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Note

Quantitative high-performance liquid chromatographic analysis of Dabsyl-amino acids within 14 min

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Since the introduction of a liquid chromatographic method for the analysis of amino acids by Spackman *et al.*[1] in 1958 attempts have been made to increase both the sensitivity and the speed of this technique. The availability of small particle sulphonated polystyrene supports and the replacement of the original postcolumn detection reagent, ninhydrin, with *o*-phthalaldehyde (OPA)[2] have been partially successful in achieving these aims[3]. It would appear, however, that the chromatographic properties of the ion-exchange resins are not suitable for the rapid analysis expected from modern high-performance liquid chromatography (HPLC). Additionally, the detection with OPA has a major drawback in that proline cannot be detected.

Alternative liquid chromatographic methods have been sought to replace those based on the conventional technique. The most successful ones have involved a precolumn derivatization of the amino acids which can then be detected by fluorescence or absorbance after their separation by reversed-phase (RP) HPLC. Reports concerning precolumn labelling with OPA[4], Dns-Cl[5], phenyl isothiocyanate (PITC)[6] and 4-dimethylaminoazobenzene-4'-sulphonyl chloride (Dabsyl-Cl)[7] have appeared. Each have claimed sensitivities at the picomol level and analysis times more rapid than in the ion-exchange technique.

Here we report that the gradient elution of Merck Hibar LiChrocart Supersphere RP-18 columns with an increasing concentration of propanol–acetonitrile (3:2) in 33 m*M* sodium acetate achieves a complete separation, in only 14 min, of all Dabsyl derivatives of the common amino acids found in peptide hydrolysates. The

The program for the separation of dabsyl-amino acids was as follows:

Time (min)	*Flow-rate (ml/min)*	*% Buffer A*	*% Buffer B*	*Gradient profile*
Initial	1.0	95	5	
0.1	1.0	90	10	Linear
4.0	1.0	80	20	Linear
8.0	1.0	68	32	Linear
9.0	1.0	40	60	Linear
12.0	1.0	40	60	Linear
13.0	1.0	95	5	Linear

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sensitivity is at the low pmol level and the standard deviation at 10 pmol for different standard aliquots is typically 0.3 pmol. The results of analyses of hydrolysed peptides previously separated by HPLC are in agreement with those obtained from an ion-exchange/postcolumn OPA HPLC procedure. Column lifetime is good and the separation is reproducible between different columns of the same type.

MATERIALS AND METHODS

Dabsyl chloride was from Fluka (Buchs, Switzerland) and was recrystallized from acetone before use. Acetonitrile and 2-propanol were HPLC grade from Fluka. Other chemicals were of analytical grade from either Fluka or Merck (Darmstadt, F.R.G.). The amino acid standard mixture was from Pierce (Rockford, IL, U.S.A.).

The HPLC system was from Waters and consisted of a WISP 710 B automatic sample injector, two Model 510 pumps, a model 680 controller and an UV detector Model 440 equipped with a 436-nm filter. Hibar LiChrocart Supersphere RP-18 columns (125 mm × 4 mm) were from Merck and all experiments were performed with these, directly coupled to a LiChrocart guard column (4 mm × 4 mm) containing LiChrosorb 100 RP-18.

Buffer A was prepared by adding 2 ml of acetic acid to 1 l of water and this was titrated to pH 6.3 using 4 *M* sodium hydroxide. One part of acetonitrile was added to five parts of this buffer, and before use the solution was filtered through a 0.2 μm filter. Buffer B was 2-propanol–acetonitrile (3:2).

Derivatization with Dabsyl-Cl was performed by adding 20 μl of a reagent solution in acetonitrile (4 nmol/μl, 1.3 mg/ml) to the sample dissolved in 10 μl of 50 m*M* sodium bicarbonate (titrated to pH 9.0 with sodium hydroxide). After heating at 70°C for 10 min, 80 μl of 50 m*M* sodium phosphate pH 7.0–ethanol (1:1 v/v) were added and the samples were then directly placed into the WISP for injection. The pH of both buffer A and the phosphate buffer was uncorrected after the addition of acetonitrile or ethanol. Standard mixtures were derivatized in aliquots containing not more than 500 pmol per amino acid. Peptides were hydrolysed in the WISP injection tubes (previously pyrolyzed) with 6 *M* hydrochloric acid–water vapour at 120°C for 15 h *in vacuo*.

