Journal of Chromatography, 213 (1981) 275–286
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,924

SYSTEMATIC SEPARATION OF MEDIUM-SIZED BIOLOGICALLY ACTIVE PEPTIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

HISAO MABUCHI* and HISAMITSU NAKAHASHI
Department of Internal Medicine and Nephrology, Nishijin Hospital, Kamigyo-Ku, Kyoto 602 (Japan)
(Received March 6th, 1981)

SUMMARY

The systematic separation of medium-sized biologically active peptides by high-performance liquid chromatography (HPLC) is described. Three steps are involved: first, high-performance sodium dodecyl sulphate (SDS) gel chromatography on a newly developed column, TSK-GEL 2000SW; secondly, ion-pair reversed-phase HPLC using stepwise elution with mobile phases containing SDS and tetrabutylammonium phosphate; thirdly, high-performance cation-exchange chromatography on a Partisil SCX column for purification, using stepwise gradient elution with volatile buffers. Removal of SDS was possible in the final step. This systematic method is fast, reproducible and gives excellent separations and recoveries.

INTRODUCTION

In recent years many reports have been published on the application of high-performance liquid chromatography (HPLC) for the separation of proteins and peptides (for a review, see ref. 1). However, the use of HPLC in the field of peptide separation has generally been restricted to the final step of separation[2–8]. The systematic separation of biologically active peptides has not been reported. Until now, tedious and time-consuming traditional methods such as Sephadex gel filtration followed by ion-exchange chromatography have been used for this purpose.

We report here the first HPLC procedure for the systematic separation of medium-sized biologically active peptides having molecular weights above 500.

EXPERIMENTAL

Chemicals

Cytochrome *c* and aprotinin were obtained from Mochida (Tokyo, Japan), ovalbumin from Miles Labs. (Elkhart, IN, U.S.A.), ferritin from Boehringer (Mannheim, G.F.R.), α-chymotrypsinogen A from Sigma (St. Louis, MO, U.S.A.) and porcine MC insulin and glucagon from Novo (Bagsvaerd, Denmark). All other peptides were from the Peptide Institute (Minoh, Osaka, Japan). Sodium dodecyl

0021-9673/81/0000–0000/$02.50

[^{35}S]sulphate ([^{35}S]SDS) (12.4 mCi/mmol) and [^{3}H]leu-enkephalin (26.8 Ci/mmol) were from New England Nuclear (Boston, MA, U.S.A.). [^{32}P]Phosphoric acid (carrier free) was obtained from Japan Atomic Energy Research Institute (Ibaragi, Japan). Aqueous counting scintillant, ACS II, was from Amersham (Arlington Heights, IL, U.S.A.). All other reagents were obtained from Nakarai Chemical Co. (Kyoto, Japan).

All reagents were of analytical or HPLC grade. The solvents used were filtered through a 0.45-μm Millipore filter (Millipore, Bedford, MA, U.S.A.) and deaerated before use.

General methods

A Shimadzu Model LC-3A HPLC system (Shimadzu, Kyoto, Japan) was used which included a Model SIL injector, a Model SGR-1A step gradient former, a Model GRE-2B gradient former, a Model CRD-5A chemical reaction detector, a Model SPD-2A variable wavelength UV detector equipped with an 8-μl flow cell and a Model RF-500LC spectrofluorometer equipped with a 12-μl flow cell. Fractions were collected in a Gilson Model FC-220K fraction collector equipped with a flow interrupter (Gilson, Middleton, WI, U.S.A.). The column effluent was monitored by UV spectrophotometry at 210 or 280 nm, or by post-column fluorescence derivatization with fluorescamine[9,10]. All chromatograms were run at room temperature (*ca.* 20–23°C). Radioactivity was counted in a Aloka Model LSC-700 liquid scintillation counter (Aloka, Tokyo, Japan).

HPLC (Step I)

A prepacked TSK-GEL 2000SW column[11–14] (particle size 10 $\pm$ 2 μm; 60 $\times$ 0.75 cm) was obtained from Toyo Soda (Tokyo, Japan). According to the manufacturer, this column has fractionation ranges of mol. wt. 500–60,000. Samples were dissolved in deionized, distilled water. Analyses were performed by using a mobile phase of 0.05 *M* sodium phosphate buffer, pH 7.2, containing 0.3% (w/v) SDS, at a flow-rate of 0.3 ml/min. The void volume (V_0), and the total permeation volume, V_t, were determined by using ferritin and alanine, respectively[12]. The distribution coefficient, K_d, is defined as

$$K_d = (V_e - V_0)/(V_t - V_0)$$

where V_e is the elution volume of the sample.

Preliminary experiments revealed that the addition of SDS to the mobile phase alleviated adsorption of peptides on the column. The resolution was dependent chiefly on the concentration of SDS, the most suitable concentration being in the range 0.3–0.5% (10.7–17.3 m*M*). In this method, no pretreatment of samples was required, except for ferritin.

