Journal of Chromatography, 553 (1991) 123–133
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2129

Advantages and limitations of pre-column derivatization of amino acids with dabsyl chloride

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ABSTRACT

This paper describes a procedure for the derivatization of amino acids using dabsyl chloride and the high-performance liquid chromatographic determination of the resulting dabsyl-amino acids. The dabsyl derivatives have a number of advantages over other derivatives, including a simple derivatization procedure, very good stability, good reproducibility and a good limit of detection for the method, complete high-performance liquid chromatographic separation of all the amino acids and specific detection at a wavelength in the visible region. The derivatization parameters were optimized to obtain a fast and practical detection method. The method presented here has been applied to a large number of different projects and an example is given for each application.

INTRODUCTION

Several pre-column derivatization methods have been reported for the determination of amino acids using high-performance liquid chromatography (HPLC). These methods use derivatization with reagents such as phenylisothiocyanate [1,2], dansyl chloride [3,4], *o*-phthalaldehyde [5,6], dabsyl chloride [7–11] and 9-fluorenylmethylchloroformate [12]. After extensive testing of several pre-column derivatization methods, it was found that the derivatizations with dabsyl chloride [13] and with 9-fluorenylmethylchloroformate [14] gave the best results, especially with respect to the stability and consequently the reproducibility of the derivatives. The amino acid derivatives of phenylthiohydantoin [15] and especially of *o*-phthalaldehyde were found to be unstable.

This paper describes a procedure for the derivatization of amino acids using dabsyl chloride and the HPLC determination of the resulting dabsyl-amino acids. A number of applications in which amino acids are involved are given.

EXPERIMENTAL

Chemicals

The solvents used were obtained from Merck (Darmstadt, Germany). Dabsyl chloride and the amino acid standards were obtained from Pierce Europe (Oud

0021-9673/91/$03.50

Beijerland, The Netherlands). The amino acids were dissolved in 0.05 mol/l hydrochloric acid and stored at −20°C before use.

HPLC equipment

The HPLC equipment consisted of the following components: an automatic injector (Model 231, Gilson, Meyvis, Bergen op Zoom, The Netherlands); two solvent delivery systems (Model 2150, LKB, Woerden, The Netherlands) controlled by a gradient controller (Model 2152, LKB); a UV–visible detector (Model 200, Linear, Analytical, Maasdijk, The Netherlands); and a computing integration system (Model CI-10B, LDC/Milton Roy, Interscience, Breda, The Netherlands).

Derivatization

The derivatization reagent consisted of a solution of 4 nmol/l 4-dimethylaminoazobenzene-4′-sulfonyl chloride (DABS-Cl) in acetonitrile. This solution was prepared daily. Acetonitrile (40 μl) and dabsyl chloride reagent (40 μl) were added to 20 μl of the standard solution. For each sample, 100 μl of sample were added to 100 μl of dabsyl chloride reagent. The mixtures were heated for 10 min at 70°C in an oven. A 900-μl volume of a solution of sodium hydrogenphosphate (0.1 mol/l, pH 7.0)–acetonitrile–methanol (70:20:10, v/v/v) was added to the reaction mixture. A 20-μl volume of this mixture was injected on to the HPLC column.

Sample preparation and HPLC conditions

Determination of standard amino acids. A standard solution of amino acids (39 mmol/l) was diluted five-fold in 0.05 mol/l hydrochloric acid. To 20 μl of the diluted standard solution, 980 μl of sodium hydrogen carbonate (0.05 ml/l) were added. After derivatization, 20 μl of the resulting solution were injected on to the HPLC system. The HPLC column consisted of two cartridges (100 × 3.0 mm) in a Chromsep holder with Hypersil ODS or another reversed-phase material such as Chromspher C_{18}, Spherisorb ODS-2, Nucleosil C_{18} or Zorbax ODS (Chrompack, Middelburg, The Netherlands). For all other applications an HPLC column with an I.D. of 4.6 mm was used. The column was thermostated at 30°C. Mobile phase A consisted of 25 mmol/l sodium acetate (pH 6.5)–acetonitrile–methanol (70:20:10, v/v/v). Mobile phase B consisted of 25 mmol/l sodium acetate (pH 6.5)–acetonitrile–methanol (10:45:45, v/v/v). The elution was performed as follows: from 0 to 20 min, a linear gradient from 0 to 50% B; from 20 to 27 min, a linear gradient from 50 to 100% B; from 27 to 32 min, 100% B. The flow-rate was 1 ml/min. The UV–visible detector was adjusted to 456 nm.

