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Note

Automated high-performance liquid chromatographic method for the determination of iodotyrosines and iodothyronines

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Research at the Experimental Pediatric Endocrinological Laboratory involves various aspects of thyroid metabolism. One of the research projects is the study of the iodination of thyroglobulin, catalysed by TPO*. Thyroglobulin is a protein with a molecular weight of 660 000. In a thyroglobulin molecule, there are about 120 tyrosine residues, of which maximally 40 can be iodinated to MIT and DIT[1]. This iodination reaction is catalysed by TPO in the presence of iodide and hydrogen peroxide[2]. In addition to this iodination reaction, TPO catalyses the formation of the thyroid hormone thyroxine from two DIT residues, the so-called coupling reaction. The reactions take place within the thyroglobulin molecule. Thyroxine is released after an enzymatic hydrolysis of the thyroglobulin molecule[3]. MIT, DIT and T4 are not the only reaction products. The formation of other (iodinated) components such as T0, T2, T3 and rT3 is possible. In order to study the kinetics of the different reactions catalysed by TPO, we required a method of analysing the possible reaction products in one chromatographic run. As kinetic studies involve many samples, automation was necessary. Several (HPLC) procedures for the separation of these compounds have been described since 1974[4–8]. These methods were usually developed to assess the purity of pharmaceutical preparations or to study and quantitate thyroid hormones and their metabolites in extracts of biological samples, such as serum and urine. In most instances DIT and T0 are not separated. Most of the quoted procedures involve complicated off-line sample preparation.

We present here a method for the determination of six iodotyrosines and io-

* Abbreviations: Tyr, tyrosine; MIT, 3-monoiodotyrosine; DIT, 3,5-diiodotyrosine; T0, L-thyronine; T0, 3,5-diiodo-L-thyronine; T3, 3,3′,5-triiodo-L-thyronine; rT3, 3,3′,5′-triiodo-L-thyronine; T4, thyroxine; HSA, heptanesulphonic acid; TPO, thyroid peroxidase (E.C. 1.11.1.7).

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dothyronines in reaction mixtures of the model substrate tyrosine and thyroid peroxidase used for kinetic studies, in urine samples from patients and in samples of enzymatically hydrolysed thyroglobulin, isolated from thyroid glands. The method described is a fully automated reversed-phase ion-pairing HPLC procedure and includes sample clean-up and preconcentration on a small C_{18} precolumn.

EXPERIMENTAL

Chemicals

Acetonitrile and methanol were of analytical-reagent grade from J. T. Baker (Deventer, The Netherlands). HPLC-quality water was obtained from doubly distilled water, further purified in a Milli-Q (Millipore, Bedford, MA, U.S.A.) purification system with a Norganic filter. Tyr, MIT, DIT, T3, rT3 and T4 were purchased form Sigma (St. Louis, MO, U.S.A.) and T2 from Serva (Heidelberg, F.R.G.).

Standard solutions of the (iodo)amino acids were prepared in HPLC-quality water with a few drops of concentrated ammonia solution added. Standards were kept in the dark at −20°C.

Buffer solution (Titrisol, pH 4) and orthophosphoric acid (89% pure) were obtained from Merck (Darmstadt, F.R.G.) and HSA disodium salt from Sigma. Pronase P and E were bought from Serva.

Liquid chromatography

The set-up for automated sample clean-up and preconcentration and subsequent LC analysis is shown in Fig. 1. The apparatus consisted of the following components: a Model 9208 HPLC pump, used for loading and flushing the precolumn (Kipp, Delft, The Netherlands); a Perkin-Elmer (Norwalk, CT, U.S.A.) Series 10

