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COMPLETE AMINO ACID ANALYSIS OF PROTEINS BY DABSYL DERIVATIZATION AND REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the quantification of the primary amino acids in protein hydrolysates by dabsylation and high-performance liquid chromatography. Improvements in the established conditions for the formation and storage of amino acid dabsyl derivatives and the use of new reversed-phase columns allow the chromatographic analysis in 25 min of all the proteinogenic amino acids in well resolved peaks of homogeneous and highly reproducible size. The method permits the simultaneous quantification of tryptophan residues by previous hydrolysis of the protein with sulphonic acids. The other acid-labile residues, asparagine and glutamine, can also be analysed by previous conversion into diaminopropionic and diaminobutyric acids, respectively, by treatment of the protein with [bis(trifluoroacetoxy)iodo]-benzene. An extended chromatographic gradient programme allows the separation of many modified amino acids, naturally occurring or produced after chemical modification of proteins. The above characteristics together with a demonstrated high reproducibility (relative standard deviation 2.1%), flexibility, sensitivity (below 100 pmol), and inertness to extraneous chromatographic contamination make this improved method a good alternative to other currently used chromatographic methods for amino acid analysis.

INTRODUCTION

The development of a method based on pre-column derivatization with dabsyl chloride (dimethylaminoazobenzenesulphonyl chloride) and high-performance liquid chromatographic (HPLC) separation of derivatives on reversed-phase columns by Chang *et al.*[1–3] has been one of the most significant improvements in the field of amino acid analysis in recent years. The very high molar absorptivity of the dabsyl derivatives in a clean spectral region, their stability with time and the differential behaviour of these derivatives on reversed-phase HPLC columns allows amino acid analyses to be carried out in a simple and economical way, free from interferences and with high sensitivity (picomole range). However, in the day-to-day application of this method we observed several problems that limit its use. Thus, the extent of

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derivatization of di-dabsylated amino acids (lysine, tyrosine and histidine) varied from one analysis to another. This problem had already been recognized in the past with histidine derivatives[1]. Moreover, the area of the different chromatographic peaks obtained from an equimolar mixture of amino acids was not homogeneous, with low areas for the peaks corresponding to glutamic and aspartic acid, serine, threonine and arginine. These problems reduce the reproducibility and precision in the quantification of amino acids.

In order to improve the reliability of the quantitative amino acid analysis by dabsylation and HPLC, we have investigated the conditions used in the derivatization (pH, nature of buffer, proportion of organic solvent, temperature) and storage in order to obtain a stable, complete and quasi-homogeneous dabsylation for all the amino acids and, therefore, to eliminate most of the problems mentioned. In addition, we have extended the dabsylation methodology to the analysis of the acid-labile amino acids tryptophan, glutamine and asparagine in proteins, and to other amino acid derivatives usually found in proteins or produced after specific modifications or cleavage. Finally, we have greatly shortened the chromatographic separation of dabsyl-amino acids with respect to the original method by using a reversed-phase resin with a higher resolving power and different elution gradients. The final method allows routine quantitative analyses of all proteinogenic amino acids and many derivatives in a reliable, sensitive and rapid manner, which compares favourably with other traditional[4–7] and recently published[8–10] methods.

EXPERIMENTAL

Materials

Dabsyl chloride was purchased from Fluka and recrystallized from acetone before use. Acetonitrile (HPLC grade) was obtained from Carlo Erba. Highly purified water was produced passing water obtained with a Milli-R purification system (Millipore) through a Norganic resin (Waters–Millipore). Proteinogenic amino acid standards and 6 *M* hydrochloric acid were purchased from Pierce. Methanesulphonic acid, lysozyme (chicken egg), ribonuclease A (bovine), free natural and modified amino acids, polyamino acids, DABA (2,4-diaminobutyric acid) and DAPA (2,3-diaminopropionic acid) were Sigma products. *p*-Toluensulphonic acid was purchased from Merck and was maintained under vacuum overnight on sodium hydroxide pellets before use. BTI {[bis(trifluoroacetoxy)iodo]benzene} was purchased from Aldrich (lot 4127TK) and Fluka (lots 32997 483 and 33405 784). All other reagents were of analytical-reagent grade.

Chemical treatment of the samples

The preparation of some modified amino acids not purchased commercially was required for the analysis of their dabsyl derivatives. A mixture of homoserine and homoserine lactone was obtained by treatment of methionine hydrochloride (Sigma) with cyanogen bromide in 70% formic acid followed by freeze-drying[11]. Homogeneous homoserine was prepared by treatment of the mixture with pyridine acetate at 105°C for 1 h[11]. Carboxymethylcysteine and carboxymethylhistidine were obtained from cysteine and histidine hydrochlorides (Sigma)[12].

