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PEPTIDE CHARACTERIZATION WITH A SULFOETHYL ASPARTAMIDE COLUMN

DAN L. CRIMMINS*, JOHN GORKA, RICHARD S. THOMA and BENJAMIN D. SCHWARTZ
Howard Hughes Medical Institute Laboratories and Department of Medicine, Division of Rheumatology, Washington University School of Medicine, St. Louis, MO 63110 (U.S.A.)

SUMMARY

A strong cation-exchange (SCX) high-performance liquid chromatography column (sulfoethyl aspartamide, 200 × 4.6 mm) was used to analyze more than 50 peptides, ranging in length from 5 to 20 residues. These data show that the elution positions of the peptides increase monotonically with the number of positively charged residues. [A 60-min linear gradient of 0 to 100% eluent B at 1 ml/min was used, where eluent A is 5 m*M* phosphate (pH 3.0)–acetonitrile (75:25) and eluent B is eluent A + 0.5 *M* sodium chloride.] A comparison of SCX with a standard C_{18} reversed-phase (RP) column [60-min linear gradient of 0 to 60% B at 1 ml/min, where eluent A is 0.1% trifluoroacetic acid (TFA), and eluent B is 0.095% TFA–acetonitrile (10:90)] further demonstrates the utility of SCX in peptide characterization. SCX separated an $(Arg)_3$-containing peptide from the Arg-deleted peptide while RP could not. In addition, SCX and RP resolved the methionine oxidation products of ACTH (4–10) (RP: Met [O] < Met [O_2] < Met; SCX: Met [O] < Met < Met [O_2]), suggesting a mixed-mode mechanism for the ion-exchange system. Finally, SCX separated the sulfated and non-sulfated forms of cholecystokinin (26–33) and Leu-enkephalin as well as the N-terminal acetylated forms of neurotensin (8–13) and angiotensinogen (1–14) from the respective unmodified peptides.

INTRODUCTION

The widespread use and success of solid-phase peptide synthesis has allowed peptides to become essential reagents in biochemical research. These synthetic peptides are now routinely used as antigens, enzyme substrates/inhibitors, and affinity reagents. The increased demand for peptides has resulted in a need for fast, selective, and effective purification techniques. High-performance liquid chromatography (HPLC), particularly reversed-phase (RP) HPLC has been extensively used to purify peptides, especially those of a hydrophobic nature. Situations arise however, where alternative chromatographic methods are required, *e.g.*, when the peptide has limited solubility in the solvents typically employed in RP-HPLC.

Ion-exchange chromatography provides an alternative method of purification, as most peptides possess net charges either at a functional residue or an N- or C-

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terminus. Strong cation-exchange (SCX) silica-based packing materials offer certain advantages over anion-exchange silica-based resins and are thus well suited for routine peptide analysis. At pH $\leqslant$ 3, peptide carboxylate functions are expected to be protonated so that separation via SCX should primarily be a function of the number of basic residues (including the N-terminus), if conditions are used to minimize hydrophobic interaction with the silica matrix. Note that the converse situation, deprotonation of the basic residues, followed by chromatography on a silica-based anion-exchanger is impossible, owing to the chemical instability of silica under alkaline conditions. Even polymer-based anion-exchangers would be ineffective for Arg-containing peptides, because at the pH required to deprotonate this amino acid (pH > 12), the functional group on the packing itself becomes deprotonated and thus ineffective as an anion exchanger.

Several research groups have used a low pH mobile phase to analyze and purify peptides by SCX[1–4]. Recently, a silica-based SCX column packing (sulfoethyl aspartamide) has been introduced, which shows excellent selectivity for peptides[1]. We have extended this work by analyzing over 50 unmodified peptides, ranging in nominal positive charge at pH 3 from +1 to +7. When a sodium chloride gradient including 25% acetonitrile in each mobile phase is used, it is found that the retention time is monotonically related to the number of nominal positive charges at that pH. In addition, SCX chromatography is shown to resolve peptides containing modified amino acids, such as an acetylated N-terminus, an amidated C-terminus, a sulfated tyrosine, or an oxidized methionine (both the sulfoxide and sulfone). Finally, data are presented which show that SCX chromatography unequivocally separates an Arg-deleted peptide from the parent peptide while standard C_{18} RP-HPLC could not.