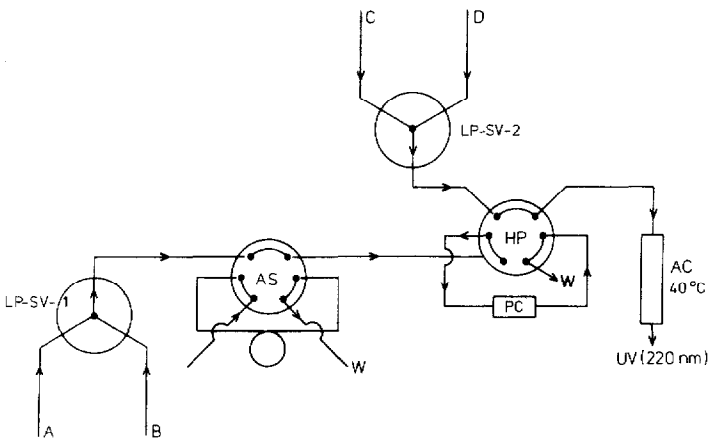

Fig. 1. Schematic representation of the eluent streams used for preconcentration and LC analysis. A = 2.5 m*M* HSA in 0.03 *M* phosphate buffer (pH 2.2); B = 2.5 m*M* HSA in methanol; C = methanol–buffer/HSA (30:70, v/v); D = methanol–acetonitrile–buffer/HSA (34:17:49, v/v/v); LP-SV = low-pressure selector valve; HP = high-pressure six-port switching valve, equipped with a C_{18} precolumn; AS = autosampler high-pressure six-port switching valve, equipped with sample loop; AC = analytical column; W = waste; UV = UV detector.

eluent pump; a Perkin-Elmer LC-235 photodiode array UV–VIS detector; a Gilson ASPI 232-401 autosampler, equipped with four programmable valves, [three Rheodyne 7010 (Cotati, CA, USA) high-pressure injection valves and one Rheodyne 5011 low-pressure solvent selection valve]. A 150-μl sample loop was used for loading the sample on the precolumn; a 15 x 3.2 mm I.D. MPLC New Guard RP-18 precolumn (Brownlee, Santa Clara, CA, U.S.A.) and an analytical (AC) column (150 x 4.6 mm I.D.), laboratory-packed with 5-μm Hypersil ODS (Shandon, Runcorn, U.K.). The column was kept at 40°C in a thermostatted water-bath.

The carrier solvent, A, used for precolumn concentration, was 0.03 *M* sodium phosphate buffer (pH 2.2), containing 2.5m*M* HSA. The clean-up solvent, B, was methanol, containing 2.5 m*M* HSA. The buffer used with the analytical column was a sodium phosphate buffer (pH 2.5) containing 2.5 m*M* HSA; it is referred to as "buffer/HSA" throughout this paper. The following mobile phases were used with the analytical column: eluent C was a mixture of methanol and buffer/HSA (30:70, v/v), containing 0.25% acetone in order to level the absorption of eluent C with solvent D; eluent D was methanol–acetonitrile–buffer/HSA (34:17:49, v/v/v). At the start of the analysis, a sample was injected into the precolumn and flushed with 1 ml of solvent A. After 1 min, the precolumn was switched on-line with the analytical column and desorbed for 6 min with eluent C. Then the eluent for both columns was changed (by switching the valve) to eluent D for 13 min. Twelve minutes after the start of the chromatographic run, the precolumn was switched off-line and cleaned with the clean-up solvent, B, for 3 min, while the analytical run continued. After that, the selector valve 1 was switched back to the initial position, carrier solvent A. On completion of the analytical run (after 18 min), the analytical column was reconditioned for 7 min with 30% of methanol in buffer/HSA (eluent C).

TABLE I

CHARACTERIZATION OF THE UV SPECTRA OF (IODO)TYROSINES AND (IODO)THYRONINES

Compound	*Peak height at selected wavelength**				*Solvent***
	230 nm	*240 nm*	*254 nm*	*280 nm*	
Tyr	0.54	0.03	0.04	0.14	1
MIT	0.36	0.10	0.03	0.11	1
DIT	0.60	0.31	0.06	0.08	1
T0	1.0	0.54	0.13	0.18	1
T2	0.84	0.40	0.14	0.11	2
T3	0.84	0.38	0.12	0.10	2
rT3	0.76	0.46	0.12	0.11	2
T4	0.80	0.43	0.12	0.10	2

* Peak heights are expressed in arbitrary units. Spectra were recorded with a photodiode array detector after injection of 10 μl of a standard solution of 1 mg/ml of each component into a C_{18} column.

** The spectra were measured in (1) methanol–0.05 *M* ammonium acetate buffer (pH 5.0) (30:70; v/v) and (2) methanol–0.05 *M* ammonium acetate buffer (pH 5.0) (60:40, v/v).

RESULTS AND DISCUSSION

Optimization of the analytical LC separation

As the methods described in the literature[4–8] did not meet our requirements, we developed another HPLC procedure, by using a systematic approach, based on the eluent optimization strategy described by Schoenmakers *et al.*[9] and Drouen *et al.*[10]. Because of the chemical nature of the compounds, it can be expected that the pH of the eluent will influence their retention. During optimization, the pH, the composition of the mobile phase and the nature of the stationary phase were chosen as variables. At this stage, we used a photodiode array detector to trace the compounds in the chromatogram. All eight compounds have similar UV spectra, with a pronounced broad band at 200–230 nm and no significant additional absorption maxima. The measured relative absorption values, at selected wavelengths, are given in Table I.