The samples to be analysed for their Asn and Gln content were previously

treated with BTI according to the method of Soby and Johnson[13]. The protein was initially dissolved in water at a concentration of 2 mg/ml and made 5 *M* in guanidinium chloride and 0.1% in trifluoroacetic acid. Then, 1 volume of BTI (36 mg/ml in dimethylformamide) was added. After incubation ast 60°C for 4 h, the protein solution was dialysed overnight against 1000 volumes of deionized water (twice). Subsequently, the protein solution was extracted three times with *n*-butyl acetate and the aqueous phase was freeze-dried.

Protein hydrolysis. Proteins were hydrolysed with 6 *M* hydrochloric acid (Pierce), 3 *M* *p*-toluenesulphonic acid or 4 *M* methanesulphonic acid (containing 0.2% of tryptamine in both of the last two instances), according to the methods of Liu and Chang[14] and Simpson *et al.*[15] in the last two instances. About 1 nmol of protein dissolved in 25 μl of the acid solution was placed in a small borosilicate glass tube (0.4 × 6 cm) and the tube was flame-sealed under deep evacuation. After keeping the sample at 110°C for the desired time (22, 48 or 72 h), the tube was opened and the hydrolysate freeze-dried in the hydrochloric acid treatment, or adjusted to pH 9.0 by careful addition of 3 *M* sodium hydroxide solution in the methanesulphonic or *p*-toluensulphonic acid treatment. In the latter instances, a parallel experiment of pH adjustment on a larger sample of acid facilitated the proper addition of alkali to the small samples of hydrolysates. Subsequently, the hydrolysate was adjusted to 100 μl with freshly prepared 0.30 *M* sodium hydrogen carbonate solution to give a final salt concentration of 0.15 *M* and dabsylated according to the method described for amino acid standards (see below).

Standard dabsylation procedure

Derivatization with dabsyl chloride was usually carried out in glass-stoppered tubes (0.6 × 2.5 cm). Usually, 100 μl of a 15 m*M* solution of dabsyl chloride in acetone were added to 100 μl of amino acid mixture [containing 10 nmol of each amino acid in 0.15 *M* sodium hydrogen carbonate solution (pH 9.0)]. A suitable pH of the buffer and ratio of aqueous buffer to acetone solution were selected after many trials under different conditions (see Results and discussion). The tightly closed tube was incubated at 70°C for 15 min and great care was taken to avoid evaporation of acetone in the incubation step. Usually, the sample was diluted to 500 μl with ethanol–40 m*M* sodium phosphate buffer (pH 6.5) (1:1, v/v). Subsequently, the derivatization mixture was centrifuged at 5000 *g* for 5 min and the supernatant was loaded on to the HPLC column or stored at −20°C.

Amino acid analysis

Compositional analyses of amino acids were performed by reversed-phase HPLC. The Waters Assoc. analyser used consisted of a 710 WISP autosampler, a 680 gradient controller, two 6000A pumps, a model 440 detector (fixed at 436 nm) and a Data Module integrator/register unit. Two octadecyl-bonded columns were used to separate dabsyl-amino acids: a LiChrosorb RP-18 (5 μm irregular particles) (25 × 0.45 cm I.D.) supplied by Merck and a Novapak C_{18} (4 μm spherical support) (15 × 0.45 cm I.D.) supplied by Waters Assoc. The latter column allowed a higher resolution and quicker analysis and was adopted in the later steps of this work. A pre-column (2.5 × 0.6 cm I.D.) of Corasil C_{18} pellicular support (Waters Assoc.) was used throughout.

Different chromatographic gradient programmes were used for the particular separation requirements. The original 12 m*M* sodium phosphate (pH 6.5)–acetonitrile gradient programme reported by Chang *et al.*[2] was followed in the separation performed with the LiChrosorb RP-18 column. Three distinct modified programmes were used with the Novapak C_{18} column, which allowed a high-speed analysis (25 or 32 min run time) or a high-resolution analysis (75 min run time) (see Results and Discussion), both based on a 9.0 m*M* sodium phosphate (pH 6.5 or 6.6, respectively)–acetonitrile gradient in the presence of 4% dimethylformamide. In all instances the column was maintained at 50°C and a flow-rate of 1 ml/min was used. The chromatographic line, consisting of 0.020 in. I.D. tubing, measured 85 cm from the injector to the column and 60 cm from the column to the detector. A 10-μl detector cell (Taper Cell, Waters Assoc.) was used in the analyses. Two Rheodyne 7060 valves were placed at the column ends.