EXPERIMENTAL

Chemicals and reagents

Water was purified by sequential passage through an ion-exchange and a carbon canister and then through a Milli-Q system (Continental Water System, St. Louis, MO, U.S.A.) with a 0.2-μm final filter. HPLC-grade solvents were purchased from American Burdick & Jackson (Muskegon, MI, U.S.A.); HPLC-grade salts from Fisher (St. Louis, MO, U.S.A.); peptides from Penisula Labs. (Belmont, CA, U.S.A.) or Sigma (St. Louis, Mo, U.S.A.); phenol, fluorescein mercuric acetate, 5,5′-dithiobis-(2-nitrobenzoic acid), N-acetyl L-tryptophanamide and L-α-amino-*n*-butyric acid also from Sigma. Ampoules (1 ml) of 6 *M* hydrochloric acid, phenylisothiocyanate (PITC), amino acid standard H; the mobile phase additives triethylamine and trifluoracetic acid (TFA), and the endoproteases aminopeptidase M and carboxypeptidase Y were purchased from Pierce (Rockford, IL, U.S.A.); two additional endoproteases, carboxypeptidase A and carboxypeptidase B from Boehringer Mannheim (Indianapolis, IN, U.S.A.); authentic tyrosine O-sulfate from Adams Chemical (Round Lake, IL, U.S.A.); and N-methyl-mercaptoacetamide from Fluka (Ronkonkoma, NY, U.S.A.). ABI (Foster City, CA, U.S.A.) was the source for all peptide synthesis and protein/peptide sequencing reagents, except for the hydrofluoric acid cleavage scavengers, anisole and dimethyl sulfide, which were purchased from Aldrich (Milwaukee, WI, U.S.A.)

HPLC instrumentation and chromatography

Chromatography hardware included the following Waters (Milford, MA, U.S.A.) equipment: Model 510 pumps, Model 680 gradient controller, Model 710B WISP autosampler, Model 1122 column oven, and either a Model 490 detector operating at 214 nm and 280 nm or a Model 440 detector with a 254-nm filter kit. Data were acquired and analyzed using a Nelson Analytical (Cupertino, CA, U.S.A.) 763SB A/D box and a Hewlett-Packard (purchased from Nelson Analytical) 9000 Series 216 computer with Nelson 4400 series software.

Vydac C-18 RP analytical (250 × 4.6 mm) and preparative (250 × 22 mm) columns as well as the analytical sulfoethyl aspartamide SCX (200 × 4.6 mm) column were purchased from the NEST Group (Southborough, MA, U.S.A.). For analytical RP-HPLC, we employed standard TFA–acetonitrile conditions: eluent A = 0.1% aq. TFA; eluent B = 0.095% TFA–acetonitrile (10:90); 60-min linear gradient from 0 to 60% B, at 1 ml/min and 35°C. Preparative RP chromatography (ambient temperature) was usually performed by simply scaling up the flow-rates. SCX chromatography was carried out with a 60-min linear gradient from 0 to 100% B, at 1 ml/min and 28°C [eluent A = 5 m*M* phosphate (pH 3.0)–acetonitrile (75:25); eluent B = 5 m*M* phosphate/0.5*M* sodium chloride (pH 3.0)–acetonitrile (75:25)]. In the early stages of this work, an Altex (San Ramon, CA, U.S.A.) C-18-PTH (250 × 4.6 mm) column was used for the chromatography of PITC derivatized amino acids, while later, a Brownlee C-18-PTC (220 × 2.1 mm) column (obtained from ABI) was used.

