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Kinetic Measurement of T_4 Following Column Chromatography

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A kinetic measurement of the eluates from the column purification of T_4 is described.

The choice of cation exchange chromatography to reduce interfering X-ray contrast substances, and the influence of protein in the reaction have been evaluated.

Data for precision, inter and intra assay reproducibility, detection limit and normal values are reported.

Using sample volumes of 0.5 ml and an analysis time of one minute, the proposed method compares favourably with published methods.

Eine kinetische Messung von Eluaten, welche man bei der Säulenchromatographie der T_4-Bestimmung erhält, wird beschrieben.

Der Einfluß von Eiweiß in den Eluaten bei Verwendung der Kationenaustauschermethode, die durch Röntgenkontrastmittel weniger gestört wird als die Anionaustauschermethode, wurde untersucht.

Angaben über die Genauigkeit, Reproduzierbarkeit innerhalb einer Serie, von einem Tag zum anderen, Nachweisgrenze der Methode und Normalwerte werden mitgeteilt.

Die Methode, die nur 0,5 ml Serum und eine Meßzeit von einer Minute erfordert, schneidet, verglichen mit den bis jetzt veröffentlichten Methoden, günstig ab.

Radioactive methods (competitive protein binding or specific radioimmuno assay) for determining T_4[1]) levels are easy to perform but demand high investments. In addition reproducibility is insufficient for a reliable control of minimal alterations in T_4 levels encountered during thyroid function tests (1, 2). The kinetic measurement of the classic Kolthoff–Sandell reaction, which is proposed here, combines two advantages: high reproducibility and low investment.

Materials and Methods

Separation of T_4 from serum was performed by a cation exchange procedure (3), modified by *Kreutzer* (4).

Cation exchange resin

Dowex 50 W X 2,200–400 mesh, H^+form (Baker)

Glass columns

Inner diameter 6 mm, provided with a G_1 filter at the bottom and a reservoir of 10 ml at the top.

Borate buffer (pH = 8.5); *Arsenious acid solution* (0.06 mol/l) and *Ceric ammonium sulfate solution* (0.03 mol/l) were prepared as described by *Backer* et al (3).

Sodium hypochlorite solution (0.067 mol/l): 67 ml of a NaClO solution (0.5 mol/l) "low in bromine" (British Drug Houses Ltd. Poole, England) is diluted with distilled water to 500 ml.

Standards

Aqueous T_4 solutions of *L*-thyroxine (Sigma, Chem. Comp. St. Louis, Mo U.S.A.) prepared according to *Passen* & von *Saleski* (5).

Preparation of columns

After initial purification of the resin by washing with NaOH (0.1 mol/l), distilled water and HCl (1.0 mol/l) respectively, columns are filled with the resin to a height of 30 ± 2 mm and distilled water is passed through until eluates show neutrality on litmus paper. Then 0.5 ml of serum or standard serum is applied on the columns and the columns are washed with the following sequence of solutions: 2 ml HCl (1.0 mol/l), 4 ml NaCl solution (58.5 g/l), 8 ml borate buffer and 4 ml distilled water.

Finally T_4 is selectively eluted by adding two times 1.25 ml NaOH (0.02 mol/l).

This eluate (2.5 ml) is collected in a clean receptacle. A second eluate is collected and measured only if the first eluate gives reason to suspect a contamination with X-ray contrast substances.

Columns are regenerated by eluting with additional volumes of 10 ml NaOH (0.02 mol/l), 10 ml distilled water, 10 ml HCl (1.0 mol/l) and distilled water until the washings are neutral to litmus paper.

Apparatus

For the kinetic measurement we use a LKB reactionrate analyser 8600 provided with 10 mm disposable cuvets. Adjustment of the analyser: wavelength 425 nm; background slide 0.7; recorder range 0.2 Ext. for full scale; decrease measurement; measuring time one minute; pump volume 100 μl; speed of the recorder paper 60 mm per minute; the pumping system is filled with ceric ammonium sulfate solution. After thorough mixing

[1]) T_4 = 3, 5, 3', 5'-tetraiodothyronine (thyroxine)

0.5 ml of the eluate is pipetted into the disposable cuvet and optimum reaction conditions are achieved by adding the following amounts of reagents: 100 µl H_2SO_4 (2.5 mol/l) and 100 µl NaClO (0.067 mol/l). After mixing, the reaction mixture is allowed to stand for ten minutes.

