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RAPID, SMALL-SCALE PREPARATION OF GASTROINTESTINAL HORMONES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON A C_{18} COLUMN

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SUMMARY

A rapid reversed-phase high-performance liquid chromatography method was developed for the isolation of small quantities of biologically active gastrointestinal hormones, using a Varian MCH-10 C_{18} column. Biologically active secretin was isolated from contamination with other hormones, including cholecystokinin, gastrin, motilin, and vasoactive intestinal polypeptide, from samples of the acid perfusate of canine duodenum and from the crude acetic acid extract of canine antral mucosa containing less than 100 picomoles of secretin. The method also appeared to be suitable for the isolation of cholecystokinin octapeptide and motilin.

INTRODUCTION

Ever since the oldest gut hormone, secretin, was found in 1902[1], more than 20 peptides have been found to exist in mammalian gastrointestinal tract. Some of them including secretin, a 27-amino acid polypeptide[2] have been established to play important roles on the regulation of gastrointestinal function. In our previous observations, duodenal acidification resulted in an increased amount of several gut peptides including secretin-, cholecystokinin (CCK[1])-, motilin- and vasoactive intestinal polypeptide (VIP)-like immunoreactivities[3,4]. To establish the biological significance of a hormone found at low concentration in body fluids, plasma, or a particular tissue, it is necessary that the hormone, usually determined by radioimmunoassay, be isolated and shown to be biologically active. The hormonal content of biological fluids and/or tissue extracts is very heterogeneous and many gut hormones exist with similar structures or with overlapping immunological and biological activities. Therefore, it is often necessary to isolate a peptide free of contaminants with immunologically or biologically crossreactive peptide(s) before determination of its bioactivity. In the present report, we will present a quick reversed-phase high-performance liquid chromatography (HPLC) method capable of achieving the separation of many gastrointestinal hormones and the isolation of biologically active secretin from duodenal juice and from antral mucosa of the dog.

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EXPERIMENTAL

Materials

Synthetic porcine cholecystokinin octapeptide (CCK_8) was obtained from Dr. M. Ondetti, Squibb Institute, NJ, U.S.A. Pure natural porcine secretin, VIP and cholecystokinin triacontatripeptide (CCK_{33}) were obtained from Dr. Viktor Mutt, Karolinska Institutet, Stockholm, Sweden. Natural porcine motilin was purchased from Dr. J. C. Brown, University of British Columbia, Vancouver, Canada. All HPLC grade solvents were purchased from Fisher, and all solvent mixtures were prepared fresh daily and filtered through a 0.2-μm Millipore filter before use. All antisera used in this study were prepared in our laboratory. Other reagents were of the best commercially available quality.

Preparation of canine duodenal perfusate

Canine duodenal perfusate was prepared by infusion of 0.1 *N* hydrochloric acid in saline, as described previously[3]. The protein of the perfusate was precipitated by saturating it with sodium chloride and was then collected by centrifugation at 6800 *g* for 1 h at 4°C. The supernatant fluid was discarded and the precipitate was collected by holding the centrifuge bottle upside down and blotting the excess supernatant with Whatman No. 1 filter paper. It was either stored at −20°C or used immediately.

Preparation of the extract of canine antral mucosa

Antrum of the dog was collected as described[5]. The mucosal layer was isolated, separated into 5-g portions and immediately boiled in 5 volumes of water for 10 min. After having cooled to room temperature, the tissue was minced and homogenized in a polytron in the water used to boil the tissue. The homogenate was then adjusted to pH 9.2 with ammonia, stirred at room temperature for 2 h, and centrifuged at 10 000 *g* for 90 min. The supernatant, containing more than 90% of the tissue gastrin, was removed and stored. The tissue precipitate was resuspended in 0.5 *N* acetic acid in a glass homogenizer, equipped with a PTFE pestle, stirred at 4°C for 2 h, and then centrifuged to collect the supernatant fluid which contained more than 90% of the tissue secretin. This supernatant was freeze dried and stored at −20°C before use.