RESULTS AND DISCUSSION

Improvement of derivatization yield and homogeneity

As already mentioned, under the standard conditions of the original method of Chang *et al.*[1–3], the peak areas of certain amino acid dabsyl derivatives are not reproducible from one experiment to another, and an overall lack of peak area homogeneity is observed for the derivatives of the different proteinogenic amino acids in the same chromatogram. We found that most of these problems can be solved by careful analyses and adjustment of the conditions of derivatization. Thus, when the final concentration of dabsyl chloride in the dabsylation mixture is increased 2- to 3-fold above the level used in the standard method of Chang *et al.* (2- to 3-fold 2.66 m*M*), the peak areas of several amino acid derivatives increase considerably (2- to 3-fold for some of them), particularly with the derivatives that formerly gave a low peak response (Glu, Asp, Ser, Thr, His, Tyr) (Fig. 1A). It is interesting that under these conditions the peak response of the didabsylated derivatives (Lys, His, Tyr) is nearly double that of the monodabsylated derivatives, and the variability usually found for the peak response of histidine and tyrosine completely disappears. Moreover, a higher homogeneity of derivatization is observed for the other amino acids.

When the derivatization is carried out at final concentrations higher than 7.5 m*M* in dabsyl chloride a progressive decrease in the peak area of most amino acid derivatives is observed, particularly for didabsylated amino acids (results not shown). This is probasbly due to the appearance of insoluble aggregates of the reagent, the concentration of which is above its solubility limit in the water–acetone mixture used (1:1 to 1:2, v/v). The aggregates of dabsyl chloride probably bind or entrap the most hydrophobic derivatives, removing them from solution.

A further slight improvement in the homogeneity of the derivatization of all the amino acids was found to occur when the pH of the derivatization mixture was lowered from 9.0 to 8.5. Studies at pH 8.5, 9.0 and 9.5 indicate that the maximum yield of the process is achieved at pH 9.0, in agreement with previously results of Chang *et al.*[1], but the maximum general homogeneity for most derivatives is achieved at pH 8.5. However, at pH 8.5 the extent of dabsylation of His and Tyr is clearly lower than that of Lys (about 20%), which was one of the problems in the original method. On the other hand, a sharp decline in the derivatization yield is observed at

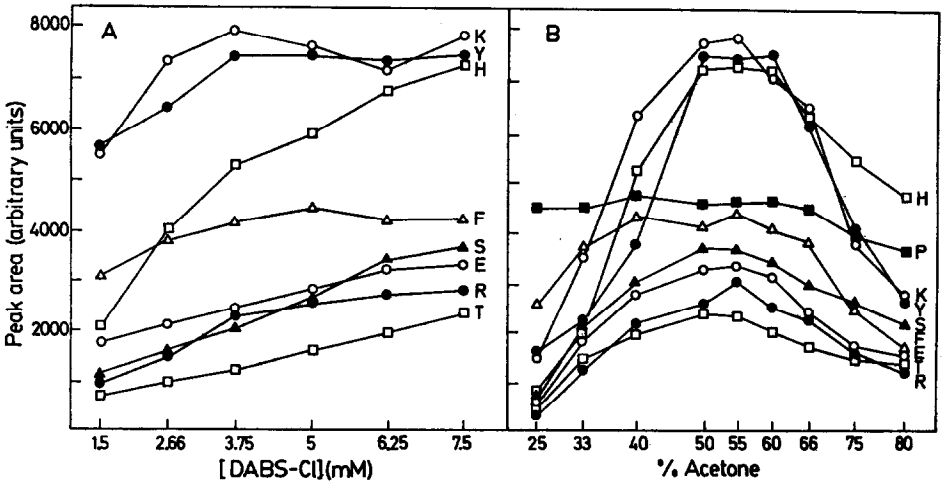

Fig. 1. Variability in the dabsylation of proteinogenic amino acids. Influence of the concentration of dabsyl chloride (A) and the percentage of acetone (B) in the final derivatization mixture. In (A), 100 μl of dabsyl chloride solution (at concentrations ranging from 3.0 to 15.0 m*M* in acetone) were added to 100 μl of amino acid mixture (0.06 m*M* of each amino acid in 0.132 *M* sodium hydrogen carbonate solution, pH 9.0) and the mixture was subsequently maintained at 70°C for 15 min. In (B) progressively increasing volumes of dabsyl chloride solution were added to progressively decreasing volumes of amino acid standard solution. Concentration of amino acids, buffer and pH in the final mixture, and the derivatization conditions, were the same as in (A), and the final concentration of dabsyl chloride was 7.5 m*M*. See ref. 26 for one-letter notation.

pH 9.5 for many different amino acids, particularly those which show a relatively low peak response under the original conditions (Glu, Asp, Thr, His). This behaviour suggests that the degradation of derivatives proceeds more efficiently at higher pH and opposes the derivatization reaction, as do other related sulphonylation reactions[16].