Ion-pair reversed-phase HPLC (Step II)

A prepacked octadecylsilane column, Cosmosil 5C_{18} (particle size 5 μm; 15 $\times$ 0.46 cm), was obtained from Nakarai. Samples were dissolved in 0.05 *M* sodium phosphate buffer, pH 7.2, containing 0.3% SDS, which was used as the mobile phase in Step I. The separation of peptides was performed by a modification of the method

described by Hancock *et al.*[15,16], using stepwise elution at a flow-rate of 1.5 ml/min. The program of mobile phases was as follows: (1) acetonitrile–water (50:50, v/v) containing 10 m*M* phosphoric acid and 15 m*M* SDS, 10 min; (2) acetonitrile–water (60:40) containing 15 m*M* SDS, 10 min; (3) acetonitrile–water (75:25) containing 15 m*M* SDS, 10 min; (4) acetonitrile–water (50:50) containing 15 m*M* SDS and 1 m*M* tetrabutylammonium phosphate (TBA), 10 min.

UV detection was not suitable for flow monitoring over 25 min due to significant baseline drift at high sensitivities.

High-performance cation-exchange chromatography (Step III)

A prepacked Partisil SCX column (25 × 0.46 cm) was obtained from Whatman (Maidstone, Great Britain). Samples were dissolved in 75% acetonitrile–water containing 15 m*M* SDS. The separation was performed by a modification of the method described by Radhakrishnan *et al.*[17], using stepwise gradient elution with pyridine–acetate buffers. The program of mobile phases and flow-rates was as follows: (1) distilled water or 0.005 *M* pyridine–0.04 *M* acetic acid at a flow-rate of 1.5 ml/min, 15 min; (2) 3.0 *M* pyridine–0.5 *M* acetic acid at a flow-rate of 0.5 ml/min, 30 min.

Pyridine–acetate buffers exhibit UV absorbance, therefore the UV detector is unsuitable in this case. The detection system used was the fluorescamine method. Some peptides such as neurotensin having amino terminal prolyl or pyroglutamyl residues are weakly reactive or undetectable with fluorescamine[18]. Fractions obtained were applied in Step II for the detection of these peptides.

Removal of SDS was estimated with [^{35}S]SDS. The sample solutions were prepared by incubating the peptide samples, at concentrations of 5–20 nmol/ml, in acetonitrile–water (50:50) containing 1% SDS and $9.6 \cdot 10^{-4}$% [^{35}S]SDS at *ca.* 20°C overnight.

Recovery

Recoveries of peptides were estimated by using [^{3}H]leu-enkephalin.

RESULTS AND DISCUSSION

Step I

Adsorption of peptides on the column could not be alleviated by adding salts such as sodium hydroxide or sodium sulphate to the mobile phase[12]. Kato *et al.*[14] have reported that the separation range of TSK-GEL columns was not extended below mol. wt. 10,000 even by use of a 2000SW column which has a smaller pore size, and the elution behaviour of samples was greatly affected by sodium phosphate concentration in the eluent. They used a 0.05–0.2 *M* sodium phosphate buffer containing 0.1% SDS as the eluent. Frenkel and Blagrove[19] have reported the gel filtration of proteins and peptides in denaturing solvents (6 *M* urea and 0.5% SDS) over controlled pore glass (CPG) for molecular weight determination in the range of 3500–12,000. Adsorption of samples on the CPG column could be alleviated by increasing the concentration of SDS to 0.5% and including 6 *M* urea in the phosphate buffer. Our preliminary experiments revealed that the addition of 0.3–0.5% SDS was effective in alleviating the adsorption of peptides on the TSK-GEL 2000SW column.

The elution volumes, K_d values and molecular weights of samples are shown in Table I. Pretreatment with SDS had no significant effect on the elution behaviour of samples, except for ferritin. The reproducibility of results was better than ±2% (relative standard deviation). The elution order of samples seems to follow that of the molecular weights. This rule, of course, cannot be generalized. A semilogarithmic plot of molecular weight *versus* K_d for several samples is shown in Fig. 1. The linearity could not be extended below mol. wt. 5000. It was possible to estimate the relative molecular weights of proteins and peptides in the range of 1000–45,000 despite the deviation of several samples.

