Journal of Chromatography, 224 (1981) 239—248
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 877

CATALYTIC DETECTION PRINCIPLE FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: DETERMINATION OF ENANTIOMERIC IODINATED THYRONINES IN BLOOD SERUM

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(First received December 11th, 1980; revised manuscript received February 24th, 1981)

SUMMARY

A method for the trace determination of iodinated thyronines with differentiation of the optical isomers by high performance liquid chromatography (HPLC) is described. The detection is effected by means of a catalytic principle based on the iodide-catalysed reaction of chloramine-T and N,N'-tetramethyldiaminodiphenylmethane, producing a coloured complex that can be measured spectrophotometrically at 600 nm. Owing to the selectivity of the catalytic reaction, iodine-containing compounds can be easily determined in a complex matrix such as blood plasma. The sensitivity is sufficient for the detection of plasma levels of iodinated thyronines. The limit of detection for thyroxine is in the sub-nanogram range. The enantiomers of thyronines can be separated on commercial reversed phases after pre-column synthesis of diastereomers. For this derivatization the reagent *tert.*-butyloxycarbonyl-L-leucine-N-hydroxysuccinimide ester is used. The coupling of the stereospecific HPLC separation with the catalytic detector offers the possibility of determining both D- and L-thyroxine in human plasma.

INTRODUCTION

The determination of iodinated thyronines in plasma is of great importance. The human thyroid gland produces the L-isomer of thyroxine and the D-isomer is being produced synthetically. D-Thyroxine was shown to reduce serum cholesterol levels significantly, acting by increasing the rate of degradation and

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oxidation of cholesterol [1—5]. Additionally, a thyrotropine suppression caused by D-thyroxine has been described [6]. For these reasons D-thyroxine is of pharmacological importance.

As the concentrations of iodinated thyronines in plasma are very low, only trace methods can be applied for their determination. Radioimmunoassay [7, 8], fluorescence immunoassay [9], enzyme immunoassay [10] and competitive protein binding assay [11] are most frequently used. A selective determination of enantiomeric thyronines is not possible with these techniques. The cross-reaction of L-thyroxine antibodies with D-thyroxine for a radioimmunoassay was found to be up to 100% [6].

Chromatographic separations of thyroid hormones can be carried out using gel chromatography [12, 13], ion-exchange chromatography [14, 15], gas chromatography (GC) [16—18] and high-performance liquid chromatography (HPLC) [19—21]. Separation of enantiomeric amino acids is possible by both GC and HPLC. Recently the determination of D,L-triiodothyronine and D,L-thyroxine by derivatization liquid chromatography was described [22, 23]. The D,L-thyronines are derivatized with *tert.*-butyloxy-L-leucine-N-hydroxysuccinimide ester and the resulting diastereomeric peptides are separated by ion-pair chromatography on reversed-phase columns. This method was applied successfully to the control of the purity of pharmaceuticals and its application to human plasma seemed to be promising.

For the analysis of plasma samples by HPLC a prior separation from the serum proteins is necessary. A simple method is precipitation with organic solvents such as tetrahydrofuran (THF). At the same time, extraction of the iodinated thyronines is achieved. The THF extract is used for the subsequent derivatization.

UV detection after HPLC separation is not sensitive enough for plasma levels of thyroid hormones. A possibility for improving the sensitivity is to use UV or fluorescence derivatization [24]. Owing to the presence of many amino acids in plasma, a suitable detection principle for iodinated thyronines must be both sensitive and highly selective. Both requirements can be met by making use of the catalytic effect of iodine. Knapp and Spitzy [25] described a modified form of the catalytic Sandell—Kolthoff reaction for the trace determination of thyroid hormones. This reaction was coupled to a liquid chromatograph by Nachtmann et al., and a sub-nanogram detection limit for thyroxine was achieved [26]. Owing to the corrosive reagents necessary for this technique, its routine application causes problems. Feigl and Jungreis [27] described the catalytic effect of iodide on the reaction of N,N'-tetramethyldiaminodiphenylmethane (tetrabase) with chloramine-T. The high sensitivity of the reaction was applied to the titrimetric and colorimetric determination of iodine [27—30]. The reaction was used as the basis for the development of a new catalytic detector for HPLC.

By combining the separation efficiency of modern HPLC with the catalytic reaction detector, new possibilities for the determination of iodinated thyronines in human plasma are offered.