Chromatography of duodenal perfusate on SP-Sephadex column

The salt precipitate of the duodenal perfusate (200 ml) was redissolved in 40 ml of 20 m*M* sodium phosphate, pH 5.5 containing 0.1% bovine serum albumin (BSA) and centrifuged at 10 000 *g* for 60 min to precipitate the small amount of insoluble material. The supernatant was then chromatographed on a column of SP-Sephadex C-25 (25 × 1.6 cm), previously equilibrated in the same buffer. The column was then washed with 50 ml of the starting buffer, containing 50 m*M* sodium chloride, before eluting with a linear gradient of sodium chloride varying from 50 to 500 m*M* in the same buffer with 100 ml in each reservoir and finally with 100 ml of 500 m*M* sodium chloride. Fractions of 3 ml were collected after sample application. All of the above procedures were performed at 4°C. The fractions were monitored for secretin, CCK, VIP, and motilin by corresponding specific radioimmunoassay (RIA) methods.

High-performance liquid chromatography

Before subjecting it to HPLC, the redissolved salt precipitate of duodenal perfusate, freeze-dried extract of dog antral mucosa, and the pooled fractions of SP-Sephadex chromatography were extracted by the XAD-2 adsorption method described previously[6,7]. Briefly, 5 ml of sample was allowed to percolate through 3 ml of dry XAD-2 resin (J. T. Baker), packed in a 5-ml disposable pipet, and recycled 3 times. The column was then washed twice with each 2 ml 0.9% sodium chloride and water and then dried under a stream of air. The column was eluted with 3 × 3 ml of freshly prepared, ethanol–ethyl acetate–0.02 *N* hydrochloric acid (2:3:1, v/v/v). The eluate was freed of organic solvents by evaporation under reduced pressure and the aqueous phase was freeze-dried. The sample was then redissolved in 1% trifluoroacetic acid (TFA) adjusted to pH 2.5 with triethylamine (glass-distilled) and then passed through a 0.2-μm Millipore filter using a syringe type instrument. The sample was subjected to HPLC by using a Varian LC-5000 system, equipped with an automatic injector, a short Whatman C_{18} guard column, and a 30-cm Varian MCH-10 C_{18} column. The sample was either injected in a single or multiple application in 80-μl aliquots through a 100 μl loop, into the column being equilibrated with 20% solvent B. After injection, the column was eluted for 5 min with 20% solvent B, a linear gradient of 20 to 35% B over 50 min, followed by 35 to 60% over 10 min, and then maintained at 60% B for 10 min at a flow-rate of 0.5 ml/min. The composition of the solvents were: Solvent A, 1.0% TFA–triethylamine, pH 2.5; B, 1% TFA in 2-propanol, containing the same amount of triethylamine as solvent A. Column eluates were collected every minute with a fraction collector. Following each run the column was repeatedly washed with cycles of solvent gradient of 40 to 60% B over 15 min to ensure a clean baseline in the subsequent run. The position of the peptides was monitored by a specific radioimmunoassay (see below) after evaporating fraction to dryness *in vacuo* and reconstituting them in the RIA buffer.

Radioimmunoassay and bioassay methods

The radioimmunoassay methods for secretin, VIP, motilin[6], gastrin[8] and CCK[7] were carried out as described previously. For bioassay of secretin, fractions under immunoreactive secretin-like peak from each HPLC run were pooled (60–63 min to minimize CCK contamination) and freed of the eluent by diluting with RIA buffer and then extracting on a XAD-2 resin column. The dried XAD-2 extract was then reconstituted in Dulbecco's phosphate-buffered saline and bioassayed in anesthetized rats by monitoring the stimulation of pancreatic volume flow after i.v. administration of a secretin sample, as described previously[3,5]. Each sample was tested in at least 4 rats by administration in randomized order, and the stimulated pancreatic secreion during the first 20 min after administration of the test sample was compared with the secretion the 20 min basal period immediately before sample administration. The data were subjected to statistical analysis by the paired Student *t* test. Changes with a $P < 0.05$ are regarded as significant.

