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ISOTACHOPHORETIC ANALYSIS OF PEPTIDES

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SUMMARY

Operating conditions for the qualitative and quantitative determination of plasma kinins, human angiotensins, reduced and oxidized glutathiones and oligopeptides with the same amino acid residues by capillary-tube isotachophoresis using a potential gradient detector have been established.

The potential unit (PU) value is proposed as a qualitative index for capillary-tube isotachophoresis with a potential gradient detector. As it is unaffected by changes in the operating conditions, the PU value permits inter-laboratory comparison of data. The coefficient of variation of this index under the various operating conditions used was less than 1.69%.

INTRODUCTION

The technique of isotachophoresis, the fundamental theory of which was developed by Haglund[1] and Martin and Everaerts[2], is now attracting attention as a simple and rapid method of analytical separation[3–6]. Conductivity[7,8], thermal[9], ultraviolet[9,10] and potential gradient[6,11] detectors have been developed for use in isotachophoresis. Everaerts *et al.*[12] made a detailed evaluation of the characteristics of these detectors.

A potential gradient detector (PGD) has the advantage that it is a general detector which can detect any charged substance, theoretically with a sensitivity as high as with a conductivity detector. At present, however, the practical sensitivity of the PGD is as low as one hundredth of the theoretical value. It is necessary to select an appropriate electrolyte system, taking the chemical structures of the components into consideration, that gives a satisfactory separation of sample components, and to collect experimental data for reference.

Potential gradients, on which qualitative isotachophoresis is based, are fundamentally different from the relative mobilities used in conventional electrophoresis, and the results deviate considerably depending on the operating conditions and the type of apparatus used; it is difficult to obtain universal constants for identification. There are thus many problems to be solved.

The physiological actions of physiologically important peptides are altered completely when some of their amino acid residues are changed and it is therefore

necessary to separate these related substances. As they are amphoteric and of low volatility, peptides are difficult to analyze directly by gas chromatography (some peptides with a few amino acid residues have been gas chromatographed after derivatization into volatile compounds[13]. High-performance liquid chromatography also has the disadvantage that it is necessary to exclude compounds that fluoresce and colouring reagents in order to achieve general detection.

Isotachophoresis may be suitable for the analytical separation of physiological peptides. The only application of this technique reported so far is that by Kopwillem *et al.*[14], which refers to the qualitative determination of synthetic peptides. This paper describes a study of the application of isotachophoresis to the separation of oligopeptides that have very similar structures. We also propose to modify the qualitative index proposed by Deml *et al.*[6] to a new index that is unaffected by changes in the inner diameter of the capillary tube or the conditions for migration, such as migration current.

EXPERIMENTAL

Samples and reagents

Kallidin, bradykinin, methionyllysylbradykinin (Met-lys-bradykinin) and human angiotensin I and II were obtained from Protein Research Foundation (Osaka, Japan). Reduced (GSH) and oxidized (GSSG) glutathiones were purchased from E. Merck (Darmstadt, G.F.R.). L-Alanine (Ala), L-alanyl-L-alanine (Ala_2), L-alanyl-L-alanyl-L-alanine (Ala_3), glycine (Gly), glycylglycine (Gly_2), glycylglycylglycine (Gly_3), L-alanyl-L-tyrosine (Ala-Tyr), L-alanyl-L-valine (Ala-Val), L-alanyl-L-glutamic acid (Ala-Glu), glycyl-L-alanine (Gly-Ala), glycyl-L-valine (Gly-Val), glycyl-L-tyrosine (Gly-Tyr) and glycyl-L-leucine (Gly-Leu) were purchased from Sigma (St. Louis, Mo., U.S.A.).

The following reagents were used as electrolytes and in their preparation: barium hydroxide, L-methionine, L-glutamine, 2-amino-2-methyl-1-propanol, 2-amino-2-methyl-1,3-propanediol (Amediol), tris(hydroxymethyl)aminomethane (Tris), hydrogen chloride, phenol and L-β-alanine. Methylcellulose (25 cps) was used to prevent electroendosmosis. Table I shows the constituents of the leading and terminating electrolytes.

Instruments

A Shimadzu IP-1B isotachophoretic analyzer (capillary-tube type), equipped with a PGD-1 potential gradient detector and a counter-flow attachment (Shimadzu Seisakusho, Kyoto, Japan), was used. The detector cells were of I.D. 0.5 mm and length 0.05 mm and I.D. 0.8 mm and length 0.5 mm. The migration currents were 50, 75, 100, 125 and 150 μA.

RESULTS AND DISCUSSION

Separation of oligopeptides with the same amino acid residues

Ala, Ala_2, Ala_3, Gly, Gly_2 and Gly_3 were analyzed as model compounds. The pK_1' and pK_2' values are 2.34 and 9.69 for Ala, 3.30 and 8.14 for Ala_2 and 3.39 and 8.03 for Ala_3, respectively. Thus the pK_1' value increases and the pK_2' value decreases

with increase in the number of alanine residues. From these data, there would be expected to be a useful difference in their mobilities in electrophoresis.

Leading electrolytes with various pH values were examined for their migration towards the anode and it was found that acidic or neutral solutions did not give good separations. A good separation was obtained under alkaline operating conditions, as shown in Table I, because the positive charge was decreased in alkaline solution. Fig. 1 shows the electropherogram obtained. The migration was faster at pH 9.63 than at pH 8.97.

