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Purification of Glomerulopressin (42610)

R. BONETTO, E. ARANY, E. DEL CASTILLO, AND J. URANGA

Instituto de Biología y Medicina Experimental, Vuelta de Obligado 2490, 1428 Buenos Aires, Argentina

Abstract. The present investigation was undertaken to attempt the purification of glomerulopressin. Isolated rat livers were perfused with Krebs–Ringer bicarbonate, and the perfusate was concentrated at reduced pressure, extracted with *n*-butanol, and subjected to thin-layer chromatography (TLC) with different solvent systems. For monodimensional TLC, (a) acetic acid or (b) chloroform/methanol/formic acid (65:30:5, v/v) was used. For bidimensional TLC solvent (b) was used as the first mobile phase and as the second mobile phase three different solvent systems were employed as follows: ethyl acetate/*n*-butanol/formic acid (40:57:3; v/v), pyridine/methanol/formic acid (50:49:1, v/v), and acetic acid/*n*-butanol/water (5:50:10, v/v). The activity of the spots viewed under uv light was studied in three different biological assays: increase of the glomerular pressure index (GPI) in the toad and of the glomerular filtration rate (GFR) in the rat and increase of the tonic tension contraction in the rat stomach strip (TTC). In monodimensional TLC developed with system (a), three spots were detected, and with system (b), seven spots were observed. The biological activity of these seven spots was studied. Only the substance showing R_f 0.47 was active. This R_f 0.47 spot subjected to bidimensional TLC with the three different solvent systems moved in the second direction as only one spot. To confirm whether the R_f 0.47 spot was composed by only one substance, reverse-phase HPLC was performed in a Waters radial compression unit using three different elution systems and in a Hewlett–Packard model equipped with an ultrasphere ODS column. With these different HPLC columns and elution systems, only one peak was observed. This is the first attempt to purify a substance showing a biological activity similar to that of glomerulopressin.

The ultrafiltrate of liver vein blood from toads (1), rabbits (2), and dogs (3) contains a hormone/factor which was called glomerulopressin (1) due to its effect in increasing glomerular pressure in the toad. Glomerulopressin is also produced by isolated perfused rat liver (4–7). This hormone/factor has also been found in pancreatectomized dogs (3), in dogs with intraportal infusion of glucagon (8), in normal humans treated with glucagon, and in newly diagnosed Type 1 (insulin-dependent diabetic patients) (9).

The ultrafiltrate containing glomerulopressin increases the glomerular pressure index (GPI) in toads (1), enhances glomerular filtration rate (GFR) in rats (2) and in dogs (3), but does not influence systemic arterial pressure (10). It also increases the contractile tension developed by isolated strips of rat stomach fundus (TTC), this action being blocked by indomethacin (11). Glomerulopressin increases mesenteric blood flow (10) and decreases ovarian blood flow in normal dogs. Again, these effects are inhibited by treatment with indomethacin (12).

The aim of this study was to purify glomerulopressin by solvent extraction and the use of several chromatographic methods. A purified substance with glomerulopressin activity was isolated.

Materials and Methods. *Obtainment of ultrafiltrate containing glomerulopressin.* Sprague–Dawley male rats, weighing 200–300 g, were anesthetized with inactin (100 mg kg^{-1} ip, Byk). The portal vein was cannulated *in situ* and infused with Krebs–Ringer bicarbonate (KRB) solution at a flow rate of 5 ml min^{-1} and a pressure of 22 mm Hg. The solution was aereated continuously with 95% O_2–5% CO_2 and maintained at 38°C. The composition of the KRB (concentrations in m*M*) was NaCl, 120.5; KCl, 4.83; $CaCl_2$, 1.59; $MgSO_4$, 1.34; $KHPO_4$, 1.21; $NaHCO_3$, 24.2; and glucose, 11.1; pH 7.4. Glucagon (60 pg ml^{-1}, Eli Lilly and Co.) was added to the solution to increase the production of glomerulopressin (4, 8). The liver was isolated by clamping the hepatic artery and inferior vena cava and the hepatic vein was cannulated for collection of perfusate during 20 min. The liver was kept at 38°C during the time of perfusion. The criteria used in determining success of the perfusion was

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the pale uniform color in all the hepatic lobes. The perfusate was ultrafiltered through YC-05 Diaflo membranes. These membranes allow the passage of substances with a molecular weight lower than 500 Da; therefore, glucagon is retained by the membrane. The ultrafiltrate was kept at −20°C until used.

