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Buffer and pH dependence of the retention of phenylthiocarbamylamino acids in reversed-phase high-performance liquid chromatography

Magdolna Morvai, Valéria Fábián and Ibolya Molnár-Perl*

Institute of Inorganic and Analytical Chemistry, L. Eötvös University, P.O. Box 32, H-1518 Budapest 112 (Hungary)

ABSTRACT

An exhaustive study was made to find the optimum conditions for the determination of phenylthiocarbamyl (PTC)-amino acids. The effects of the type of salt dissolved in the eluent (sodium or ammonium acetate), the pH of the eluent, the presence of additives such as triethylamine and phosphate and the maximum storage time of the dissolved derivatives were investigated. On Hypersil or Nucleosil columns (15 cm × 4.6 mm I.D.; 5 μm) the determination of 21 PTC-amino acids [including cyst(e)ine, tryptophan and ornithine] require 22 min. The reproducibility of the measurments was < 5.0% (relative standard deviation).

INTRODUCTION

The separation of amino acids after precolumn derivatization with phenyl isothiocyanate (PITC) and their subsequent determination by reversed-phase high-performance liquid chromatography (HPLC) has gained wide acceptance (Table I). The determination of the components of protein hydrolysates and those of free amino acids as phenylthiocarbamyl (PTC) derivatives has considerable advantages over other derivatization methods, including the quantitative reaction of all common amino and imino acids with PITC, the high stability of the PTC-amino acids, the relatively good sensitivity and reproducibility for the resulting PTC derivatives and the relative ease and reasonable cost of their preparation and HPLC separation.

After pioneering work [1,2] numerous papers [3–13] have dealt with the improvement of the initial methods. In this work, we made a systematic study in order to optimize the pH, the salt type and the maximum detector responses of 21 amino acids, including cyst(e)ine, tryptophan and ornithine, using the same two linear-increment gradient-type elution systems, to find the differences (if any) between two different stationary phase-containing columns of the same size, to define the stability of the PTC-amino acid derivatives after their dissolution in buffer and to examine the role of the presence of triethylamine and phosphate in the eluents.

EXPERIMENTAL

Materials

Triethylamine (TEA), PITC, amino acids and proteins were obtained from Sigma (St. Louis, MO, USA) and from Serva (Heidelberg, Germany). HPLC-grade acetonitrile and methanol were purchased from Reanal (Budapest, Hungary). All other reagents were of the highest purity available. Hydrolysis and derivatization tubes were supplied with the Pico-Tag Workstation (Waters Assoc., Milford, MA, USA).

Derivatization of amino acids with PITC

Standards of free amino acids in a mixture containing 12.5, 25.0 or 37.5 nmol of each amino acid were placed in the 50 × 6 mm I.D. tubes and dried under vacuum. Free amino acids were redried, adding 10 μl of ethanol–water–TEA (2:2:1) to each tube. Thereafter to each redried sample 20 μl of derivatization reagent [ethanol–TEA–water–PITC

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TABLE I

HPLC CONDITIONS SUGGESTED FOR THE DETERMINATION OF PTC-AMINO ACIDS

Column (cm × mm I.D.)	Particle size (μm)	Manufacturer	pH	Temp. (°C)	Eluent[a]		No. of amino acids measured	Analysis time (min)[b]	Ref.
					A	B			
15 × 3.9	3	Waters, Picotag	6.4	38	0.14 *M* Na.ac. + 0.5 ml/l TEA	ACN–H_2O (6:4)	18	12	1,6
25 × 4.6	5	IBM, DuPont, Altex	6.8	52	0.1 *M* Amm. ac.	ACN–Met–H_2O (44:10:46 and others)	16	35	2
25 × 4.6	5	Waters, Picotag and others	6.4	45	0.7 *M* Na.ac. + 2.5 ml/l TEA	H_2O; C = ACN–H_2O (80:20)	23	30	3,4
10 × 4.6	3	Thomson Instrument (Newark, DE, USA)	6.4	36	0.05 *M* Na.ac. + 2.25 ml/l TEA	Solvent A: ACN–Met (5:4:1)	17	20	5
15 × 3.9	3	Waters, Picotag	6.4	?	0.14 *M* Na.ac–ACN (9.4:0.6) + 1 ml/l TEA	ACN–H_2O (6:4)	18	14	7
15 × 3.9	3	Waters, Picotag	5.7	33	0.14 *M* Na.ac. + 0.7 ml/l TEA	ACN–H_2O (6:4)	20	16	8
25 × 4.6	5	Hypersil (?)	6.5	55	0.15 *M* Na.ac.	ACN	16	20	9
30 × 3.9	?	Waters, Picotag	6.4	46	0.14 *M* Na.ac.–ACN (9.4:0.6) + 0.05 ml/ml TEA	ACN–H_2O (6:4)	29	20	10
25 × 4.6	5	Dynamex C_{18}, Rainin (Emeryville, CA, USA)	5.7	45	0.1 *M* Na.ac. + 0.5 ml/l TEA	ACN–H_2O (6:4)	18	45	11
25 × 4.6	5	Hypersil, Jones (Hengoed, UK)	6.4	?	0.01 *M* Na.ac.	0.01 *M* Na.ac–ACN (4:6)	17	60	12
25 × 4.6	5	Vydac C_{18} (?)	5.0-6.8	40-60	0.05 *M* Amm.ac.	0.023 *M* Amm.ac.–ACN–H_2O (44:46:10)	17	50	13
15 × 4.6	5	Hypersil (Shandon) Nucleosil (Macherey Nagel)	7.2	amb.	0.05 *M* Na.ac. or 0.05 *M* Amm.ac.	0.1 *M* Na.ac (or Amm.ac.)–ACN–Met (46:44:10)	21	22	This work