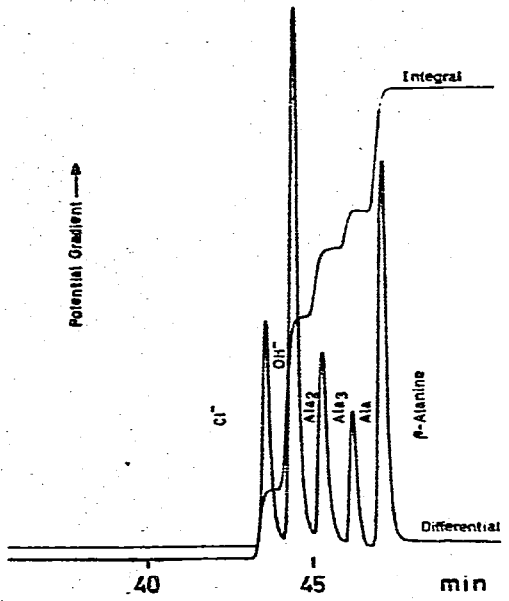

Fig. 1. Isotachophoretic separation of a mixture of Ala, Ala_2 and Ala_3. Conditions as in Table I (e). Capillary tube of I.D. 0.57 mm and detector cell of I.D. 0.8 mm and length 0.5 mm were used. Amounts injected: Ala, 3.6 μg; Ala_2, 6.4 μg; Ala_3, 8.6 μg. Temperature of electrolyte: 18°.

Dipeptides and tripeptides with glycine residues were also analyzed. The pK_1' and pK_2' values are 2.34 and 9.60, respectively, for Gly, 3.12 and 8.17 for Gly_2 and 3.26 and 7.91 for Gly_3. The same electrolytes as with the alanines were used and it was found to give a good separation. When the amount of sample was increased 2.5-fold, however, a mixed zone was formed between Gly_2 and Gly_3, as shown in Fig. 2. This mixed zone could be eliminated, as shown in Fig. 3, by allowing the leading electrolyte to flow at 200 μl/h for 12 min by means of a counter-flow attachment[15], and a good separation was then achieved.

Standard curves for these peptides were prepared by measuring each zone length. As shown in Fig. 4, good linearity was obtained between zone length and concentration.

Next, a mixture of the three alanine and three glycine compounds was analyzed simultaneously. When the pH values were 8.97 and 9.63, it was found that the peak

TABLE I

ELECTROLYTE SYSTEMS FOR ISOTACHOPHORESIS

System	*Sample*	*Leading electrolyte*	*Terminating electrolyte*	*Current (μA)*	*Capillary tube*		*Sensitivity*	
					I.D. (mm)	*Length (cm)*	*Differential (V)*	*Integral (mV)*
a	Bradykinin Kallidin	0.01 *M* $Ba(OH)_2$, methionine, pH 9.55	0.02 *M* Tris, 0.005 *M* HCl, pH 8.0	75	0.57	20	30	512
b	Kallidin Met-lys-bradykinin	0.01 *M* $Ba(OH)_2$, glutamine, pH 9.25	0.02 *M* Tris, 0.005 *M* HCl, pH 8.0	125	0.57	20	30	256
c	Oxidized and reduced glutathiones	0.02 *M* Amediol, 0.5% methylcellulose, HCl, pH 6.52	0.005 *M* phenol, $Ba(OH)_2$, pH 10.0	125	0.57	15	30	512
d	Angiotensin I and II	0.02 *M* Amediol, 0.5% methylcellulose, HCl, pH 7.05	0.01 *M* phenol, $Ba(OH)_2$, pH 10.0	100	0.57	20	20	256
e	Ala, Ala_2 and Ala_3 Gly, Gly_2 and Gly_3	0.05 *M* 2-Amino-2- methyl-1-propanol, 0.5% methylcellulose, HCl, pH 8.97	0.01 *M* β-alanine, $Ba(OH)_2$	150	0.57	15	20	128
f	Ala, (Ala_2 Gly_3), Ala_3, Gly and Gly_2	0.05 *M* Amediol, 0.5% methylcellulose, HCl, pH 9.63	0.01 *M* phenol, 0.5% methylcellulose, $Ba(OH)_2$, pH 10.15	100	0.57	20	20	256

