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Comparison of high-performance liquid chromatographic detection methods for thyronine and tyrosine residues in toxicological studies of the thyroid

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ABSTRACT

Four high-performance liquid chromatographic methods for the detection of thyroid hormones (iodinated thyronines) and precursors (iodinated tyrosines) have been developed and evaluated. Two methods consist of direct determination of the parent compounds with detection at ultraviolet wavelength (230 nm) and with electrochemical detection. The two other methods consist of a pre-column derivatization (with fluorenylmethyl chloroformate and dabsyl chloride) prior to high-performance liquid chromatographic analysis. The various methods were evaluated based on their practical use and sensitivity. The method with direct ultraviolet detection turned out to be the most practical method. With this method analyses of thyroid homogenates have been performed from rats from a toxicological experiment.

INTRODUCTION

In the thyroid gland synthesis of the thyroid hormones 3,3′,5′-triidothyronine (T3) and 3,3′,5,5′-tetraiodothyronine (T4) takes place in the thyroidal follicle [1]. The formation of the precursors 3-monoiodotyrosine (MIT) and 3,5-diiodotyrosine (DIT) and the coupling of both precursors in the formation of T3 and T4 is controlled by thyroid peroxidase [2]. Thyroglobulin is the major storage of these hormones and the precursors.

The relative concentrations of these compounds are an important parameter for the study of toxicological effects on the thyroid [3,4]. In the present study, a number of detection methods have been developed for the thyroid hormones, precursors and metabolites using high-performance liquid chromatography (HPLC) [5–7]. Four HPLC methods will be compared: the determination of the parent compounds with UV and electrochemical detection and the determination of the compounds after precolumn derivatization with dabsyl chloride [8] and fluorenylmethyl chloroformate (FMOC-Cl) [9]. Results will be shown for a number of assay parameters such as limit of detection and practical applicability.

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EXPERIMENTAL

Materials

HPLC solvents were of analytical grade (Westburg, Leusden, The Netherlands). Water was taken from a Millipore Milli-Q filtration unit. Dabsyl chloride was obtained from Sigma, Brunschwig (Amsterdam, The Netherlands). FMOC-Cl and 1-adamantylamine were obtained from Aldrich (Brussels, Belgium). The following tyrosine and thyronine standards were used: tyrosine (TYR) obtained from Merck (Darmstadt, Germany), 3-MIT, DIT, thyronine (T0), 3,5-diiodothyronine (T2), T3, 3,3′,5-triiodothyronine (rT3) and T4, all obtained from Sigma.

HPLC with direct UV detection

The HPLC equipment consisted of the following components: an autoinjector (Varian Model 9000), two solvent delivery systems (Perkin Elmer Series 10), two low-pressure three-way valves (Rheodyne 5301), a high-pressure valve (Rheodyne 7010), a UV–VIS detector (Linear Model 200), a data acquisition system (Axxiom, Analytica, Maasdijk, The Netherlands). The column system consisted of a cartridge of Chromspher C_{18} (100 × 3 mm I.D., 5 μm) with a concentrating column (15 × 3.2 mm I.D., 7 μm) (Brownlee Newgard RP-18, Inacom Instruments, Veenendaal, The Netherlands) and was maintained at 35°C with an electric column oven (LKB Model 2155). The following HPLC conditions were used: after injection the concentrating column was eluted for 2 min with mobile phase A (phosphate buffer, 0.05 *M*, pH 2.2, containing 2.5 m*M* heptanesulphonic acid). Then the analytical column was put in line with the concentrating column and eluted for 3.5 min with mobile phase C (a mixture of phosphate buffer, 0.05 *M*, pH 2.5, containing 2.5 m*M* heptanesulphonic acid–methanol, 65:35, v/v) and for 14.5 min with mobile phase D (a mixture of phosphate buffer, 0.05 *M*, pH 2.5, containing 2.5 m*M* heptanesulphonic acid–methanol, 42.5:57.5, v/v). At 11.5 min the concentrating column was switched off and eluted with mobile phase B (methanol containing 2.5 m*M* heptanesulphonic acid). The total time of analysis was 20 min.

