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CATALYTIC DETECTION PRINCIPLE FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new detection principle for high-performance liquid chromatography is described in which the separation column is coupled to a catalytic detection system. The catalytic reaction is based on the redox reaction between cerium(IV) and arsenic(III) that is catalyzed by trace amounts of iodine. The decoloration of cerium(IV) is measured spectrophotometrically at 365 nm. The reaction was studied for continuous operation using triiodothyronine as substrate. The speed of reaction was decreased by all of the organic solvents tested. The detection principle permits trace determinations of iodine-substituted molecules. The limits of detection for the hormones tetra- and triiodothyronine are in the sub-nanogram range, which should be sufficient for their determination in human plasma.

INTRODUCTION

The most sensitive commercial detectors suitable for routine analysis in high-performance liquid chromatography (HPLC) are UV and fluorescence detectors, but many substances of interest cannot be detected with sufficient sensitivity by these. Great efforts have been made to effect derivatizations to improve the UV and fluorescence detection properties of such substances[1–5]. The hormones of the thyroid gland belong to the group of molecules for which the sensitivity of detection is not sufficient for the determination of trace amounts, as found for example in human plasma.

In most instances tetraiodothyronine (T_4) and triiodothyronine (T_3) are determined by competitive protein-binding analysis[6] or radioimmunoassay[7–12]. No separation is necessary because of the high specificity of these methods. Another possibility for the determination of the hormones is a combination of a chromatographic procedure with a non-specific detection principle. Chromatographic procedures also have the advantage that, in addition to T_3 and T_4, other iodinated thyronines are detectable. One possibility is a gas chromatographic determination following derivatization[13–15]. The derivatization procedure must be carried out in a non-aqueous medium, and therefore this method is suitable only in special cases.

An alternative is a separation of the iodinated amino acids by classical

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column liquid chromatography[16–18]. For detection, the catalytic activity of iodine (Sandell–Kolthoff reaction) is used in such instances. The main problem with these methods is the low separation efficiency and the long analysis time. The use of HPLC could overcome these disadvantages. UV detection of the hormones is not sensitive enough, as already mentioned, and therefore it has been attempted to optimize a modified form of the Sandell–Kolthoff reaction[19] as a post-column reaction system before UV detection of the eluent.

EXPERIMENTAL

Reagents and materials

The reference substances L-T_3 (sodium salt) and L-T_4 were provided by Merck (Darmstadt, G.F.R.). RT_3 was a gift from Warner Lambert (Morris Plains, N.J., U.S.A.). All reagents and solvents were of analytical-reagent grade (Merck). For the catalytic reaction, cerium(IV) and arsenic(III) solutions are necessary.

Cerium(IV) solution. $Ce(SO_4)_2 \cdot 4H_2O$ (1800 g) is dissolved in 13 ml of sulphuric acid (96%) and the solution diluted with water to 500 ml. This solution is stable for several weeks. Two parts of this solution are mixed with seven parts of nitric acid (65%) to give a solution ready for use. It must be prepared freshly every day.

Arsenic(III) solution. As_2O_3 (1800 g) is dissolved in 20 ml of 1 *N* sodium hydroxide solution and the solution diluted with water to 500 ml. A 1-ml volume of hydrochloric acid (37%) is added. This solution is ready for use and is stable for several weeks.

Stationary phases. LiChrosorb RP-2, LiChrosorb RP-8 (Merck) and Nucleosil C_{18} (Macherey, Nagel & Co., Düren, G.F.R.) were used as stationary phases in HPLC.

Instruments

For HPLC determinations, a Siemens S 200 liquid chromatograph equipped with a Zeiss PM 4 spectrometric detector and a Waters Assoc. M 6000A pump in combination with a Schoeffel SF 770 spectrophotometric detector were used. The Zeiss PM 4 had to be modified with a glass cell, but the PTFE cells with quartz windows in the Schoeffel detector could be used without change. The cerium(IV) and arsenic(III) reagents were pumped by a PMP 10 Duo pump (Ismatec, Zurich, Switzerland) and an adapted Perfusor V infusion pump (Braun, Melsungen, G.F.R.). For the study of the catalytic reaction, the mobile phase was transported by an mP 1 squeezing pump (Bühler, Tübingen, G.F.R.). The mixing devices were home-made from PTFE. A Zeiss PMQ II spectrophotometer with a glass cell (100 μl) was used for detection. PTFE capillaries (0.5 mm I.D.) were used for all connections.