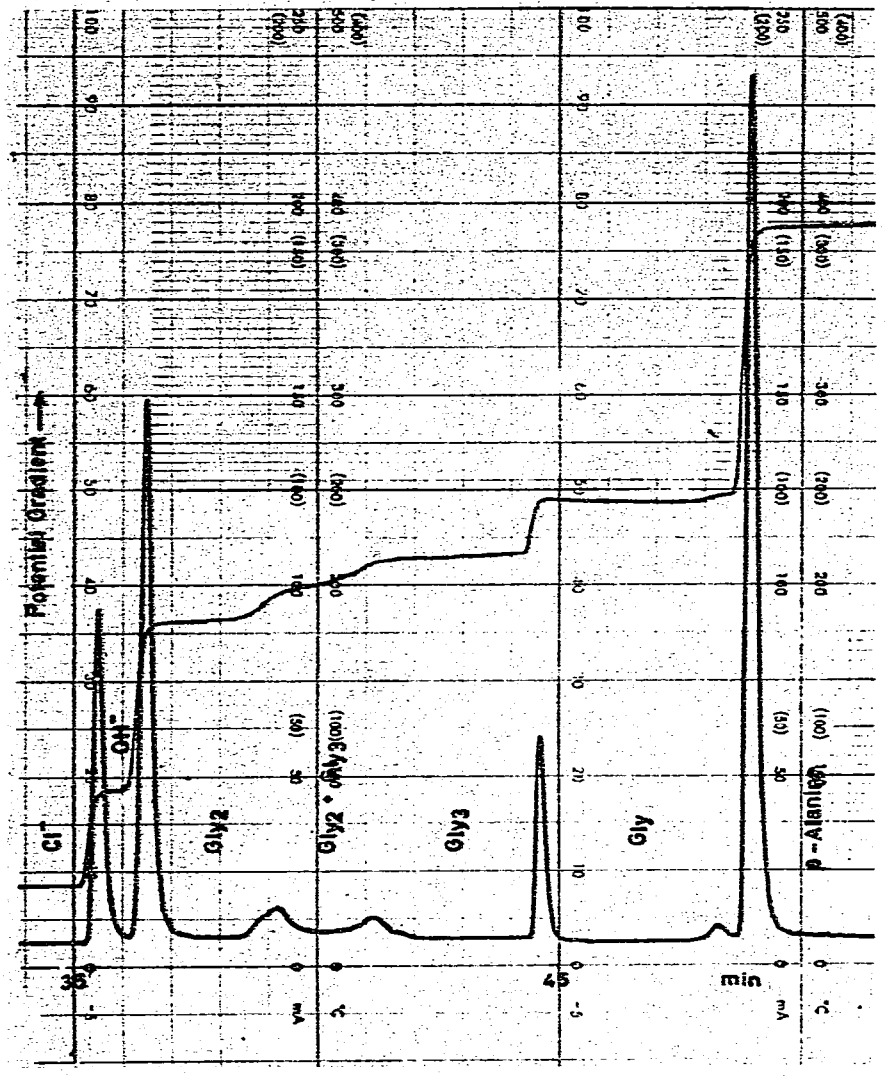

Fig. 2. Isotachopherogram of a mixture of Gly, Gly_2 and Gly_3. A mixed zone of Gly_2 and Gly_3 was found. Conditions as in Fig. 1. Amounts injected: Gly, 7.5 μg; Gly_2, 13.2 μg; Gly_3, 19.1 μg. Temperature of electrolyte: 25°.

of Ala_2 overlapped with that of Gly_3 and that only five zones were recorded. Fig. 5 shows the isotachopherogram obtained at pH 9.63. Although higher pH values were used, separation into six zones was not achieved.

Separation of biologically important peptides (bradykinin, kallidin and methionyllysyl-bradykinin)

The plasma peptides released from kininogens in blood by plasma and glandular enzymes are bradykinin, kallidin and methionyllysylbradykinin[16–18]. Bradykinin