Determination of glycine in preparations of anti-thymocyte globulin. A 25-μl volume of a solution with valine (0.3 mol/l) as an internal standard, 55 μl of 0.1 mol/l hydrochloric acid and 1900 μl of sodium hydrogencarbonate buffer (0.05 mol/l) was added to 20 μl of the preparation of anti-thymocyte globulin. A 50-μl volume of this solution was added to 150 μl of acetonitrile to precipitate the proteins. A 100-μl volume of the supernatant was used in the derivatization procedure. After derivatization, 20 μl were injected on to the HPLC system. The HPLC column (150 × 4.6 mm) was packed with Hypersil ODS (5 μm). The mobile phase consisted of 25 mmol/l sodium acetate (pH 6.5) and acetonitrile (72:28, v/v). The elution was performed isocratically from 0 to 15 min. The column was then cleaned of the tightly bound components with 100% methanol for 2 min. The flow-rate was 1 ml/min at room temperature. The UV–visible detector was adjusted to 436 nm.

Determination of amino acids in cell culture media. A 200-μl volume of acetonitrile was added to 100 μl of a sample of culture medium to precpitate the proteins. A 40-μl volume of the supernatant was used in the derivatization procedure, to which 40 μl of dabsyl reagent were added. After heating, 240 μl of a mixture of phosphate buffer (0.05 mol/l, pH 7.0) and methanol (1:1) were added. From this mixture, 20 μl were injected on to the HPLC system. The HPLC column (150 × 4.6 mm) was packed with Hypersil ODS (5 μm) and was thermostated at room temperature. Mobile phase A consisted of 100 mmol/l sodium acetate (pH 6.5)–acetonitrile–methanol (56:16:28, v/v/v). Mobile phase B consisted of 100% methanol. The elution was performed as follows: from 0 to 5 min, 0% B; from 5 to 30 min, a linear gradient from 0 to 40% B; from 30 to 35 min, 40% B; from 35 to 40 min, a linear gradient from 40 to 100% B; and from 40 to 43 min, 100% B. The flow-rate was 1 ml/min. The UV–visible detector was adjusted to 436 nm.

Determination of hydrophobic amino acids in human serum. A 400-μl volume of acetonitrile was added to 200 μl of a serum sample to precipitate the proteins. A 40-μl volume of the supernatant was used in the derivatization procedure, to which 40 μl of dabsyl reagent were added. After heating, 70 μl of a mixture of phosphate buffer (0.05 mol/l, pH 7.0) and methanol (1:1) were added. From this mixture, 20 μl was injected on to the HPLC system. The HPLC column (150 × 4.6 mm) was packed with Hypersil ODS (5 μm). Mobile phase A consisted of 100 mmol/l sodium acetate (pH 6.5)–acetonitrile–methanol (56:16:28, v/v/v). Mobile phase B consisted of 25 mmol/l sodium acetate (pH 6.5)–acetonitrile (71:29, v/v). The elution was performed as follows: from 0 to 16 min, 0% B; from 16 to 17 min, a linear gradient from 0 to 100% B; and from 17 to 35 min, 100% B. The flow-rate was 1 ml/min at room temperature. The UV–visible detector was adjusted to 436 nm.

Determination of tyrosine and thyronine derivatives. A 200-μl volume of acetonitrile was added to 100 μl of sample of thyroid tissue to precipitate the proteins. A 100-μl volume of the supernatant was used in the derivatization procedure, to which 100 μl of dabsyl reagent were added. After heating, 400 μl of a mixture of phosphate buffer (0.05 mol/l, pH 7.0) and methanol (1:1) were added. From this mixture, 20 μl were injected on to the HPLC system. The HPLC column (150 × 4.6 mm) was packed with Hypersil ODS (5 μm). Mobile phase A consisted of 25 mol/l sodium acetate (pH 6.5)–methanol (44:56, v/v). Mobile phase B consisted of 100% methanol. The elution was performed as follows: from 0 to 15 min, a linear gradient from 20 to 65% B; from 15 to 18 min, 65% B; from 18 to 19 min, a linear gradient from 65 to 100% B; and from 19 to 21 min, 100% B. The flow-rate was 1 ml/min at room temperature. The UV–visible detector was adjusted to 436 nm.