Optimization of the catalytic reaction

A scheme of the experimental procedure is shown in Fig. 1. The mobile phase is pumped from the solvent reservoir by pump A. The sample is injected at B and mixed with the reagent solution at C. The two reagents are transported by pump D and mixed in a small mixing vessel (E). The mixing device (C) is coupled to the reaction capillary (F), which is connected to the spectrophotometer (H). The reaction capillary and the reservoirs of the mobile phase and the reagent solutions are placed in a thermostat (G).

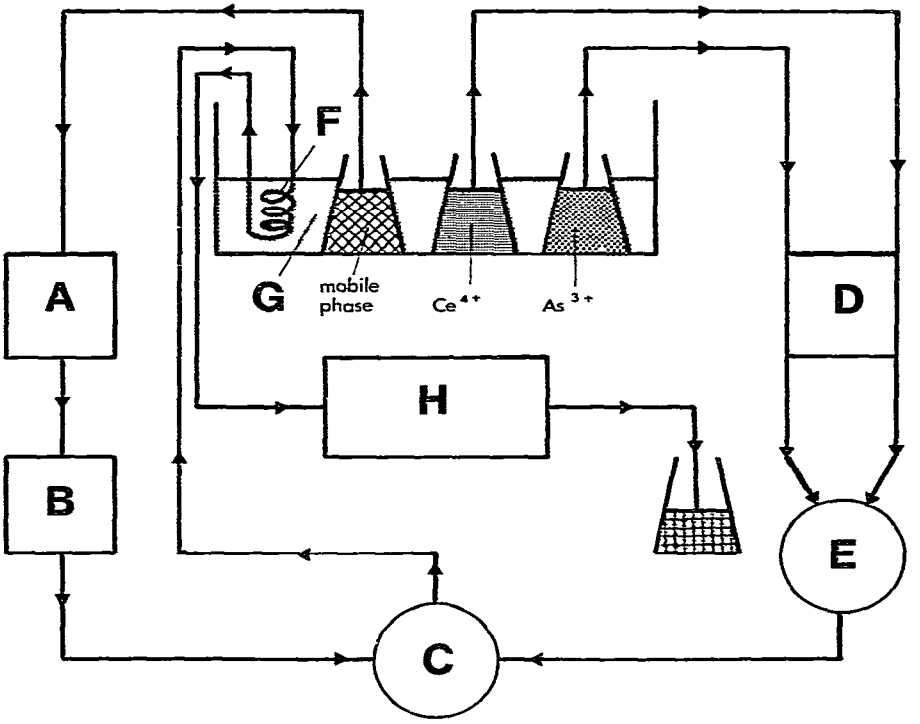

Fig. 1. System for optimization of the catalytic reaction. A = Pump for mobile phase; B = injection system; C = mixing device; D = pump for reagent solutions; E = mixing device; F = reaction capillary; G = thermostat; H = spectrophotometer.

Coupling of HPLC catalytic detection system

The procedure shown in Fig. 1 has to be slightly modified. The analytical column is placed in the thermostat and connected with the mixing device (C) by a PTFE capillary. The columns used (15 cm × 3.2 mm I.D.) were made of steel.

RESULTS AND DISCUSSION

Theoretical

The redox reaction between cerium(IV) and arsenic(III), which is catalysed by iodine, can be described by the equation

$$2\,Ce^{4+} + As^{3+} \xrightarrow{I} 2\,Ce^{3+} + As^{5+} \quad (1)$$

The reaction is pseudomonomolecular[19]. The kinetics are described by the equation

$$-\frac{d\,[Ce^{4+}]_t}{dt} = k\,[Ce^{4+}]_t \cdot [I] \quad (2)$$

Integration gives

$$\ln[Ce^{4+}]_t = \ln[Ce^{4+}]_{t=0} - k[I]t \quad (3)$$

The concentration of Ce^{4+} can be monitored by a spectrophotometer, and we can write

$$\ln A_t = \ln A_0 - k_A[I]t \tag{4}$$

where A = absorbance.