TABLE I

ELUTION VOLUMES (V_e), K_d VALUES AND MOLECULAR WEIGHTS OF SAMPLES CHROMATOGRAPHED ON A TSK-GEL 2000SW COLUMN

No.	*Sample*	V_e *(ml)*	K_d	*Mol.wt.*
1	Ferritin (V_0)	12.6	0	480,000
2	Ovalbumin	12.6	0	45,000
3	α-Chymotrypsinogen A	13.2	0.058	25,700
4	Cytochrome *c*	14.5	0.183	12,400
5	Aprotinin	14.5	0.183	6520
6	Insulin (porcine)	14.6	0.192	5782
7	Glucagon	14.8	0.211	3485
8	Tetracosactide	14.8	0.211	2934
9	α-Endorphin	15.9	0.317	1746
10	Neurotensin	15.0	0.231	1673
11	α-Melanocyte stimulating hormone	15.2	0.250	1665
12	Somatostatin	15.4	0.270	1638
13	Substance P	15.1	0.240	1348
14	Luteinizing hormone releasing hormone	14.9	0.221	1182
15	Bradykinin	15.2	0.250	1060
16	Angiotensin I	15.0	0.231	1297
17	Angiotensin II	15.8	0.308	1046
18	Angiotensin III	15.6	0.288	931
19	Oxytocin	15.0	0.250	1007
20	Vasopressin	15.2	0.250	1056
21	Arg-vasotocin	15.3	0.260	1022
22	β-Casmorphin	17.5	0.471	790
23	β-Casmorphin (1–5)	19.0	0.615	580
24	Thymopoietin active fragment (32–36)	16.8	0.404	680
25	Met-enkephalin	20.2	0.731	574
26	Leu-enkephalin	19.0	0.615	556
27	Tuftsin	16.4	0.365	501
28	Arginine	21.0	0.808	174
29	Alanine (V_t)	23.0	1.000	89
30	Other amino acids	>22.8	–	–

This method is rapid and simple compared with SDS polyacrylamide gel electrophoresis[20,21] in the estimation of peptide molecular weights. The fluorescamine system can detect as little as 10 pmol peptides[9,22], therefore high sensitivity is obtainable.

Regnier *et al.*[23] have reported that the ratio $V_t - V_0/V_0$ in gel permeation

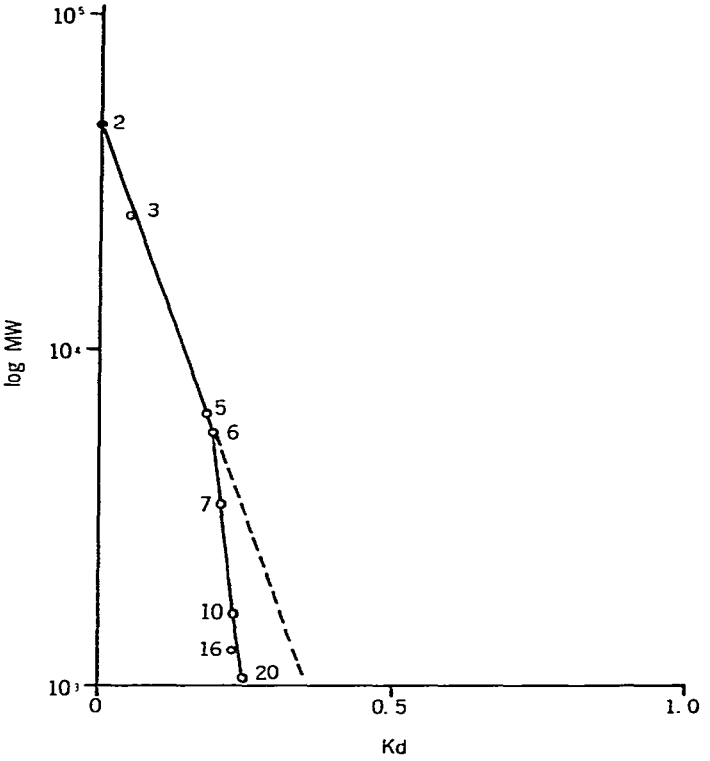

Fig. 1. Semilogarithmic plot of molecular weight (MW) *versus* K_d for several samples chromatographed on a TSK-GEL 2000SW column. Sample numbers as in Table I.

chromatography cannot exceed 1.3. The ratio obtained from our results is about 0.83. However, other phenomena such as partition are more likely to be predominant in the separation of peptides having molecular weights below 5000.

Recently, we have reported[24] the analysis of medium-molecular-weight peptides in normal and uremic body fluids using the method reported here.

Step II

The results are shown in Table II. The reproducibility was better than $\pm 3\%$ (relative standard deviation). Using this program for the stepwise elution, samples having molecular weights above 6000 were not eluted. It was possible, however, to elute these samples using other programs of mobile phases (data not shown). Several linear gradients tested gave poor resolutions and lacked reproducibility. The samples dissolved in solutions containing SDS showed different behaviours from the samples dissolved in distilled water when other ion-pairing reagents such as phosphoric acid[15,25] were used, and some samples were strongly retained on the column. Therefore, we chose SDS as the anionic ion-pairing reagent.