HPLC with electrochemical detection

The HPLC equipment consisted of the following components: a manual injector (Rheodyne Model 7125) equipped with a sample loop of 20 μl, a solvent delivery system (Waters Model 590), an electrochemical detector (Antec, Leiden, The Netherlands) operating at 0.8 V and an recorder (Kipp en Zonen BD-41, Delft, The Netherlands). The column (150 × 4.6 mm I.D.) with 2-μm frits and Valco fittings (Chrompack, Middelburg, The Netherlands) was packed with Hypersil ODS (particle size 5 μm, Shandon, Zeist, The Netherlands) using a column-packing instrument (Shandon) according to the manufacturers instructions. The following HPLC conditions were used: the column was eluted isocratically with a mixture of phosphate buffer (0.05 *M*, pH 2.5, containing 2.5 m*M* heptanesulphonic acid and 60 mg/l sodium EDTA)–methanol (40:60, v/v). The flow-rate was 1 ml/min at room temperature.

Derivatization with FMOC-Cl

To a 100-μl sample or standard were added 100 μl of borate buffer (0.5 *M*, pH

7.7) and 100 μl of a solution of FMOC-Cl (2.5 m*M* in dry acetone). After a reaction time of 45 s at room temperature, 200 μl of a solution of 1-adamantylamine (12 m*M* in acetonitrile) was added to stop the derivatization by reaction with excess of FMOC-Cl.

HPLC separation of FMOC derivatives

The HPLC equipment consisted of the following components: an automatic injector (Model WISP, Waters, Etten Leur, The Netherlands), two solvent delivery systems (LBK Model 2150) controlled by a gradient controller (LKB Model 2152), a UV–VIS detector (Linear Model 200), a data acquisition system (Axxiom, Analytica). The column consists of a cartridge of Chromspher C_{18} (100 × 3 mm I.D., 5 μm) with a pre-column (10 × 3 mm I.D., 30 μm) (Chrompack) and was maintained at 35°C with an electric column over (LKB Model 2155). Detection was performed with a UV–VIS detector at 260 nm (Linear Model 200, Analytica) or with fluorescence with excitation at 260 nm and emission at 320 nm (Shimadzu Model RF530, Lamers and Pleuger, 's Hertogenbosch, The Netherlands).

The following HPLC conditions were used: from 0 to 40 min a linear gradient from 100% mobile phase A (a mixture of sodium acetate buffer, 0.05 *M*, pH 4.2–methanol, 60:40, v/v) to 100% mobile phase B (a mixture of sodium acetate buffer, 0.05 *M*, pH 4.2–methanol–acetonitrile, 20:60:20, v/v/v), from 40 to 45 min 100% mobile phase B. The flow-rate was 0.7 ml/min.

Derivatization with dabsyl chloride

To a 20-μl sample or standard were added 40 μl of carbonate buffer (0.05 *M*, pH 8.5) and 40 μl of a solution of dabsyl chloride (4 m*M* in acetonitrile). After a reaction time of 15 min at 70°C, 100 μl of a sodium acetate buffer (25 m*M*, pH 6.5) were added to stop the derivatization.

HPLC separation of dabsyl derivatives

The HPLC equipment used for separation and detection of dabsyl derivatives is similar to that described for the separation of FMOC derivatives. The wavelength of the UV–VIS detector was set to 436 nm.

The following HPLC conditions were used: from 0 to 15 min a linear gradient from 100% mobile phase A (a mixture of sodium acetate buffer, 0.025 *M*, pH 6.5–methanol, 30:70, v/v) to 100% mobile phase B (a mixture of sodium acetate buffer, 0.025 *M*, pH 6.5–methanol, 10:90, v/v), from 15 to 20 min 100% mobile phase B. The flow-rate was 0.7 ml/min.

RESULTS

Four different detection methods for thyrosine and thyronine residues will be compared with respect to HPLC separation, time of analysis, limit of detection and practical applicability.