RESULTS

Derivatization

The pH dependence of the derivatization procedure was tested for five amino acids: Glu, Ala, Val, Leu and Lys. Between pH 7.5 and 9.0 no significant differences were observed in either the relative or absolute peak heights. The time and temperature dependence of the derivatization was investigated in the same experiment. In the initial experiments the derivatization was stopped by a ten-fold dilution of the mobile phase of the HPLC procedure. At derivatization times longer than 15 min, a decrease in the

peak heights was observed, even at room temperature. Therefore, in all further experiments the derivatization was stopped by the addition of ammonia to remove the excess of dabsyl chloride. In all experiments the HPLC peak heights were compared with those obtained at 70°C for 15 min. The derivatization at 20°C was complete after only 30 min for the following amino acids: Gly, Ile, Val, Leu, Lys, Met, Phe, Pro and Trp. For five amino acids, Asn, Cys, Ser, His and Gln, the peak heights were even higher than those obtained by derivatization at 70°C (data not shown). Almost the same derivatization behaviour was obtained at 37°C (see Table I). At 50°C the derivatization of most of the amino acids was complete after 30 min. At 70°C, all twenty amino acids showed a derivatization behaviour which was a function of time. The optimum time of derivatization at 70°C was 15–30 min.

As the amino acid standards were dissolved in dilute hydrochloric acid, the influence of the concentration of chloride ions was investigated. An increase from 50 to 150 mmol/l chloride ions has an effect on the derivatization behaviour of the amino acids (Table II).

Most of the peak heights decreased in intensity to a maximum of 15% of the original value as the chloride ion concentration increased. A few amino acids showed an increase in peak height to a maximum value of 30% (*e.g.*, Ile).

TABLE I

EFFECTS OF TEMPERATURE AND TIME OF DERIVATIZATION OF AMINO ACIDS WITH DABSYL CHLORIDE

The peak heights of the dabsyl derivatives are shown after various derivatization times (5, 10, 15, 30 and 60 min) at 37, 50 and 70°C. The data are presented as a percentage of the standard condition (15 min at 70°C).

Amino acid	Derivatization time at 37°C (min)					Derivatization time at 50°C (min)					Derivatization time at 70°C (min)				
	5	10	15	30	60	5	10	15	30	60	5	10	15	30	60
Asp	28	46	59	84	97	48	72	83	93	101	73	98	100	104	108
Asn	46	72	88	112	116	61	85	94	106	114	83	96	100	102	108
Gly	100	100	103	103	99	99	99	100	98	102	115	103	100	104	102
Ile	98	104	99	88	78	104	101	98	95	98	115	101	100	97	97
Cys	24	59	85	129	149	70	99	120	136	139	26	92	100	105	111
Glu	41	67	79	102	113	62	88	95	105	102	78	99	100	105	102
Ala	69	89	99	105	102	80	90	96	97	110	89	98	100	102	99
Val	89	101	100	98	90	93	97	98	96	109	95	99	100	98	99
Leu	83	100	101	102	93	90	95	99	97	109	98	105	100	105	103
Lys	64	95	102	109	93	86	94	96	96	110	94	102	100	105	107
Ser	66	93	108	116	139	75	95	102	108	106	83	103	100	106	107
Met	103	123	130	126	131	97	106	104	101	101	97	102	100	101	100
Phe	91	104	107	104	111	91	97	100	99	100	93	101	100	105	100
His	44	79	101	47	13	63	96	109	116	118	73	102	100	108	101
Gln	57	86	94	111	109	71	92	92	104	103	81	92	100	100	96
Pro	103	101	98	99	96	98	97	93	99	97	98	98	100	103	96
Tyr	15	30	41	53	59	45	76	74	91	91	66	96	100	104	99
Thr	54	81	92	102	103	67	84	96	101	103	46	99	100	104	108
Arg	53	81	91	104	109	68	89	97	103	105	42	98	100	102	103
Trp	112	114	107	103	99	111	107	106	107	108	80	108	100	108	106

TABLE II

EFFECT OF THE CONCENTRATION OF SODIUM CHLORIDE ON THE DERIVATIZATION OF AMINO ACIDS WITH DABSYL CHLORIDE

The data are shown as a percentage of the standard condition (15 min at 70°C with a concentration of sodium chloride of 50 mmol/l).

