alkaline phosphate buffer T_4 is eluted before T_3. The same is true for the L-Leu derivatives of the iodinated thyronines.

A baseline separation of all compounds was not possible with the solvent system tested. The addition of citrate buffer results in an improvement in the separation selectivity (Fig. 2). The various pH values were reached by mixing 0.2 *M* phosphate buffer (pH 8.0) with 0.1 *M* citrate buffer (pH 2.2) to give the desired pH value. The change in the order of elution of T_3 and T_4 and the derivatives of the D- and L-forms occurs at similar pH values to that in the phosphate buffer system. A better separation between the six substances is obtained. At pH 6.4 a baseline separation is easily possible, as shown in Fig. 3. This chromatographic system is very useful for the analysis of mixtures of D,L-T_3 and D,L-T_4.

The quantitative determination of small residues of the L-isomer in D-T_4 for

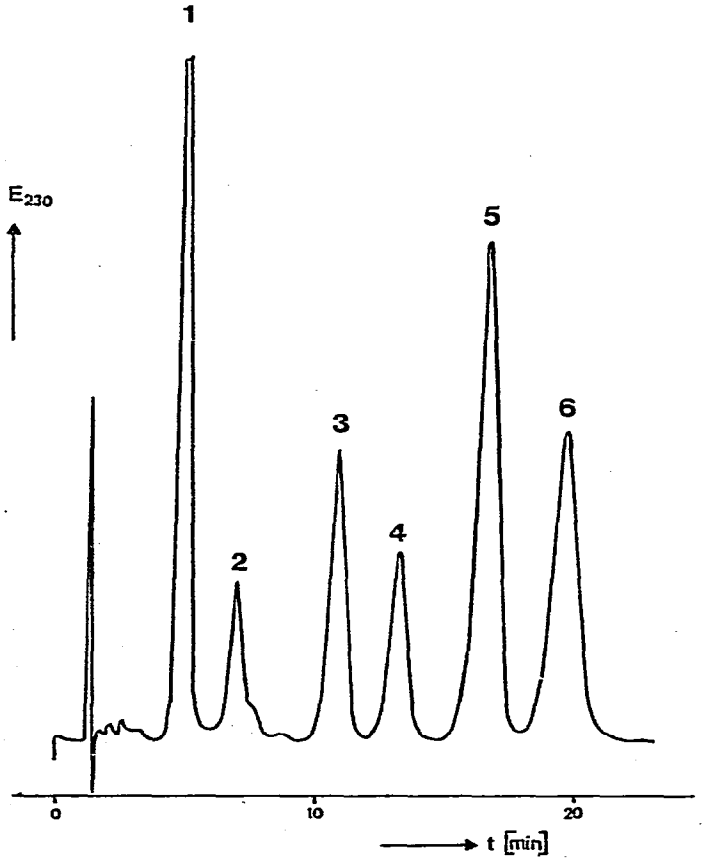

Fig. 3. Separation of D,L-T_3 (1), D,L-T_4 (2), L-Leu-D-T_3 (3), L-Leu-L-T_3 (4), L-Leu-D-T_4 (5) and L-Leu-L-T_4 (6). Column: LiChrosorb RP-18 (7 μm), 250 × 3.2 mm I.D. Mobile phase: methanol–phosphate–citrate buffer (0.085 *M*), pH 6.4 (60:40). Flow-rate: 1 ml/min. Injection volume: 30 μl. The unmarked peaks are unknown impurities.

purity tests is not possible with the mobile phases discussed because of the formation of by-products during the derivatization steps that cannot be separated from the peak of L-Leu-L-T_4. Such separations can be carried out with methanesulphonic acid as ion-pairing agent in the mobile phase. The calculation of the percentage of the L-isomer in the D-form is easily possible by comparison of the peak areas of the corresponding L-Leu derivatives. A presupposition for reproducible results is a good integration system such that the slope sensitivity can be optimized in small steps.

Quantitative determinations of as little as 0.2% of the L-form in D-T_4 are possible with a relative standard deviation of 8%. The detection limit is 0.05% of L-T_4 in D-T_4. Recovery studies were carried out by adding known amounts of L-T_4 to D-T_4. The concentration range was 0.1–1% of L-T_4 and the recoveries were 90–104%.

Chromatograms showing the enantiomeric purity of commercial L-T_4 and D-T_4 are given in Figs. 4 and 5. In L-T_4 the D-form is not detectable (<0.05%); the content of the L-isomer in the D-T_4 tested (Fig. 5) was 0.2%. Three different batches of D-T_4 were analysed in parallel by using the described HPLC method and an enzymatic method based on the work of Neudecker and co-workers[24,25]. For the final colour reaction 4-aminodiphenylamine-2-sulphonic acid was used instead of *o*-dianisidine. The results are summarized in Table II. The results of the two methods showed a good correlation.

