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ESTIMATION OF MOLECULAR WEIGHTS OF PEPTIDES BY DETERMINATION OF HEIGHT EQUIVALENT TO A THEORETICAL PLATE IN SIZE-EXCLUSION CHROMATOGRAPHY

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SUMMARY

The analysis of the flow-rate required to obtain the optimum height equivalent to a theoretical plate, HETP, has been used to estimate the molecular weight, MW, of peptides in size-exclusion chromatography. A straight line is obtained when log MW is plotted against the flow-rate which gives the optimum HETP. This relationship holds even for peptides which adsorb to column packings. The typical quantity of peptide required is 1 nmol and analysis time was less than 2 h. The method is applied to peptides of 200–10,000, and the precision is $\pm 20\%$. The method may be applicable to the analysis of MW for all classes of compounds.

INTRODUCTION

The estimation of the molecular weight, MW, of proteins can be performed by either size-exclusion chromatography[1–4] or by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis[5]. However, these methods cannot always be applied for peptides of small MW. Gel electrophoresis is not suitable because of too large a pore size. Many column packing materials have been developed for size-exclusion chromatography, but there are inherent difficulties in their use to estimate small MW. Because ionic, hydrophobic interactions between solutes and column packing materials become more pronounced in the separation, the estimation of low MWs is not easily performed from the elution volume. Addition of salts, organic solvents or detergents to prevent anomalous elution in size-exclusion chromatography is not always satisfactory.

However, in a size-exclusion analysis, some chromatographic factors such as Eddy and longitudinal diffusion and the time to reach equilibrium may be MW dependent, and the diffusion or equilibrium should occur more rapidly for substances of small MW. If this holds true, the flow-rate to obtain the optimum height equivalent to a theoretical plate, HETP, should depend on the MW of the solute considered. In this paper we describe the relationship between the MW of the solute and the flow-rate required to obtain the optimum HETP.

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EXPERIMENTAL

Chemicals

Bovine γ-globlin, bovine serum albumin, cytochrome c, insulin, insulin A and B chain S-sulphonate, melittin and gramicidine J were obtained from Sigma (St. Louis, MO, U.S.A.), glucagon was from Calbiochem (Los Angeles, CA, U.S.A.) and secretin was a gift from Dr. S. Tachibana (Eisai Pharmaceutical Ltd., Tokyo, Japan). The peptides mentioned above were of natural origin; other peptides were synthetic. ACTH was a gift from Dr. R. Matsueda (Sankyo Pharmaceutical Ltd., Tokyo, Japan), and insulin C-peptide and vasoactive intestinal peptide (VIP) were gifts from Dr. N. Yanaihara (Shizuoka College of Pharmaceutical Sciences, Shizuoka, Japan). Other synthetic peptides were obtained from Protein Research Foundation (Osaka, Japan). The peptides studied are listed in Table I. Other chemicals including solvents for chromatography were obtained from Yoneyama Pharmaceutical Ltd. (Osaka, Japan). All were used without further purifications.

Size-exclusion chromatography

The high-performance liquid chromatographic (HPLC) system used was a TSK 805 with a TSK GEL G2000SW size-exclusion column (10 μm, 7.5 × 600 mm; Toyo Soda Ltd., Tokyo, Japan). Post-column detection of peptides was carried out by the *o*-phthalaldehyde (OPA) method according to Benson and Hare[6]. The fluorometer used was a FLD-1 (Shimadzu, Kyoto, Japan). The typical quantity of peptide needed for one chromatogram was about 0.2 nmol. The void volume was determined from the elution volume of Blue Dextran.

RESULTS AND DISCUSSION

Elution of peptides from the size-exclusion column

Over 50 peptides (MW 200–10,000, as shown in Table I) were tested to determine whether there were good relationships between MW and elution volumes (capacity factors), using some elution solvents. As shown in Table II, the elution volumes (capacity factors) of the peptides differed from one solvent to another. In the tested solvent systems, 0.15 *M* phosphate buffer (pH 7.4) containing 1 *M* NaCl, 20% methyl cellosolve and 1% SDS eluted peptides from the column in approximate order of MW as shown in Fig. 1. However, two groups of peptides were eluted in unexpected positions. One group adsorbed to the resin and eluted later than expected, and contained Gly–Trp, angiotensin I and II and insulin A and B chain S-sulphonate. The second group were eluted faster than expected, and contained melittin and mastoparan. The reason for the faster elution was not clear. Thus the estimation of the MW of unknown peptides from simple size-exclusion chromatography is not promising.