Next 200 µl arsenious acid (0.06 mol/l) are added, the reaction mixture is mixed again and transferred to the reaction-rate analyser.

Results

Figure 1 shows typical tracings of the method.

Linearity of the tracings is achieved within a few seconds. Figure 2 shows the influence of albumin during the reaction time. Linearity of the standard curve, tested with 0.5 ml samples of T_4 solutions which were run through the entire ion exchange procedure is shown in Figure 3.

Sensitivity of our procedure at various wavelengths is illustrated in Table 1.

To determine the reproducibility of our method two serum pools were run twenty times.

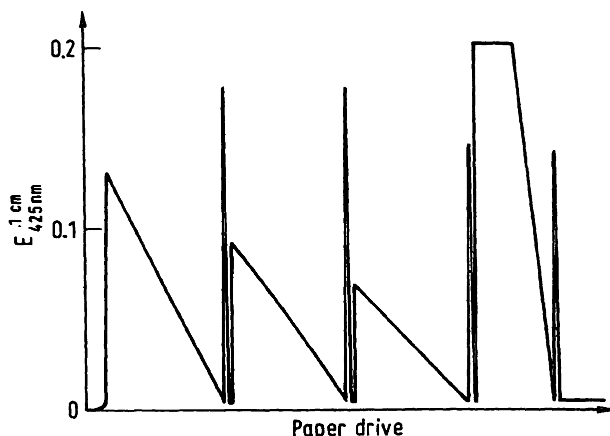

Fig. 1. Representative tracings of some serum eluates.

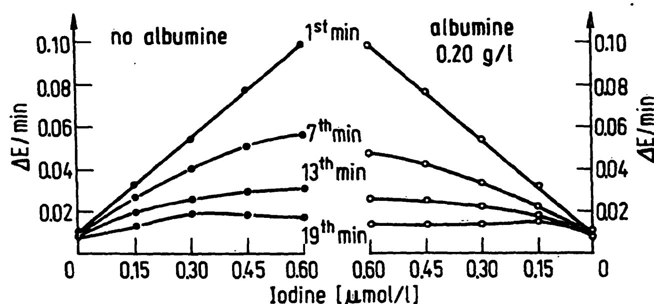

Fig. 2. Standard lines, as obtained by measuring the reaction-rates of five aqueous T_4 solutions, during the first minute (straight lines), 7 th, 13 th and 19 th minute (declining lines) of the reaction in albumin-free samples on the left, with addition of albumin (0.20 g/l) on the right.

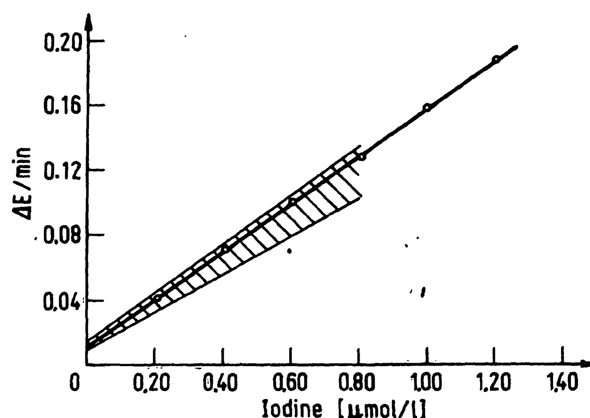

Fig. 3. Standard curve obtained by the described method by measuring eluates of aqueous T_4 solutions with indicated concentrations up to 1.20 µmol l/l.
Shaded area:
Collection of the standard curves from more than 200 series, performed in the course of three years by the proposed method.

Tab. 1. Sensitivity of the proposed method at various wavelengths.

As a measure of sensitivity, the ΔE/min of a 0.80 µmol l/l sample minus the ΔE/min of a column blank sample is taken.

Wavelength [nm]	Sensitivity [ΔE/min]
406	0.191
425	0,116
457	0.027

Mean values of these pools were 0.225 µmol l/l and 0.752 µmol l/l with corresponding standard deviations 0.0045 µmol l/l (C.V. 2.0 %) and 0.013 µmol l/l (C.V. 1.8 %). Day to day reproducibility of a serum pool determined on ten different days was slightly less: mean value 0.415 µmol l/l with standard deviation 0.010 µmol l/l (C.V. 2.4 %). Similar experiments carried out on two other serum pools with a competitive protein-binding method gave mean values of 0.302 µmol l/l and 0.655 µmol l/l with corresponding standard deviations 0.018 µmol l/l (C.V. 6.0 %) and 0.045 µmol l/l (C.V. 6.9 %).