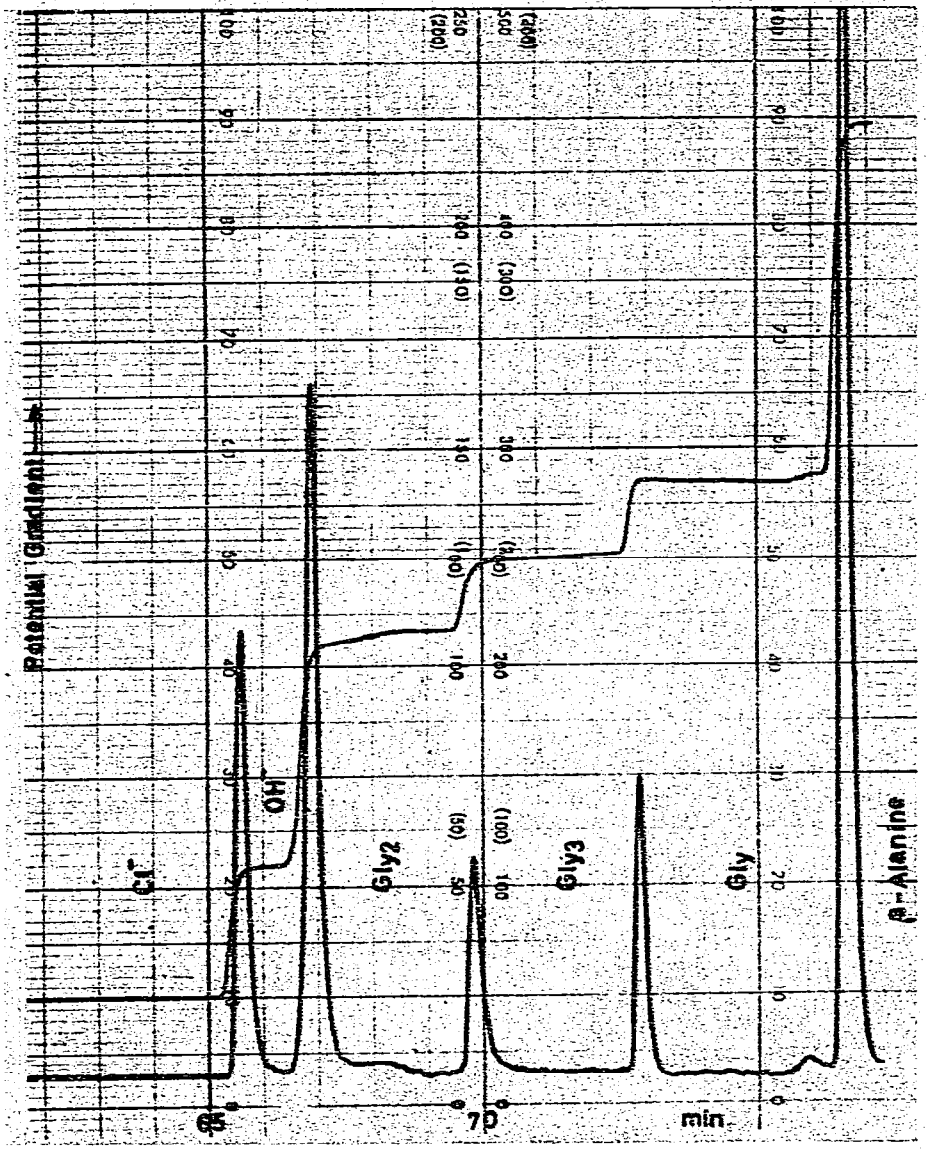

Fig. 3. Isotachophoretic separation of a mixture of Gly, Gly_2 and Gly_3 by using a counter-flow attachment. Counter electrolyte (leading electrolyte) flowed at 200 μl/h for 12 min. Conditions as in Figs. 1 and 2.

is released by plasma kallikrein and trypsin, while kallidin is released by glandular kallikrein. Both of these peptides have important physiological roles. As shown in Table II, they have similar amino acid sequences, differing only in the amino acid residues at the N-terminal position in bradykinin.

As these peptides contain arginine and lysine residues, we applied the operating conditions adopted by Kopwillem *et al.*[14] for their separation, but the result was

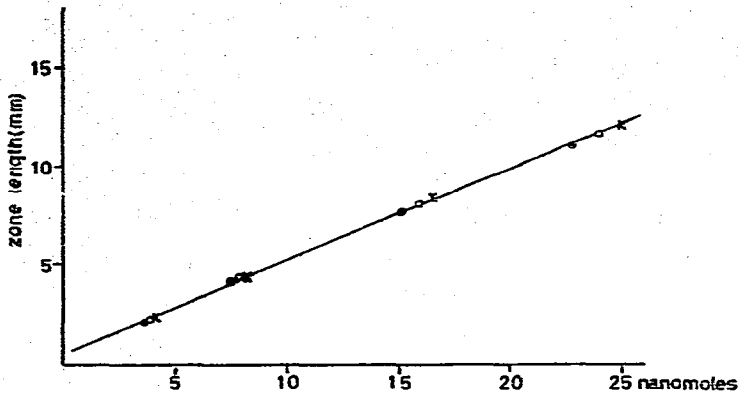

Fig. 4. Standard curves for Gly (○), Gly_2 (●) and Gly_3 (×). Electrolytes as in Table I (e). Capillary tube of I.D. 0.25 mm and length 15 cm and detector cell of I.D. 0.5 mm and length 0.05 mm were used. Conditions: migration current, 75 μA; integral sensitivity, 32 mV; differential sensitivity, 10 V; chart speed, 10 mm/min; temperature of electrolyte, 22°.

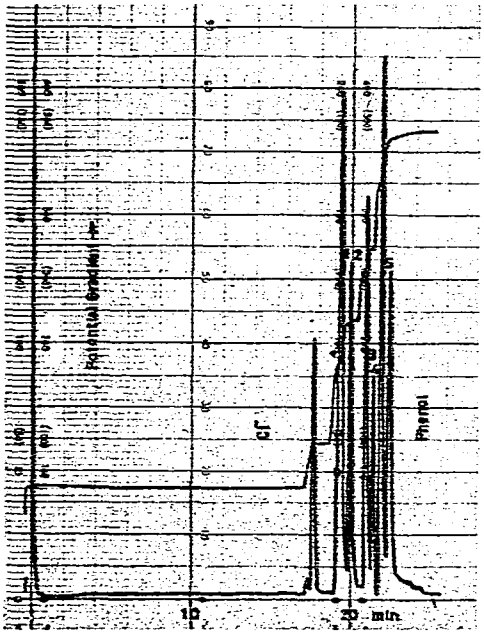

Fig. 5. Isotachophoretic separation of a mixture of Ala (5), (Ala_2, Gly_3) (2), Ala_3 (3), Gly (4) and Gly_2 (1). Conditions as in Table I (f). Amounts injected: Ala, 1.1 μg; Ala_2, 1.6 μg; Ala_3, 2.3 μg; Gly, 0.9 μg; Gly_2, 1.6 μg; Gly_3, 2.3 μg. Temperature of electrolyte: 22°.

unsatisfactory. Bradykinin and kallidin were separated well on migration towards the cathode based on a positive charge, as shown in Fig. 6, with Ba^{2+} as the leading ion, and with the pH adjusted to 9.55, as shown in Table I. However, methionyllysyl-bradykinin was not separated from kallidin. This separation was attempted repeated-

TABLE II

AMINO ACID SEQUENCES OF BRADYKININ, KALLIDIN, METHIONYLLYSYLBRADYKININ, ANGIOTENSIN I, ANGIOTENSIN II, REDUCED GLUTATHIONE AND OXIDIZED GLUTATHIONE