Amino acid	Concentration of NaCl (mmol/l)			
	50	75	100	150
Asp	100	93	97	91
Asn	100	92	90	87
Gly	100	98	95	90
Ile	100	104	120	131
Cys	100	107	112	108
Glu	100	103	102	99
Ala	100	107	103	101
Val	100	106	100	97
Leu	100	115	115	115
Lys	100	98	92	86
Ser	100	87	92	85
Met	100	93	96	88
Phe	100	115	108	109
His	100	94	92	89
Gln	100	102	96	96
Pro	100	101	91	90
Tyr	100	99	87	84
Thr	100	102	100	97
Arg	100	98	93	85
Trp	100	114	109	115

Characterization of dabsyl derivatives

The dabsyl derivatives of the various amino acids show absorption maxima ranging from 448 to 468 nm. The individual data are summarized in Table III, together with the relative intensities of the dabsyl derivatives. Three amino acid derivatives show a substantially higher signal than the remainder, which is a result of, the occurrence of a double-substituted dabsyl moiety on the side-chain amino group (Lys), the phenolic hydroxyl group (Tyr), and the NH of the imidazole moiety (His). Low intensities are observed for Gln and Cys.

The stability of the dabsyl amino acid derivatives is very good. The HPLC signal of five derivatized amino acids (Tyr, Val, Ile, Trp and Phe) was followed for 7 days. The average signal height was 97.2% after 1 week. The limit of detection of the dabsylated amino acids is also very good. An average value of 100 pg (about 1 pmol) was obtained with Ser, Ala, Pro and Cys with a signal-to-noise ratio of 3 using the Linear 200 variable-wavelength detector with a wolfram lamp at 456 nm.

High-performance liquid chromatographic separations

To obtain the best separation of all twenty naturally occurring amino acids, combinations of methanol, tetrahydrofuran and acetonitrile were tested within a 25 mmol/l sodium acetate buffer as the aqueous phase. The pH of the buffer was varied

TABLE III

MAXIMUM WAVELENGTHS OF THE UV–VISIBLE SPECTRUM AND RELATIVE INTENSITIES OF PEAK AREAS (RELATIVE TO Ala = 100%) OF TWENTY NATURALLY OCCURRING AMINO ACIDS

Amino acid	Maximum wavelength (nm)	Intensity (%)
DABS-OH	456	–
Trp	462	83.2
Thr	464	88.8
Ser	464	96.4
Pro	464	108.0
Phe	458	92.8
Met	464	80.5
Lys	448	203.8
Asn	464	56.4
Arg	464	83.0
Ala	464	100
Tyr	456	273.8
Leu	462	94.2
Ile	464	90.5
Glu	468	82.7
Cys	450	32.5
Gly	464	100.0
His	458	134.2
Val	462	87.7
Asp	466	85.0
Gln	468	52.3

from 5.5 to 7.5; the best result was obtained with the acetonitrile–methanol buffer at pH 6.5. Attempts to use chemometric models for the calculation of the best separation failed because of the unusual parabolic behaviour of the amino acids which occurred as the percentage of methanol and acetonitrile was varied. This is shown in Fig. 1, where mobile phase B was acetonitrile and A was methanol. The effect of small amounts of

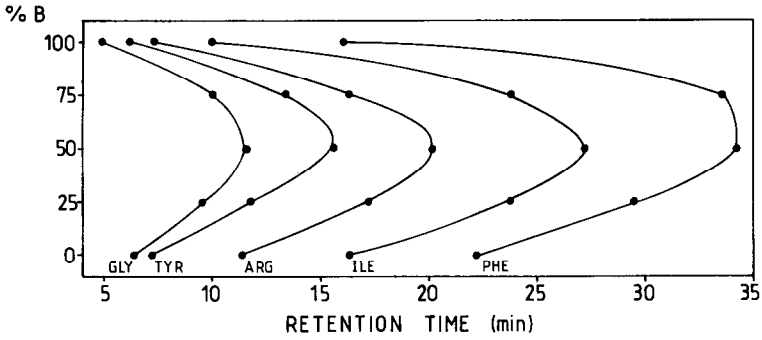

Fig. 1. Retention time behaviour of dabsylated amino acids as a function of solvent composition. Solvent A is methanol and solvent B is acetonitrile (A + B = 100%).