Day to day reproducibility of a serum pool (stored at 4° C) determined on six successive days with the same method[2]) gave a mean value of 0.462 µmol l/l with standard deviation 0.0471 µmol l/l (C.V. 10.2%). These latter data agree well with values found in the literature.

Precision of our method was illustrated by the fact that a calibration curve, obtained by running aqueous T_4

[2]) Res-O-Mat T_4 kit manufactured by Byk-Mallinckrodt, Chemische Produkte GmbH D-6051 Dietzenbach-Steinberg.

solutions was identical with a calibration curve obtained by running a Hyland Special sample (0.32 µmol l/l range), a Hyland Abnormal sample (0.65 µmol l/l range), and a sample of an equal mixture of both standard sera and a column blank.

Addition of known amounts of T_4 to a serum pool of 0.225 µmol l/l, resulting in samples with concentrations of 0.225; 0.425; 0.625; 0.825 and 1.025 µmol l/l, also gave the same calibration curve.

The detection limit of the method, expressed as two standard deviations of a tenfold determination of a blank value, was 0.013 µmol l/l.

Comparison with results from two publications which describe a kinetic measurement of T_4 eluates, both in connection to an anion exchange procedure (7, 8), is illustrated in table 2. Data for comparison of the sensitivities are corrected for different sizes of serum samples, total volume of eluate, volume of eluate used in the reaction and the final volume in which the reaction rate was determined.

The constancy of the sensitivity of our method is well illustrated by the shaded area presented in Figure 3. Normal values of the method, initially based on sera of fifty euthyroid persons, were 0.22–0.51 µmol l/l and these were maintained over the last three years.

Discussion

We have replaced the initially used anion exchange chromatography by a cationexchange procedure, because the latter method is far less sensitive to X-ray contrast substances (6). The influence of protein, which, in the cation exchange procedure, appears in the eluate to a maximum of 0.20 g/l (4) was evaluated.

As can be concluded from Figure 2 no measurable influence of the protein during the first minute of the reaction (which is routinely measured) is encountered. Later on, in the 7th and consecutive minutes of the reaction however, the reaction-rate in the albumin samples is considerably lower than in the albumin free samples.

From Table 1 it can be seen that shifting to lower wavelengths enhances the sensitivity of our method.

For practical reasons the wavelength of 425 nm was considered to be the most suitable.

Besides the indicated different circumstances of the three compared methods in Table 2, the choice of halogenation reagent may be an important reason for the observed differences in sensitivities.

In conclusion it may be stated that the proposed modification leads to a method which can be applied in cases where minimal alterations of T_4 have to be measured with sufficient reproducibility.

Tab. 2. Comparison of the proposed method with the two kinetic measurements so far published.

	Hathaway (ref. 7)	*Arcq* (ref. 8)	Proposed method
Wavelength [nm]	360	405	425
Reactions temperature [°C]	37	25	35
$[As\,O_3]^{3-}$final [mmol/l]	12	8	12
$[Ce]^{4+}$final [mmol/l]	0.6	30	3
Reproducibility (C.V.%)	not stated, day to day 8.8	3,8	2.0
Sensitivity (calc. as in table 1) [ΔE/min]	0.060	0.040	0.116

References

1. Melsen–van der Vis, M. E. (1974), Thesis on applications of oral Thyrotropin Releasing Hormone stimulation, p. 101–104 Free University Amsterdam, the Netherlands.
2. de Vries, H. P. & Wiener, J. D. (1974), Journal Endocrin. (in press).
3. Backer, E. T., Postmes, Th. J. & Wiener, J. D. (1967) Clin. Chim. Acta *15*, 77–85.
4. Kreutzer, E. K. J. (1971), Thesis on Determination of Hormonal Iodine in serum, p. 59–61, University of Amsterdam, the Netherlands.
5. Passen, S. & von Saleski, R. (1969), Amer. J. Clin. Pathol. *51*, 166–176.
6. Wiener, J. D. & Backer, E. T. (1968), Clin. Chim. Acta *20*, 155–163.
7. Hathaway, J. A.; Sanella, J. J. & de Witt–Hunter, T. (1970), Amer. J. Clin. Pathol. *53*, 635–640.
8. Arcq, J. C. & Arcq, A. (1973), Clin. Chim. Acta *48*, 287–289.

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