EXPERIMENTAL

Reagents

All solvents were of analytical-reagent grade (Merck, Darmstadt, G.F.R.). Triiodothyronine (T_3) and thyroxine (T_4) were produced by Sanabo (Kundl, Austria). L-[3′,5′-^{125}I]Thyroxine was purchased from the Radiochemical Centre (Amersham, Great Britain). The derivatization reagent *tert.*-butyloxy-L-leucine-N-hydroxysuccinimide ester (BOC-L-Leu-SU) was purchased from Fluka (Buchs, Switzerland), chloramine-T from Merck and N,N′-tetramethyldiaminodiphenylmethane (tetrabase) from Merck-Schuchardt (Darmstadt, G.F.R.). N,N′-Tetramethyldiaminodiphenylmethane was recrystallized in ethanol; all other reagents were used without further purification.

Apparatus

For HPLC a Waters Type 1000A pump in combination with a WISP 710A injection system was used. Stainless-steel columns (150 × 3.2 mm I.D.) packed with Nucleosil 5 C-18, particle size 5 μm (Macherey, Nagel & Co., Düren, G.F.R.) were employed. The separations were carried out isocratically at room temperature, thermostating being unnecessary. The detection system consisted of a Technicon AutoAnalyzer II proportioning pump with flow-rated Solvaflex pump tubes and a Zeiss PM 2A spectrophotometer. The cell volume was 70 μl. Peak integration and calculation of data were performed with a Hewlett-Packard 3380A integrator and a Waters 730 data module.

Procedure

Determination of total thyroxine in plasma. Ethanol (200 μl) containing an internal standard (30 μg of triiodothyronine per 100 ml) is pipetted into a vial, 100 μl of plasma are added, the vial is tightly closed, shaken well for 2 min and centrifuged at 2700 *g* for 3 min. An aliquot of the clear supernatant liquid is injected into the liquid chromatograph.

Stereoselective determination of D- and L-thyroxine in plasma. A 0.5-ml volume of plasma is pipetted into a vial containing 1 ml of THF and the vial is tightly closed, shaken well for 2 min and centrifugated at 2700 *g* for 2 min. A 1-ml volume of the supernatant liquid is transferred into another tube, 50 μl of sodium hydrogen carbonate solution (1 *M*) and 50 μl of the derivatization solution (20 mg of BOC-L-Leu-SU per 1 ml of THF) are added. The solvents are removed by evaporation and 100 μl of trifluoroacetic acid are pipetted on to the dry residue. After 15 min at room temperature, 0.5 ml of sodium hydrogen carbonate solution (1 *M*) is added and the mixture is centrifuged at 4500 *g* for 10 min. The clear solution is removed and the precipitate is treated with 200 μl of ethanol and 100 μl of 0.5 *M* sodium hydroxide solution containing the internal standard (1 μg/ml of triiodothyronine). The insoluble part of the precipitate is removed by centrifugation and an aliquot of the clear solution is injected into the liquid chromatograph.

RESULTS AND DISCUSSION

Sample preparation

As plasma cannot be injected directly into an HPLC column, the thyroid hormones have to be extracted from the matrix. One possibility is to use short ion-exchange columns, which have already been applied successfully for this purpose [31]. A simpler and faster alternative involves precipitation of the plasma proteins with an organic solvent. In this step the iodinated thyronines are extracted at the same time. After centrifugation the clear extract can be used directly for further analysis.

For the optimization of this technique, plasma samples spiked with [^{125}I]T_4 were used. In this way the extraction yields could be determined radiometrically. The extraction yield using ethanol as solvent was 63.4 ± 2.7%, using acetone 68.4 ± 3.2% and using THF 86.2 ± 3.5%. For each solvent twelve tests were performed, adding 200 μl to 100 μl of serum and measuring the clear extract after centrifugation. For the HPLC determination of total T_4 this extract was used.

After several injections, blocking of the columns can occur due to the co-extraction of many other non-polar compounds into the solvent. These problems can be minimized by using ethanol for the extraction.