RESULTS

HPLC separation of standard hormones

The results shown in Fig. 1A represent the typical separation of standard

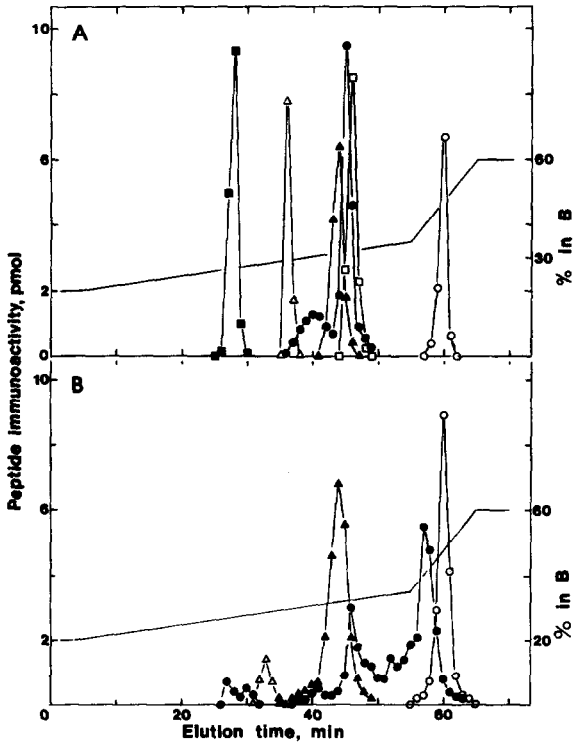

Fig. 1. Reversed-phase chromatogram of standard gastrointestinal hormones and acetic acid extract of canine antral mucosa on a MCH-10 C_{18} column. (A) Chromatogram of six standard hormones: ○, Secretin; ●, CCK_{33}; △, motilin; ▲, VIP; □, gastrin-I; ■, CCK_8; (B) Chromatogram of the acid extract of antral mucosa. The symbols represent immunoreactivities of secretin, ○, CCK, ●; motilin, △; and VIP, ▲. The actual concentration of VIP was 10 times as high as plotted. The line across each panel represents the change of solvent composition from 20 to 60% B, as described in the text. The baseline values between peaks are not plotted.

CCK_8, motilin, CCK_{33}, VIP, gastrin and secretin in the amount less than 25 pmol under our HPLC conditions. The elution times were 28, 36, 44, 45, 46 and 60 min for CCK_8, motilin, VIP, CCK_{33}, gastrin and secretin, respectively. The recovery of each peptide varied between runs but usually was in the range of 60–90%. Part of the loss of peptides appears to be due to losses in the injection loop. When the volume of the injected sample was smaller than the capacity of the loop, the recovery of the peptide improved. The distinct separation of secretin, motilin and CCK_8 from other peptides suggested that this procedure may be a useful step for isolation of these peptides from tissue extracts and biological fluids.

Isolation of secretin-like immunoreactivity from canine duodenal perfusate and extract of antral mucosa

The feasibility of using the above HPLC procedure for isolation of secretin-like material was tested with dog duodenal perfusate and mucosal acid extract of canine antrum. These had previously been demonstrated in our laboratory to contain biologically active, secretin-like immunoreactivity possessing similar molecular size