Extraction of glomerulopressin from the ultrafiltrate. The exposure of the ultrafiltrate to a stream of O_2 decreases its activity; this loss of activity was prevented by the addition of mercaptoethanol to the ultrafiltrate. For the preparation of each sample 300 ml of ultrafiltrate containing mercaptoethanol (final concentration 1 m*M*) was evaporated to dryness at reduced pressure. The dry residue was dissolved in 10 ml water and the pH was lowered to 4.0 with concentrated HCl, saturated with NaCl, and extracted six times with 0.6 ml *n*-butanol. The extract was washed three times with water at pH 4.0 and evaporated to dryness under a stream of N_2 in a bath at 30°C.

Thin-layer chromatography. The dry residue was dissolved in 0.2 ml ethanol and spotted on TLC plates (DC Alufolien Kieselgel F 254, Merck). Monodimensional and bidimensional TLC were performed. For monodimensional TLC, acetic acid or chloroform/methanol/formic acid (65:30:5, v/v) was used. For bidimensional TLC the first mobile phase was chloroform/methanol/formic acid (65:30:5, v/v) and the second mobile phase was ethyl acetate/*n*-butanol/formic acid (40:57:3, v/v), pyridine/methanol/formic acid (50:49:1, v/v), or acetic acid/*n*-butanol/water (5:50:10, v/v). Mercaptoethanol (final concentration 1 m*M*) was added to all the solvent systems. The spots were visualized with a Fotodyne, Inc. uv transilluminator. For the bioassays monodimensional TLC was performed: the bands were scrapped from the plates, placed in small columns, eluted with ethanol, and evaporated to dryness under a stream of N_2 at 30°C.

uv Absorption. The samples obtained by elution of monodimensional TLC bands, evaporated to dryness, were dissolved in ethanol or water at pH 3 or 9. Ultraviolet absorption spectra were scanned between 200 and 350 nm using a Beckman DB-G spectrophotomer equipped with a hydrogen lamp.

High-performance liquid chromatography. Reverse-phase HPLC was performed using a Waters system consisting of a Model U6K injector, a Model 6000A solvent delivery pump, a Model 450 variable wavelength detector, and a Z module radial compression unit containing a Waters radial pak 5-μm C_{18} cartridge of 100-mm length. Three different isocratic elution systems were used: water/methanol (80:20; v/v, taken to pH 4.0, with acetic acid), water/methanol (80:20, v/v, with sodium acetate 0.05 *M*, pH 7.5), and water/methanol (20:80, v/v). The samples were injected in water/methanol (80:20 v/v, pH 4.0). Elution was performed with a flow rate of 1 ml min^{-1} at room temperature. HPLC was also performed with a Model 1084 B Hewlett–Packard equipped with an ultrasphere ODS column (Altex, 10 and 250 mm; particle size, 5 μm) and variable wavelength detector. The elution system was methanol/water (70:30, v/v) with a flow rate of 3 ml min^{-1}. Samples were injected in methanol. The elution profile was followed by monitoring at 220 and 280 nm. All solvents were filtered through an Ultipore NR 0.2-μm filter, deareated by vacuum, and sonicated prior to use.

The biological activity of the HPLC eluates was determined by the TTC assay.

Tonic tension contraction of the rat stomach fundus. Sprague–Dawley female rats weighing 130–140 g were killed by a blow on the head. The stomach fundi were removed and placed in petri dishes containing KRB, and longitudinal segments of 0.4 × 0.8 cm were cut. One end of the strip segments was attached to a glass holder and immersed in a tissue chamber filled with 270 μl KRB solution (9). This medium was continuously bubbled with O_2:CO_2 gas mixture and maintained at 37°C. The other end of the tissue was connected to a force transducer Gould Statham UC 3 with a UL 5 Micro scale and registered by a Grass 7D polygraph. After a resting tension of 700 mg was applied to the preparations by means of a micrometric device, the tissues contracted in isometric conditions. Their mechanical activity was then determined in terms of tension of tonic contraction (TTC), i.e., the sustained levels of contractile force (in mg) developed above the resting or basal tension.

