[Chem. Pharm. Bull. 19(8)1691—1695(1971)]

UDC 547.466.1.05 : 615.21.011.5

Selective Condensation of Plasma Kinins from Biological Fluids with Siliconised Silica Gel

Hisanobu Yoshida, Kojiro Matsumoto, Terumi Nakajima, and Zenzo Tamura

Faculty of Pharmaceutical Sciences, University of Tokyo[1]

(Received February 24, 1971)

The method for condensation of plasma kinins with high selectivity in biological samples was presented using siliconised silica gel. The method may be applied for kinins in urine, plasma, amuniotic fluid and lymph. The condensed kinin fraction was free from inorganic salts, urea, active amines and major proteins in plasma, and was charged directly on column chromatographic separation in the next step. Some of the kinins in biological fluid was purified as a single fluorescent band by dansylation on thin-layer chromatography within the two steps. The recovery of bradykinin (1 to 100 μg) was 70 to 90% during the procedure.

Plasma kinins (bradykinin and its related compounds) generally exist in plasma or urine at very low concentration of 10^{-11}mole/ml. Solvent extraction[2] or ion exchange resin adsorption[3] were often employed for condensation of these kinins from such biological fluids, but the results were not effective because of coexixtence of large amounts of proteins, urea or inorganic salts. We have investigated selective adsorbents for plasma kinins in biological fluids, as an aid for detection of these kinins with dansyl method. This report deals with screening of adsorbents, investigation of their selectivity, recovery of kinins and application of the procedure to biological fluids.

Experimental

Materials——Peptides and Other Chemicals: Bradykinin was purchased from Protein Research Foundation, Osaka University. Kallidin, a gift from Dr. J. Pisano, National Institutes of Health, U.S.A. Angiotensin II, a gift from Dr. S. Sakakibara, Institute for Protein Research, Osaka University. Oxytocin (Teikoku Zoki Co.) and vassopressin (Park Davis Co.) were used without any purification. Serotonin, histamine and other chemicals were all guaranteed grade.

Adsorbent: The following adsorbents were used: Silica gel H (E. Merck), Silica gel (Wakogel Q-23, 100—200 mesh, Wako Chemicals Co.), Kaolin (Koso Chemicals Co.), Magnesol (Fluka AG Chemische Fabrik), Acidic terra alba (Wako Chemicals Co.), Aluminum oxide (E. Merck), glass beads (Shibata Chemicals Co.).

Siliconisation of Silica Gel: Silica gel of 100 g, dried previously at 100° for 2 to 3 hr in an oven, was poured into 300 ml of toluene in a round bottled flask and evacuated to remove air. Twenty ml of dimethyldichlorosilane was added to the slurry, and the mixture was kept for 2 to 3 hr at room temperature with occational shaking. Sufficient siliconisation was checked by detecting excess of the reagent in the supernatant. Treated silica gel was collected on a Buchner funnel, washed with methanol of ten fold excess, then dried *in vacuo*.

Assay Method: Bradykinin, kallidin, angiotensin II, oxytocin, vassopressin, histamine and acetylcholine were assayed by Magnus method[4] using rat uteri. Serotonin was assayed fluorometrically according to Bogdanski, *et al.*[5] Urea and amino acids were assayed by amino acid analyzer (Yanagimoto LC-2).

1) Location: *Hongo, Bunkyo-ku, Tokyo.*
2) K. Abe, N. Watanabe, N. Kumagai, I. Mouri, T. Seki, and K. Yoshinaga, *Tohoku J. Exp. Med.*, **89**, 1031 (1966).
3) M.E. Webster and J.P. Gilmore, *Biochem. Pharmacol.*, **14**, 1161 (1965).
4) I. Trautshold, "Handbook of Experimental Pharmacology," Bradykinin, Kallidin and Kallikrein, Vol. XXV, ed. by E.G. Erdos, Springer-Verlag, New York, 1970, p. 55.
5) D.F. Bogdanski, A. Pletscher, B.B. Brodie, and S. Udenfriend, *J. Pharmacol. Exptl. Therap.*, **117**, 82 (1956).

Other inorganic salts such as sodium chloride or ammonium formate were tested by 1% silver nitrate or ninhydrin reaction, respectively. Albumin was measured by absorbance at 280 mμ.

All procedures were carried out with polyethylene-made vessels or siliconised glass equipments.