For differentiation of the enantiomers of T_4 the plasma extract is derivatized. Diastereometric dipeptides are formed from D,L-amino acids with the reagent BOC-L-Leu-SU [22, 32]. The reaction is carried out in mixtures of THF and sodium hydrogen carbonate solution [32] or methanol and sodium hydrogen carbonate solution [22, 33]. Optimization studies with [^{125}I]T_4 showed that the reaction is nearly quantitative in THF solutions. Mild reaction conditions are necessary owing to the chemical lability of iodinated thyronines. Most important is the purity of the reaction and extraction medium THF, which must be free from peroxides. The commercial analytical-reagent grade material, stabilized with 2,6-di-*tert.*-butyl-4-methylphenol, can be used without further purification. A 0.5-ml volume of serum is added to 1 ml of THF, the mixture is shaken for 2 min and the precipitate is centrifuged at 2700 *g*. For the derivatization 1 ml of the extract is necessary.

Human plasma contains many amino acids that react with BOC-L-Leu-SU, and therefore the amount of reagent had to be investigated empirically. A 50-μl volume of sodium hydrogen carbonate solution (1 *M*) and various amounts of the reagent solution (20 mg of BOC-L-Leu-SU per 1 ml of THF) were added to 1 ml of the THF extract and the solvents removed by evaporation. After splitting off the BOC groups the solutions were analysed by HPLC. Fig. 1 shows the peak height of the L-Leu derivative of L-T_4 as a function of the amount of reagent. Under the conditions described, 1 mg of reagent is sufficient for the quantitative derivatization of 400 ng of L-T_4 per 1 ml of plasma. The sodium hydrogen carbonate solution must be added in order to obtain the optimal pH value for the reaction. It is important to keep the temperature below 30°C during the evaporation, otherwise artifacts are formed [22]. The time necessary for the evaporation (about 30 min) is sufficient for the reaction equilibrium to be attained.

The selectivity of the liquid chromatographic separation is greatly improved by splitting off the BOC groups from the derivatives [22, 33]. The optimal reagent proved to be trifluoroacetic acid (TFA), which splits off the BOC groups at room temperature without causing decomposition of the thyronine derivatives. A 100-μl volume of TFA is added to the dry sample. The reaction is quantitative after 15 min at room temperature (20—22°C). The TFA must be removed prior to chromatography. Therefore, the peptides are precipitated by addition of 0.5 ml of sodium hydrogen carbonate solution (1 *M*) and separated by centrifugation. In order to remove non-polar substances that are insoluble in the mobile phase of the HPLC system, the liquid is aspirated off and 200 μl of ethanol and 100 μl of 0.5 *M* sodium hydroxide solution are added to the precipitate. After centrifugation, the clear extract is injected into the HPLC column. This sample clean-up results in effective purification of the sample. Several hundred injections into the same column are possible without a decrease in the separation efficiency.

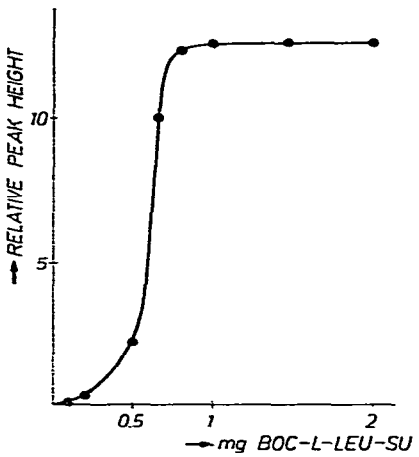

Fig. 1. Relative peak height of the L-Leu derivative of L-T_4 (400 ng/ml serum) as a function of the reagent concentration (20 mg BOC-L-Leu-SU/ml THF). External standard: L-T_4 (6 ng per injection, without derivatization).

The catalytic reaction detector

The reaction detector is based on the catalytic effect of iodide on the oxidation of N,N'-tetramethyldiaminodiphenylmethane (tetrabase). According to Feigl and Jungreis [27], the reaction is described by three equations (Fig. 2). As shown in Fig. 2, hypochlorite is liberated during the hydrolysis of chloramine-T (A). The hypochlorite is able to oxidize iodide ions to give elemental iodine (B). Iodine oxidizes the tetrabase and is itself reduced to iodide again (C). The oxidized form of the tetrabase can be determined spectrophotometrically at 600 nm.