other organic modifiers such as isopropanol, dimethylformamide and triethylamine was also tested. On the addition of 1% isopropanol, only small changes were observed, especially in the region Pro–Val–Arg. The addition of 1% dimethylformamide or triethylamine resulted in poorer separations; an increase in the buffer strength from 25, to 100 mmol/l sodium acetate did not improve the separation. All the amino acids moved to longer retention times, except Arg.

The optimum conditions were: a biphasic linear gradient from 25 mmol/l sodium acetate (pH 6.5)–acetonitrile–methanol (70:20:10, v/v/v) to 25 mol/l sodium acetate pH 6.5)–acetonitrile–methanol (10:45:45, v/v/v), as described under Experimental. Under these conditions five different reversed-phase packing materials were tested in commercially available cartridges of 20 cm length; these were Hypersil ODS, Chromspher C_{18}, Spherisorb ODS-2, Nucleosil C_{18} and Zorbax ODS. The retention times are summarized in Table IV. In Fig. 2 it is shown that under the experimental conditions described, the best separation is achieved with Zorbax ODS. As the di-substituted derivatives of His and Tyr are low in intensity, the peaks marked with an asterix in Fig. 2 may originate from the mono-substituted analogues.

TABLE IV

RETENTION TIME BEHAVIOUR OF ALL TWENTY NATURALLY OCCURRING AMINO ACIDS ON CARTRIDGES PACKED WITH DIFFERENT REVERSED-PHASE MATERIALS

Experimental conditions are as described in the text.

Amino acid	Retention time (min)				
	Nucleosil C_{18}	Zorbax ODS	Chromospher C_{18}	Spherisorb ODS-2	Hypersil ODS
Trp	17.39	17.47	14.43	14.87	15.93
Thr	11.17	11.11	8.57	8.73	9.77
Ser	10.49	10.35	7.97	8.07	9.15
Pro	14.13	13.63	11.29	11.57	12.37
Phe	18.29	18.47	15.49	15.77	16.69
Met	14.79	15.57	12.59	12.27	13.79
Lys	26.85	26.77	25.29	25.61	25.83
Asn	9.21	8.93	6.81	6.87	7.89
Arg	13.47	12.19	11.27	12.19	11.65
Ala	12.17	11.95	9.53	9.55	10.49
Tyr	28.29	28.27	27.13	27.25	27.35
Leu	16.91	17.01	14.35	14.35	15.21
Ile	16.37	16.49	13.77	13.75	14.61
Glu	4.81	5.19	3.15	2.63	4.07
Cys	21.69	22.05	19.23	18.33	20.21
Gly	11.87	11.49	9.29	9.21	10.25
His	27.53	27.53	26.23	26.33	26.67
Val	14.29	14.39	11.67	11.69	12.65
Asp	4.27	4.81	2.93	2.47	3.81
Gln	10.03	9.85	7.69	7.57	8.97