Result

Selection of Adsorbents

Bradykinin (5 μg) was dissolved in 1 ml of water saturated with sodium chloride or lower phase of aqueous *n*-butanol, then 5 mg of adsorbents (see Table I) were added to the solution. After 10 min, remaining bradykinin activity in the supernatant was assayed. Silicic acid polymer including glass, generally adsorbed bradykinin as shown in Table I.

Table I. Some Adsorbents for Bradykinin

Adsorbent	Sat. NaCl	H_2O	Aqueous *n*-BuOH
Siliconised silica gel	##	##	##
Silica gel	##	##	##
Magnesol	##	##	##
Kaoline	##	##	##
Acidic terra alba	##	##	##
Glass powder	±	±	##

tested by batch system
solvent 1 ml
bradykinin 5 μg
adsorbent *ca.* 5 mg

##: adsorbed above 80%
+: 50—80%
±: 10—50%

Bradykinin was adsorbed not only in saturated sodium chloride but also in 5% trichloroacetic acid, 0.1 M triethylamine, 80% ethanol, urine or plasma. Adsorbed bradykinin was not removed from the adsorbents by washing with water, but with the mixture of acetic acid: pyridine (3:1). Silica gel and siliconised silica gel were the most useful among these adsorbents for less residues in acetic acid: pyridene (3:1) eluae which might be disturbed for further analysis, but the latter was the better for specificity.

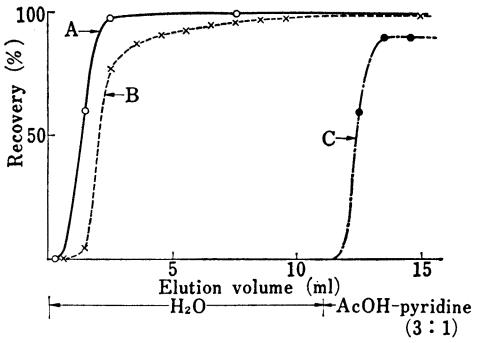

Fig. 1. Recovery of Each Material from Siliconized Silica Gel Column

column size: 6×0.75 cm
sample: H_2O 1 ml
NaCl, urea, albumin 10 mg
amines, amino acids 1 mg
bradykinin 10 μg
type A: Cl^-
type B: urea, amino acids, amines, albumin
type C: bradykinin

Chracteristics of Siliconised Silica Gel

Silica gel did not adsorb sodium chloride, urea, acidic and neutral amino acids, and serum albumin, but adsorbed basic amino acids, serotonin, histamine, acetylchloine, and polypeptides including oxytocin, vassopressin, angiotensin II and plasma kinins.

While siliconised silica gel adsorbed these polypeptides. Amines of similar biological activity, basic amino acids, bovine kininogen II and enzymes of plasma kinin formig and destroying activity in plasma, were eluted by washing with water.

Each 10mg of sodium chloride, urea, serum albumin, 1 mg of active amines, amino acids and 10 μg of bradykinin in 1 ml of water was charged on 3 ml of siliconised slica gel column, the recovery of each materials is shown in Fig. 1.

Bradykinin (200μg) in 0.1M ammonium formate was the maximum charging amount for 1 ml of siliconized silica gel.

Recovery of Bradykinin

After adsorption of bradykinin of 1 to 10μg on 2ml of siliconised silica gel column, recovery in acetic acid: pyridne (3:1) eluate was between 70 to 90%, but recovery of 50 ng of bradykinin was between 60 to 80%. The more minute amount was resulted in the poor recovery of less than 50%, which was considered to be adsorption of the kinin on siliconised glass or some other equipments during the procedure. In fact, after dissolving 20 ng of bradykinin in a siliconised glass test tube and evaporating *in vacuo*, the activity was decreased about 50% of the original.

TABLE II. Kinins in Body Fluids and Recovery of Added Bradykinin

	Body fluids	Kinin content per ml	Recovery of added bradykinin (50 ng/ml)
Human	urine	10—10 ng as bradykinin	60—80%
Dog	amuniotic fluid	5—10 ng as bradykinin	60—80%
Rat	plasma	trace	40—80%
Dog	lymph	trace	40—80%
Dog	duodenal juice	none	trace
	H_2O	50 ng as bradykinin	60—80%

Recovery of added bradykinin (50 ng) into some biological fluids is shown in Table II, in which the decreased recovery in plasma, lymph or duodenal juice is based on the decomposition of the peptide by protease in these samples.