Relationship of MW and the rate of attainment of equilibrium

Using model peptides of approximately the same MW but of different elution volumes from the size-exclusion column, the relationships between the flow-rate and the HETP were obtained as shown in Fig. 2. Clearly, when the MW of the peptides were approximately the same, the flow-rates required to obtain optimum HETP were

TABLE I

THE PEPTIDES TESTED

Substance	*MW*	*Log MW*	*Isoelectric point*	*Hydrophobicity**	*Biological activity*
1 γ-Globulin	160,000	5.20			–
2 Albumin	70,000	4.85	4.0		–
3 Cytochrome *c*	12,384	4.09	10.0		+
4 Insulin	5750	3.76	5.0	36.1	+
5 ACTH	4500	3.65	4.7		+
6 Glucagon	3550	3.55	6.0	8.5	+
7 Insulin C-peptide	3306	3.52	6.1	13.5	?
8 Vasoactive intestinal peptide (VIP)	3325	3.52	6.4	15.1	+
9 Insulin B chain S-sulphonate	3040	3.48	3.8	22.7	–
10 Secretin	2956	3.47	9.0	9.9	+
11 Melittin	2848	3.45	6.2	17.5	+
12 α-Endorphin	1684	3.23		6.9	+
13 Insulin A chain S-sulphonate	1653	3.22	6.4	13.4	–
14 Somatostatin	1638	3.21	6.3	10.7	+
15 Mastoparan	1479	3.17	6.8	14.6	+
16 Substance P	1348	3.13	10.9	7.9	+
17 Vespakinin	1344	3.13	9.6	10.7	+
18 Met–Lys–bradykinin	1319	3.12	9.4	8.6	+
19 Physalaemin	1266	3.1	7.1	9.0	+
20 Angiotensin I	1297	3.1	6.8	8.9	+
21 Gramicidin J	1369	3.14	7.2	13.4	+
22 Lys–bradykinin	1188	3.07	11.9	7.5	+
23 Bradykinin potentiator B	1181	3.07	8.5	9.6	+
24 Bradykinin	1060	3.03	9.4	7.0	+
25 Angiotensin II	1046	3.02	7.4	7.1	+
26 Val^1–Thr^6–bradykinin	1017	3.01	8.3	8.7	+
27 Angiotensin III	931	2.97	6.9	7.2	?
28 Eledoisin related peptide	707	2.85	9.6	7.8	+
29 Glutathione oxidized	613	2.79	5.9	0.0	?
30 [Leu^5]–enkephalin	556	2.74	7.2	5.9	+
31 Tuftsin	501	2.70	8.7	1.3	+
32 $(Gly)_6$	360	2.56	6.0	0	–
33 Liver cell growth factor	340	2.53	7.0	0.3	+

(Continued on p. 344)

TABLE I *(continued)*

Substance	*MW*	*Log MW*	*Isoelectric point*	*Hydrophobicity**	*Biological activity*
34 Reduced glutathione	307	2.49	5.9	0.0	?
35 $(Gly)_3$	303	2.48	6.0	0.0	–
36 Gly–Gly–Arg	288	2.46	8.3		–
37 Gly–Gly–His	269	2.43	5.8	– 0.2	–
38 $(Glu)_2$	275	2.44	3.2	– 2.2	–
39 His–Leu	268	2.43	5.8	1.8	–
40 Gly–Gly–Leu	245	2.42	6.0	1.9	–
41 $(Gly)_2$	246	2.39	6.0	0.0	–
42 Leu–Gly–Gly	245	2.39	6.0	1.9	–
43 Gly–Trp	260	2.42	6.0	2.3	–
44 Gly–Phe	222	2.35	5.7	2.2	–
45 Gly–His	212	2.33	5.8	– 0.2	–
46 Gly–Lys	203	2.31	7.4	0.5	–
47 $(Gly)_3$	189	2.28	6.0	0.0	–
48 Gly–Leu	188	2.27	6.0	1.9	–
49 Leu–Gly	188	2.27	6.0	1.9	–
50 $(Gly)_2$	132	2.12	6.0	0.0	–

* Relative lipophilicities of the side chains according to Rekker[9], excluding Arg and Orn residues.

also the same, even so the optimum HETP value for each peptide was different due to different elution mechanisms. Peptides, which adsorbed to the column and were eluted later showed smaller HETP. Fig. 3 shows the relationship between MW and the flow-rate required to obtain optimum HETP for the model peptides. It is clear that the optimum flow-rate is inversely and linearly related to log MW. The relationship holds even for peptides which were eluted from the column at unexpected positions from the viewpoint of size-exclusion chromatography. This relationship was also true when the elution solvent was a simple phosphate–saline, although the retention volumes were larger for many peptides due to strong adsorptive effects and the time required for one analysis was increased.