UV detection of parent compounds

The thyroid hormones and precursors can be detected directly at a wavelength of 230 nm. For the separation of all seven compounds, a reversed-phase HPLC

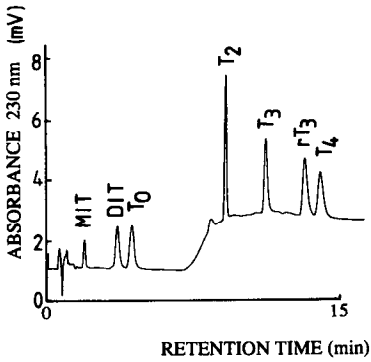

Fig. 1. HPLC profile of a standard mixture of tyrosines and thyronines as detected directly at 230 nm. A cartridge of Chromspher C_{18} (100 × 3 mm I.D., 5 μm) was used and eluted for 3.5 min with a mixture of phosphate buffer, 0.05 *M*, pH 2.5, containing 2.5 m*M* heptanesulphonic acid–methanol (65:35, v/v) and for 14.5 min with a mixture of phosphate buffer, 0.05 *M*, pH 2.5, containing 2.5 m*M* heptanesulphonic acid–methanol (42.5:57.5, v/v).

system has been developed with a stepwise gradient. In addition a pre-concentration column is used with column switching in order to avoid the appearence of matrix components of the analytical column. In Fig. 1 the chromatogram of the separation of all seven components is shown. The limits of detection are in the range 310–900 pg per injection. The individual data are listed in Table I.

Electrochemical detection of parent compounds

In general, phenolic compounds are suitable for electrochemical detection. This sensitive detection method has been applied here on four thyroid hormones, T2, T3, rT3 and T4, as shown in Fig. 2. Limits of detection have been determined at 800 mV as 0.7, 1.0, 0.2 and 0.4 pg per injection, respectively. These detection limits can probably be increased at higher voltages. For instance, at 900 mV the electrochemical responses are increased by factors of 6, 3, 2 and 1.5. Although electrochemical detec-

TABLE I

LIMIT OF DETECTION (SIGNAL-TO-NOISE RATIO = 3) FOR THYRONINE AND TYROSINE RESIDUES WITH SEVERAL DETECTION METHODS

Compound	UV (230 nm)		Dabsyl		FMOC UV		Electrochemical	
	pg	pmol	pg	pmol	pg	pmol	pg	pmol
TYR	360	2.0	80	0.44	–	–	–	–
MIT	360	1.2	120	0.39	650	2.1	–	–
DIT	900	2.1	470	1.1	800	1.9	–	–
T0	730	2.7	100	0.36	500	1.8	–	–
T2	310	0.6	190	0.36	1000	1.9	2000	3.8
T3	590	0.9	230	0.35	2300	3.5	3000	4.6
rT3	840	1.3	330	0.51	1300	2.0	250	0.38
T4	960	1.2	300	0.39	1200	1.6	1000	1.3

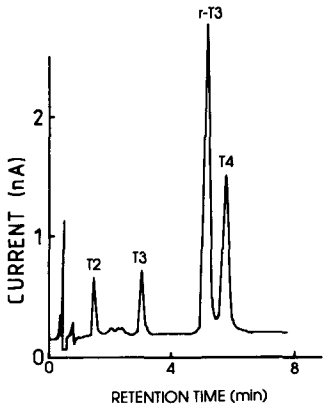

Fig. 2. HPLC profile of a standard mixture of thyroid hormones as determined with electrochemical detection. A cartridge of Chromphere C_{18} (100 × 3 mm I.D., 5 μm) was used and eluted isocratically with a mixture of a phosphate buffer (0.05 *M*, pH 2.5, containing 2.5 m*M* heptanesulphonic acid and 60 mg/l sodium EDTA)–methanol (40:60, v/v).

tion using the new Antec detector was intended as a ready-to-use and practical method of detection, this technique seems to be less suitable for automatic unattended HPLC analysis.

Fluorescence and UV detection of FMOC derivatives

Firstly, the derivatization yield was determined as function of pH. Between pH 6.5 and 9.0 no significant changes in the formation of FMOC derivatives were found. Derivatization with FMOC-Cl results mainly in mono-substituted derivatives. To a lesser extent di-substituted derivatives are formed, which can be observed in the HPLC profile at higher retention times. This may interfere with correct quantitation of the compounds. The FMOC derivatives of thyroid hormones and precursors can be detected by UV at 260 nm, with fluorescence with excitation at 260 nm and emission at 320 nm. In Fig. 3 the two detection techniques have been compared. In our hands UV detection gave the best results with respect to background signals. The fluorescence may be more sensitive but in the chromatogram high peaks also appeared in blank samples without any amino acid. This phenomenon can probably be ascribed to the preparation of FMOC-Cl which was only 95% pure. Limits of detection with UV detection ranged from 0.5 to 2.3 ng per injection. The data for the individual compounds are listed in Table I. The limits of detection with fluorescence could not be determined, as mentioned before.