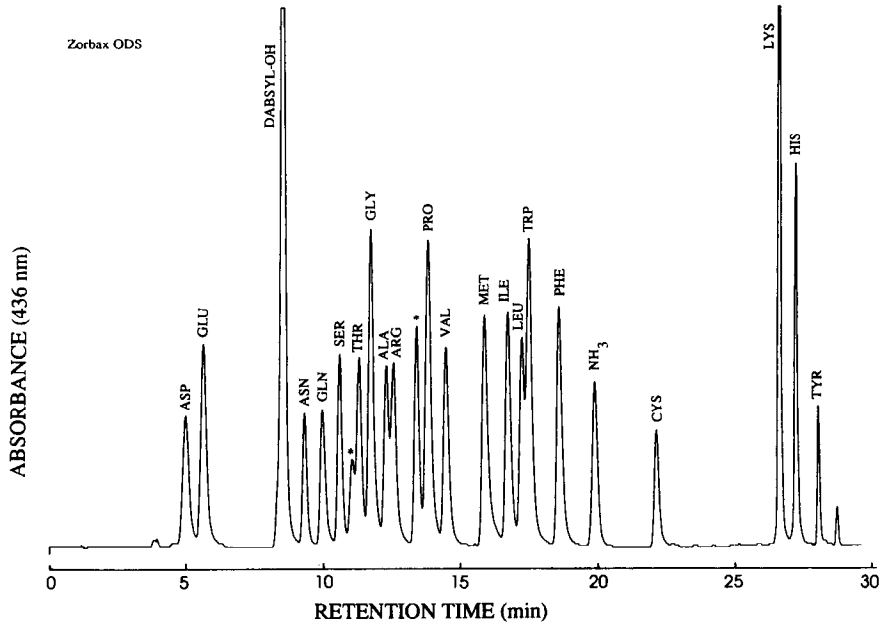

Fig. 2. Chromatogram of the separation of the dabsyl derivatives of all twenty naturally occurring amino acids on a cartridge of Zorbax ODS. The elution conditions are described in the text. The peaks indicated with an asterix have not been identified.

Applications

In our institute, the quantitative determination of amino acids by dabsylation followed by HPLC determination has been used in a number of applications. A brief description and an example are given for each application.

The characterization of proteins and peptides obtained by chromatographic or electrophoretic isolation procedures can be carried out by sequencing or total amino acids analysis. In Fig. 3 the HPLC profile is shown for dabsyl amino acids after the acid hydrolysis of bovine serum albumin. It should be noted that Gln and Asn are respectively converted to the corresponding amino acids Glu and Asp. Amino acid analysis after hydrolysis is used in our institute to support mass spectrometric sequencing measurements and to control the composition of synthetic peptides.

Another application is the off-line monitoring of the consumption of essential amino acids in cell culture media. In the culture of animal cells for the production of monoclonal antibodies, the concentration of ten amino acids was followed. Fig. 4 shows HPLC profile of a typical amino acid mixture taken from the culture medium, which has been optimized for this application.

The dabsylation of amino acids is used also for the control of buffer components in preparations of anti-thymocyte globuline, which is produced by our institute. In these preparations glycine must be identified and determined; this is carried out using valine as an internal standard.

Another study in which the dabsylation of amino acids was used to determine amino acids was directed to the problem of premenstrual complaints. One of the most

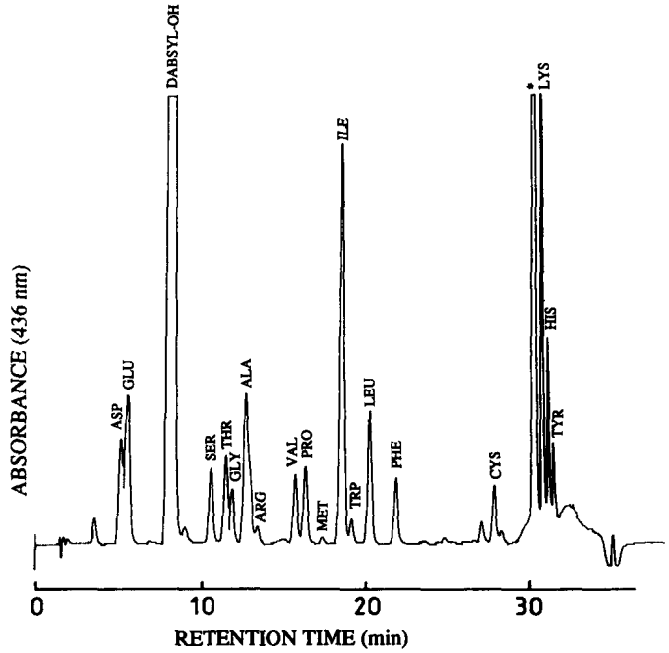

Fig. 3. Chromatogram of the separation of the dabsyl derivatives of amino acids from an acid hydrolysate of bovine serum albumin. The elution conditions are described in the text.