Another factor to be taken into account is the relative amount of acetone added to the derivatization mixture. As shown in Fig. 1B, the maximum dabsylation yield for most amino acids is observed for water to acetone ratios between 1:1 and 1:1.25 (v/v), conditions which deviate from those selected by Chang *et al.* (1:2, v/v)[1–3]. This fact limits the increase in the relative amount of organic solvent in the reaction mixture and, therefore, the possible increase in the final concentration of dabsyl chloride used. The change in the peak response of His and Tyr derivatives when the acetone concentration varies is dramatic and illustrates the need to adjust the dabsylation conditions carefully and to avoid evaporation of acetone during the derivatization procedure. In conclusion, a dabsyl chloride concentration of 7.5 m*M*, pH 9.0 and water–acetone (1:1 or 1:1.25) were chosen as the final conditions for obtaining maximum homogeneity and dabsylation yields for all the proteinogenic amino acids.

Stability and storage of dabsyl-amino acids

One of the advantages of the dabsylation–HPLC method over some other methods with pre-column derivatization is the great stability of the dabsyl derivatives. According to Chang *et al.*[1–3], the peak response of the different dabsylated amino

acids, kept at intermediate pH, is maintained for several months when the samples are stored at room temperature. However, we have observed that this general stability does not hold for some derivatives, such as those of Glu, Asp, His and Tyr, the peak areas of which decrease as much as 30% after being kept at room temperature for 15 days. A similar behaviour is observed when the derivatives are kept at 4°C. In contrast, if these derivatives are kept at −20°C in the original derivatization solution [water–acetone (1:1)] or in the freeze-dried state, the peak areas of all the amino acid derivatives are maintained within an error of 1–2% during the same or even longer periods of time.

It was also found that the dilution of dabsylated amino acids with ethanol–phosphate buffer (pH 6.5) (1:1 v/v), a common practice after derivatization and before chromatography[1,2], does not affect the stability of the derivatives after storage at −20°C. However, the chromatographic separation of these derivatives after storage is spoiled when pure ethanol (a previously recommended solvent[3]) is used as the diluting agent instead of the ethanol–phosphate mixture.

Dabsylation is a particularly useful derivatization method for automated pre-column amino acid analysis owing to the great stability of the dabsyl-amino acids obtained. The decrease in the peak area of the less stable derivatives at room temperature during a whole working day (about 1%) is lower than the precision threshold of the quantification procedure. The combined use of an autosampler and a high-speed separation programme (see below) permits the analysis of about 40 amino acid samples in a day with negligible errors due to degradation of the dabsyl derivatives. The insertion of standard reference samples after every five to ten analysed samples improves the reliability and precision of the analysis. If the samples cannot be analysed on the same day, they can be stored at −20°C and analysed much later without suffering noticeable degradation. Conversely, the derivatization of the samples can be carried out many days before their analysis, provided they are subsequently stored at −20°C.

High-speed and high-resolution chromatography of dabsyl derivatives

Different chromatographic programmes are required to separate the dabsyl derivatives according to speed requirements and the number and nature of the amino acids in the initial mixture. For routine analysis, where high speed is needed, it has been found that the 20 primary amino acids can be cleanly resolved in 25 min (see Fig. 2), about 60% of the chromatographic time required in the original method of Chang *et al.*[2,3]. This improvement was obtained by using a spherical C_{18} support, of 4 μm particle size, in a short column (15 cm), and the following gradient (in linear steps unless indicated otherwise) between 9.0 m*M* sodium phosphate (pH 6.5) in water (solvent A) and acetonitrile (solvent B), both containing 4% of dimethylformamide: from 91% A and 9% B at 0 min to 77% A and 23% B at 8 min with a hyperbolic step (curve 5 in the M680 controller); 37% B at 16 min; 46% B at 17 min; 49% B at 20 min and 60% B at 21 min.

If these steps are shortened it is possible to reduce the chromatographic separation time to less than 20 min, but in this instance the homogeneous distribution of the peaks in the profile and their size homogeneity is lost, causing problems with the integration system. No efforts were made to improve the chromatographic resolution by decreasing the length and inner diameter of the chromatographic line, to-