Using a C_{18} column, we found that separation in a single isocratic chromatogram was not possible. There were two groups of peaks present in the chromatogram. The first group, Tyr, MIT, DIT and T0, could be eluted with a low percentage of methanol in the eluent, whereas for the second group, T2, T3, rT3 and T4 a stronger eluent was needed. The peaks of this last group were tailing in all isocratic chromatograms and also with linear gradient elution. We then varied the stationary phase. On a C_8 column the two groups of peaks are positioned closer together, as was expected, but the peak shapes were still not very good. On a C_1 column (Hypersil SAS), the peaks could be separated isocratically, using a mixture of 0.01 *M* ammonium acetate buffer (pH 5.0) and methanol (60:40, v/v). However, owing to bad batch-to-batch reproducibility of the C_1 stationary phase used, this method, unfortunately, had to be rejected. Remarkably, with acetonitrile as modifier, poor peak shapes were observed on all stationary phases tested. Next, ion-pair chromatography was investigated, with HSA as the ion-pair reagent. Because HSA is negatively charged, the compounds must be present as positively charged ions in the solution. This is achieved at low pH. A phosphate buffer of pH 2.5 was used as the aqueous phase and methanol was chosen as modifier. Again, we started with a Hypersil ODS column and again we found two groups of peaks in isocratic runs. However, all eight compounds could be separated in one ion-pair chromatogram with a one step-gradient solvent programme. The retention times were Tyr, 3.08 $\pm$ 0.06; MIT, 5.42 $\pm$ 0.16; DIT, 9.36 $\pm$ 0.008; T0, 9.61 $\pm$ 0.07; T2, 12.02 $\pm$ 0.10; T3, 16.38 $\pm$ 0.13; rT3, 19.70 $\pm$ 0.17; T4, 22.60 $\pm$ 0.21 min (n = 10). We preferred to use stepwise gradient elution over other forms of gradients, as this requires the least costly equipment (one isocratic pump and a low-pressure solvent selector valve) and also because we have the experience, from previous work, that a better reproducibility of eluent conditions is obtained. A one-step gradient from 30 to 60% of methanol in the aqueous buffer mixture, changing after elution of the first group of peaks, gave the required result.

After this result we did not further investigate ion-pair chromatography on other stationary phases, such as C_8, which also is a good candidate, because of the implications that this would have for the precolumn conditions. First, when using a C_8 column, the modifier content necessary for the separation would probably not be higher than 15–20%. This would limit the choice of solvent conditions for the clean-up step within the range 0–15 (20)% of modifier and would thus not be favourable in

the clean-up of actual samples. Second, in order to avoid unnecessary band broadening, retention on the precolumn should not be higher than that on the analytical column. A C_8 precolumn would have a lower volumetric loading capacity than a C_{18} precolumn. The use of a C_8 precolumn would mean a lower clean-up efficiency than is attainable with C_{18} and also a lower concentration factor. Another practical argument in favour of the use of C_{18} material was that we have had good experience with the stability of this type of C_{18} phase in previous ion-pair chromatographic applications and none with C_8 phases.

Optimization of on-line sample preparation

From the descriptions in the literature, it was expected that the real samples would contain a large number of interfering compounds, present in high concentrations. At the Laboratory of Organic/Analytical Chemistry of the RIVM, the experience and equipment for on-line preconcentration and clean-up in HPLC are available. In analogy with our previously published methods for on-line sample preparation[11,12], a precolumn sample pretreatment method was developed. All compounds involved could be sorbed from a buffered aqueous solution on a C_{18} precolumn, equilibrated with 0.03 *M* phosphate buffer (pH 2.2)–2.5 m*M* HSA ("buffer/HSA"). On this column, the first-eluted compound, Tyr, had a retention volume of 2ml, when eluted with this buffer–HSA mixture. We therefore concluded that the boundary condition for sampling without loss of analytes on the precolumn was *ca.* 1 ml of buffer–HSA (a 1-ml margin was arbitrarily adopted). The compounds sorbed could be quantitatively desorbed from the precolumn with 30% methanol in buffer–HSA.