Methods and quality control

Peptides were synthesized with an ABI Model 430 peptide synthesizer using Boc chemistry and standard cycle conditions, except that Boc-N-imidazole-CBZ-L-His (Bachem, Philadelphia, PA, U.S.A.) was used for His additions via an Ala-coupling cycle. Cleavage from the phenylacetamidomethyl resin by hydrofluoric acid, with anisole and dimethyl sulfide as scavengers, resulted in a crude product which was subsequently purified as described above. For quality control, the purified peptides were subjected to gas phase sequencing with an ABI Model 470A protein sequencer, using standard 03CPTH cycles and on-line Model 120A PTH Analyzer. In addition, gas phase acid hydrolysis (1 h at 150°C with 6 *M* hydrochloric acid containing 1% phenol) was performed with a Waters Work Station. Subsequent PITC derivatization and analysis (L-α-amino-*n*-butyric acid as internal standard), performed essentially as in ref. 5 then provided the amino acid composition data. Cysteine was determined with 5,5′-dithiobis-(2-nitrobenzoic acid), as described in ref. 6 with slight modifications, and cystine was estimated by fluorescein mercuric acetate titration at 499 nm with minor changes[7]. The Trp content was assessed by comparison with UV scans (210–330 nm) of known concentrations of N-acetyl-L-tryptophanamide. Methionine sulfoxide-containing peptides were converted to the corresponding methionine peptides with N-methyl-mercaptoacetamide, as adapted from ref. 8. The oxidation state of methionine was determined after aminopeptidase M or carboxypeptidase A, B, or Y digestion, followed by PITC derivatization and analysis[9]. Tyr-O-sulfate was determined by base hydrolysis with barium hydroxide and subsequent PITC derivatization and analysis[10].

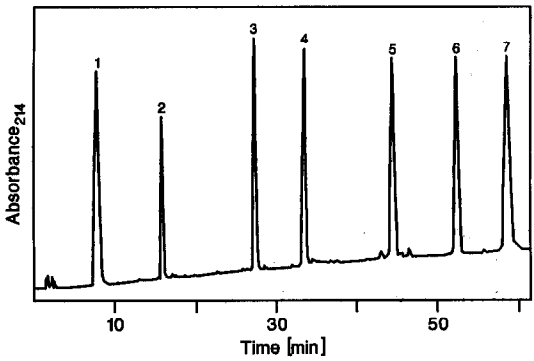

Fig. 1. SCX chromatogram of a mixture of seven peptides. The number of nominal positive charges at pH 3.0 is shown above each peak. The peptides from Table I are listed in increasing order of elution: 2, 14, 29, 42, 48, 53 and 54; 205 mV full scale (mV F.S.).

RESULTS AND DISCUSSION

One goal of this study was the characterization of the selectivity of this new cation exchanger for peptides which differ in the number of basic residues. Fig. 1 shows the chromatogram of a mixture of seven peptides, ranging in nominal positive charge at pH 3.0 from +1 to +7*. The resolution is excellent and indicates the monotonic nature of the separation, when a pH 3.0 mobile phase and salt gradient elution are used. Omission of 25% acetonitrile resulted in some peak broadening. This is in agreement with a previous observation[1] and suggests that the column can be operated in a mixed mode, if desired.

A summary of SCX chromagraphy of 47 unmodified peptides, ranging in size from 5 to 20 residues, and of 15 modified peptides is presented in Tables I and II, respectively. The retention times for these peptides follows the trend illustrated in Fig. 1. Some variation of retention times within a charge group is observed, indicating that additional factors, such as hydrophobicity and number of acidic residues, affect column selectivity. In general, reasonable predictions of the retention time for peptides with a known number of basic residues can be made.