[a] ACN = Acetonitrile; Met = methanol; TEA = triethylamine; Na.ac. = sodium acetate; Amm.ac. = ammonium acetate.
[b] Time from injection to elution of the last PTC derivative.

TABLE II

DETECTOR RESPONSES AND REPRODUCIBILITY STUDY OF PTC-AMINO ACIDS IN MODEL SOLUTIONS OBTAINED WITH (A) SODIUM ACETATE- AND (B) AMMONIUM ACETATE- CONTAINING ELUENTS OF VARIOUS pH VALUES

Values in parentheses and in quotation marks have been omitted from averages: (i) data in quotation marks indicate that the detector responses of glycine and alanine obtained with different eluents (A and B) are different; (ii) values in parentheses are higher (threonine, proline; pH = 7.6; eluent A) or lower [cyst(e)ine, lysine; pH = 7.0; eluents A and B] than the average.

Eluent	pH	Detector response (arbitrary units/pmol of amino acid)																			
		1 Aspartic acid	2 Glutamic acid	3 Hydroxyproline	4 Serine	5 Glycine	6 Histidine	7 Threonine	8 Alanine	9 Proline	10 Arginine	11 Tyrosine	12 Valine	13 Methionine	14,15 Cyst(e)ine	16 Isoleucine	17 Leucine	18 Phenylalanine	19 Ornithine	20 Tryptophan	21 Lysine
A	7.6	168	182	190	181	180	170	(214)	219	(226)	217	213	207	205	133	208	216	208	310	231	294
	7.2	165	176	181	179	174	162	189	215	208	192	201	192	197	122	208	206	202	304	226	287
	7.0	163	170	178	178	174 "176"	159	172	206 "213"	207	197	200	201	193	(111)	189	217	198	302	217	(260)
B	7.6	165	170	177	175	210	175	186	199	208	202	203	200	200	123	192	192	200	309	231	297
	7.2	167	174	178	179	212	169	171	182	195	203	207	201	208	129	195	199	198	309	231	283
	7.0	151	167	179	171	203 "208"	161	175	183 "188"	197	215	194	180	200	(101)	194	198	199	302	215	(264)
Average		163	173	181	177		166	179		203	204	203	197	201	127	198	205	201	306	225	290
Standard error		6.2	5.4	4.9	3.6		6.3	8.3		6.4	9.9	6.5	9.5	5.4	5.2	8.3	10.2	3.8	3.7	7.4	6.4
Standard error (%)		3.8	3.1	2.7	2.0		3.8	4.7		3.1	4.8	3.2	4.8	2.7	4.1	4.2	5.0	1.9	1.2	3.3	2.2

pH	Found amounts (% of the quantitative values)																			
7.0	100	100	100	100	100	100	100	100	100	100	100	100	100	84	100	100	100	100	96	90
6.8	91	93	95	96	94	96	98	96	91	95	90	91	92	80	90	94	93	95	95	85
6.4	89	95	96	94	95	96	98	95	92	95	90	92	90	78	91	95	92	96	94	88
6.0	90	92	94	94	91	96	98	91		90[a]	91	89	87	73	87	93	89	96	93	82
5.6	84	89	90	91	86	95	99	88		90[a]	91	90	85	54	87	90	79	95	86	81

[a] Could not be calculated separately.

(7:1:1:1)] were added and vortex mixed. They were prepared according to the Waters Pico-Tag Workstation manual.

The derivatized standards were dissolved in 1.00 ml of 0.05 *M* sodium acetate solution (pH 7.2). Hence 20-μl aliquots of standards contained 250, 500 and 750 pmol of each amino acid.