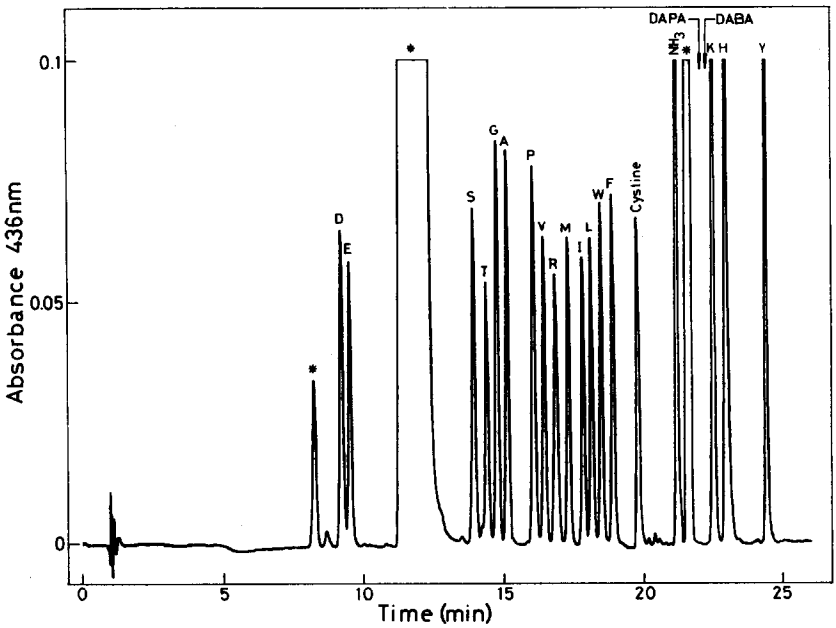

Fig. 2. Rapid analysis of dabsyl derivatives from primary amino acids on a reversed-phase column. DAPA and DABA refer to the respective transformed products of asparagine and glutamine after BTI treatment. A proteinogenic amino acid standard (containing 1 nmol of each amino acid) was treated with 3 *M* *p*-toluensulphonic acid at 110°C for 22 h, as in the standard hydrolysis procedure. After pH adjustment and dabsylation, 1/12th of the sample was loaded on to the column. The analysis was performed on a Novapak C_{18} column (15 × 0.46 cm I.D.), 4 μm reversed-phase support, at pH 6.5 and 50°C, and using the short chromatographic programme. The asterisks refer to excess of reagent and side reaction products. See Fig. 1 and text for amino acid abbreviations.

gether with the use of a smaller flow cell detector and a smaller pre-column filled with the same resin as the column (see Experimental for details).

When the protein composition is greatly unbalanced for some amino acids (*e.g.*, Gly and Pro in collagen and Lys and Arg in histones and protamines), the use of a shallower gradient to separate the major components cleanly is recommended: 91% A and 9% B at 0 min; 40% B at 24 min; 46% B at 25 min; 55% B at 31 min and 60% B at 33 min. On the other hand, if the separation of a greater number of derivatives is desired, such as those of post-translational covalent modifications or artificial modifications (see below), a much shallower gradient is required, *e.g.*, 93% A and 7% B between 0 and 5 min; 19% B at 32 min; 24% B at 42 min; 34% B at 55 min; 38% B at 56 min; 53% B at 78 min and 60% B at 80 min.

With this high-resolution programme, the separation of primary and modified amino acids, shown in Fig. 3, is quicker than with many other analytical methods based on traditional ion-exchange chromatography and post-column derivatization, either in the extended mode (*i.e.*, for physiological fluids[5]) or even in the standard (quick) modes[17]. In order to separate the earlier eluted derivatives, this high-resolution chromatography was performed at pH 6.6, 0.1 pH unit above the conditions of the rapid and standard methods.

As already indicated by Chang *et al.*[2,3], the chromatographic position of cer-