If all of the parameters in a continuous system are kept constant, the concentration of iodine is proportional to the change in absorbance:

$$\begin{aligned} &\Delta \ln A = b[I] \\ &\Delta \ln A = \ln A_0 - \ln A_t \\ &k_A t = b \end{aligned} \tag{5}$$

Eqn. 5 is the basis of this investigation. The term b represents the sensitivity of the reaction, if it is used for the determination of iodine. As b contains the specific reaction rate and the reaction time, some conditions must be applied in order to obtain reproducible analytical results: constant composition of all reagents and solvent mixtures, constant temperature and constant reaction time, which requires pumps with a constant flow delivery.

Optimization of the catalytic reaction in flowing systems

In order to obtain results that can be used for a detection system applicable to HPLC, conditions that are present in the eluent of a liquid chromatograph were simulated. The scheme of the experimental procedure is shown in Fig. 1 and has been described above. As a test substrate the hormone T_3 (sodium salt) was used. Solutions in water were injected at B (Fig. 1), with concentrations of 0–32 ng per injection (50 μl). An exact description of the injection system is shown in Fig. 2; the whole system is made of PTFE.

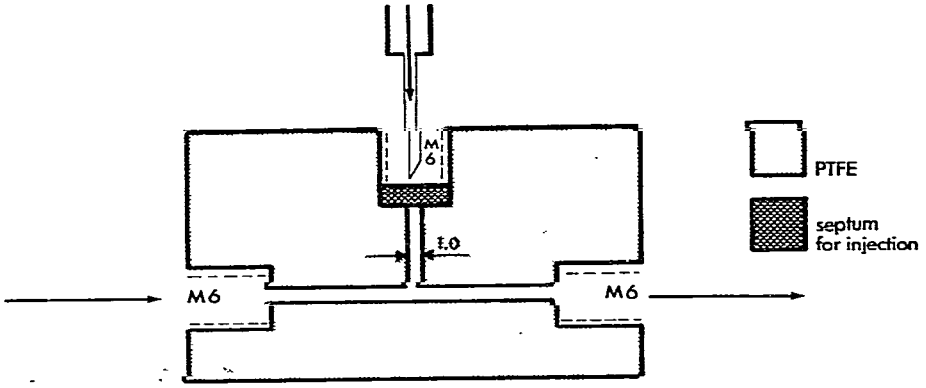

Fig. 2. Injection system.

Great attention was paid to the optimization of the mixing devices. The solutions of cerium(IV) and arsenic(III) cannot be mixed in the reservoir, because a slow redox reaction also takes place in the absence of iodine, so that the reagents must be mixed immediately before use. The mixture should be completely homoge-

neous in order to prevent pulsations in the detector, and a special mixing vessel with a magnetic stirrer (Fig. 3) was constructed for this purpose. This vessel, made of PTFE, has a volume of slightly more than 1 ml and guarantees a homogeneous mixture within a short time. The stirrer is a small iron wire embedded in polyethylene. The vessel is closed with a PTFE disk, which is pressed to the vessel by an aluminium disk that can be fixed by three screws.

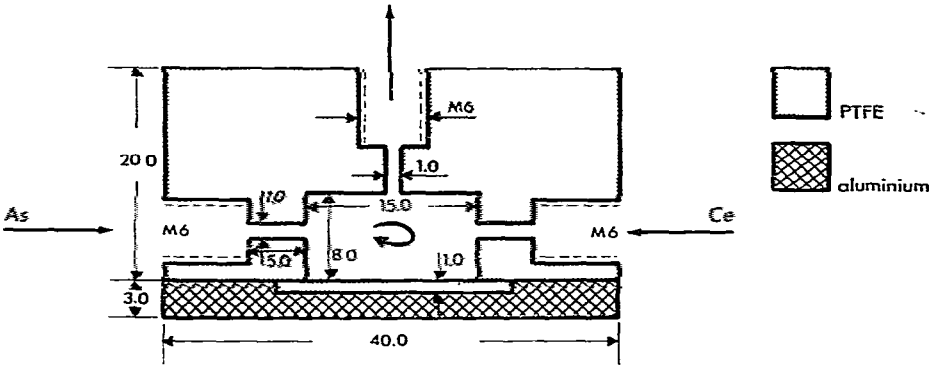

Fig. 3. Mixing chamber for the reagent solutions.