The actual solvent program for clean-up of the samples with the use of a precolumn was derived empirically, within these two boundary solvent conditions. The

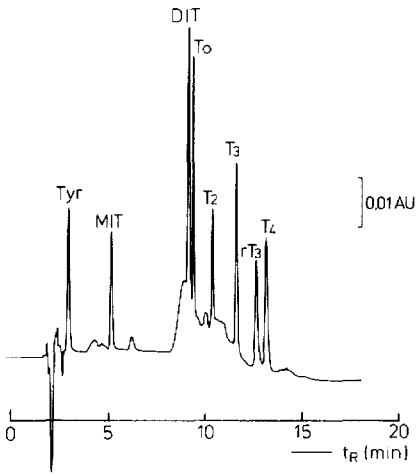

Fig. 2. LC separation of 75 μl of a standard solution containing 1 μg/ml of each of the iodoamino acids in water, carried out by the precolumn switching and ion-pair chromatography procedure described under Experimental. Flow-rate 1 ml/min. Clean-up was accomplished by flushing the loaded precolumn with 1ml of buffer/HSA. Detection, UV at 220 nm, 10 mV, 0.1 a.u.f.s.

result from the experimental optimization was the solvent program we described under Experimental.

With the method described, a test sample of thyroglobulin, enzymatically hydrolysed with pronase P (a protease mixture), was run. The first-eluted analyte, Tyr, could not be seen because of interference from unknown compounds, eluted at t_0. Fortunately, for our study the determination of Tyr is not essential. Further, the chromatogram showed an interfering peak (a compound with the same retention time as T3, but with a different UV spectrum). In order to improve the selectivity of the system towards this peak pair, without having to change the procedure essentially, the use of an iso-eluotropic eluent was indicated, in the step in which T3 is eluted[9,13]. Acetonitrile and THF are the most commonly used alternatives for methanol[9,13,14]. As elution with acetonitrile was found to give poor peak shapes (see above), we only partially replaced methanol with acetonitrile, thus creating a ternary mobile phase, as the second part of the analytical step-gradient procedure. The optimal composition of the ternary eluent was found by trial and error. Satisfactory results were obtained with methanol–acetonitrile–0.05 *M* buffer/HSA (34:17:49, v/v/v). With this eluent the resolution (R_s) between T3 and the unknown peak was 1.1.

The chromatographic behavior and potential interference of other UV-absorbing aromatic amino acids (proline, phenylalanine, histidine and tryptophan) was also investigated. None of them appeared in the chromatogram, because they were not trapped by the precolumn. Standard mixtures of the iodoamino acids can be analysed in a maximum volume of 1000 μl (see above). Samples containing thyroglobulin hydrolysate were in one instance limited to a maximum volume of 100μl, because of a matrix component being eluted close to T3.

A typical chromatogram of a standard mixture is shown in Fig. 2, where the baseline rise indicates the solvent switch from eluent C to D (the step gradient started at $t = 5$ min; the dead volume of the system was 3 ml).

The chromatographic behaviour of the analytes was significantly influenced by the thyroglobulin hydrolysate. Retention values were increased by 0.1 min for MIT, DIT and T0, by 0.5 min for T2, by 1.5 min for T3, by 2 min for rT3 and by 2.5 min for T4 owing to the presence of the matrix. This behaviour was repeatable for Tyr, MIT, DIT and T0, with relative standard deviations of 2–2.5%. The relative standard deviations for the later eluting compounds, T2, T3, rT3 and T4 (peaks 5–8 in Fig. 2), however, are larger (up to 4.25%). Therefore, when analysing samples, measurement of a reference sample (with standards added) after every fifth run, is strongly recommended. No matrix effects were observed in the analysis of tyrosine incubation mixtures.

Quantitative analysis

Plots for 50-μl standard injections of each of the iodoamino acids into the automated clean-up–chromatography system were measured in the ranges 10–180 ng for Tyr, 10–600 ng for DIT and MIT, 5–300 ng for T2 and 10–1000 ng for T3, rT3 and T4 (quantitation of T0 was, unfortunately, not possible owing to the lack of a standard solution of known concentration). Linearity was observed when either peak height or peak area was plotted *vs.* concentration ($R \geqslant 0.98$). The repeatability of the retention values for each compound in the chromatographic precolumn switching–analysis system were determined from ten injections of *ca.* 50 ng of each of the

analytes. The relative standard deviations were less than 1% (0.73–0.93%) for DIT, MIT, T0, T2, T3, rT3 and T4 (see above). The retention times of the two compounds eluted first, Tyr and MIT, showed a much higher variability, 1.95 and 2.95% (R.S.D.), respectively. This can be explained by the susceptibility of the least retained compounds to minor fluctuations in the eluent conditions and the fact that the procedure involves several switches in solvent composition during the precolumn flushing steps.