Hancock *et al.*[15] have used lower concentrations of SDS (< 5 mM) in an effort to minimize the problems of removal of SDS from the samples. We used higher concentrations of SDS (15 mM) in order to minimize band broadening or tailing and

TABLE II

RETENTION TIMES OF SAMPLES CHROMATOGRAPHED ON A COSMOSIL $5C_{18}$ COLUMN

Samples as in Table I. ND = Not done; NE = not eluted.

No.	*Retention time (min)*	*No.*	*Retention time (min)*
1	ND	16	25.1
2	ND	17	14.8
3	ND	18	27.1
4	NE	19	3.9
5	NE	20	11.3
6	35.6	21	2.4
7	35.3	22	3.3
8	35.2	23	2.3
9	6.4	24	16.2
10	24.1	25	3.1
11	23.2	26	3.8
12	21.1	27	15.7
13	28.6	28	6.7
14	11.0	29	ND
15	24.2	30	<5.8

for resolution. Hancock *et al.*[16] have also shown that an equimolar mixture of the anionic (SDS) and cationic (dodecylammonium acetate) reagents resulted in rapid elution of peptide and protein samples. Our preliminary experiments, however, revealed that the addition of as little as 1 m*M* of the cationic ion-pairing reagent TBA to acetonitrile–water (50:50) containing 15 m*M* SDS is effective for the elution of larger peptides such as insulin.

Step III

All peptides tested were eluted within 40 min as sharp peaks. SDS was eluted with distilled water, therefore it was readily removed. An example of an elution profile is given in Fig. 2. The baseline elevation and ghost peak were essentially inevitable. The removal of [^{35}S]SDS within 15 min was 98.7 ± 1.4% (mean ± S.D., n = 3). Acetonitrile, monitored with a UV detector at 210 nm, was eluted as a sharp peak near the void volume. Moreover, phosphoric acid could be eliminated and the removal of [^{32}P]phosphoric acid within 6 min was 99.4 ± 3.6% (mean ± S.D., n = 4) (Fig. 3). The removal of TBA and the removal rate of acetonitrile were not estimated in this study.

However, large peptides such as insulin were eluted as two peaks, that is, a SDS–peptide complex peak and a native peptide peak (Fig. 4A). The dissociation of the SDS–peptide complex may thus be incomplete in distilled water. For these peptides, instead of distilled water, 0.005 *M* pyridine–0.04 *M* acetate buffer was effective in dissociating the SDS–peptide complex (Fig. 4B). The important question to consider is whether or not SDS truly binds to small peptides. Most proteins bind 1.44 g SDS per g of protein[26,27]. However, the degree of binding of SDS to small peptides has not been reported. Assuming that small peptides bind 1.44 g of SDS per g peptide, the net amounts of bound SDS are small on a molar basis compared with large

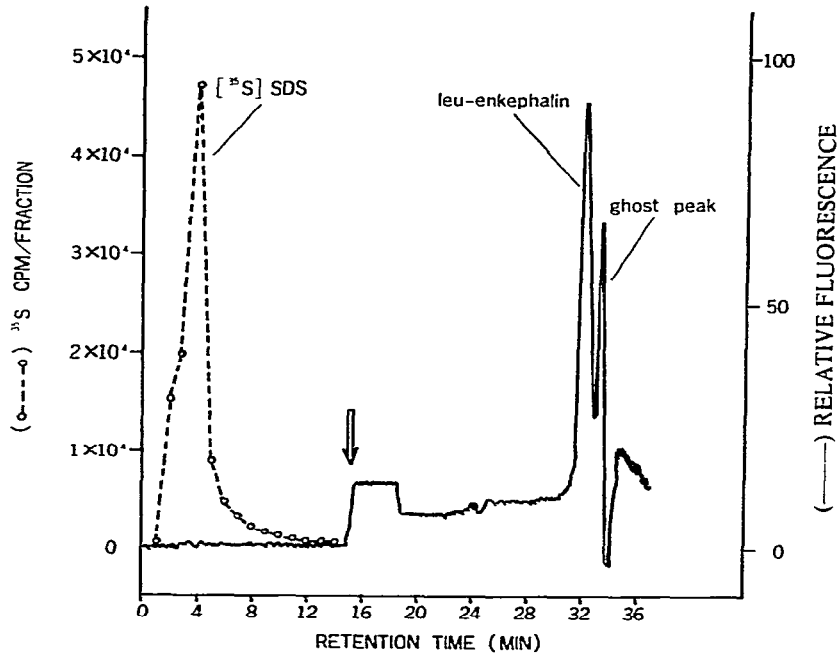

Fig. 2. Elution profile of leu-enkephalin dissolved in a solution containing SDS and [^{35}S]SDS chromatographed on a Partisil SCX column with a stepwise gradient elution. Mobile phase: (1) distilled water at a flow-rate of 1.5 ml/min, 15 min; (2) 3.0 *M* pyridine–0.5 *M* acetic acid at a flow-rate of 0.5 ml/min, 30 min. The arrow indicates the beginning of the elution with phase 2. Radioactivity was determined by collecting eluate fractions at 1-min intervals.