Peptide	*Amino acid sequence*
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg·3 AcOH·5 H_2O
Kallidine	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg·3 AcOH·4 H_2O
Met-lys-bradykinin	Met-lys-bradykinin·5 AcOH·4 H_2O
Angiotensin I (human)	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu·2 AcOH·4 H_2O
Angiotensin II (human)	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe·AcOH·4 H_2O
Reduced glutathione	γ Glu-Cys-Gly
Oxidized glutathione	γ-Glu-Cys-Gly | S | S | γ-Glu-Cys-Gly

ly, using electrolytes with lower pH values, and it was found that an electrolyte of pH 9.25 gave a good separation, as shown in Fig. 7. Under these conditions, however, it was impossible to obtain an isotachopherogram on which all of these three peptides were separated simultaneously, because bradykinin migrated more slowly than the terminating ion.

Separation of angiotensin I and II

Kallikrein–kinin and renin–angiotensin play important physiological roles, because the former has the effect of lowering blood pressure and the latter of raising it. Angiotensin I is formed from angiotensinogen by renin, and is converted into angiotensin II under the influence of a converting enzyme[19]. It has long been desirable, therefore, to develop a simple and rapid method for separating and identifying them in order to clarify the mechanism of homeostatis.

As the difference between angiotensin I and II is that the former has an L-leucine residue and the latter an L-phenylalanine residue at the C-terminal position, their separation was attempted by using an electrolyte system shown in Table I, and a good separation was obtained (Fig. 8).

Potential unit (PU) value

The qualitative index for isotachophoresis with a PGD is based on the magnitude of the electric gradient, which is specific for each compound. As the index is greatly influenced by the operating conditions, however, Deml *et al.*[6] proposed the "inverse relative mobility" as a new qualitative index. It was found that this index has excellent reproducibility under identical operating conditions but is considerably influenced by the current, inner diameter of the capillary tube and re-preparation of the electrolyte. The index proposed by Deml *et al.*[6] is therefore only as reliable as the relative retention time in gas chromatography: it differs with the apparatus and

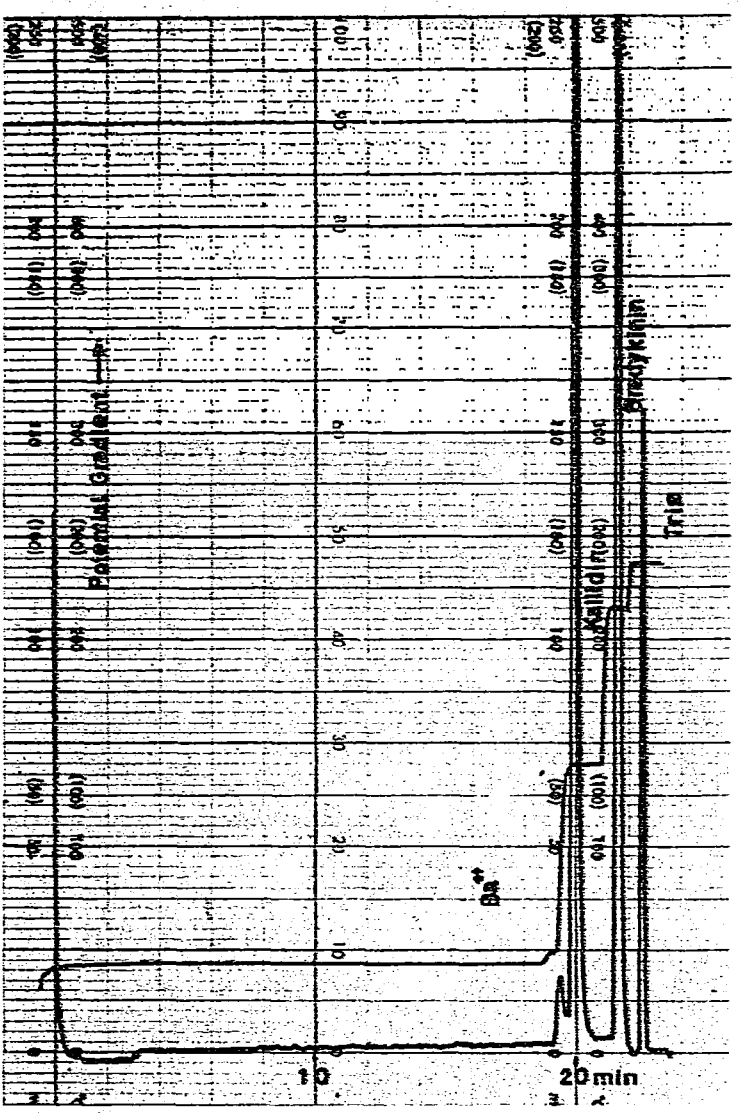

Fig. 6. Isotachophoretic separation of kallidin and bradykinin. Conditions as in Table I (a). Amounts injected: kallidin·3 AcOH·4 H_2O, 30.0 μg; bradykinin·3 AcOH·5 H_2O, 20.8 μg. Temperature of electrolyte: 25°.

laboratory used. Consequently, it seems to be unsuitable as a practical qualitative value specific to an individual compound. We therefore propose a new index that is relative to the leading and terminating ions, in the same manner as the methylene unit (MU) value[20] in gas chromatography, which is the retention time relative to two standard substances. We have termed this new index the "PU value". The PU value is defined as $(PG_S - PG_L)/(PG_T - PG_L)$, where PG_S is the potential gradient of the sample, PG_L the potential gradient of the leading ion and PG_T the potential gradient of the terminating ion, as illustrated in Fig. 9.