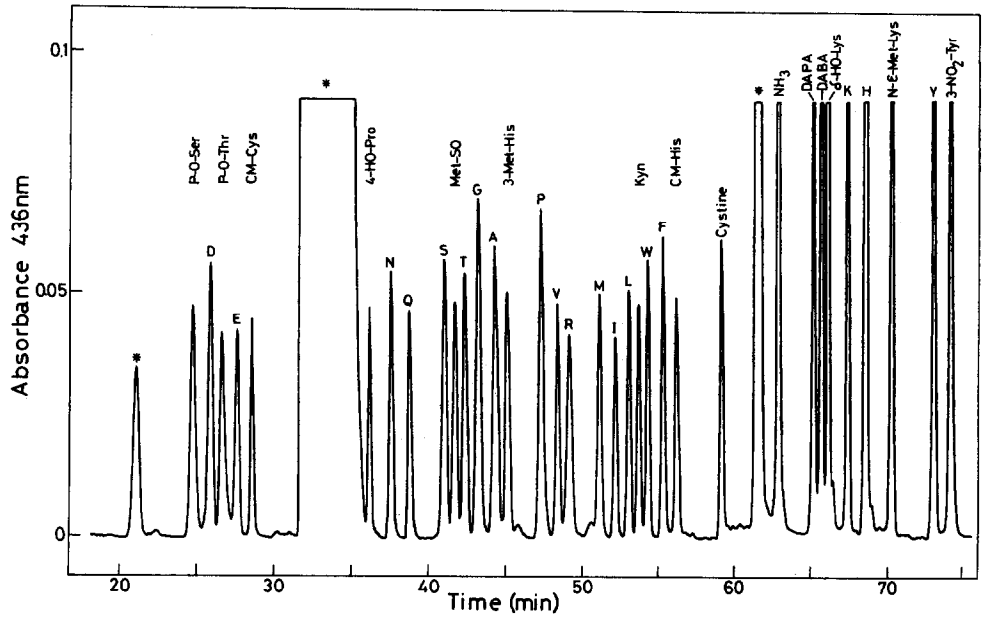

Fig. 3. High-resolution analysis of dabsyl derivatives from primary and modified proteinogenic amino acids on a reversed-phase column. A proteinogenic amino acid standard in 0.15 *M* sodium hydrogen carbonate solution (pH 9.0) was complemented by addition of individual modified amino acids at equimolar ratios. This mixture, 0.1 m*M* in each amino acid or derivative, was dabsylated according to the standard dabsylation procedure. Separation of dabsyl derivatives at 100 pmol level was performed on a Novapak C_{18} column (15 × 0.46 cm I.D.) at pH 6.6 and 50°C, using the high-resolution chromatographic programme. Abbreviations: P-O-Ser, phosphoserine; P-O-Thr, phosphothreonine; CM-Cys, carboxymethylcysteine; 4-HO-Pro, 4-hydroxyproline; Met-SO, methionine sulphoxide; 3-Met-His, 3-methylhistidine; Kyn, kynurenine; CM-His, carboxymethylhistidine; δ-HO-Lys, δ-hydroxylysine; N-ε-Met-Lys, N-ε-methyllysine; 3-NO_2-Tyr, 3-nitrotyrosine. All other abbreviations as in text and Fig. 1.

tain derivatives (*e.g.*, Glu, Asp and Arg) is slightly dependent on the particular column and its age, and some adjustments of eluent composition have to be made. With the Novapak C_{18} column the position of the Arg derivative is highly sensitive to the ionic strength of the eluent, moving from covering the Pro peak at 15 m*M* phosphate to covering the Met peak at 8 m*M* phosphate. We found that about 9.0 m*M* phosphate is a suitable ionic strength in the above column as under these conditions the position of the dabsyl-Arg is between dabsyl-Val and dabsyl-Met.

It is interesting to note that with the present dabsylation–chromatographic methods, as in the other classical methods[4,11], it is advisable to determine cysteine after it has been converted into a more stable derivative. In this work we converted it into carboxymethylcysteine or cysteic acid, as these are very stable and their dabsyl derivatives elute in a clean chromatographic region, whereas the cysteine derivative elutes between valine and arginine (see Table I). In contrast, the natural oxidized form cystine can be determined without conversion before dabsylation as it is stable to hydrolysis with sulphonic acids and its dabsyl derivative elutes cleanly between phenylalanine and ammonia derivatives.

TABLE I

HPLC RETENTION TIMES OF DIFFERENT DABSYL DERIVATIVES OF NATURAL AND CHEMICALLY MODIFIED AMINO ACIDS IN A HIGH-SPEED (A) AND A HIGH-RESOLUTION (B) CHROMATOGRAPHIC SYSTEM

Amino acid	*Retention time (min)*		*Amino acid*	*Retention time (min)*	
	A	*B*		*A*	*B*
Phosphoserine		24.90	Cysteine		49.01
Cysteic acid		25.66	Arginine	17.31	49.65
Aspartic acid	9.17	26.05	Methionine	17.79	51.62
Phosphothreonine		26.80	Isoleucine	18.34	52.67
Glutamic acic	9.50	27.82	4-PEP**		52.92
Phosphotyrosine		28.10	Leucine	18.63	53.57
CM-cysteine*		28.82	Kynurenine		54.22
4-Hydroxyproline		36.42	Tryptophan	19.03	54.82
Asparagine		37.53	Norleucine		54.87
Glutamine		38.54	Phenylalanine	19.47	55.82
Serine	14.18	41.45	CM-histidine***		56.72
Methionine sulphoxide		42.15	Cystine	20.39	59.65
Homoserine		42.63	NH_3	21.86	63.40
Methionine sulphone		42.68	DAPA§		65.67
Threonine	14.69	42.77	DABA§§		66.20
Glycine	15.07	43.67	δ-Hydroxylysine		66.65
Alanine	15.48	44.80	Lysine	23.15	67.97
3-Methylhistidine		45.47	Histidine	23.61	69.12
β-Alanine		45.60	N-ε-Methyllysine		70.87
Proline	16.47	47.77	Tyrosine	24.82	73.65
Homoserine lactone		48.01	3-Nitrotyrosine		74.75
Valine	16.87	48.90			

* Carboxymethylcysteine.
** S-β-(4-Pyridylethyl)-DL-penicillamine.
*** Carboxymethylhistidine.
§ 2,3-Diaminopropionic acid.
§§ 2,4-Diaminobutyric acid.