Preparations of the tissues were allowed to equilibrate for 60 min and thereafter 30 μl of the sample was added to the tissue bath in order to give a final dilution of 10% (v/v).

Toad bioassay. Mildly dehydrated male *Bufo arenarum* Hensel weighing between 140 and 160 g were used. They were anesthetized

with 20% urethane, of which 1 ml/100 g body weight was injected into a lymphatic sac. A PE 10 cannula was placed in the aorta above the renal arteries for infusion, and the aorta was ligated caudally to the renal arteries. A PE 50 cannula was placed in one urether; this cannula was connected to a Gould P 23 ID transducer and the pressure was recorded in a 7D Grass polygraph. The samples were infused at 0.013 ml min^{-1} with a Harvard pump. Previous experiments showed that this rate of infusion with KRB does not alter the toad's glomerular pressure. The pressure of the occluded urether in the toad equals the pressure in Bowman's capsule and responds rapidly and precisely to its variation (13); therefore it was considered as a GPI.

Rat assay. Female Sprague–Dawley rats weighing 280 g and allowed free access to a standard pellet diet and water were anesthetized with inactin (100 mg kg^{-1} ip). The rats were maintained at a constant body temperature of 36–37°C. The trachea was cannulated and the rat was allowed to respire spontaneously. Catheters were placed in the jugular vein, carotid, femoral arteries, and bladder. Blood pressure was monitored through the carotid catheter using a Gould Model P23 ID transducer attached to a Grass 7D polygraph recorder. At the beginning of the experiment a bolus injection of 2 ml inulin (36 mg ml^{-1}) was given through the jugular vein, followed by a substaining infusion at a rate of 0.02 ml min^{-1}. KRB was infused through the femoral artery at this same rate. Infusions were made with constant infusion pumps (Harvard Apparatus Co., Inc.). After an equilibration period of 30 min, urine was collected during 30 min, considered as a control period, and then the femoral KRB infusion was changed for the experimental sample. After 15 min infusion, a second urine sample was collected during 30 min. Urine volumes were determined gravimetrically. At midpoint of each urine collection 0.2 ml of blood was extracted. Urine and plasma inulin was determined with the method of Schreiner (14).

Statistical analysis. Results are given as means ± SE. The Wilcoxon matched-pairs signed rank test (15) was used for the statistical analysis of the variation of GFR because we considered our data not normally distributed.

Results. *n-Butanolic extract.* The concentrated ultrafiltrate has a very high salt content that would interfere with the TLC and HPLC studies. The treatment with *n*-butanol removed almost all the salts (16). The redissolved butanolic extract had 75% of the activity of the ultrafiltrate. The activity of the ultrafiltrate in the TTC assay before extraction was 475 ± 65.8 mg (n = 12) and that of the dry extract redissolved to its original volume was 357 ± 52.4 mg (n = 15). This redissolved butanolic extract was also active on the toad assay, increasing the GPI by 6, 10, 19, and 29 mm Hg in four toads.

Thin-layer chromatography. The fluorescence of the plates permitted the detection of spots by uv transillumination. In monodimensional plates developed with acetic acid three spots were detected (R_f values 0.67, 0.53, and 0.34) (Fig. 1), whereas when the plates were developed with chloform/methanol/formic acid seven spots were observed (Table I). Biological activity was studied in all the spots developed by this system. Of the seven spots only one (R_f 0.47) had biological activity producing a contraction of 312 ± 68 mg (n = 5) in the TTC assay, increasing the GPI by 7, 14, 16, and 19 mm Hg in the four toads assayed and increasing the GFR by 43 ± 16.8% of the rat ($P < 0.02$, n = 25). In the TTC assay