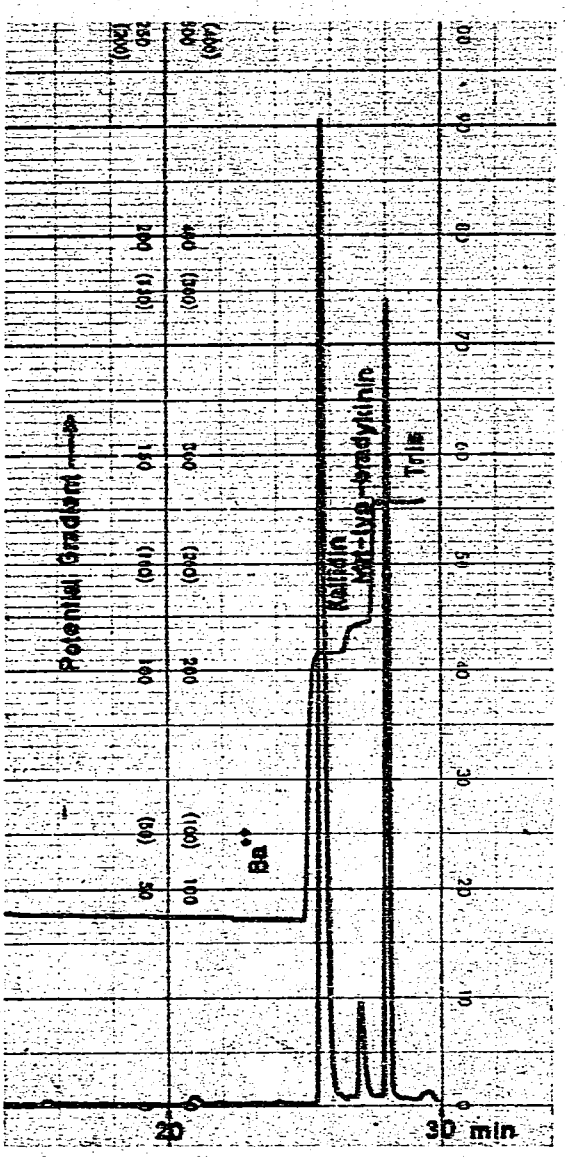

Fig. 7. Isotachophoretic separation of kallidin and methionyllysylbradykinin. Conditions as in Table I (b). Amounts injected: kallidin·3 AcOH·4 H_2O, 30.0 μg; Met-lys-bradykinin·5 AcOH·5 H_2O, 24.0 μg. Temperature of electroiyte: 25°.

The new PU value and the index proposed by Deml *et al.*[6] were investigated by using Gly, Gly_2 and Gly_3 as samples with various currents, inner diameters of the capillary tubes, re-preparation of the electrolyte and samples of different composition. The results (Table III) show that the index proposed by Deml *et al.*[6] is influenced by these factors, while the PU value is almost unaffected. The coefficients of variation for Gly, Gly_2 and Gly_3 under the above conditions were 4.95 and 4.56% for the index