General Procedure for Selective Condensation of Kinins

Siliconised silica gel is suspended in 50% methanol, then poured into a small glass tube (5 mm diameter) to make 1 ml of column. The column is washed with 5 ml of water. The sample solution containing 0.2 to 100 μg of kinin activity is passed through the column and the column was washed with 5 to 10 ml of water. Absorbed kinin is eluted with 2 ml of acetic acid: pyridine (3:1) and the eluate is evaporated *in vacuo* under 30°. A few drops of water is added to the residue and the evaporation is repeated until the smelling of the solvent is not detected. The sample which is likely to contain some proteases, may be treated with 5% trichloroacetic acid, heated at 100° for 15 min, or diluted by addition of ethanol to 80% to block the enzyme activity.

Application of the Method to the Kinins in Activated Plasma

It has been known that kinin forming enzyme system in plasma is readily activated by freezing,[6] contact to glass,[7] to casein[8] or to foreign compound such as organic solvent[8] or acidification[8,9] of plasma. This method was applied to condensation of plasma kinins in these treated plasma.

Acetone Activation——Rat plasma (7.5 ml) mixed with 0.75 ml of acetone, was incubated for 30 min at room temperature. In the series of these experiments, 1 mg/ml of 8-hydroxyquinoline was added to plasma to inhibit kininase activity.

6) D. Armstrong and G.L. Mills, *J. Physiol.* (London), **179**, 89 (1965).
7) D. Armstrong, J.B. Jepson, C.A. Kede, and J.W. Stewert, *J. Physiol.* (London), **135**, 350 (1957).
8) U. Eisen, *J. Physiol.* (London), **166**, 496 (1963).
9) D.F. Elliott, and G.P. Lewis, *Biochem. J.*, **95**, 437 (1965).

Casein Activation——Rat plasma (7.5 ml) was added to 0.7 g of casein and incubated for 30 min at room temperature.

Glass Activation——About 0.5 g of glass powder added to 12.0 ml of rat plasma, was incubated for 30 min at room temperature.

Acidification——Rat plasma (7.5 ml) was diluted to 50 ml with 0.2% acetic acid and kept for 30 min at room teperature, then adjusted the pH to 7.8 with 1N NaOH and 0.1M tris buffer, and kept again for 30 min at room temperature.

The liberated kinin was adsorbed completely on siliconised silica gel column and any activity was not detected in nonadsorbed fraction or in water eluate. Thus condensed kinin showed a fine separation on SE-Sephadex column chromatography in the next step. A typical chromatogram are shown in Fig. 2.

The activity was eluted at the similar position to bradykinin as shown in Table III. The purity of the peak area of activity was checked by dansyl method.[10] The fluorescent band on thin-layer chromatography of silica gel H with the solvent system of isopropanol: methylacetate: 28% ammonia (9:7:4), was only dansylbradykinin in the acidfied plasma,

Table III. Liberated Kinins in Rat Plasma

Activation	Rat plasma (ml)	Liberated kinin (μg as bradykinin)	Elution from SE-Sephadex (concentration of $HCOONH_4$) (M)	Identification by DNS[a] method
Acetone	7.5	2.3	0.21—0.23	bradykinin+unidentified fluorescent band
Glass	12.0	4.6	0.20—0.22	bradykinin+unidentified fluorescent band
Casein	7.5	2.6	0.22—0.24	not identified
Acidification	7.5	6.4	0.20—0.23	bradykinin

a) DNS: dansyl=(1-dimethylaminonaphthalene-5-sulfonyl)

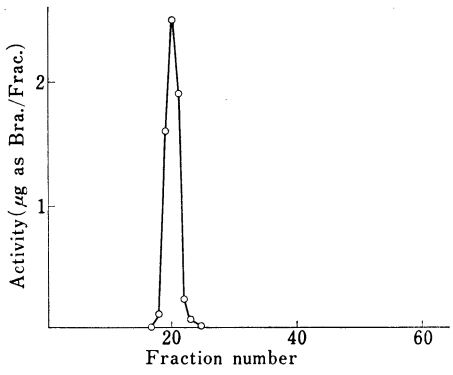

Fig. 2. SE-Sephadex Column Chromatogram of the Condensed Kinin

activation: acidification
column size: 45×0.75 cm
elution: 0.1M $HCOOHN_4$ pH 7.0 (150 ml)—0.4M $HCOONH_4$ pH 7.0 (150 ml)
fraction volume: 5 ml