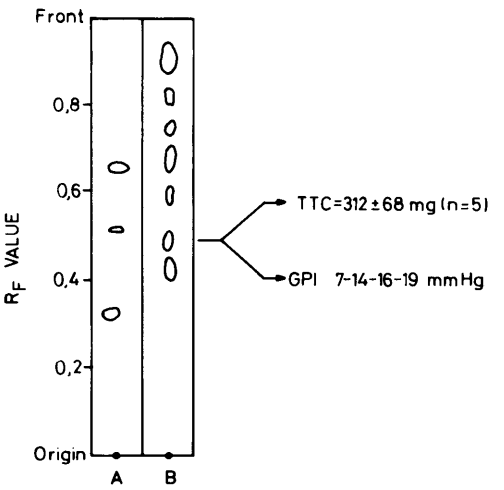

FIG. 1. Thin-layer chromatograms of butanolic extracts of concentrated rat liver perfusate. (A) TLC plate developed with acetic acid. (B) TLC plate developed with chloroform/methanol/formic acid (65:30:5, v/v). The spots were visualized by uv transillumination. The data in the figure show the biological activity of R_f 0.47 spot on the TTC assay and in the toad assay (GPI).

TABLE I. TLC CHROMATOGRAPHY DEVELOPED WITH CHLOROFORM/METHANOL/FORMIC ACID (65:30:5, v/v)

Spot	R_f
1	0.88 ± 0.01
2	0.82 ± 0.01
3	0.75 ± 0.02
4	0.67 ± 0.02
5	0.60 ± 0.02
6	0.47 ± 0.01
7	0.40 ± 0.01

Note. Values are means ± SE in 11 plates.

it is necessary to use 30 μl of ultrafiltrate, per stomach fundus, to obtain a contraction, while only 1.5 μl of purified glomerulopressin produces a contraction of the stomach fundus. The other spots were inactive both in the TTC and in the toad assay.

To verify whether the active spot was composed by one or several substances a two-dimensional TLC was carried out. The solvent system chloroform/methanol/formic acid was used as the first mobile phase. When ethyl acetate/*n*-butanol/formic acid was used as second mobile phase the R_f 0.47 spot has a small displacement (R_f 0.11) in the second direction. When pyridine/methanol/formic acid was used for the second run the R_f 0.47 spot had a larger displacement (R_f 0.52). With the solvent system acetic acid/*n*-butanol/water the R_f 0.47 spot showed an R_f 0.32. With these three solvent systems the R_f 0.47 spot did not dissociate and moved in the second direction as only one spot.

uv Absorption spectra. When the R_f 0.47 spot was dissolved in ethanol the absorption spectrum showed two peaks, one at 220–224 nm and another at 275–280 nm. When the R_f 0.47 spot was dissolved in water it presented the same spectrum. The change in pH from 9.0 to 3.0 showed no batochromic effect.

High-performance liquid chromatography. To confirm the possibility that the R_f 0.47 spot contained a single substance reverse-phase HPLC elution profile was studied with two different systems.

With the radial compression column using a mobile phase water/methanol (80:20, v/v) taken to pH 4.0 with acetic acid, no peak was observed after 15 min, which suggests that the substance was retained in the column. With a mobile phase water/methanol (80:20, v/v, with sodium acetate 0.05 *M* at pH 7.65) the elution profile showed a single peak with a retention time of 6.08 min. The last 5-min flow was increased to 3 ml min^{-1} to observe whether any other substance was retained; however, no other peak was obtained. When the elution profile was monitored at 220 and 280 nm simultaneously, both wavelengths showed the same single peak (Fig. 2). When the peak with a 6.08 retention time was recycled, a single peak, lower and broader than before, was detected (Fig. 2). With a mobile phase water/methanol (20:80, v/v) only one peak with a 3.94-min retention time was observed.

Using a column with a higher resolution power (Ultrasphere ODS, 5 μm) with a mobile phase water/methanol (30:70, v/v) and a flow of 3 ml min^{-1} a single peak with a 10.63 retention time was obtained.