Analysis of acid-labile amino acids in proteins

The dabsylation–HPLC method can be extended to the determination of Trp, Gln and Asn in proteins by preceding both techniques with one of the already known methods of protein hydrolysis that do not destroy these amino acids, provided that no interferences occur. We have found that the prior hydrolysis of the protein by the methods of Liu and co-workers[14,15], with either 3 *M* *p*-toluensulphonic acid or 4 *M* methanesulphonic acid (with 0.02% tryptamine as scavenger), allows the subsequent direct dabsylation of all the released amino acids, including Trp, which is destroyed when the standard hydrolysis method with 6 *M* hydrochloric acid is used. Neither contaminant peaks nor apparent changes appear in the HPLC separation patterns of any of the released amino acids or in their derivatization yields with respect to an amino acid standard not treated with sulphonic acid. After dabsylation, Trp gives a peak response very similar to those of other apolar amino acids and it is cleanly eluted between Leu and Phe (see Fig. 2). However, the need to adjust the sample carefully to pH 9.0 (with sodium hydroxide and sodium hydrogen carbonate) after

the hydrolysis with the sulphonic acid and before its dabsylation must be stressed. A separate trial of neutralization with a large volume of the same sulphonic acid solution makes it possible to establish the exact volume of alkali to be added to each sample of hydrolysate in order to neutralize it. The whole method is very rapid and avoids the need to eliminate acid after hydrolysis, a time-consuming step and a possible source of contamination and losses in the hydrolysis method with 6 *M* hydrochloric acid[17,18].

The analysis of Asn and Gln in proteins by the dabsylation–HPLC method can be carried out after treatment of the protein with the reagent [bis(trifluoroacetoxy)iodo]benzene (BTI)[13] before acid hydrolysis. This reagent converts those carboxamide residues into their corresponding diamines, that is, into diaminopropionic acid (DAPA) and diaminobutyric acid (DABA), preventing their subsequent conversion into Asp and Glu during acid hydrolysis. We have found that after this chemical treatment most of the amino acids of a protein can be analysed by dabsylation–HPLC in the usual way and without interferences. The observed decrease in the peak area of dabsyl-Asp and dabsyl-Glu in a BTI-treated sample with respect to the untreated sample and the appearance of two specific peaks for dabsyl-DAPA and dabsyl-DABA (between ammonia and lysine derivatives) should allow the quantification of these acidic amino acid residues in the protein. However, in our hands the conversion of Glu and Asp in DABA and DAPA was not always quantitative when the method was tested on proteins such as lysozyme and ribonuclease A. Work is in progress to clarify this point. Moreover, the treatment of the protein with BTI converts several residues, such as Met, Trp, His and Tyr, giving rise to the disappearance or shift of their dabsyl derivatives from the chromatogram. This is probably due to oxidation and iodination reactions[19] promoted by the reagent. Therefore, a duplicate analysis, *i.e.*, on BTI-untreated and on BTI-treated protein, is still required in the analysis of all the proteinogenic amino acids.

Analysis of modified amino acids

In addition to the 20 primary amino acids encoded by DNA and RNA, proteins contain more than 100 natural secondary amino acids which are produced by post-translational covalent modifications[20,21]. Moreover, during the isolation of proteins and after various treatments to study their sequence, conformation and function, many different modified amino acids are generated. Many different methodologies are also required in order to determine differentially all these modified amino acids[21]. The dabsylation–HPLC method can be a simple and rapid alternative to certain of these methodologies. Thus, Table I and Fig. 3 illustrate the chromatographic behaviour on a reversed-phase column of several modified amino acids frequently found in native proteins or generated after common chemical treatments to which these proteins are submitted. The extended high-resolution chromatographic programme was used to resolve the dabsyl derivatives of these modified amino acids from those of the primary amino acids. The reported information can be particularly useful in interpreting unusual peaks or peak shoulders in the routine analysis of proteins by the method discussed here.

It is important to note that the non-proteinogenic amino acids norleucine and S-β-(4-pyridylethyl)-D,L-penicillamine cannot be used as internal standards in the present method as is usual in the classical ion-exchange method[22], as they are eluted

in a chromatographic region crowded with peaks of primary amino acids. Instead, we suggest the use of the amino acid derivative nitrotyrosine as an internal standard as it is eluted in a clean region (see Table I and Fig. 3), and is easily quantified spectrophotometrically at 381 nm (an isosbestic point with respect to pH of $\varepsilon = 2200$ $l\ mol^{-1}\ cm^{-1}$)[23]. Further, this derivative is very stable to acid hydrolysis, provided that protecting agents such as 2-mercaptoethanol and phenol (0.05% of each) are present during hydrolysis.

Sensitivity of the method

In most experiments carried out in this work 1 nmol of protein was used and, after hydrolysis and derivatization, 100 pmol of its hydrolysate (or amino acid standard) were chromatographed. According to Chang *et al.*[1–3], the great sensitivity of the chromatographic method allows the routine determination of 5–20 pmol of each amino acid. However, when the starting amount of protein to be hydrolysed and analysed is smaller than 100 pmol, we found it advisable to take stringent precautions to avoid extraneous contamination of the sample[17,24] and losses of amino acids and derivatives by adsorption on the tubes[25].