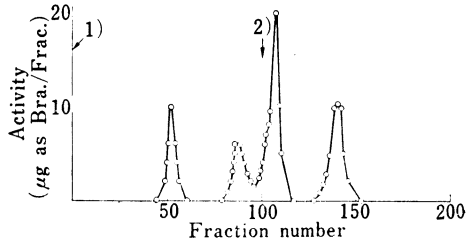

Fig. 3. SE-Sephadex Column Chromatogram of the Condensed Kinins in Human Urine

column size: 40×2.9 cm
elution: stepwise linear gradient
1) 0.1M $HCOONH_4$ pH 3.0 (500 ml)—0.5M $HCOONH_4$ pH 5.0 (500 ml)
2) 0.5M $HCOONH_4$ pH 5.0 (500 ml)—1.0M $HCOONH_4$ pH 7.5 (500 ml)
fraction volume: 10 ml

10) W.R. Gray and B.S. Hartley, *Biochem. J.*, **89**, 379 (1963).

but in the other activation systems, some other fluorescent bands were observed in addition to dansylbradykinin. The chracterisation of these bands is in progress.

Application to Human Urinary Kinins

In 1968 Miwa, *et al.*[11] have separated the urinary kinins into three kinds and estimated these kinins as bradykinin, methionyl-lysyl-bradykinin and kallidin respectively based on the chromatographic pattern comparing to the authentic samples. We have found that 1 ml of siliconised silica gel adsorbed all of the kinin activity in 100 ml of urine, and applied this method to the separation of urinary kinins. Fresh urine of 30 liters which was heated immediately at 100° for 15 min, was passed through 500 g of siliconised silica gel column. After washing the column with water, the column was eluted with 300 ml of acetic acid: pyridine (3:1). This kinin fraction was free from salt and urea to be charged directly to SE-Sephadex column. The chromatogram was also a fine resolution of four peaks of activity (Fig. 3). Two peaks out of four which were expected to bradykinin and kallidin by the elution position, showed single fluorescent bands by dansylation and were identified as bradykinin and kallidin respectively.

Another two peaks were unsuccessful for complete purification by this step. On the methionyl-lysyl-bradkinin which is expected to be eluted between bradykinin and kallidin by SE-Sephadex column chromatography, the authors could not find the activity in this region, but two peaks which were eluted before bradykinin fraction. Investigation whether the possibility of artifacts or the new type of kinin is in progress.

Discussion

The mechanism of the selective adsorption of these active peptides is not obvious, but kinins were purified to about 1000 times by weight with this method. By combination with SE-Sephandex column chromatography, some of the kinins were observed as a single band of dansylpeptide on thin-layer chromatography. The separation of kinin from biological fluids was therefore finished in two steps. Kinins of less than 50 ng were, however, poor recovery by adsorption to vessels. The method may be applicable to more than 100 ng of kinins.

The by-product formation was observed in evaporation process of acetic acid–pyridine elute. When the eluate was evaporated over 30°, kinin was acetylated. For example, 20% of bradykinin (1 mg) in acetic acid: pyridine (3:1) solution was acetylated by standing at 75° for 4 hr.

Siliconised silica gel was incubated with plasma for 30 min at room temperature, the silica gel was collected to a small column and eluted with acetic acid: pyridine (3:1), but any activity was not detected in the eluate. This fact indicated that siliconised silica gel did not activate the kinin forming enzyme systm. The kinin-like active substance in rat plasma producing after treatment with trypsin, *i.e.* Dinitz's kinin,[12] was hardly adsorbed on siliconised silica gel to need four fold volume of the adsorbent. Furthermore, about a half of the activity did not adsorb on SE-Sephadex column in the next step, but another half was identified as bradykinin. The result may suggest a production of the another type of active substance.

Acknowledgement The authors express their deep gratitudes to Dr. J.J. Pisano and Dr. S. Sakakibara for the kind gifts of active peptides. Thanks are also due to Miss. S. Kayaoka for useful discussion on biological assay method.

11) I. Miwa, E.G. Erdös, and T. Seki, *Life Sci.*, **7**, 1339 (1968).
12) C.R. Dinitz, I.F. Carvalbo, J. Ryan, and M. Rocha Silva, *Nature* (London), **192**, 1194 (1961).