Estimation of the MW

First a working plot of the relationship between log MW and the optimum flow-rate is drawn using at least three peptides of different MW on the column being used, as follows. By use of three flow-rates, ranging from 0.2 to 1.0 ml/min, each HETP is calculated. As $HETP = A + B/u + Cu$ (refs. 7 and 8), where u is the flow-rate and A, B and C are constants for each peptide under the same analytical conditions including temperature, from three HETP values from different flow-rates, A, B and C can be calculated. For the optimum HETP, the flow-rate should be $\sqrt{B/C}$.

The optimum flow-rate for the sample peptide is then determined by the same procedure.

From the working plot obtained in the first step and the optimum flow-rate for the sample obtained in the second step, the MW of the sample can be estimated.

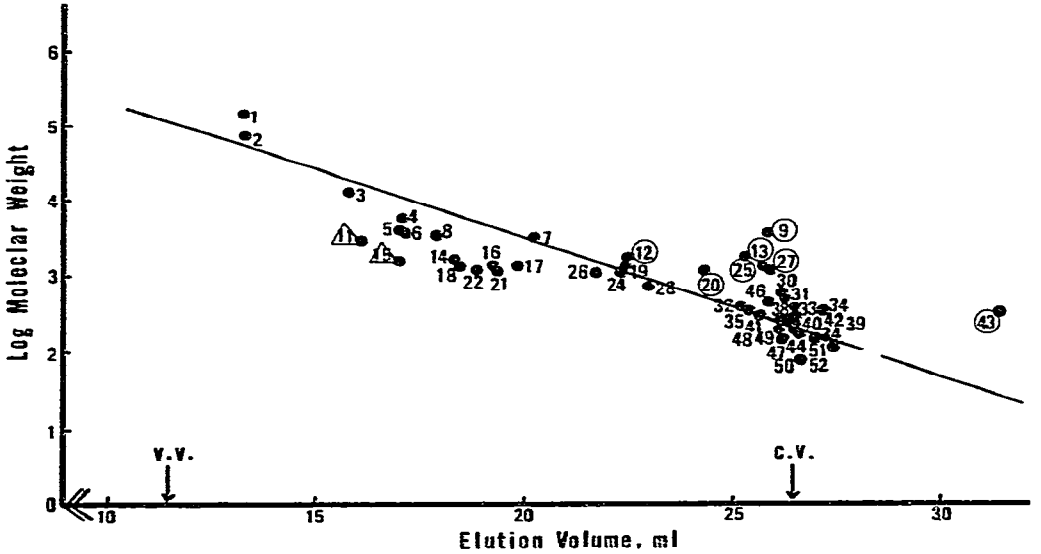

Fig. 1. The relationship between log MW and elution volumes of peptides. Column: TSK GEL G2000SW (7.5 × 600 nm). Elution: 0.15 *M* phosphate buffer (pH 7.4) containing 1 *M* NaCl, 20% methyl cellosolve and 1% SDS. Temperature: 22°C. Flow-rate: 0.9 ml/min. V.V. = Void volume; C.V. = column volume. Numbers correspond to the peptides in Table I; 51 = norleucine; 52 = glycine; ○ and △, see Fig. 3.

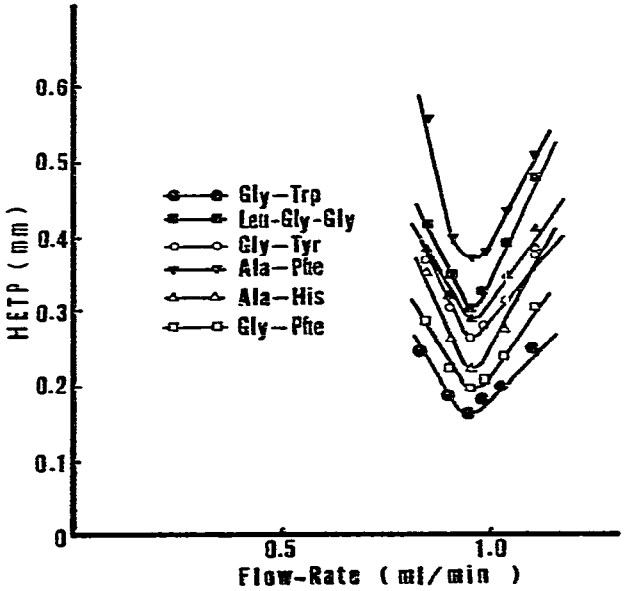

Fig. 2. The relationship between flow-rate and HETP. Column: TSK GEL G2000SW (7.5 × 350 mm). Elution: 0.15 *M* phosphate buffer (pH 7.4) containing 1 *M* NaCl, 20% methyl cellosolve and 1% SDS. Temperature: 22°C. ▲—▲, $(Gly)_4$.