The vessel described is not suitable for a mixture of the reagents with the mobile phase. The mixing devices tested for this purpose (Fig. 4) were made of PTFE, all holes having an I.D. of 1 mm. Types b and c were optimal. Similar results were described by Frei *et al.*[5] for the post-column derivatization of primary amines with fluorescamine. Mixing device b was used in subsequent investigations.

It is essential for the whole detection system that the reagents should not come into contact with metals, as this would lead to a reduction of cerium(IV) and decoloration of the solution.

With the system described, the parameters temperature, reaction time and the role of organic solvents in the mobile phase, which influence the sensitivity of the reaction, were investigated. The flow-rate was kept constant and the reaction time was varied by varying the length of the reaction capillary. The flow-rate of each of the three streams [mobile phase, cerium(IV) solution and arsenic(III) solution] was 0.7 ml/min, so that the flow-rate in the reaction capillary was 2.1 ml/min. One PTFE capillary was 3 m long, with a calculated reaction time for the given flow-rate of 16 sec, which is too short for a useful reaction[19]. Some capillaries have to be coupled, and the sensitivity of the reaction was investigated for three, four and five capillaries (9, 12 and 15 m) at 30°. The mobile phase was water–acetonitrile (5:1) containing 1% of acetic acid.

T_3 was determined in the concentration range 0–16 ng per injection and the results are shown in Fig. 5. The sensitivity (*b*) was calculated from calibration graphs using eqn. 5. All calibration graphs were linear in the investigated range with regression coefficients ranging from 0.97 to 0.99. The sensitivity increases with increasing reaction time. As would be expected from theoretical considerations, this increase is not a linear function. On the other hand, a long reaction time causes in-

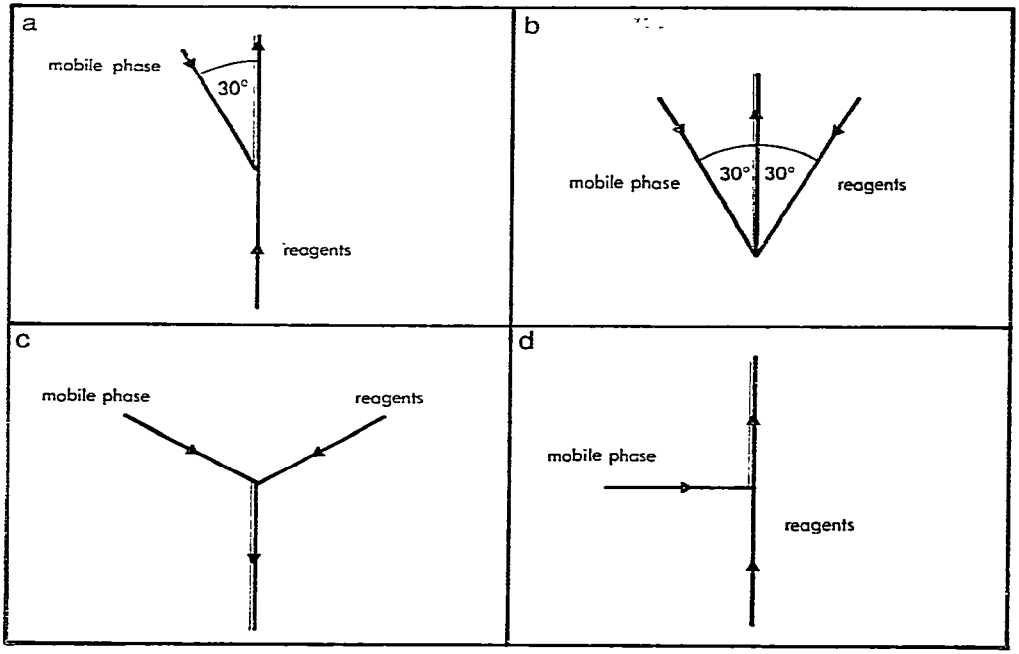

Fig. 4. Mixing devices for mobile phase and reagents.

creased peak broadening due to the great length of the reaction capillary, and a greater length than 15 m cannot be recommended for this reason.