Chromatography

The system was a Liquochrom Model 2010 liquid chromatograph (Labor MIM, Budapest, Hungary), which consisted of two Liquopump 312/1 solvent-delivery systems and a Type OE-308 UV detector with a wavelength range of 195–440 nm. Samples were injected in 20-μl volumes using an injector supplied by Labor MIM. The columns (BST, Budapest, Hungary) were 15 cm × 4.6 mm I.D. containing Hypersil ODS bonded phase (5 μm) (Shandon) or Nucleosil 5 C_{18} (5 μm) (Macherey–Nagel). Eluents were kept under a blanket of nitrogen.

The two eluent systems each consisted of two components: (A) = 0.05 *M* sodium acetate or 0.05 *M* ammonium acetate (pH 7.2) and (B) = 0.1 *M* sodium acetate or 0.1 *M* ammonium acetate–acetonitrile–methanol (46:44:10) (mixed in volume ratios and titrated with glacial acetic acid or 50% sodium hydroxide to pH 5.6, 6.0, 6.4, 6.8, 7.2 and 7.6), henceforth called sodium acetate- or ammonium acetate-containing eluents). The gradient which was optimized for the separation was from 100% A to 100% B in 22 min; for 5 min a washing step with 100% B was applied, followed by a return to 100% A in 2 min. After an additional 4 min, elution with solvent A was performed. Thereafter the system was equilibrated for the next injection. Thus, the total time required from one injection to the next is 32 min. Both the derivatization and elution procedures were carried out at ambient temperature.

RESULTS AND DISCUSSION

The pH dependence of the elution order of PTC derivatives applying sodium acetate- or ammonium acetate-containing eluents of the same composition with various pH values (5.6, 6.0, 6.4, 6.8, 7.2 and 7.6) was tested. As shown in Figs. 1 and 2 and Table II, the detector responses obtained with eluents of pH 7.2–7.6 are independent of the nature of the salts dissolved. Thus, in reproducibility studies all values with the exception of those for cyst(e)ine and lysine, obtained with eluents of pH 7.0–7.6, could be taken into account [Table II, values of averages, standard deviations (S.D.) and relative standard deviations (R.S.D., %)].

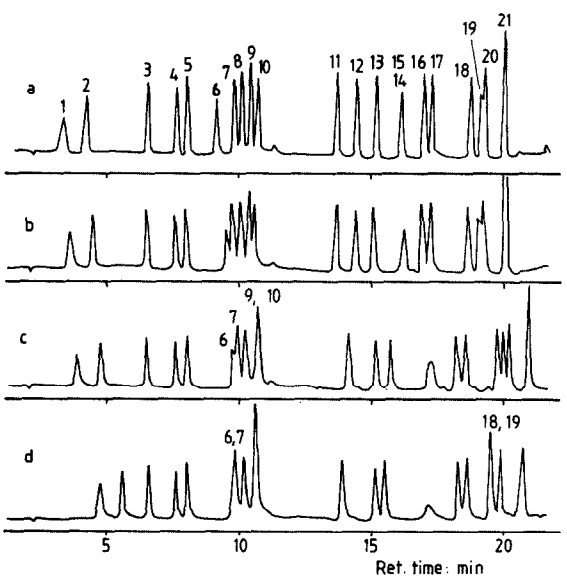

Fig. 1. HPLC with sodium acetate-containing eluents of various pH values: (a) 7.2; (b) 6.4; (c) 6.0; (d) 5.6. For peak identification, see Tables II and III. Column: Hypersil.

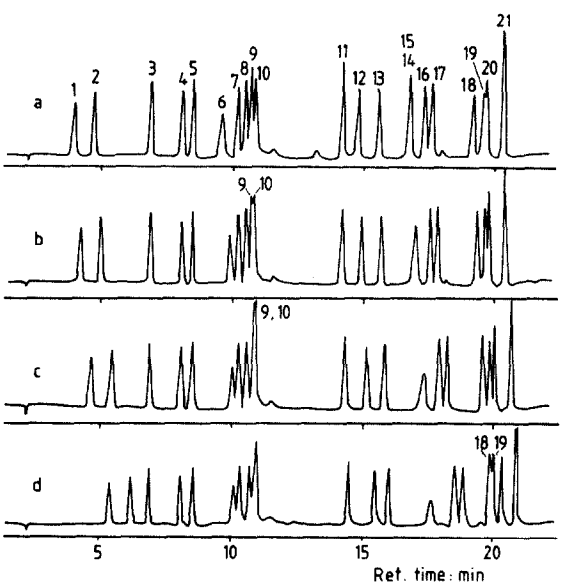

Fig. 2. HPLC with ammonium acetate-containing eluents of various pH values: (a) 7.2; (b) 6.4; (c) 6.0; (d) 5.6. For peak identification, see Tables II and III. Column: Hypersil.