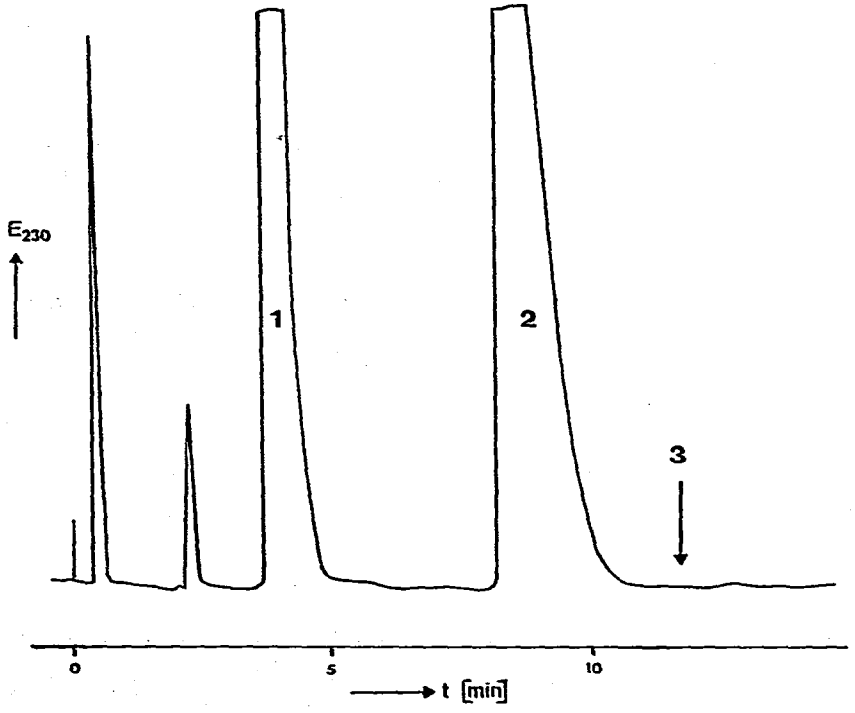

Fig. 4. Determination of the D-isomer in L-T_4. Column: LiChrosorb RP-18 (7 μm), 150 × 3.2 mm I.D. Mobile phase: methanol–water (60:40) + 0.05% of methanesulphonic acid. Flow-rate: 1 ml/min. Injection volume: 15 μl. Peaks: 1 = D,L-T_4 (underivatized); 2 = L-Leu-L-T_4; 3 = L-Leu-D-T_4. The other peaks are unknown impurities.

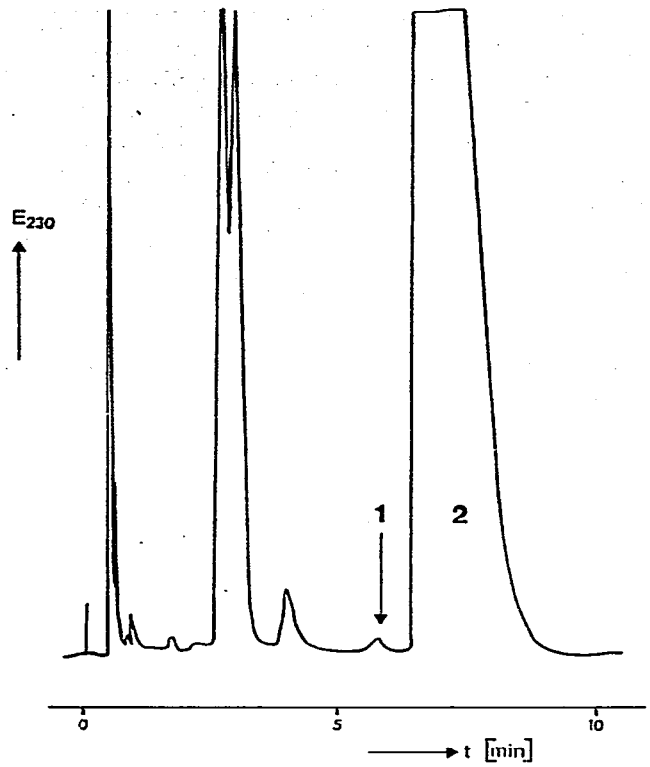

Fig. 5. Determination of the L-isomer in D-T_4. Column: LiChrosorb RP-18 (7 μm), 150 × 3.2 mm I.D. Mobile phase: methanol–water (63:37) + 0.05% of methanesulphonic acid. Flow-rate: 1 ml/min. Injection volume: 15 μl. Peaks: 1 = L-Leu-L-T_4; 2 = L-Leu-D-T_4.

TABLE II

COMPARISON OF THE DETERMINATION OF THE L-ISOMER IN D-T_4 USING HPLC AND AN ENZYMATIC METHOD

Batch	*L-Isomer content (%)*	
	HPLC	*Enzymatic analysis*
1	0.19	0.22
2	0.20	0.21
3	0.24	0.23

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