RESULTS AND DISCUSSION

Over the past years, amino acid analysis has been performed in this laboratory by applying HPLC techniques to the separation of amino acids on ion-exchange resins, followed by detection with OPA[3,9]. This has produced adequate quantitation at the 50 pmol level; however, the failure to detect proline and the relatively long analysis time (inject-to-inject time: 80 min) forced us to search for an alternative means of analysis. Our first attempt was the precolumn derivatization procedure using PITC[6]. Efforts to obtain a reliable quantitative system on anything but standards were unsuccessful and thus we decided to attempt precolumn derivatization with Dabsyl-Cl, a procedure reported by Chang *et al.*[7] in 1981.

Recently, we reported[8] a complete separation of phenylthiohydantoin (PTH)-amino acids within 12 min on a LiChrocart Supersphere cartridge using gradients of 2-propanol in sodium acetate buffer. The success of this procedure encour-

aged us to attempt the separation of Dabsyl-amino acids under similar conditions. From initial experiments, we observed that the Dabsyl derivatives were, on average, more strongly adsorbed on the column than the PTH derivatives. Replacing the 2-propanol–water (3:2) used as buffer B for the PTH derivatives by a mixture of 2-propanol–acetonitrile and raising the pH to above 6.3 achieved a workable system.

An ever present problem in the separation of the Dabsyl derivatives is the large methyl orange peak (produced by the hydrolysis of excess of reagent) and two extraneous peaks which elute between cysteine and lysine. In the PTH analysis, histidine and arginine are often problematical and their elution positions are usually adjusted by varying the salt concentration[8]. In the Dabsyl-Cl method, only arginine presents

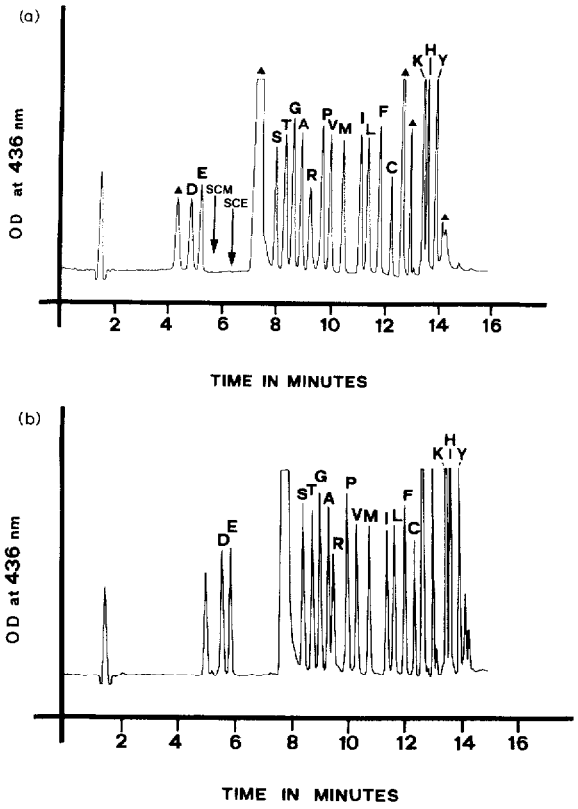

Fig. 1. (a) Separation of a standard Dabsyl-amino acid mixture on LiChrocart Supersphere. The column (125 mm × 4 mm, plus guard column) was eluted with increasing (linear) concentrations of 2-propanol–acetonitrile (3:2, buffer B) in 33 m*M* sodium acetate, pH 6.3–acetonitrile (5:1, buffer A). A flow-rate of 1 ml/min was used at a temperature of 37°C. See Materials and methods for details. A standard mixture containing 500 pmol of each amino acid was derivatized and 10% taken for injection. Carboxymethylcysteine (SCM) and carboxyethylcysteine (SCE) are eluted after Glu (E) as indicated by the arrows. Lys (K), His (H) and Tyr (Y) are present as their bis Dabsyl derivatives. The inject-to-inject time was 18 min. Closed triangles indicate extraneous peaks which do not contain amino acids. (b) Separation of Dabsyl-amino acids on a second column of LiChrocart Supersphere. Conditions identical to those in Fig. 1a with the exception that the sodium acetate concentration in buffer A was reduced to 30 m*M* in order to effect a better separation of Ala (A) from Arg (R).