Temperature also has a great influence on the speed of reaction. Using a reaction capillary of 15 m and conditions as described above, the influence of temperature (25, 30, 35 and 40°) was investigated. At 25, 30 and 35°, linear calibration graphs

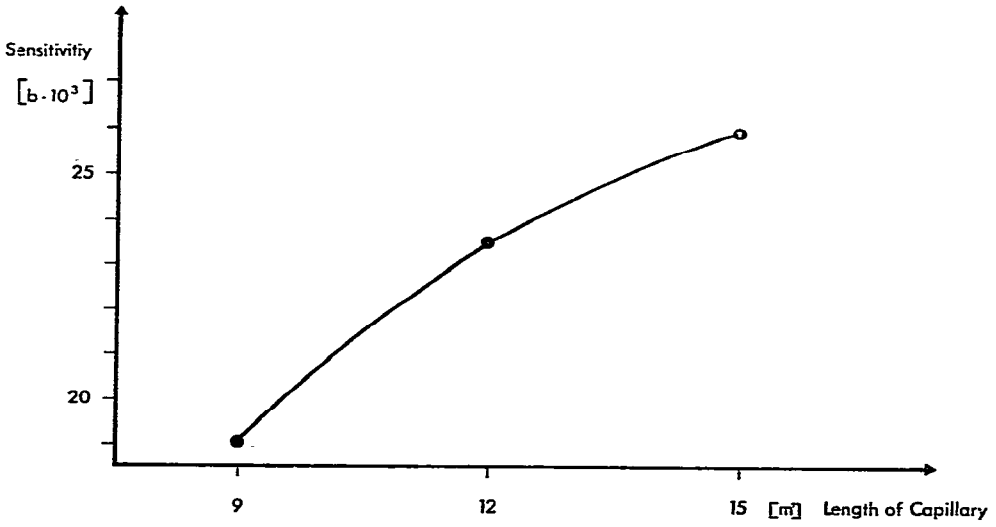

Fig. 5. Influence of the length of the reaction capillary on the sensitivity of the reaction.

were obtained from 0 to 16 ng of T_3 with regression coefficients of 0.98–0.99. At 40° the calibration graph was linear only up to 10 ng of T_3. The results are shown in Fig. 6. The sensitivity increases from 25° to 35°, but further increases in temperature lead to no further improvement. The increase in sensitivity is small and the linear range decreases.

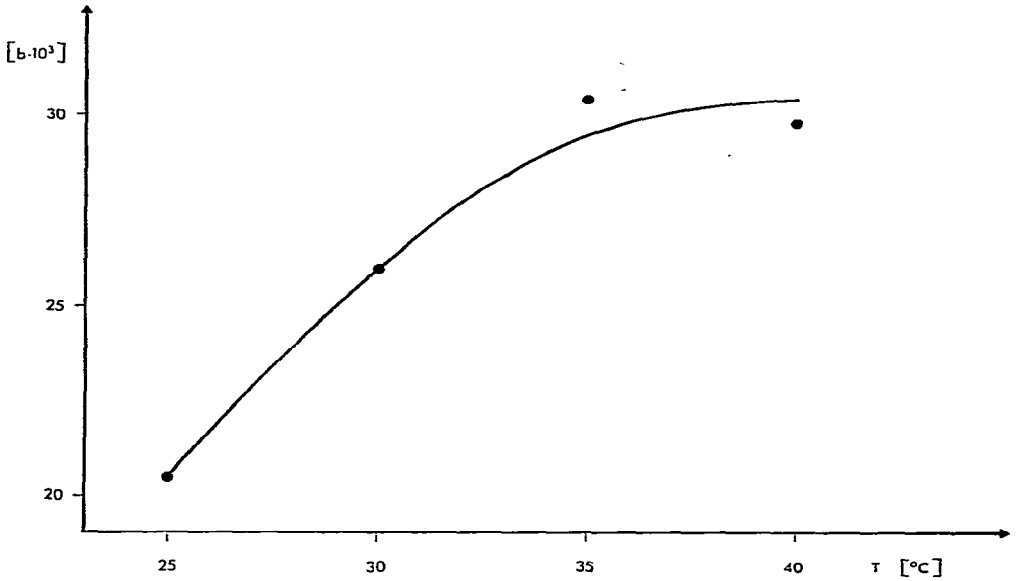

Fig. 6. Influence of temperature on the sensitivity of the reaction.