In order to realize an on-line detection system for HPLC the reaction conditions had to be optimized with respect to a continuous flow system. Chloramine-T was dissolved in dilute acetic acid because water is needed for the

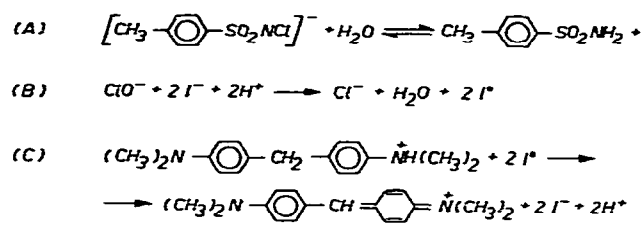

Fig. 2. Catalytic reaction of iodide with chloramine-T and N,N'-tetramethyldiaminodiphenylmethane (tetrabase).

hydrolysis and protons are necessary for the oxidation of iodide. Tetrabase is poorly soluble in water, and ethanol was chosen as a solvent.

The schematic arrangement of the detector is shown in Fig. 3. The reagents are pumped by means of a Technicon peristaltic pump and the appropriate flow-rated pump tubes to the mobile phase. The peak broadening is minimized by air segmentation (0.1 ml/min). As a result of optimization studies the best sensitivity of the reaction was found for a concentration of 300 mg of chloramine-T per litre of 0.15% acetic acid and 2 g of tetrabase per litre of ethanol. The chloramine-T solution is pumped at a flow-rate of 2 ml/min and the tetrabase solution at a flow-rate of 0.23 ml/min. The reaction is performed at 20—22°C; thermostating is not necessary.

The separation of iodinated amino acids and the corresponding L-leucine derivatives is achieved by ion-pair chromatography on reversed phases [22]. The optimal mobile phase for a C-18 column is a mixture of methanol and water (67:33) with the addition of 0.05% of methanesulphonic acid. The alcohol in the mobile phase must not be replaced by solvents with other

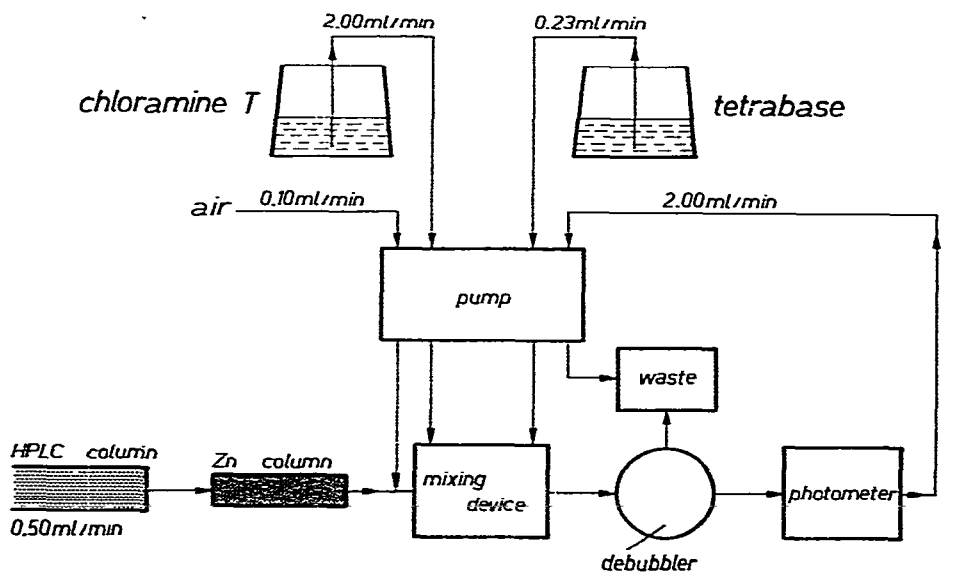

Fig. 3. Configuration of the catalytic reaction detector.

functional groups such as acetonitrile, as they are not compatible with the catalytic detection system. Instead of methanesulphonic acid, phosphate buffers or phosphate—citrate buffers can be used [22].

Iodine bound covalently in iodinated thyronines shows only a low catalytic activity. Therefore, the effluent of the HPLC columns is passed through a small glass column (50 × 1 mm I.D.) packed with zinc powder (75—150 μm, purity 99.999%; Goodfellow Metals, Cambridge, Great Britain). The column is closed with glass-wool at both ends. By pumping the acidic mobile phase through this column, hydrogen is produced. This hydrogen in statu nascendi splits off the iodine from the phenyl groups of the iodinated thyronines. The resulting iodide ions show high catalytic activity. As zinc is dissolved in this reaction, the zinc column must be renewed daily.