Detection of dabsyl derivatives

The optimization of the derivatization with dabsyl chloride has been described in detail elsewhere [10]. The dabsyl conjugates of the thyroid hormones are all di-substituted derivatives. Besides the amino group, the phenolic hydroxyl group is also susceptible to conjugation. As a result the dabsyl conjugates elute at a much higher methanol concentration from the C_{18} column than the other naturally occurring amino acids. In Fig. 4 a separation of eight tyrosine and thyronine derivatives is

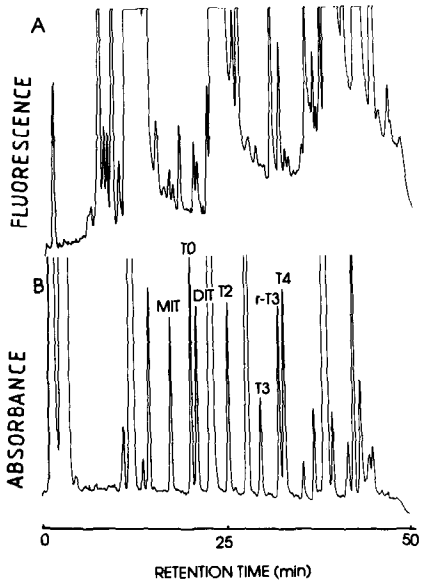

Fig. 3. HPLC profiles of a standard mixture of FMOC derivatives of tyrosines and thyronines as detected with fluorescence (A) and UV (B). A cartridge of Chromspher C_{18} (100 × 3 mm I.D., 5 μm) was used and eluted from 0 to 40 min with a linear gradient from a mixture of sodium acetate buffer, 0.05 *M*, pH 4.2–methanol (60:40, v/v) to a mixture of sodium acetate buffer, 0.05 *M*, pH 4.2–methanol–acetonitrile (20:60:20, v/v/v).

shown with a complete baseline separation. The limits of detection are very good, ranging from 80 to 470 pg injected on the column with a signal-to-noise ratio of 3. The individual detection limits are listed in Table I.

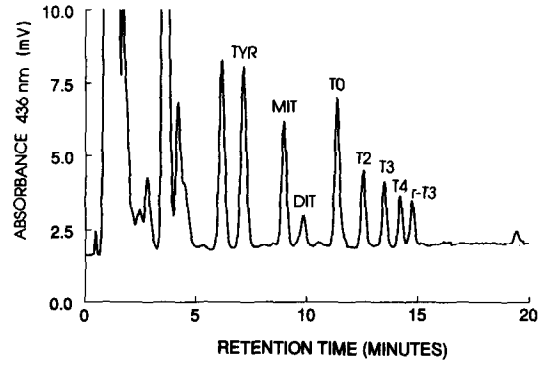

Fig. 4. HPLC profile of a standard mixture of dabsyl derivatives of tyrosines and thyronines as detected at 436 nm. A cartridge of Chromspher C_{18} (100 × 3 mm I.D., 5 μm) was used and eluted from 0 to 15 min a linear gradient from a mixture of sodium acetate buffer, 0.025 *M*, pH 6.5–methanol (30:70, v/v) to a mixture of sodium acetate buffer, 0.025 *M*, pH 6.5–methanol (10:90, v/v).

Samples from toxicological experiments

The HPLC method with UV detection at 230 nm was selected as the most practical method of analysis. With this method a number of toxicological experiments on the thyroid have been performed. Thyroid precursors and hormones have been determined in the thyroidal protein called thyroglobulin, which consists to a large extent of tyrosine residues. In this protein the synthesis of the precursors MIT and DIT takes place by iodination of tyrosine, and the synthesis of the hormones T3, rT3 and T4 by the coupling of the various thyrosines to thyronines by thyroid peroxidase. A method was developed for total proteolytic hydrolysis of thyroglobulin [11] followed by HPLC analysis of the residue to determine the concentrations of the precursors and the thyroid hormones. In Fig. 5 an example of a typical chromatogram is shown from the hydrolysate of the thyroid from a rat which has been treated with sodium bromide. Experimental conditions and detailed data of analysis will be published elsewhere.