TABLE I

ANALYSIS OF STANDARD AMINO ACID MIXTURES

	*Amino acid**																
	D	*E*	*S*	*T*	*G*	*A*	*R*	*P*	*V*	*M*	*I*	*L*	*F*	*C*	*K*	*H*	*Y*
Standard deviation (pmol) at 10 pmol**	0.4	0.4	0.4	0.2	0.2	0.2	0.6	0.4	0.3	0.3	0.4	0.4	0.3	0.2	0.3	0.3	0.4
Average values obtained at 50 pmol***	47	55	47	54	49	51	52	50	49	53	53	53	53	56	51	51	54
Standard deviation	3	3	3	2	4	2	3	4	2	2	2	2	2	3	1	2	2

* For explanation of the one-letter notation for amino acids, see ref. 10.

** Seven aliquots containing 100 pmol of each amino acid were separately derivatized and 10% of the sample was injected, carboxyethyl cysteine was used as an internal standard.

*** Seven aliquots of 500 pmol were derivatized and 10% of each sample was analysed, results were calculated using a calibration table obtained from the derivatization at 100 pmol (see above).

a problem and is positioned as in the PTH analysis, *i.e.*, a decrease in the salt concentration increases its retention time. The bis derivative of histidine is formed and is eluted at the end of the chromatogram. As seen in Fig. 1a, all the common amino acids are resolved within 14 min which allows an analysis to be made every 18 min. It should be noted that the three major contaminating peaks are well separated from all the amino acid derivatives.

Columns of the same type, even among the same batch, are known to display different separation abilities. Thus, we tested a second Supersphere column under exactly the same conditions. The chromatogram obtained was identical with the exception that alanine and arginine were almost co-eluted. Reduction of the salt concentration in buffer A from 33 to 30 m*M* produced the satisfactory chromatogram shown in Fig. 1b.

Amino acid analysis requires not only a perfect resolution but also an excellent degree of quantitation. To test the accuracy of the method, seven standards containing 100 pmol of each amino acid were derivatized separately using the rapid and simple procedure described in materials and methods. A 10-pmol amount of each was injected and the results, based on carboxyethylcysteine as an internal standard, are shown in Table I. The average standard deviation (0.3 pmol for a 10-pmol injection), though not as accurate as that obtained by ion-exchange and postcolumn detection with OPA (10 pmol for a 1-nmol injection)[3], is adequate for many applications. Presumably, the results could be improved by the use of automatic derivatization. The linearity of the response was measured by derivatizing seven standards of 500 pmol and using the calibration table formed from the seven determinations made at 10 pmol. From Table I, the average standard deviation is a respectable 2.5 pmol for 50-pmol injections. In agreement with the findings of Chang *et al.*[7], we found that it is essential to have at least a four-fold excess of Dabsyl-Cl over amino groups.

In our studies on the interaction of the secretory component with immunoglobulin A dimers, we have produced peptide digests containing 45 or more components which were separated by two-dimensional HPLC[9]. Hydrolysates of these peptides were analysed in parallel by Dabsyl-Cl and ion-exchange/OPA techniques. As is seen from Table II, the two methods produced strikingly similar results. It should be noted, however, that an approximately 50-times larger amount of material was injected into the ion-exchange column.

Peptide hydrolysates containing amounts less than 50 pmol per amino acid have been derivatized with Dabsyl-Cl and successfully analysed. The limit of the sensitivity has, however, not been determined. The data for a representative chromatogram obtained from a peptide hydrolysate are shown in Fig. 2 and Table II (peptide 6). The complete separation of the component amino acids, the non-interference by side-products and the ease by which the peaks can automatically be integrated at 20 pmol per component are worthy of note.

Column lifetime is in excess of 600 injections (to date we have not lost a column) and the resolution does not diminish. The only precautions taken were that the column was stored in the organic solvent and the guard column changed every 200 injections.

It can be argued that any precolumn derivatization procedure cannot be as accurate as the analysis performed on the free amino acids when detected by a post-

TABLE II

COMPARISON OF AMINO ACID COMPOSITIONS OBTAINED USING PRECOLUMN DERIVATIZATION WITH DABSYL-Cl WITH THOSE FROM POSTCOLUMN REACTION WITH OPA

N.D. = Not determined.