TABLE II

CAPACITY FACTORS OF PEPTIDES ON TSK GEL G2000SW WITH SEVERAL SOLVENTS

PB = 0.15 *M* Phosphate buffer; NaCl = 1 *M* NaCl; MeOH = 20% methanol; MC = 20% methyl cellosolve; Brij = 1% Brij-35; Triton = 1% Triton X-100; SDS = 1% sodium dodecyl sulphate.

Substance	*Distilled water*	*PB*	*PB NaCl*	*PB NaCl MeOH*	*PB NaCl MC*	*PB NaCl Brij-35*	*PB NaCl MeOH Brij-35*	*PB NaCl MeOH Triton*	*PB NaCl MC Triton*	*PB NaCl MC SDS*
3 Cytochrome	0.28	0.80	0.59	0.90	0.78	0.64	0.80	0.90	0.76	0.40
16 Substance P	0.48	0.97	3.28	2.26	1.25	3.01	1.18	1.22	1.27	0.68
20 Angiotensin I	3.0	0.96	2.16	1.04	1.19	2.20	1.15	1.21	1.27	1.13
30 [Leu^5]-enkephalin	0.50	1.04	1.82	1.05	1.25	1.76	1.16	1.22	1.28	1.33
40 Gly-Gly-Leu	0.90	0.99	1.20	1.31	1.26	1.16	1.16	1.23	1.27	1.33
51 Norleucine	0.48	0.99	1.25	1.04	1.35	1.32	1.27	1.33	1.35	1.38

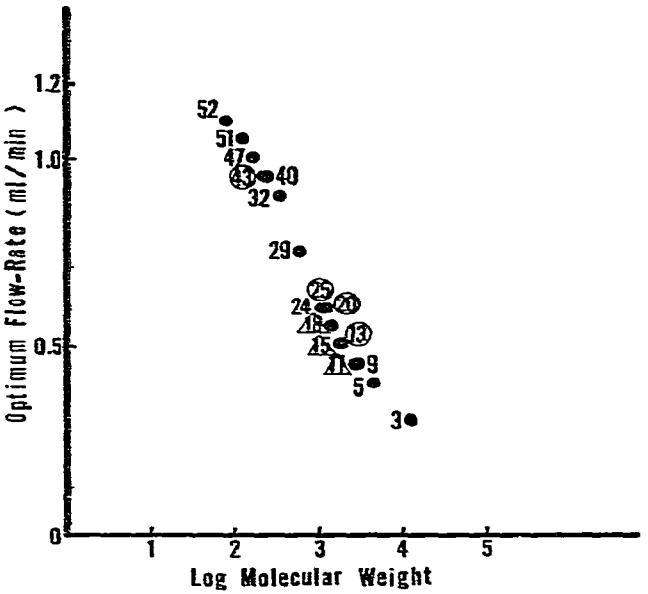

Fig. 3. The relationship between log MW and flow-rate required to obtain optimum HETP. Column: TSK GEL G2000SW (7.5 × 600 mm). Elution: 0.15 *M* phosphate buffer (pH 7.4) containing 1 *M* NaCl, 20% methyl cellosolve and 1% SDS. Temperature: 22°C. Numbers correspond to the peptides in Table I. ○, Peptides adsorbed to the column and eluted later than expected by size-exclusion chromatography with the same solvent; △, peptides eluted faster than expected by size-exclusion chromatography with the same solvent. Numbers correspond to the peptides in Table I; 51 = norleucine; 52 = glycine.

Accuracy of the method

Using the same size-exclusion column, the results of the MW estimation of some model peptides by the present method and by the conventional method are presented in Table III. The present method is superior than the conventional method, and is suitable to estimate MW in the range of 200–10,000 with an error of ±20%. The amount of sample peptide needed is a few nmol.

TABLE III

MW DETERMINATIONS BY THE DESCRIBED METHOD AND BY THE CONVENTIONAL METHOD

Substance	*MW*	*Present method**	*Conventional method***
Gly–Trp	260	260	25
$(Gly)_6$	360	400	320
Bradykinin	1060	1200	1000
Angiotensin I	1250	1300	400
Mastoparan	1480	1500	6310
Melittin	2850	3000	10,000
ACTH	4500	4500	5620

* By a calibration curve for the relationship between log MW and the optimum flow-rate.
** By a calibration curve for the relationship between log MW and the elution volume.

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