Another aspect of our work was a comparison of SCX chromatography and standard C_{18} RP chromatography in routine peptide characterization. Fig. 2 represents RP and SCX chromatograms for the methionine oxidation products of ACTH/MSH (4–10)[D-Lys^8, Phe^9]. The elution order observed for RP chromatography (Fig. 2A) is as expected from the polarity change of the peptide resulting from conversion of methionine (I) to the sulfoxide (II) and sulfone (III). However, in SCX chromatography (Fig. 2B) the sulfone derivative (III) has the longest retention time, while the relative positions of the sulfoxide derivative (II) and the unmo-

* Chromatography of peptides in this range, which spans the majority of peptides we analyzed, was optimized. However, peptides outside this range can be analyzed by simply increasing the salt concentration in eluent B (data not shown).

TABLE I

PEPTIDES ANALYZED BY SCX CHROMATOGRAPHY

Amino acids are designated by the standard one-letter code. Peptides are grouped according to the number of nominal positive charges at pH 3.0. Basic residues are underlined. For purchased peptides the common name is shown.

Charge	*Number*	*Sequence*	*Retention time (min)*
1+	1	WAGGDASGE [sleep-inducing peptide]	8.1
	2	YPFVEPI [β-casomorphin (human)]	8.2
	3	TTNTVAAY	8.5
	4	YPFPGPI [β-casomorphin (bovine)]	8.9
	5	YGGFM [Met-enkephalin]	9.4
	6	YGGFL [Leu-enkephalin]	10.0
2+	12	DDPSPAAKSAVT	13.1
	13	CPAPPQIDNGIIQGERD	13.5
	14	VLPRPTITM	16.0
	15	VAKGSYNNTSGEQMLIIW	16.7
	16	GNSQSSKSVGGQYC	16.9
	17	KLNGIPPLELGDSCIA	17.0
	18	KAYGMPPSASAGQNL	18.7
	19	YGGFLR [dynorphin A (1–6), (porcine)]	20.5
3+	21	CNSSVDCYPENRSLFLRD	22.1
	22	DDAQYSHLGGNWAR	22.1
	23	SVINQKLKDDEVAQL	23.1
	24	EDDQYSHLQGNQLR	23.5
	25	RCLPSACEVVTGSPRGDS	23.8
	26	VVTGSPRGDFQSSWK	24.0
	27	VVTGSPRGESQSSWK	24.3
	28	PDHEPRGVITY	26.0
	29	KEKLIAPVA	26.4
	30	RGDSQSSWKSVGSQC	27.1
	31	CSHGYTGIRC	27.7
	32	MEHFKFG [ACTH/MSH (4–10), D-Lys^8, Phe^9]*	29.5
	33	RPPGFSPFR [bradykinin]	29.5
	34	SYSMEHFRWG [ACTH (1–10)]	29.7
	35	YGGFLRR [dynorphin A (1–7), (porcine)]	31.7
	36	MEHFRWG [ACTH (4–10)]	32.9
	37	RRPYIL [neurotensin (8–13)]	33.3
4+	39	KGSGTTSGTTRLLSGHTC	30.4
	40	DRVYIHPFHLLVYS [angiotensinogen (1–14), (porcine)]	31.4
	41	DYGGIKKIRLPSDDVC	31.8
	42	DRVYIHPLHL [angiotensin I (human)]	33.0
	43	SKRSSVKPGYV	37.0
	44	RRLIEDAEYAARG	37.1
	45	SGRCRGKSPSDC	38.6
	46	HHLGGAKWAGDV	39.9
5+	47	FNDYEELKHLLSSVKH	40.3
	48	RQAGDDFSRRYRGDFAE	43.8
	49	RGCPNGRRSSVKPGYV	44.8
	50	RRCPDHEPRGVITY	46.7
6+	51	FEKVKILPKDRWTQHTTTGG	49.2
	52	HRIIHKSDDEIRYEC	49.4
	53	YGGFLRRIRPKLK [dynorphin A (1–13), (porcine)]	52.3
7+	54	KPVGKKRRPVKYP [ACTH (11–24)]	58.4

* Converted from the Met [O] peptide 32′ with N-methyl mercaptoacetamide.