After fractional collection of the eluant was made, the fraction corresponding to the peak

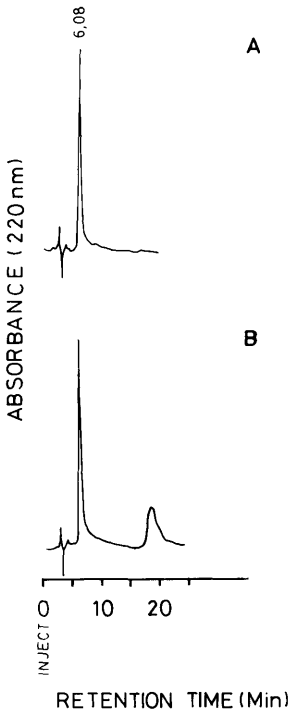

FIG. 2. RP-HPLC profile of the active spot obtained by TLC of the butanol extract of concentrated rat liver perfusate. (A) Elution profile was made at 1.0 ml min^{-1} during the first 15 min and at 3.0 ml min^{-1} the last 5 min. (B) The recycling of the peak with retention time 6.08 min again showed only one peak.

was evaporated to dryness, dissolved in 0.2 ml in KRB, and assayed in the TTC. This fraction produced a contraction of 115 ± 8.35 mg (n = 12), showing that this peak had biological activity.

Discussion. Both a diet rich in protein (17–23) and an intravenous infusion of amino acids (20, 24, 25) cause an increase in the GFR. Failure to obtain a significant effect on the GFR in the isolated perfused kidney by a solution with high amino acids concentration prompted one report to exclude a direct effect of amino acids or proteins on the kidney (26). It has been proposed by several authors (27–32) that a circulating hormone, glomerulopressin, may be responsible for the increase in the GFR.

Untreated Type 1 diabetes mellitus is also associated with glomerular hyperfiltration (33–36). In Type 1 diabetes mellitus glomerulopressin is increased in peripheral venous blood (9). We have proposed that glomerulopressin may be involved in the increase of the GFR observed in Type 1 diabetes mellitus (9).

It has previously been shown that glomerulopressin has a low molecular weight, less than 500 Da (1), that changes in pH from 8.8 to 2.2 does not alter its activity (1), and that it is soluble in ethanol but not in ethyl acetate. Passage through an Amberlite M 23 column showed that when the amount was enough to retain NaCl, glomerulopressin was also retained, thus suggesting its polar nature (38).

Glomerulopressin can be inactivated by β-glucuronidase (3, 10, 12, 16, 38, 39). When glomerulopressin is incubated with β-glucuronidase treated with a specific inhibitor it does not lose its activity (3). There is a good correlation between glucuronide concentration and glomerulopressin activity in the ultrafiltrates (3, 8). The production of glomerulopressin by isolated rat liver is enhanced by stimulation of glucuronyl transferase activity with benzo[a]pyrene and inhibited by the glucuronyl transferase inhibitor novobiocin (6). These facts suggest that glomerulopressin is a glucuronide.

Highly polar glucuronides can be extracted with n-butanol with salt addition and a low pH (40). This treatment of the concentrated ultrafiltrate permitted the extraction of the active substance with only a small amount of salts which would not greatly interfere in the biological and chemical studies.

The exposure of the ultrafiltrate to a stream of O_2 decreased its activity. This loss of activity was prevented by the addition of mercaptoethanol to the ultrafiltrate (R. Bonetto, unpublished data).

The observation that in bidimensional TLC the R_f 0.47 spot did not dissociate with three different solvent systems suggests that it is composed of only one substance.

The reverse-phase HPLC with a radial compression column and three different elution systems showed only one peak in each instance; when this peak was recycled the elution profile again showed only one peak. With a higher resolution column (Ultrasphere ODS) the R_f 0.47 spot produced only one peak in the elution profile. These results suggest that considerable purification has been obtained.

The R_f 0.47 spot was the only spot with biological activity. The activity on the toad glomerular pressure and the rat GFR and TTC assay was similar to that of the ultrafiltrates containing glomerulopressin obtained under several experimental conditions. Our data, therefore, suggest that a purified substance has been obtained that shares several biological properties with glomerulopressin.

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