A homogeneous mixture of the reagents with the mobile phase is of great importance. An incomplete mixture of the aqueous and organic solvents causes high baseline noise in the spectrophotometer. Therefore, some mixing units were tested and the signal-to-noise ratio was measured under constant conditions. The mixing systems tested are illustrated in Fig. 4. Types A, B and C were constructed as described by Nachtmann et al. [26]; type E was a mixing device as published recently by Kobayashi and Imai [34].

Table I shows the signal-to-noise ratio and the peak half-width for a signal obtained after injection of 1 ng of T_4 under the chromatographic conditions given in Fig. 5A. As the signal height was nearly constant, the signal-to-noise ratio was measured as the index of mixing efficiency and the half of the peak width was used as the index of the peak broadening. Type F, a newly constructed mixing chamber, gave the best efficiency. The cylindrical magnetic stirrer has a fin at its upper side. This mixing device was used for all further investigations.

The pumping of the reagents by a peristaltic pump would give rise to periodic oscillations of the detector baseline. Therefore, as depicted in Fig. 3, the

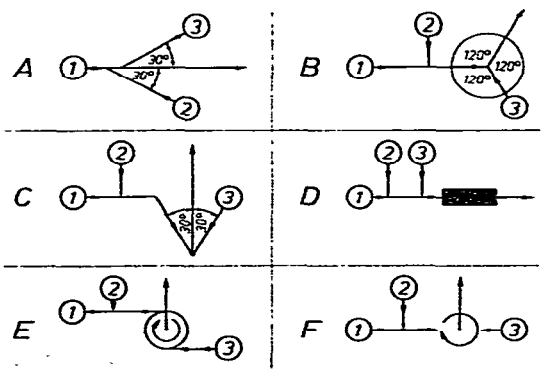

Fig. 4. Mixing devices for HPLC column effluent and reagents. A, mixing against flow direction, 1 mm I.D. bores; B, 120° mixing device, 1 mm I.D. bores; C, 30° mixing device, 1 mm I.D. bores; D, column reactor filled with glass beads (60 mesh), 50 × 2 mm I.D.; E, rotating flow mixing device, 5 × 4 mm I.D.; F, dynamic mixing chamber with magnetic stirring bar, 12 × 8 mm I.D. (cylindrical stirring bar, 11.5 × 7.7 mm). 1, Air-segmented chloramine-T solution; 2, HPLC column effluent; 3, tetrabase solution.

TABLE I

EFFECTS OF DIFFERENT MIXING DEVICES ON THE SIGNAL-TO-NOISE RATIO AND ON PEAK BROADENING AT CONSTANT ATTENUATION AND CONSTANT FLOW-RATE

Test substance: T_4. For chromatographic parameters, see Fig. 5A.

Type of mixing device (see Fig. 4)	Signal-to-noise ratio	Peak half-width (s)
A	1.5	26
B	2.8	25
C	2.9	26
D	3.0	30
E	2.9	29
F	24.0	28

solvents are sucked through the detector cell with a constant flow. By this technique the pulsations are compensated very effectively. Air bubbles and the excess of solvent are separated by means of a debubbler.

Analyses of plasma samples

For the determination of total T_4 50 μl of the ethanolic plasma extract and for the stereospecific determination of D- and L-T_4 30 μl of the derivatized extract are injected into the HPLC system. An improvement in the precision of the analysis was achieved by internal standardization. A suitable internal standard was found to be T_3, which is eluted before T_4 under the conditions described. Interferences from the T_3 level present in human plasma do not occur because the natural T_3 level is two or three orders of magnitude smaller than the concentration of the internal standard. For the determination of the total T_4 content the internal standard is dissolved in the ethanol used for the extraction of the plasma samples. The distribution coefficients for T_3 and T_4 are identical under the conditions used. In the stereospecific determination of D- and L-T_4 the internal standard is dissolved in the sodium hydroxide that is added to the samples after the removal of TFA. Addition before derivatization is not possible owing to the different solubilities of T_3 and T_4 in the precipitation steps.