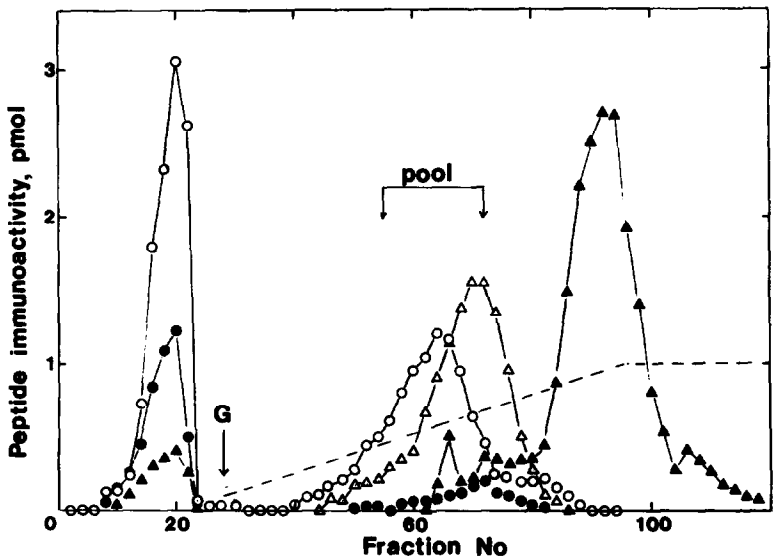

Fig. 2. Chromatogram of canine duodenal acid perfusate on SP-Sephadex C-25 column. The NaCl precipitate of canine acid perfusate was chromatographed and assayed for secretin, ○; CCK, ●; motilin, △ and VIP, ▲. The actual scale of secretin and CCK-like immunoreactivities were 10 times as high as plotted. The arrow indicates the beginning of the NaCl gradient, shown as a dashed line. The double arrow indicates fractions pooled for further separation on the C_{18} column.

and charge as natural porcine secretin[3,5]. When the XAD-2 eluate of the extract of the antral mucosa was subjected to HPLC, a substantial amount of secretin-like immunoreactivity, as shown in Fig. 1B, was eluted with the same retention time as natural porcine secretin. This material was well-separated from a large quantity of VIP-like and a small amount of CCK_8-like, CCK_{33}-like immunoreactivities as well as a yet unidentified CCK-like substance, which was eluted immediately before the secretin peak. A small amount of motilin-like immunoreactivity was also eluted at 34 min, which was 2 min earlier than natural porcine motilin. However, a similar application of the chromatographic procedure to the freeze-dried duodenal perfusate was not successful, resulting in smearing of peaks. This was probably due to interference by the mucus protein and bile salts. Therefore, the salt precipitate of duodenal perfusate was partially purified on a SP-Sephadex C-25 column before being subjected to purification by the reversed-phase HPLC method. As shown in Fig. 2, the duodenal perfusate contained substantial amount of motilin-, CCK- and VIP-like immunoreactivities. VIP-like immunoreactivity, but not CCK- and motilin-like immunoreactivity was well resolved from secretin-like immunoreactivity. Thus the pooled fractions (Fig. 2) that contained most of the secretin-like immunoreactivity retained by the ion exchanger also contained about 50% of both of the motilin and CCK-like immunoreactivities. However, these contaminants could be separated from secretin-like immunoreactivity by HPLC, as shown in Fig. 3A. The results of Fig. 3A also indicate the presence of the still unidentified CCK-like material as well as the motilin-like material, eluted at 34 min and seen in the extract of antral mucosa. A subsequent study, shown in Fig. 3B, indicated that the duodenal perfusate can be fractionated directly by the HPLC procedure if the peptides are first precipitated by