TABLE II

MODIFIED PEPTIDES ANALYZED BY SCX CHROMATOGRAPHY

All peptides were purchased and are grouped according to the number of nominal positive charges at pH 3.0. CCK = cholecystokinin.

Charge	*Number*	*Sequence*	*Retention time (min)*
1+	7′	DY(SO_3)MGWMDF(NH_2) [CCK (26–33), (sulfated)]	1.8
	6′	Y(SO_3)GGFL [Leu-enkephalin (sulfated)]	2.1
	7	DYMGWMDF(NH_2) [CCK (26–33)]	6.6
	5′	YGGFM[O] [Met-enkephalin, Met[O]^5]	8.2
	8	C̄YIQNC̄ [tocinoic acid]	8.5
	9	C̄YUSNC̄PIG(NH_2) [isotocin]	10.7
	6″	YGGFL(NH_2) [Leu-enkephalinamide]	11.5
	10	YWAWFA(NH_2)	12.3
	11	WAWFA(NH_2)	13.1
2+	37′	(Ac)RRPYIL [N-acetyl neurotensin (8–13)]	18.8
	20	C̄YFQNC̄PRG(NH_2) [vasopressin]	21.5
3+	40′	(Ac)DRVYIHPFHLLVYS [N-acetyl angiotensinogen (1–14), (porcine)]	21.9
	32′	M[O]EHFKFG [ACTH/MSH (4–10), Met[O]^4,D-Lys^8, Phe^9]	29.0
	38	RPKPQQFFGLM(NH_2) [Substance P]	29.3
	32″	M[O_2]EHFKFG [ACTH/MSH (4–10), Met[O_2]^4, D-Lys^8, Phe^9]	30.0

dified methionine (I) are unaltered. A plausible explanation is that for these peptides a mixed-mode retention mechanism operates in the SCX system even in the presence of 25% acetonitrile. The use of both RP and SCX chromatography allows both positive identification of the various methionine oxidation products, formed by reaction with reagents such as hydrogen peroxide and performic acid, and monitoring the progress of the reaction.

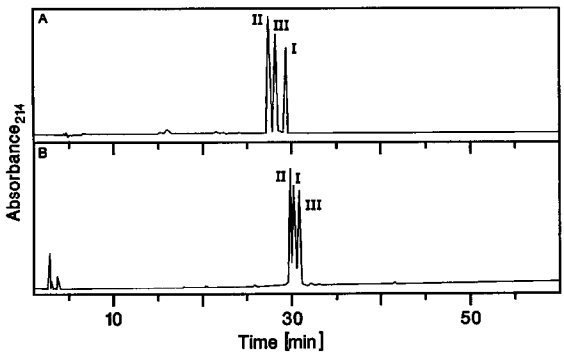

Fig. 2. RP and SCX chromatograms of a mixture of the methionine oxidation products of parent peptide 32. (A) RP chromatogram, 515 mV f.s.; (B) SCX chromatogram, 410 mV f.s. Peak I = parent peptide 32, peak II = Met [O] peptide 32′, peak III = Met [O_2] peptide 32″.