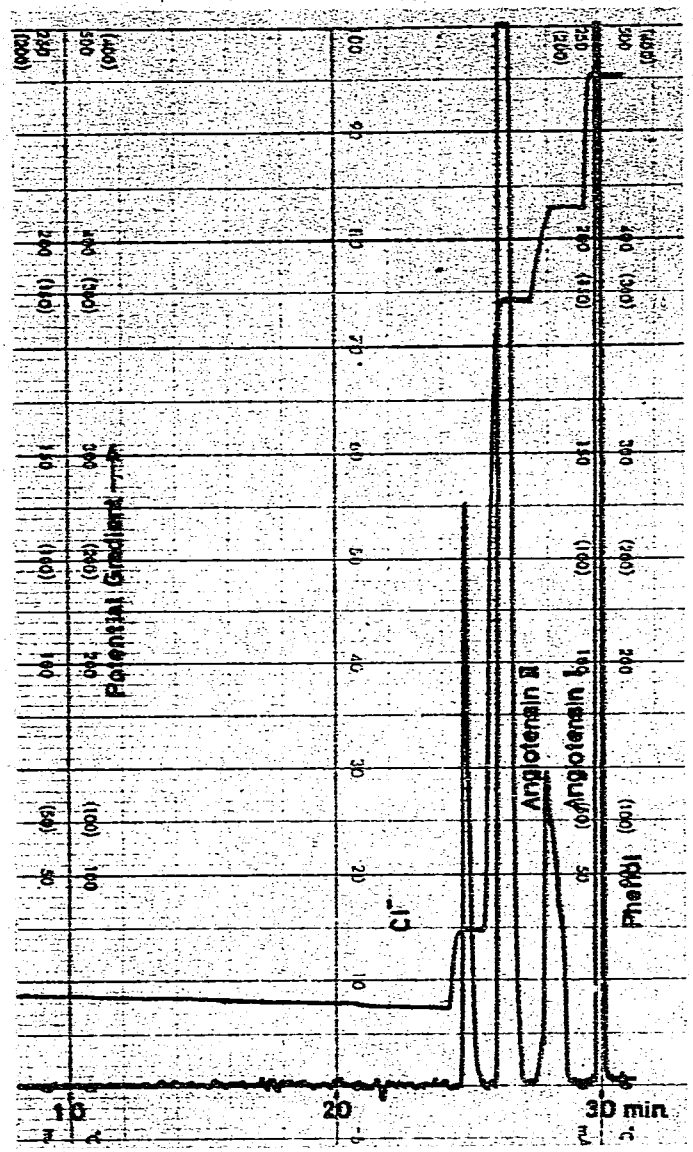

Fig. 8. Isotachophoretic separation of human angiotensin I and II. Conditions as in Table I (d). Amounts injected: angiotensin I·2 AcOH·4 H_2O, 25.0 μg; angiotensin II·AcOH·4 H_2O, 25.0 μg. Temperature of electrolyte: 25°.

of Deml *et al.*[6], and 1.69 and 1.30% for the PU value. Table IV shows the PU value for all of the substances used in this work.

Quantitative analysis

The only previous report on quantitative analysis by isotachophoresis with a PGD is that by Deml *et al.*[6]. Glutathione is known to exist in oxidized and reduced

TABLE III

COMPARISON BETWEEN INVERSE RELATIVE MOBILITY AND POTENTIAL UNIT VALUE UNDER VARIOUS CONDITIONS

Electroiytes as in Table I (f) were used. S.D. = standard deviation; C.V. = coefficient of variation.

Index	*Sample*	*Conditions*	*Factor affecting index*					$\bar{x}$	*S.D. (n = 15)*	*C.V. (%)*
			Current		*Sample*	*Capillary tube*	*New electrolyte*			
		Current (μA)	100	125	100	50	100			
		Capillary tube length (cm)	20		20	30	20			
		Capillary tube diameter (mm)	0.57		0.57	0.25	0.57			
		Sample	Mixture		Alone	Mixture	Mixture			
Inverse relative mobility	Gly_2		0.448	0.447	0.450	0.506	0.465	0.465	0.023	4.95
			0.449	0.451	0.454	0.504	0.459			
			0.450	0.458	0.451	0.513	0.468			
		$\bar{x}$	0.449	0.452	0.452	0.508	0.464			
		S.D.	0.001	0.006	0.002	0.005	0.005			
		C.V. (%)	0.22	1.23	0.44	0.98	0.99			
	Gly_3		0.385	0.381	0.395	0.429	0.403	0.401	0.018	4.56
			0.386	0.389	0.392	0.431	0.400			
			0.387	0.396	0.397	0.441	0.405			
		$\bar{x}$	0.386	0.389	0.395	0.434	0.403			
		S.D.	0.001	0.008	0.003	0.007	0.003			
		C.V. (%)	0.26	2.06	0.76	1.61	0.74			
Potential unit value	Gly_2		0.356	0.354	0.356	0.340	0.365	0.355	0.006	1.69
			0.357	0.349	0.358	0.346	0.357			
			0.358	0.354	0.357	0.349	0.362			
		$\bar{x}$	0.357	0.352	0.357	0.345	0.361			
		S.D.	0.001	0.003	0.001	0.005	0.004			
		C.V. (%)	0.28	0.85	0.28	1.33	1.11			
	Gly_3		0.462	0.454	0.468	0.453	0.472	0.462	0.006	1.30
			0.461	0.456	0.462	0.460	0.460			
			0.461	0.455	0.470	0.463	0.468			
		$\bar{x}$	0.461	0.455	0.467	0.459	0.467			
		S.D.	0.001	0.001	0.004	0.005	0.006			
		C.V. (%)	0.22	0.22	0.86	1.09	1.29			

forms and to be related to the oxidation–reduction mechanism in the body[21], and no conventional method can simultaneously determine these two forms. An attempt to separate glutathione into the two forms was carried out successfully by the isotachophoretic technique described here.

Glutathione in the reduced form, which has an SH group, is easily oxidized in slightly alkaline solution and autoxidized in the presence of a catalyst such as Cu^{2+}