The sensitivity of the spectrophotometric detection at 220 nm was determined. As little as 10 ng/ml of each of the compounds could be detected in a standard mixture after the injection of 50-μl aliquots.

Calibration graphs for 25–250 ng of each of the analytes in thyroglobulin hydrolysate samples, measured after standard addition, were also linear for all components ($R \geqslant 0.98$) with the exception of Tyr (as mentioned above, Tyr could not be detected at these levels in this matrix, as it is co-eluted with a large amount of a matrix component). The sensitivity of the method for each of the components MIT, DIT, T2, T3, rT3, and T4 in thyroglobulin hydrolysates was 20 ng/ml when 50 μl of sample were injected.

Applications

The method developed was applied to the analysis of dog thyroglobulin, treated with pronase E or with pronases obtained from other manufacturers. A typical chromatogram is shown in Fig. 3. Remarkably, these samples were all much cleaner than was the initial test sample, treated with pronase P. Therefore, the ternary eluent after the gradient step, could be changed back to the binary methanol–buffer/HSA mixture. In the chromatogram shown in Fig. 3, three peaks are present with retention

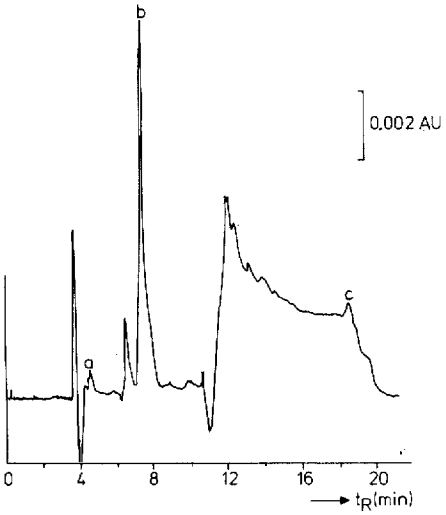

Fig. 3. LC separation of 250 μl of hydrolysed thyroglobulin (50 μg), carried out by the column switching procedure described. Details as under Experimental, except solvent D was replaced by the binary mixture methanol–buffer/HSA (60:40, v/v) and no acetone was added to eluent C. Detection, UV 220 nm, 10 mV, 0.02 a.u.f.s. Flow-rate 1 ml/min. The retention times of peaks a, b and c correspond to those of Tyr, MIT and T4, respectively.

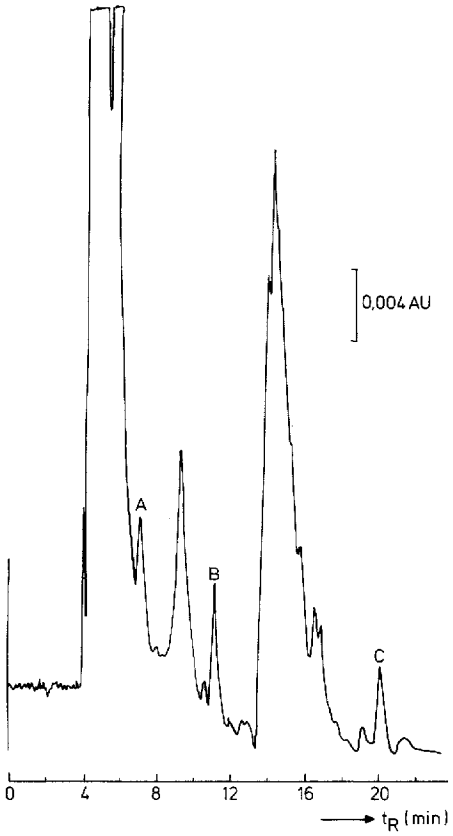

Fig. 4. LC separation of 250 μl of untreated human urine. Conditions as in Fig. 3. UV detector setting: 0.04 a.u.f.s. Flow-rate, 1 ml/min. The retention times of peaks A, B and C are identical with those of MIT, DIT and rT3, respectively.

times identical with those of Tyr, MIT and T4. Experiments are currently being undertaking to confirm the presence of these three compounds.

Another application was the analysis of a urine sample from a patient with a probable de-iodase defect. The chromatogram is shown in Fig. 4. In this chromatogram MIT, DIT and rT3 are probably present.

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