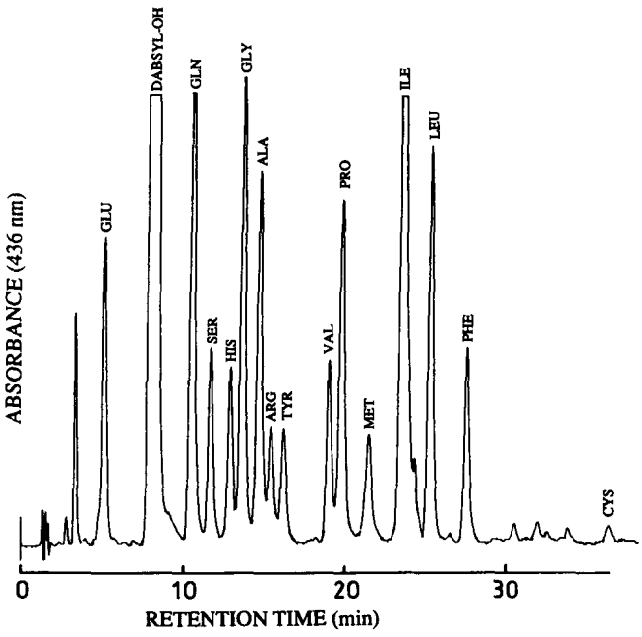

Fig. 4. Chromatogram of the separation of the dabsyl derivatives of amino acids from a cell culture medium. The elution conditions are described in the text.

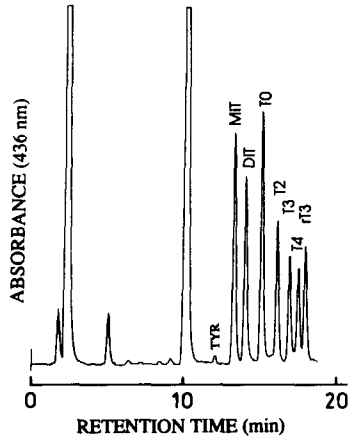

Fig. 5. Chromatogram of the separation of the dabsyl derivatives of a number of thyronine and tyrosine analogues found in the thyroid. The elution conditions are described in the text.

important parameters in this study was the ratio in serum between Trp and other hydrophobic amino acids such as Val, Ile, Leu and Phe. This ratio determines the amount of TRP which enters the brain via the blood–brain barrier. As Trp is considered to be the precursor of serotonin in the brain, the determination of the ratio of Trp and the other hydrophobic amino acid in serum is an important parameter for premenstrual complaints. The results of this work will be presented elsewhere [16].

The thyroid is an important organ which produces thyroid hormones, such as triiodothyronine (T3), reversed triiodothyronine (rT3) and tetraiodothyronine (T4). These hormones are synthesized in the thyroid follicle by the coupling of precursor hormones, such as tyrosine, monoiodotyrosine (MIT) and diiodothyrosine (T2). In toxicology the relative amounts of these hormones and precursors are an important parameter in assessing the toxicity of xenobiotic agents. As these compounds are essentially amino acids, the determination *via* dabsylation was investigated. For all components di-substituted dabsyl derivatives were formed which could be separated and quantitated by HPLC (Fig. 5).

DISCUSSION

The derivatization of amino acids with dabsyl chloride gives the best results at 70°C for 15–30 min, as was described previously [9,10]. Neither the pH nor the time of derivatization is very critical. The only restriction is with derivatizations performed at temperatures lower than 70°C. For some amino acids an incomplete derivatization was observed at such temperatures. In addition, it was observed that at lower temperatures some amino acids show higher peak heights than at higher derivatization temperatures. This might indicate that the dabsyl amino acids derivatized at 70°C are tending to decrease in intensity as a result of instablity. Therefore the conditions of incubation should be standardized at 70°C as far as possible. It was also observed that the concentration of chloride ions should be kept constant and preferably as low as possible.

The derivatives were characterized by a good stability, a good limit of detection and a maximum absorbance at 448–468 nm. At this wavelength no or little interference is expected from matrix components. The separation of all twenty naturally occurring amino acids can be performed by reversed-phase gradient HPLC within 30 min. The best mobile phase was a mixture of methanol and acetonitrile in a sodium acetate buffer at pH 6.5. Several reversed-phase materials were tested under identical experimental conditions. The best separation was observed with Zorbax ODS and Hypersil ODS.

The pre-column derivatization with dabsyl chloride has proved to be a very good and convenient method of determining amino acids. The determination of amino acids described here has already been applied for a great number of applications in our institute. The results of these applications will be the subject of further papers.

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