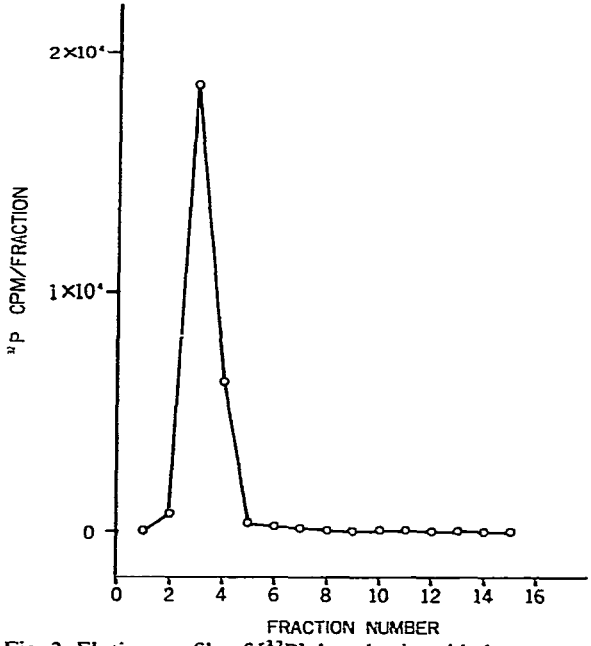

Fig. 3. Elution profile of [^{32}P]phosphoric acid chromatographed on a Partisil SCX column with distilled water at a flow-rate of 1.5 ml/min. Eluate fractions were collected at 1-min intervals.