TABLE III

STABILITY STUDY OF PTC-AMINO ACIDS DISSOLVED IN SODIUM ACETATE-CONTAINING ELUENT A AND STORED AT 4°C

The amounts of amino acids injected were all 500 pmol.

Time of storage (h)	Found amounts (%) of the quantitative values)																			
	1 Aspartic acid	2 Glutamic acid	3 Hydroxyproline	4 Serine	5 Glycine	6 Histidine	7 Threonine	8 Alanine	9 Proline	10 Arginine	11 Tyrosine	12 Valine	13 Methionine	14,15 Cyst(e)ine	16 Isoleucine	17 Leucine	18 Phenylalanine	19 Ornithine	20 Tryptophan	21 Lysine
0–7	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
18	100	100	100	96	100	93	100	97	100	91	100	100	100	95	100	100	100	97	100	100
26	100	96	100	96	97	91	100	91	100	89	100	100	100	95	100	100	100	97	100	100
44	100	97	100	95	98	87	92	92	100	85	100	96	100	90	100	100	95	97	94	93
73	93	92	100	92	96	85	89	89	100	85	90	87	95	84	95	95	95	93	95	91
120	91	91	100	92	96	82	80	87	100	85	90	82	95	79	89	89	94	93	95	90
190	80	79	93	87	92	81	72	77	92	76	79	66	80	70	81	81	89	91	91	88

Although between pH 7.0 and 7.6 the sodium acetate- and ammonium acetate-containing eluents resulted in the same detector responses, because of the perfect resolution of peaks 6–10 (Fig. 2a and d), and in order to obtain maximum detector responses even for cyst(e)ine and lysine, the sodium acetate-containing eluents of pH 7.2 are to be preferred [Table II, values for cyst(e)ine at pH 7.0].

The studies with eluents of lower pH 6.8–5.6) revealed following disadvantageous changes. The retention times of the earlier eluting members (peaks 1–9) increase in parallel with the decreasing pH values of eluents. Thus, at pH < 7, the peaks of histidine (peak 6) and threonine (peak 7), and those of alanine (peak 8) and arginine (peak 9) become increasingly closer to each other, then merge (Figs. 1 and 2). The detector responses of all amino acids decrease continuously (Table II, pH 6.8–5.6); considerable losses can be observed with phenylalanine, aspartic acid, glycine and methionine and an extremely high loss with cyst(e)ine. The last result could be the reason for the fact that about the half of the published studies do not mention the problem with cyst(e)ine [5,7,9,11,14–18].

Regarding the type of column, no differences in resolution and sensitivity were observed with the Hypersil and Nucleosil packings when applying strictly the same elution conditions.

Regarding the stability of PTC derivatives after dissolving them in buffers either no [2,5,7–9,11,12] or contradictory [1,3,4,6,10,13] literature data can be found. The statement [1,6] that no loss could be detected after 3 days (in the cold) proved to be incorrect [3,4,10,13]. On keeping the dissolved PTC amino acids at 5–8°C, their stability lasted only 16 h [3,4], whereas others [10,13] reported as *ca.* 5% loss after storage for 48 h at 4°C.

The stability of the PTC-amino acids dissolved in eluent A and stored at 4°C was checked immediately after their preparation and after 30 min and 7, 18, 26, 44, 73, 102 and 190 h (Table III). As can be seen, the most sensitive PTC derivatives, in decreasing order, are histidine, serine and cyst(e)ine. Hence, according to our experience, the maximum time for retaining the dissolved derivatives in their initial amounts was $\leqslant 7$ h.

The effects of the presence of TEA and "phosphate" in eluent A were also investigated, applying our optimum conditions, *i.e.*, sodium acetate-containing eluent of pH 7.2, completed with 5 and 50 μl/l and 1, 2, 3 and 4 ml/l of TEA or with $5 \cdot 10^{-3}$, $1 \cdot 10^{-2}$ and $2 \cdot 10^{-2}$ *M* phosphate. The data obtained showed that the use of both additives is of secondary importance. Although the presence of 50 μl/l and 1–4 ml/l of TEA increased the detector response of cyst(e)ine by 5–10%, at the same time the proline and arginine peaks became inseparable. Also, employing phosphate, increased sharpness of the histidine peak could not be approved confirmed [5] and only an increased aspartic acid response was observed.

In conclusion, it has been shown that in the determination of PTC-amino acids, the use of sodium acetate- instead of ammonium acetate-containing eluents improves the separation of histidine, threonine, alanine, proline and arginine, from the point of view of the maximum detector response the optimum pH of the eluents is 7.2–7.6 and PTC-amino acids dissolved in buffer should not be stored at 4°C for longer than 7 h.

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