Organic solvents have a great influence on the reaction velocity. Preliminary experiments showed that iodinated thyronines can be separated on reversed-phase systems with mixtures of water–methanol and water–acetonitrile with the addition of 1% of acetic acid. For this reason, the investigations were restricted to such mixtures. In comparison with water, a 1% solution of acetic acid produces an unimportant decrease in the speed of reaction. Greater inhibitions are caused by methanol and acetonitrile, both of which show qualitatively identical behaviour: a linear decrease in sensitivity with increasing solvent content, as can be seen for acetonitrile in Table I. The mixture water–acetonitrile (2:1) in comparison with water, causes a two-fold decrease in the sensitivity, but the linear range of the calibration graph increases by the same factor. Similar results were obtained with methanol, the decrease in sensitivity being smaller than that for acetonitrile by a factor of 1.2. Nevertheless, acetonitrile was preferred to methanol because it has better characteristics in the liquid chromatographic separation of thyroid hormones.

Coupling of HPLC catalytic detection system

For the separation of hormones by HPLC, only reversed-phase chromatography can be used because the catalytic reaction must be carried out in an aqueous system. Separations of the substrates T_3, RT_3 and T_4 were performed on LiChrosorb RP-2, LiChrosorb RP-8 and Nucleosil C_{18}. Nucleosil C_{18} showed the best separation

TABLE I

INFLUENCE OF ACETONITRILE ON THE SENSITIVITY OF THE REACTION

Detection wavelength, 365 nm.

Water–acetronitrile	b^*	r^{**}	*Linear range (ng T_3)*
10:0	0.0361	0.990	0–10
10:1	0.0334	0.988	0–16
10:2	0.0279	0.997	0–16
10:3	0.0247	0.994	0–16
10:4	0.0176	0.984	0–16
10:5	0.0166	0.991	0–32

* b = sensitivity of the reaction, calculated from eqn. 5.

** r = regression coefficient (linear calibration graph).

properties. A 15-cm column permits an optimal separation of the three substances with water–acetonitrile (5:2) containing 1% of acetic acid as the mobile phase at 30°. The addition of acetic acid is necessary in order to obtain symmetrical peaks. In comparison with water, this mobile phase lowers the sensitivity of detection by a factor 1.85.

Great difficulties were caused by periodic changes of the baseline, as can be seen in Fig. 7, and this chromatogram cannot be used for exact quantification of the hormones. The cause of the pulsation was found to be the Ismatec pump that transports the two reagent solutions. Unfortunately, no pump that guarantees the