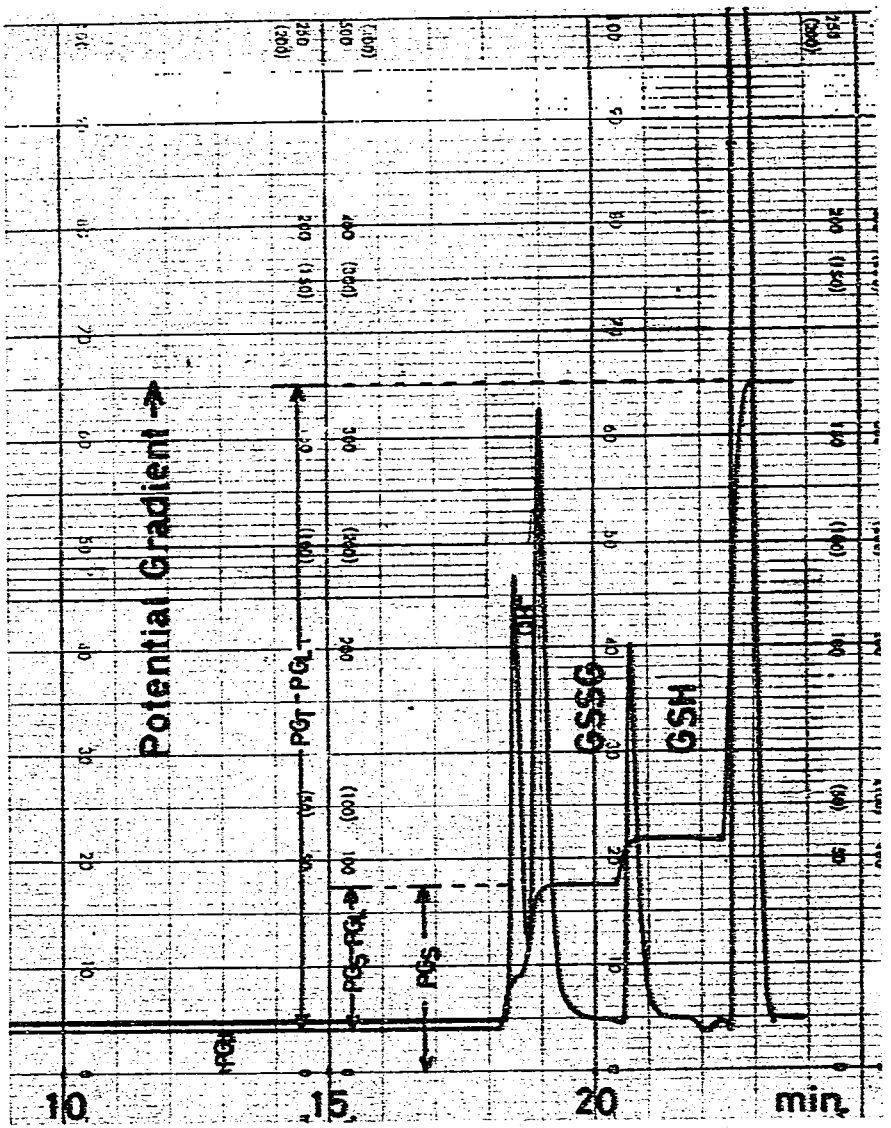

Fig. 9. Isotachophoretic separation of reduced and oxidized glutathione. Conditions as in Table I (c). Amounts injected: reduced glutathione, 20.7 μg; oxidized glutathione, 38.4 μg. Temperature of electrolyte: 17°.

TABLE IV

POTENTIAL UNIT VALUES OF VARIOUS PEPTIDES

Sample	*PU value*	*Electrolyte system**
Kallidin	0.496	a
	0.637	b
Bradykinin	0.885	a
Met-lys-bradykinin	0.701	b
Glutathione (oxidized)	0.223	c
Glutathione (reduced)	0.298	c
Angiotensin I	0.861	d
Angiotensin II	0.759	d
Ala_2	0.517	e
	0.472	f
Ala_3	0.660	e
	0.610	f
Gly_2	0.405	e
	0.357	f
Gly_3	0.506	e
	0.461	f
Ala-Tyr	0.539	e
Ala-Val	0.576	e
Ala-Glu	0.263	e
Gly-Ala	0.480	e
Gly-Val	0.557	e
Gly-Tyr	0.568	e
Gly-Leu	0.598	e

* See Table I.

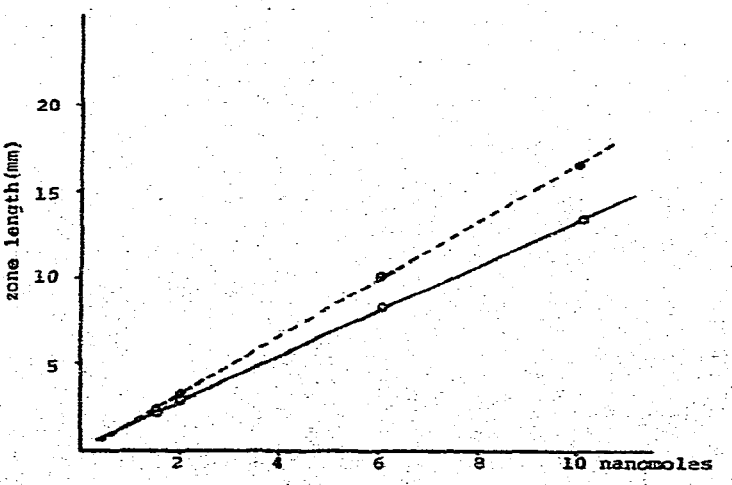

Fig. 10. Standard curves for reduced (○) and oxidized (●) glutathione. Electrolytes as in Table I (c). Capillary tube of I.D. 0.25 mm and length 15 cm and detector cell of I.D. 0.5 mm, and length 0.05 mm were used. Conditions: migration current, 50 μA; integral sensitivity, 64 mV; differential sensitivity, 20 V; chart speed, 10 mm/min; temperature of electrolyte, 22°.

(ref. 22). A neutral or slightly acidic electrolyte was used and a good separation was obtained (Fig. 9) under the operating conditions shown in Table I. The pH of the leading electrolyte was 6.52.

As no oxidized form was detected in the analysis of pure reduced glutathione under the operating conditions used, it was concluded that no oxidation of reduced glutathione occurred during the analysis. Fig. 10 shows the standard curves for glutathione in the reduced and oxidized forms. The quantitative error of our technique was a coefficient of variation of 1.65% ($n = 36$).

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