DISCUSSION

The detection of thyroid hormones and precursors such as TYR, MIT, DIT, T0, T2, T3, rT3 and T4 was investigated with four different HPLC methods. For all methods a satisfactory baseline separation can be shown. The parent compounds were investigated with two methods: UV detection and electrochemical detection. The UV detection of the parent compounds showed rather low limits of detection (from 310 to 960 pg per injection) caused by the high intrinsic extinction coefficients

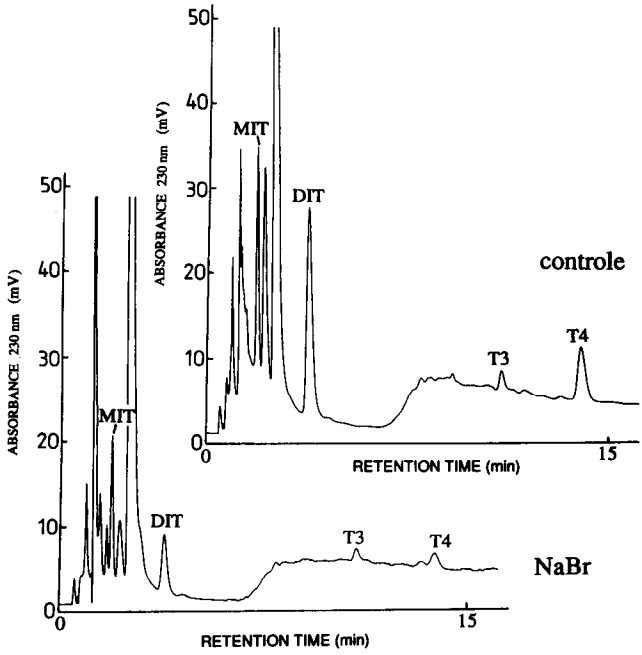

Fig. 5. HPLC profiles of the detection of thyroid tissue from a control rat and a rat treated with sodium bromide. Experimental details have been described in the Experimental section.

of the parent compounds. The detection limits for the individual compounds are listed in Table I. Electrochemical detection was a practical technique without many problems. This could probably be ascribed to the new Antec detector. The limit of detection ranged from 0.25 to 3 ng per injection and can probably be decreased by measurements at higher oxidation potentials. However, the UV detection was judged to be of more practical value.

To increase the sensitivity, two pre-column derivatization methods were investigated: dabsyl and FMOC. These were chosen because, as already described for amino acids, they yield stable derivatives. Both the FMOC and the dabsyl derivatives are stable at least for several hours or even days. In addition, both derivatives can be detected with great sensitivity. The FMOC derivatives can even be detected with fluorescence. In our experience the detection with fluorescence was not satisfactory because too many high peaks originating from the reagent blank appeared in the chromatogram. A similar observation was made in our laboratory with the derivatization of aminoglycosides with FMOC-Cl. The limit of detection of the dabsyl derivatives differed by a factor 2–10 compared with the FMOC derivatives with UV detection. In addition, the detection of the dabsyl derivatives take place at 436 or 450 nm where no disturbance peaks from matrix components are expected. For these reasons the dabsyl derivatization was preferred.

The direct detection of the parent compounds at 230 nm showed somewhat higher limits of detection (by a factor 1.5 to 7) than the dabsyl derivatives. However, for good quantitation the extra steps necessary to perform a derivatization can only decrease the reliability. In addition, a number of parameters involved in the derivatization must be investigated thoroughly, such as extent of derivatization, the influence of matrix components, the sustainability of the method with respect to pH, type of buffer, ionic strength, temperature, time of derivatization, etc. Therefore we have chosen UV detection of the parent compounds as the most practical method, with dabsyl derivatization as second best to achieve a higher sensitivity if necessary.

With UV detection a number of samples from a toxicological experiment with sodium bromide were analyzed and it was concluded that the selected method described here has also proven very suitable in practice.

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