Typical chromatograms are shown in Fig. 5. The separations take place rapidly. In peak 1 (A, B) the halide anions iodide, bromide and chloride are eluted. The sensitivity for bromide and chloride is smaller than that for iodide: the factor for bromide is 200 and that for chloride is 500. The simultaneous determination of free iodide in plasma with the method described is not successful because the large excess of chloride and bromide in normal serum [35] interferes with the signal of iodide.

In the biologically relevant concentration range of 0—310 nmol/l in serum linear calibration graphs are obtained for the L- and D-isomers and the correlation coefficients found were 0.985—0.999. With higher concentrations, dilution of the sample or reduction of the injection volume is necessary. Table II gives the relative standard deviations for the determination of total T_4 and the deter-

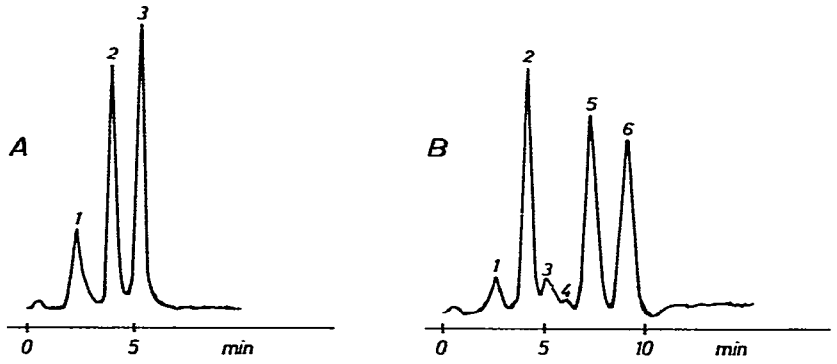

Fig. 5. Chromatographic determination of iodinated thyronines with catalytic detection. Column, Nucleosil 5 C-18 (150 × 3.2 mm I.D.); mobile phase, methanol—water (67:33) plus 0.05% of methanesulphonic acid; flow-rate, 0.5 ml/min at 1100 p.s.i.; detection wavelength, 600 nm. (A) Determination of total T_4. Injection volume, 50 µl of ethanolic serum extract. 1, Free halide ions; 2, 10 ng of T_3 (internal standard); 3, 8 ng of T_4. (B) Stereospecific determination of D- and L-T_4 in serum after derivatization. Injection volume, 30 µl. 1, Free halide ions; 2, 10 ng of T_3 (internal standard); 3 and 4, not identified; 5, L-Leu-L-T_4 (corresponding to 7 ng of L-T_4); 6, L-Leu-D-T_4 (corresponding to 7 ng of D-T_4).

TABLE II

RELATIVE STANDARD DEVIATION (S_{rel}) FOR THE DETERMINATION OF D,L-T_4 IN SERUM USING INTERNAL STANDARDIZATION

Concentration of T_4 (nmol/l serum)	S_{rel} (%) ($n = 6$)		
	Total T_4	L-T_4	D-T_4
38.6	5.3	9.5	9.8
77.2	4.8	7.4	6.9
154.4	4.2	6.8	7.5
308.8	5.1	7.1	6.4

mination of D- and L-T_4. The standards were prepared by spiking a T_3- and T_4-free plasma, which was prepared as follows: 40 ml of human plasma were pipetted on top of a glass column (300 × 25 mm I.D.) filled with Dowex 1-X2 (50—100 mesh) and the same anion exchanger (100—200 mesh) (1:1 mixture). After incubation overnight the elution was carried out with 40 ml of an isotonic sodium chloride solution. The first 5 ml were discarded, the remainder of the solution being plasma free of thyroid hormones. For regeneration the column was washed with 500 ml of water followed by 500 ml of 70% acetic acid and 500 ml of water.

The quantitative analysis of plasma samples is performed by comparison of the sample values with a calibration graph with use of the internal standard. The detection limit for D,L-T_4, taking a signal-to-noise ratio of 3:1, is 125 pg absolute or approximately 3 nmol/l in plasma.

The method described is being used for a pharmacokinetic study of D-T_4 in collaboration with the Department of Internal Medicine, University of Graz,

Austria. This method is the only one known so far that permits the stereoselective determination of D- and L-T_4 at plasma levels. The sensitivity and precision are sufficient for clinical studies. Of course, the catalytic reaction detector can also be used for the trace determination of other iodine-containing compounds.

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