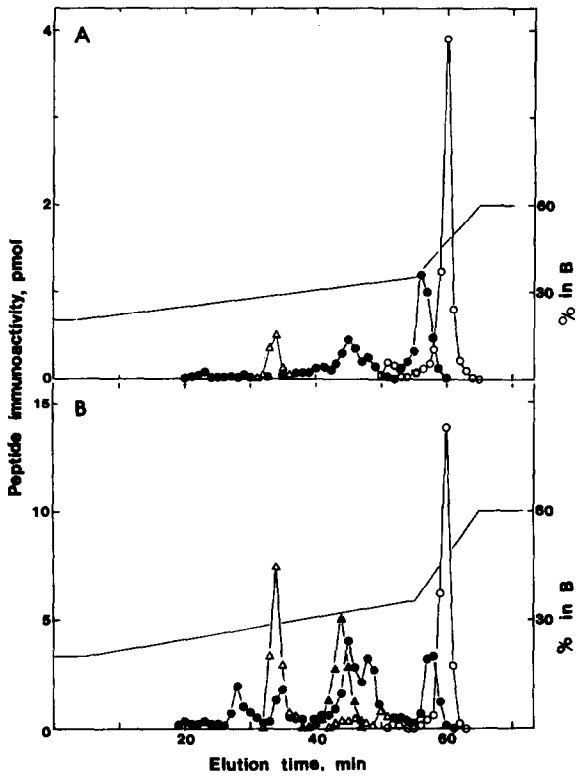

Fig. 3. Reversed-phase HPLC of canine duodenal acid perfusate on a MCH-10 C_{18} column. (A) Chromatogram of the fractions pooled in Fig. 2. (B) Chromatogram of the NaCl precipitate of canine duodenal acid perfusate before ion exchange chromatography. The experimental conditions and the symbols are the same as shown in Fig. 1B. The scale of CCK, motilin and VIP are magnified 10 times in panel B.

saturating with sodium chloride and then extracting with XAD-2 column before application to the C_{18} column. The results shown in Fig. 3B also indicate that CCK-like immunoreactivity present in the duodenal acid perfusate is highly heterogeneous. This observation is in good agreement with our previous report that the duodenal acid perfusate contains in addition to CCK_8 and CCK_{33}, species with molecular size between CCK_{33} and CCK_8 (ref. 4).

Biological activity of the isolated secretin-like immunoreactivity

The secretin-like immunoreactivity, isolated from both the duodenal perfusate and antral mucosa by HPLC, was as active as natural porcine secretin in stimulating exocrine pancreatic secretion in the anesthetized rats. Moreover, the CCK-like immunoreactivity eluted immediately before the secretin peak was inactive in a comparable dose.

DISCUSSION

The results presented in this report clearly indicate that several gastrointestinal

peptides with overlapping biological activity can be separated by a single step reversed-phase liquid chromatography on a C_{18} column. For example CCK, VIP, and secretin are all known to stimulate pancreatic bicarbonate and water secretion, although each peptide exibits different potency. Our method appears to be suitable for the quick isolation of bioactive secretin, without contamination by both CCK and VIP, from both the duodenal perfusate and the extract of antral mucosa of the dog, thereby confirming our previous observation[3,5] that these biological preparations contain secretin. This method also appears to be suitable for isolation of motilin-like immunoreactivity for bioassay. The elution time of the motilin-like immunoreactivity in the canine duodenal perfusate or antral mucosa was 2 min earlier than that of natural porcine motilin. This difference may be ascribed to the difference in the amino acid sequence in the N-terminal region between canine and porcine motilin[9]. Our method is also suitable for the isolation of CCK_8 free of contamination with other peptides of CCK family and gastrin. Although our HPLC method did not provide a clean separation of CCK_{33} from either VIP or gastrin as their peaks overlapped (Fig. 1A), separation would be possible if HPLC was carried out after partial separation using other techniques. For example, a majority of gastrin and CCK_8, which are acidic peptides, can be readily removed from antral mucosa by extraction of the boiled tissue with a slightly alkaline solution. The residual amount of these peptides in the subsequent acetic acid extract then can be separated from the basic peptides including motilin, VIP, CCK_{33} and secretin by chromatography on a column of a cation exchanger such as SP-Sephadex C-25, on which we also were able to get separation of VIP from secretin, CCK_{33} and motilin (Fig. 2). The last three peptides were subsequently separated by the reversed-phase HPLC on a C_{18} column. By the same token, these procedures may be extended to a large-scale purification of these peptides.

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