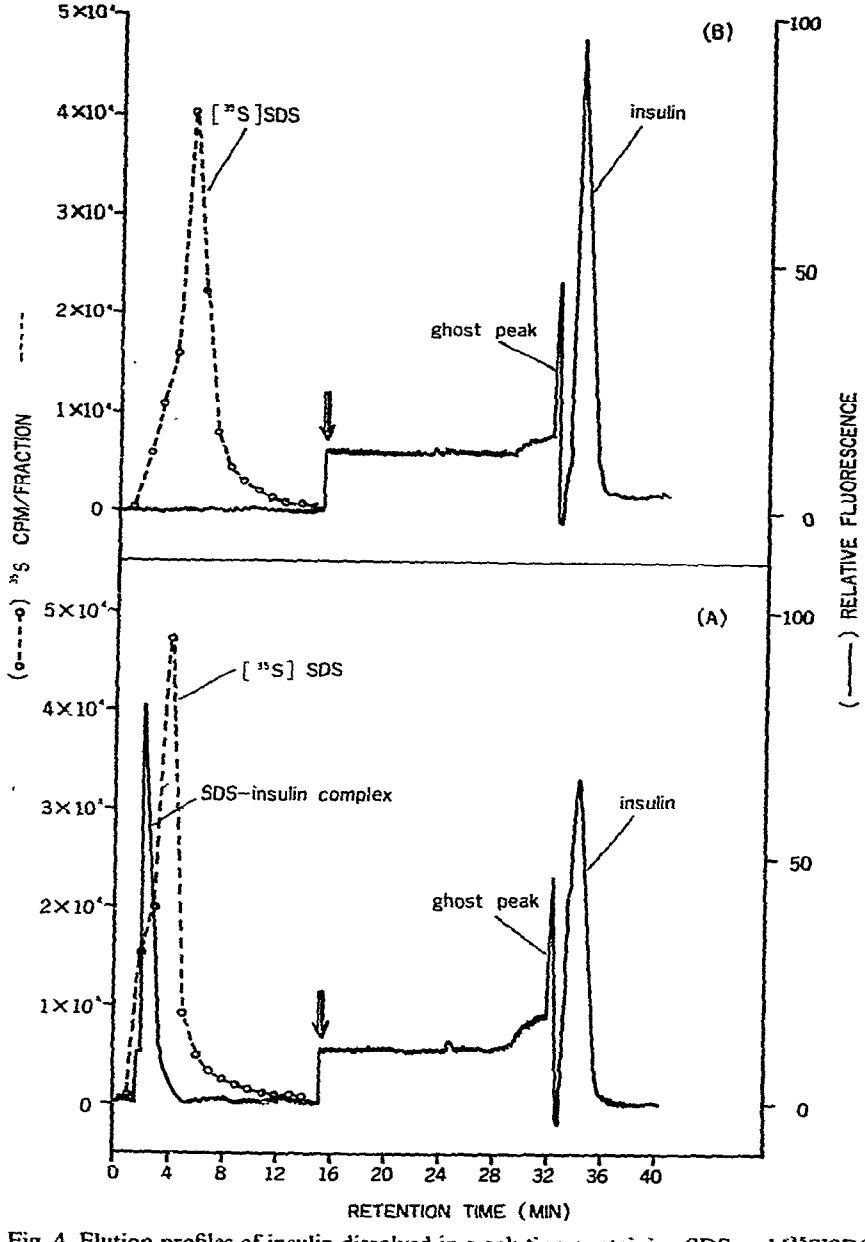

Fig. 4. Elution profiles of insulin dissolved in a solution containing SDS and [^{35}S]SDS chromatographed on a Partisil SCX column with stepwise gradient elution. Mobile phase: (A) (1), (2) as in Fig. 2; (B) (1) 0.005 *M* pyridine–0.04 *M* acetic acid at a flow-rate of 1.5 ml/min; (2) 3.0 *M* pyridine–0.5 *M* acetic acid at a flow-rate of 0.5 ml/min, 30 min. The arrows indicate the beginning of elution with 3.0 *M* pyridine–0.5 *M* acetic acid. Radioactivity was determined by collecting eluate fractions at 1-min intervals.

peptides. Probably, this is the reason why small peptides are not eluted as two peaks, but its proof remains elusive.

SDS is one of the most potent protein denaturants and solubilizing agents and has been widely used. It may be removed by prolonged dialysis. However, it is not applicable to peptide samples. The method reported here is rapid compared with previous methods[28–30]. In addition, the solvents used are readily removed from the eluted samples by lyophilization and facilitate further direct investigations of separated samples. Moreover, isocratic or gradient elutions with pyridine–acetate buffers are useful for further separations of peptides[17]. The application of this method to the removal of SDS from proteins is now under investigation.

The fluorescamine detection system is destructive to samples without the stream-splitting device described by Bohlen *et al.*[9]. This is the only disadvantage of the method.

Systematic separation

Examples of systematic separations of a mixture of samples are shown in Figs. 5 and 6. All peptides tested were well separated. If further separations of peptides are

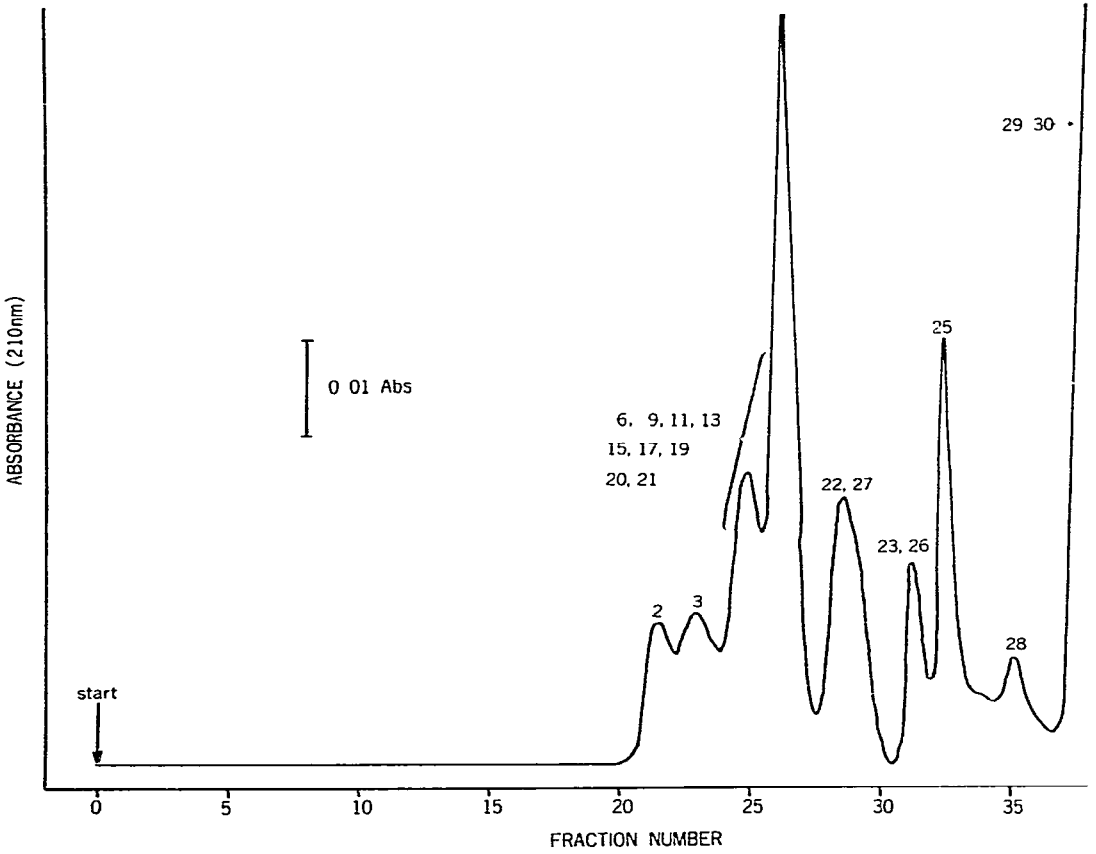

Fig. 5. Systematic separation (Step I) of a mixture containing 0.5–10 μg of each sample applied to a TSK-GEL 2000SW column. Eluate fractions were collected at 2-min intervals. Sample numbers as in Table I. See text for details.