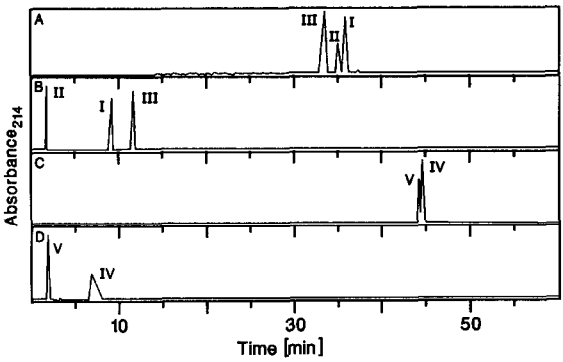

Fig. 3. RP and SCX chromatograms of sulfated and C-terminal amidated peptides. (A) RP chromatogram of peptides 6, 6′. and 6″: peak I = parent peptide 6, peak II = peptide 6′ (the sulfated derivative of peptide 6), peak III = peptide 6″ (the C-terminal amidated derivative of peptide 6); 880 mV f.s. (B) SCX chromatogram of the same peptide mixture as in Fig. 3A, 685 mV f.s. (C) RP chromatogram of peptides 7 and 7′: peak IV = C-terminal amidated parent peptide 7, peak V = peptide 7′ (the sulfated derivative of the C-terminal amidated peptide 7); 590 mV f.s. (D) SCX chromatogram of the same peptides as in Fig. 3C, 580 mV f.s.

The analysis of peptides, modified by sulfation of Tyr and C-terminal amidation, is shown in Fig. 3. Fig. 3A and B are RP and SCX chromatograms of analogues of Leu-enkephalin, respectively. Again, the elution order for RP analysis is as expected. The retention time for the amidated peptide (III) is shifted relative to the parent peptide (I) in the SCX system. This observation has been made before[1] and can be rationalized by the fact that even at pH 3.0, not all acidic functions (in this case the C-terminus) are completely protonated. Removal of this negative charge via amidation will result in a peptide which possesses a relatively greater positive charge and thus shows a greater retention time. Tyr sulfation of Leu-enkephalin (II) renders the peptide so acidic that little binding occurs for the SCX system. This result is supported by a comparison of RP (Fig. 3C) and SCX chromatography (Fig. 3D) of sulfated CCK (26–33) (V) and the unmodified peptide (IV).

N-terminal blocking, resulting from natural processes or sample manipulation during purification, is a frequent complication of the sequence analysis of peptide fragments. Fig. 4 presents the data obtained for N-acetylated neurotensin (8–13) (II), neurotensin (8–13) (I), N-acetylated angiotensinogen (1–14) (IV) and angiotensinogen (1–14) (III). While RP chromatography of both the neurotensin (8–13) pair (Fig. 4A) and the angiotensinogen (1–14) pair (Fig. 4C) resulted in good resolution, SCX analysis gave even greater separation with a concomitant shift in relative retention times (Fig. 4B and D, respectively). Thus, parallel analysis of peptides by RP and SCX chromatography allows reasonable estimates of the amount of blocked N-terminal material. It is anticipated that modification of Lys residues reft in relative retention times (Fig. 4B and D, respectively). Thus, parallel analysis of peptides by RP and SCX chromatography allows reasonable estimates of the amount of blocked N-terminal material. It is anticipated that modification of Lys residues resulting in a decreased positive charge could also be monitored by SCX chromatography. Preliminary data (not shown) indicate that reduction and alkylation of cystine-containing peptides, *e.g.*, by introduction via the modifying reagent of a negative or positive

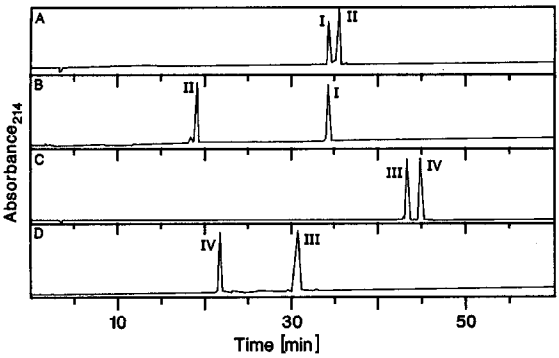

Fig. 4. RP and SCX chromatograms of N-terminal acetylated peptides. (A) RP chromatogram of peptides 37 and 37′: peak I = parent peptide 37, peak II = peptide 37′ (the N-terminal acetylated derivative of peptide 37); 140 mV f.s. (B) SCX chromatogram of the same peptides as in Fig. 4A, 165 mV f.s. (C) RP chromatogram of peptides 40 and 40′: peak III = parent peptide 40, peak IV = peptide 40′ (the N-terminal acetylated derivative of peptide 40); 555 mV f.s. (D) SCX chromatogram of the same peptides as in Fig. 4C, 235 mV f.s.

charge, can also be assessed by SCX chromatography. The retention times for three cystine-containing peptides along with the remainder of modified peptides which we have studied are listed in Table II.