TABLE II

REPRODUCIBILITY OF AMINO ACID ANALYSIS BY DABSYLATION AND HPLC

Influence of chromatography, dabsylation and storage on the quantitative analysis of a standard protein sample (chicken lysozyme) hydrolysed with *p*-toluenesulphonic acid (22 h, 110°C). Values are expressed as residues per molecule and given as means ± S.D. from seven different determinations under the following conditions: (A) a sample of lysozyme hydrolysate was dabsylated, separated into seven aliquots and analysed immediately; (B) a sample of lysozyme hydrolysate was separated into seven aliquots, which were subsequently dabsylated individually and analysed immediately; (C) seven samples containing the same amount of lysozyme were hydrolysed in different days and dabsylated immediately after hydrolysis; the dabsylated samples were kept at −20°C for 2 months and finally analysed on the same day; (D) expected values based on the sequence analysis.

Amino acid	*A* (*n* = 7)	*B* (*n* = 7)	*C* (*n* = 7)	*D*
Aspartic acid	21.81 ± 0.20	21.62 ± 0.24	21.52 ± 1.14	21
Glutamic acid	5.34 ± 0.09	5.76 ± 0.12	4.32 ± 0.47	5
Serine	9.79 ± 0.17	10.10 ± 0.16	9.67 ± 1.01	10
Threonine	6.68 ± 0.02	7.07 ± 0.23	6.12 ± 1.01	7
Glycine	12.06 ± 0.12	12.10 ± 0.05	12.77 ± 0.12	12
Alanine	11.37 ± 0.12	11.39 ± 0.18	12.00 ± 0.29	12
Proline	2.08 ± 0.06	2.19 ± 0.13	2.21 ± 0.06	2
Valine	5.45 ± 0.08	5.28 ± 0.10	5.32 ± 0.28	6
Arginine	12.07 ± 0.05	11.81 ± 0.10	11.26 ± 0.70	11
Methionine	2.11 ± 0.04	2.02 ± 0.16	2.01 ± 0.41	2
Isoleucine	5.43 ± 0.09	5.25 ± 0.14	5.71 ± 0.49	6
Leucine	8.39 ± 0.09	8.15 ± 0.14	8.56 ± 0.46	8
Tryptophan	6.14 ± 0.07	5.89 ± 0.13	6.53 ± 0.43	6
Phenylalanine	3.20 ± 0.10	3.31 ± 0.23	3.23 ± 0.32	3
Cystine	4.25 ± 0.07	4.11 ± 0.07	4.40 ± 0.45	4
Lysine	5.76 ± 0.05	5.73 ± 0.11	4.94 ± 0.70	6
Histidine	1.13 ± 0.03	1.36 ± 0.09	1.00 ± 0.18	1
Tyrosine	2.75 ± 0.06	2.76 ± 0.19	3.16 ± 0.73	3

Reproducibility of the dabsylation–HPLC amino acid analysis

In order to test the influence of the different steps of the analytical method on its overall reproducibility, three different series of amino acid analyses were carried out on *p*-toluensulphonic acid hydrolysates of chicken lysozyme, which was used as a standard protein. From results in Table II, column A, it is apparent that, in our system and at the 100 pmol level, the average reproducibility of the chromatographic procedure by itself (which takes into account the peak area variability, the reproducibility of integration and the short-term stability of derivatives when analysed sequentially with an autosampler) is about 1.3% (relative standard deviation, R.S.D.). When, in addition, the variability due to the dabsylation procedure is taken into account, as in Table II, column B, the average reproducibility for all the amino acids is 2.1% (R.S.D.). The overall reproducibility found in the latter series of analyses, which correspond to the usual standard procedure, compares well with that obtained in classical ion exchange–ninhydrin or *o*-phthalaldehyde post-column analytical methods[4,11,17].

Finally, when most of the different manipulations that can be carried out in a whole series of amino acid analyses are taken into account (hydrolysis, dabsylation and storage of derivatives at −20°C, on different days and with different batches of reagents), the reproducibility decreases to an overall level of 7.5% (R.S.D.). This is an important variability, but the extreme, unusual and diverse conditions in which the experiments were carried out for all the different samples have to be considered. However, if the different hydrolysates are not stored at −20°C but analysed immediately after dabsylation, the overall reproducibility is significatively improved (to 5.2%; results not shown). Therefore, for the high-precision quantitative analysis of *p*-toluensulphonic acid hydrolysates of proteins it is advisable to analyse the samples immediately after dabsylation, avoiding the storage of the dabsyl derivatives, in spite of the great stability previously shown for these derivatives in amino acid standards.

In conclusion, the original dabsylation–HPLC reversed-phase method in combination with the improvements proposed here constitutes a rapid, sensitive, complete, and very reproducible method for the determination of primary and modified amino acids. From an overall point of view, this method compares favourably with other currently used reliable methods for automatic amino acid analysis[4–10].

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