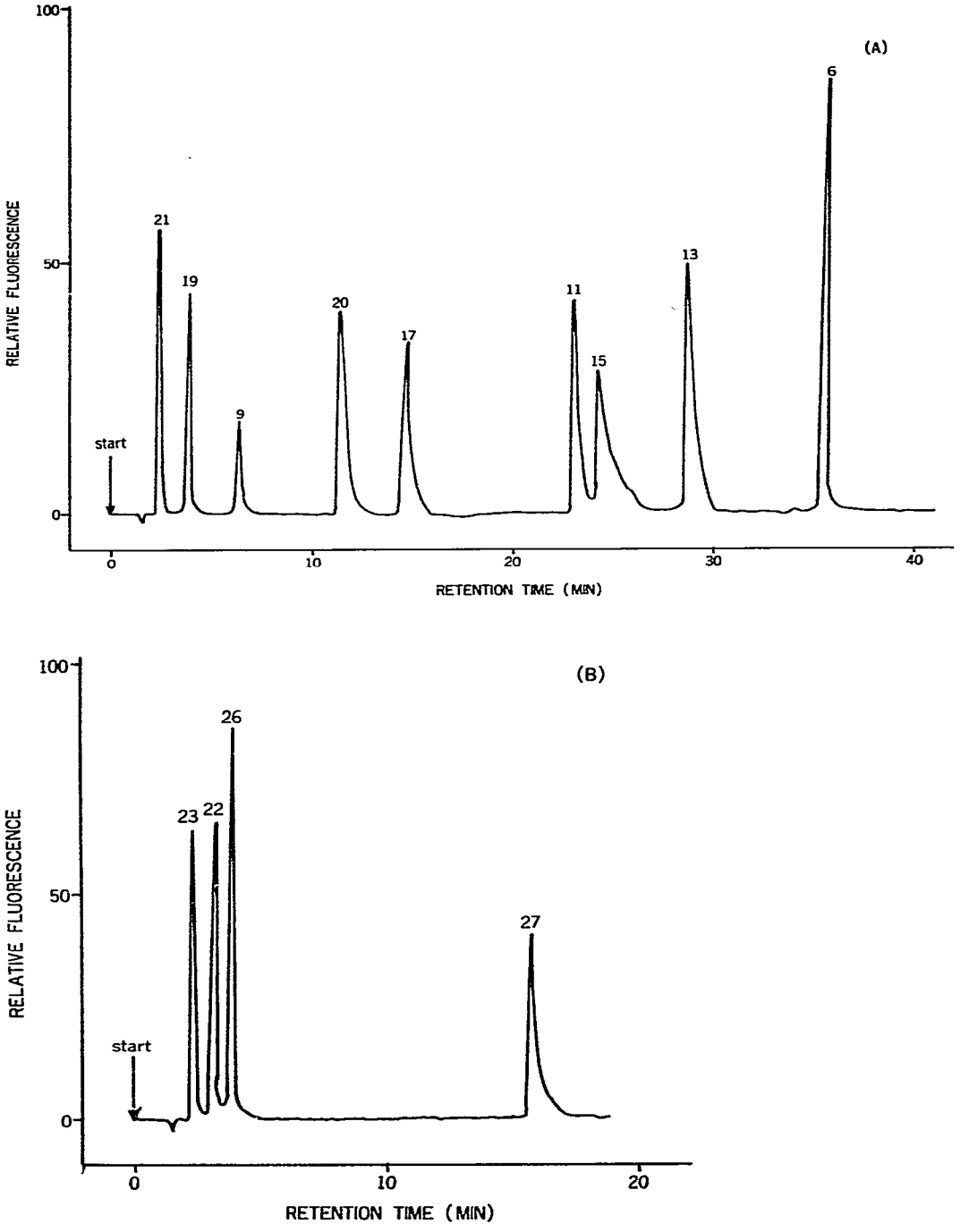

Fig. 6. Systematic separation (Step II) of fractions 25–27 (A) and 28–32 (B) obtained from TSK-GEL 2000SW applied to a Cosmosil $5C_{18}$ column. Sample sizes: 500 μl. Sample numbers as in Table I. See text for details.

necessary, isocratic or gradient elutions with pyridine–acetate buffers on a Partisil SCX column are useful. The three HPLC steps allowed the separation of peptides with an analysis time of about 150 min.