Another example of the ability of SCX chromatography to resolve peptide components is displayed in Fig. 5. Difficulties are occasionally encountered during solid-phase synthesis of Arg-containing peptides. Deletion products occur, even though double-couple cycles are standard procedure, and there is the inherent difficulty in complete removal of the tosyl protecting group by hydrofluoric acid cleavage. Fig. 5A shows a standard analytical C_{18} RP chromatogram of purified peptide 50 having an input sequence of RRCPDHEPRGVITY. Sequence analysis, and to a lesser extent amino acid composition, revealed *ca.* 30% of the synthesized peptide

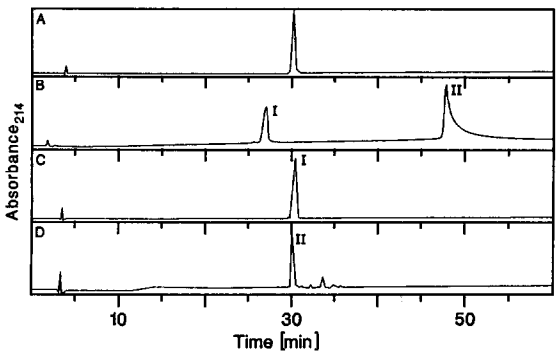

Fig. 5. RP and SCX chromatograms of a basic residue deletion peptide. (A) RP chromatogram of peptide 50, indicating apparent homogeneity; 355 mV f.s. (B) SCX chromatogram of peptide 50 showing heterogeneity; 185 mV f.s. (C) RP chromatogram of peak I from Fig. 5B; 420 mV f.s. (D) RP chromatogram of peak II from Fig. 5B; 205 mV f.s. Sequence analysis and amino acid composition confirmed that peak I = peptide 28 and peak II = peptide 50.

had amino acid deletions in the 3 N-terminal positions. The inability of both sequencing and amino acid analysis to identify cysteine positively without prior modification rendered the sequence assignment of the impurity tentative. SCX chromatography (Fig. 5B) of the same sample resolved it into two components. The two components resolved by SCX chromatography, when again analyzed by RP chromatography (Fig. 5C and D), had virtually identical retention times. Prior to sequence and amino acid analysis, each of these resolved samples was assayed for sulfhydryl residues[6]. The result of zero cysteine for peak I (peptide 28) and one cysteine for peak II (peptide 50), in conjunction with the sequence and amino acid composition data, verified the tentative sequence assignment. The above example shows that peptides with Arg deletions can be easily detected by SCX chromatography. In this case, the deleted arginines resulted from an error during the addition of the cysteine residue. In another example, (a peptide containing no cysteine 44) where identical chromatographic steps were followed, the deletion occurred at the arginine residue (data not shown). The last two results have indicated to us that SCX chromatography of Arg-containing peptides should be part of any routine quality control.

The data obtained for the sulfoethyl aspartamide SCX column indicate the general utility of this sorbent for routine peptide characterization. Most of our work compared SCX chromatograms with standard C_{18} RP chromatograms in an effort to evaluate the new column as an adjunct to commonly used RP chromatography. Parallel chromatography by both systems allowed the unambiguous assignment of the various modified amino acid residues that we examined. However, in some instances SCX chromatography provided better resolution (blocked N-terminus) and easier identification of peptide contaminants (Arg-deleted peptides) than RP chromatography. To date, more than 400 chromatograms have been developed in the SCX system without obvious signs of column deterioration. It is concluded that the sulfoethyl aspartamide column is a reliable complement to the RP column in peptide characterization.

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