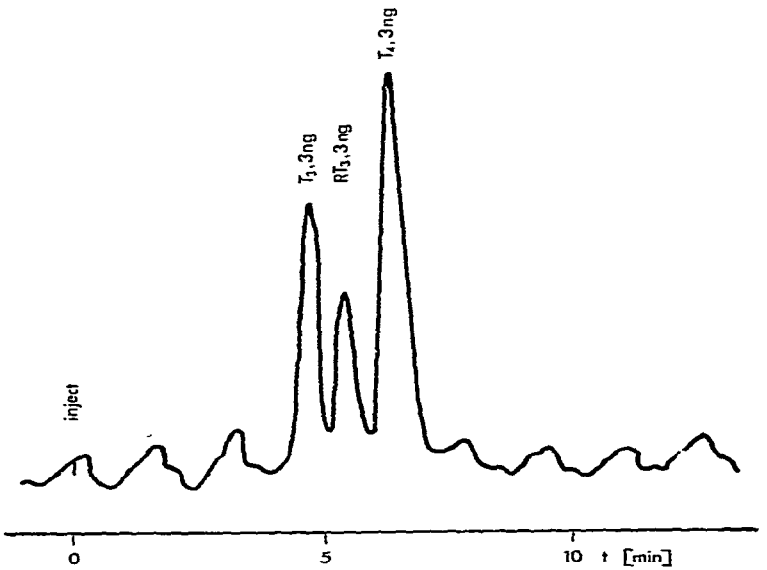

Fig. 7. HPLC separation and catalytic detection of three iodinated thyronines. Reagent pump: Ismatec PMP 10 Duo. Solvent system: water–acetonitrile (5:2) + 1% of acetic acid. Column: Nucleosil C_{18}, 5 μm, 15 cm × 3.2 mm I.D. Flow-rates: mobile phase, 1 ml/min; reagents, 1 ml/min. Injection volume: 100 μl. Temperature: 30°. Length of reaction capillary: 15 m. Detector: Schoeffel SF 770 at 365 nm, range 2.

necessary constant flow is available commercially. An adapted infusion pump (Perfusor V) gave a much better baseline. The two syringes were replaced with glass cylinders (50 ml) with PTFE pistons. The front of each glass cylinder is closed with a PTFE disk, into which is screwed the PTFE tube for filling or emptying the cylinder. The pump can be used only at pressures below 1 bar. For a reaction capillary of 6 m, the maximal flow-ratio were 0.5 ml/min for the reagents and 0.5–0.7 ml/min for the mobile phase. A longer capillary or higher flow-rates cause a back-pressure higher than 1 bar.

A determination of T_3 and T_4 using the conditions described is shown in Fig. 8. The better baseline compared with Fig. 7 is obvious. The chromatogram shows the the actual absorbance with no amplification. For the determination of hormones from plasma a large volume (200 μl) was injected. T_4 is detectable with a greater sensitivity than T_3 because of its higher iodine content. The limit of detection for both hormones is in the sub-nanogram region.

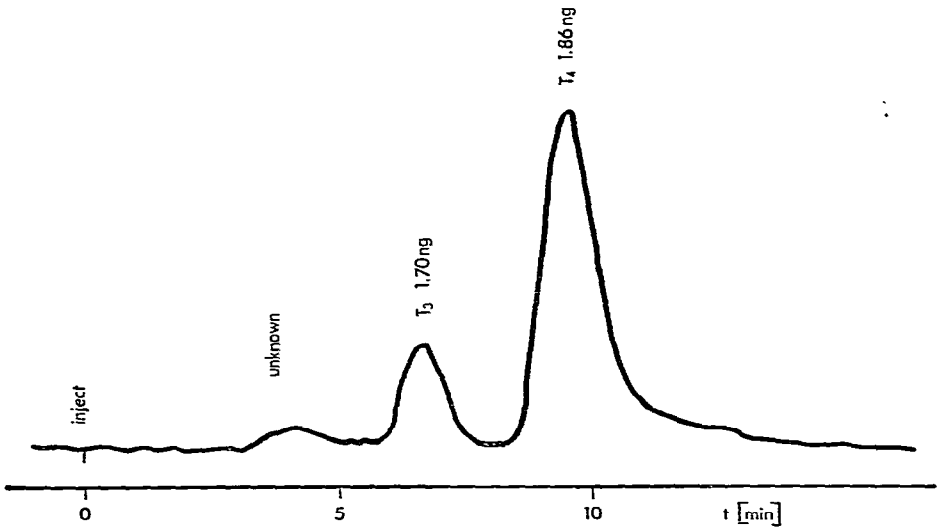

Fig. 8. HPLC separation and catalytic detection of T_3 and T_4. Reagent pump: Perfusor V, modified. Solvent system: water–acetonitrile (5:2) + 1% of acetic acid. Column: Nucleosil C_{18}, 5 μm, 15 cm × 3.2 mm I.D. Flow-rates: mobile phase, 0.7 ml/min; reagents, 0.5 ml/min. Injection volume: 200 μl. Temperature: 30°. Length of reaction capillary: 6 m. Detector: Zeiss PM 4 at 365 nm, range 1.

The detection principle presented here is one of the most sensitive described so far for liquid chromatography. The determination of T_3 and T_4 in human plasma should be possible with this method, and work on this aspect is in progress. Additionally, the method should permit trace determinations of other molecules that contain iodine.

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