Peptide	*Quantity**	*Analysis method*	*Amino acid*															
			D	*E*	*S*	*T*	*G*	*A*	*R*	*P*	*V*	*M*	*I*	*L*	*F*	*C***	*K*	*H*	*Y*
1	1: 884 2: 442	Dabsyl	1.0	2.3			2.2	1.0								1.1	1.0		0.5
	3: 442	OPA	1.0	2.0			2.2	1.0								0.7	1.0		0.5
2	1: 1660 2: 830	Dabsyl	1.0		2.6		1.0				1.0			2.1	1.0				
	3: 380	OPA	1.1		2.8		1.0				0.9			2.1	1.0				
3	1: 1200 2: 600	Dabsyl				0.8							0.9	0.9	1.0		1.0		
	3: 600	OPA				1.1							1.1	0.9	1.0		1.0		
4	1: 1144 2: 286	Dabsyl		2.4		1.0		1.0		1.0	1.0			1.0			1.0		
	3: 858	OPA		2.3		1.2		1.1		N.D.	0.9			1.0			1.0		
5	1: 520 2: 130	Dabsyl			1.5				1.0	1.0	1.1			1.3			1.0		
	3: 390	OPA			2.0				1.0	N.D.	0.9			1.0			1.0		
6	1: 1200 2: 240	Dabsyl	2.0	4.7	3.8	1.2	2.9	1.8	2.0		1.2		1.3	4.3	0.9			2.0	1.3
	3: 960	OPA	2.0	4.1	3.3	1.2	2.5	1.7	2.0		0.9		0.7	4.1	0.9			1.8	1.0
7	1: 260 2: 55	Dabsyl	7.4	6.8	4.6	3.0	4.0	2.2		1.8	3.2			5.3	3.1	0.5	1.0		0.9
	3: 205	OPA	6.6	6.4	4.3	3.2	3.1	2.0		N.D.	2.8			5.5	3.0	0.7	1.0		0.7
8	1: 680 2: 340	Dabsyl	5.0	7.0	2.0	1.5	5.0		1.0	1.0	2.8		1.0	2.9	1.0	1.0	1.0		0.9
	3: 340	OPA	5.2	6.4	2.4	1.3	5.0		1.0	N.D.	2.4		0.8	2.9	0.7	0.7	1.0		0.9

* Amounts of the peptide (pmol): 1 = hydrolysed; 2 = derivatized with Dabsyl-Cl (10% was injected except for peptide 7 of which 30% was injected); 3 = analysed by postcolumn OPA reaction.

** Determined as carboxymethylcysteine.

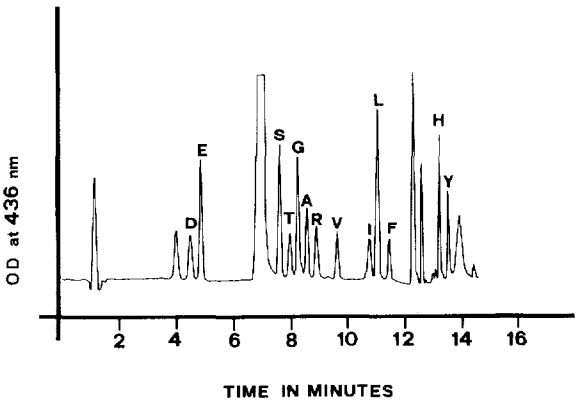

Fig. 2. Analysis of a peptide hydrolysate by Dabsyl-Cl precolumn derivatization. A tryptic digest of the rabbit secretory component was separated by RP-HPLC using an Aquapore RP-300 column and increasing concentrations of acetonitrile in 0.1% trifluoroacetic acid. A 1.2-nmol amount of one of the purified peptides was taken for hydrolysis, an aliquot containing 240 pmol was derivatized and 10% was injected. The chromatogram was obtained using the conditions described in Fig. 1a.

column reaction. The derivatization can be affected by the presence of non-peptide material within the hydrolysate and in addition the manipulations, however trivial, can only introduce errors. Here we have shown that the Dabsyl method does work simply, rapidly and with high sensitivity.

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