Recovery

The data in Table III indicate that the recovery is in excess of 90% in all the steps. The maximum sample size was not determined, however, as much as 100 μg of oxytocin were eluted without any signs of overloading.

TABLE III

RECOVERIES OF [^{3}H]LEU-ENKEPHALIN

Loading, 20 ng.

Step	*Recovery (%) (mean ± S.D., n = 4)*
I	90.4 ± 1.0
II	99.8 ± 5.5
III	90.0 ± 3.0

In conclusion, this systematic separation and purification of medium-sized biologically active peptides by HPLC may have a wide applicability in biological samples.

REFERENCES

1 F. E. Regnier and K. M. Gooding, *Anal. Biochem.*, 103 (1980) 1.
2 M. Rubinstein, S. Stein and S. Udenfriend, *Proc. Nat. Acad. Sci. U.S.*, 74 (1977) 4969.
3 S. Kimura, R. V. Lewis, A. S. Stern, J. Rossier, S. Stein and S. Udenfriend, *Proc. Nat. Acad. Sci. U.S.*, 77 (1980) 1681.
4 R. Lewis, A. S. Stern, S. Kimura, S. Stein and S. Udenfriend, *Proc. Nat. Acad. Sci. U.S.*, 77 (1980) 5018.
5 R. C. C. Chang, W. Y. Huong, T. W. Redding, A. Arimura, D. H. Coy and A. V. Schally, *Biochim. Biophys. Acta*, 625 (1980) 266.
6 B. Sharp, A. E. Pekary, N. V. Meyer and J. M. Hershman, *Biochem. Biophys. Res. Commun.*, 95 (1980) 618.
7 V. Clement-Jones, R. Corder and P. J. Lowry, *Biochem. Biophys. Res. Commun.*, 95 (1980) 665.
8 N. Minamino, K. Kanagawa, A. Fukuda and H. Matsuo, *Biochem. Biophys. Res. Commun.*, 95 (1980) 1475.
9 P. Bohlen, S. Stein, J. Stone and S. Udenfriend, *Anal. Biochem.*, 67 (1975) 438.
10 R. W. Frei, L. Michel and W. Santi, *J. Chromatogr.*, 126 (1976) 665.
11 K. Fukano, K. Komiya, H. Sasaki and T. Hashimoto, *J. Chromatogr.*, 166 (1978) 47.
12 S. Rokushika, T. Ohkawa and H. Hatano, *J. Chromatogr.*, 176 (1979) 456.
13 Y. Kato, K. Komiya, H. Sasaki and T. Hashimoto, *J. Chromatogr.*, 190 (1980) 297.
14 Y. Kato, K. Komiya, H. Sasaki and T. Hashimoto, *J. Chromatogr.*, 193 (1980) 29.
15 W. S. Hancock, C. A. Bishop, L. J. Meyer, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 161 (1978) 291.
16 W. S. Hancock, C. A. Bishop, J. E. Battersby, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 168 (1979) 377.
17 A. N. Radhakrishnan, S. Stein, A. Licht, K. A. Gruber and S. Udenfriend, *J. Chromatogr.*, 132 (1977) 552.

18 N. Nakai, C. Y. Lai and B. L. Horecker, *Anal. Biochem.*, 58 (1974) 563.
19 M. J. Frenkel and R. J. Blagrove, *J. Chromatogr.*, 111 (1975) 397.
20 R. T. Swank and K. D. Munkres, *Anal. Biochem.*, 39 (1971) 462.
21 T. Kato, M. Sasaki and S. Kimura, *Anal. Biochem.*, 66 (1975) 515.
22 S. Udenfriend, S. Stein, P. Bohlen, W. Dairman, W. Leimgruber and M. Weigele, *Science*, 178 (1972) 871.
23 F. E. Regnier, K. M. Gooding and S. H. Chang, in D. M. Hercules, G. M. Hieffthe, L. R. Snyder and M. A. Evanson (Editors), *Contemporary Topics in Analytical and Clinical Chemistry*, Vol. 1, Plenum, New York, 1977, pp. 1–48.
24 H. Mabuchi and H. Nakahashi, *J. Chromatogr.*, 224 (1981) 322.
25 M. J. O'Hare and E. C. Nice, *J. Chromatogr.*, 171 (1979) 209.
26 R. Pitt-Rivers and F. S. A. Impiombato, *Biochem. J.*, 109 (1968) 825.
27 J. A. Reynolds and C. Tanford, *Proc. Nat. Acad. Sci. U.S.*, 66 (1970) 1002.
28 J. Lenard, *Biochem. Biophys. Res. Commun.*, 45 (1971) 662.
29 G. P. Tuszynski and L. Warren, *Anal. Biochem.*, 67 (1975) 55.
30 I. P. Griffith, *J. Chromatogr.*, 109 (1975) 399.