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EXTRACTION OF CHOLECYSTOKININ PEPTIDES FROM BIOLOGICAL FLUIDS USING OCTADECYLSILANE-PACKED CARTRIDGES

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SUMMARY

Cholecystokinin is an important peptide hormone, which occurs naturally in molecular forms ranging in length from 4 to 58 amino acid residues and varying in charge from acidic to basic. In order to quantify the individual molecular forms of this hormone present in plasma or tissues, it is first necessary to efficiently extract all of the diverse forms. In this paper, we establish and validate a simple method to do this using octadecylsilane-packed cartridges. Peptide adsorption to the cartridge and subsequent elution from it is not significantly affected by the pH (3—7) or salt concentration (0—2 g per 100 ml) needed for extraction, or the protein concentration (0—10 g per 100 ml) in the applied sample. Peptides are extracted in a form which can be separated using reversed-phase high-performance liquid chromatography and subsequently quantified by a commonly available radioimmunoassay which recognizes both cholecystokinin and gastrin.

INTRODUCTION

Cholecystokinin (CCK) is a polypeptide hormone, synthesized in the gastrointestinal tract and nervous system, which possesses a wide variety of physiologic functions [1]. Since its discovery over 55 years ago [2], several species of CCK ranging in length from 4 to 58 amino acid residues have been described [1, 3]. However, a well established sensitive and specific method to quantify the molecular species of CCK in tissue and biological fluids does not currently exist. The aim of this study is to establish and validate a method to extract CCK peptides from such samples in preparation for separation by high-performance liquid chromatography (HPLC) and the subsequent quantification by radioimmunoassay (RIA).

All molecular species of CCK share a common COOH-terminal sequence. Gastrin, however, also shares the COOH-terminal pentapeptide-amide with CCK peptides. Because of this homology, gastrin and CCK cross-react with each

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other in many RIAs. In order to have a CCK-specific RIA, which does not cross-react with gastrin and which recognizes all molecular species of CCK larger than and including the predominant circulating form, CCK-8, the antiserum must be directed toward the tripeptide Asp-Tyr(SO_3)-Met adjacent to the pentapeptide-amide which is common to CCK and gastrin. Only recently has such an antiserum been described [4]. Even using this type of antiserum, however, it is necessary to separate the different forms of CCK to quantify each of the molecular species.

Reversed-phase HPLC is a particularly powerful tool to separate peptides [5]. Use of this technique to separate each of the CCK and gastrin species present in biological samples would permit identification and quantification with either the unique [Asp-Tyr(SO_3)-Met] CCK-specific RIA or the more commonly available COOH-terminal RIA which recognizes both CCK and gastrin. Tissue homogenates and many biological fluids, however, cannot be injected directly onto an HPLC column. Some investigators have successfully used octadecylsilane (ODS)-packed cartridges for the extraction of peptides from biological samples in preparation for HPLC [6—10].

In this study, we used such cartridges to extract and concentrate CCK peptides from human plasma. Since each molecular form of CCK has distinct charge and hydrophobic characteristics, it was necessary to optimize conditions so that each of the species would be adsorbed to the octadecylsilane and then eluted from it to result in high and reproducible yields. Subsequent to this, the molecular species were seaprated by HPLC and quantified by RIA.

EXPERIMENTAL

Peptides

Synthetic CCK-8 and CCK-8 desulfate were gifts from Dr. Miguel Ondetti (Squibb Institute for Medical Research, Princeton, NJ, U.S.A.). Natural porcine CCK-33 was obtained from Dr. Victor Mutt (Gastrointestinal Hormone Research Laboratory, Karolinska Institutet, Stockholm, Sweden). Synthetic CCK-4 was purchased from Peninsula Labs. (Belmont, CA, U.S.A.). Synthetic human gastrin-17-I was purchased from ICI (Cheshire, U.K.) and gastrin-34 from Fluka (Hauppauge, NY, U.S.A.).

CCK-8 was radioiodinated to a specific activity of 2000 Ci/mmol by the method we described using ^{125}I-labeled Bolton—Hunter reagent (New England Nuclear, Boston, MA, U.S.A.) [11]. CCK-33 was radioiodinated with ^{125}I-labeled Bolton-Hunter reagent to a specific activity of 1000 Ci/mmol by a modification of the method described by Rehfeld [12]. Gastrin-17 was radioiodinated to a specific activity of 600 Ci/mmol using the chloramine-T method of Hunter and Greenwood [13].

General extraction procedure

Peptide extractions were performed using ODS-packed cartridges (Sep-Pak C_{18}, Waters Assoc., Milford, MA, U.S.A.). These were activated by injection of 5 ml methanol and rinsed with 5 ml water. The peptide-containing aqueous solution was then injected at a standard rate of 1 ml/min, and the cartridge was washed with 5 ml of 0.1 *M* triethylamine (TEA) (Sequenal Grade, Pierce,

Rockford, IL, U.S.A.) adjusted to pH 5.0 with acetic acid (TEA-A) (Ultrex, J.T. Baker, Jackson, TN, U.S.A.). All effluents from the cartridge were assayed for unadsorbed peptide. Then, the peptides were eluted from the cartridge using an organic solvent (acetonitrile or methanol) (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) buffered with TEA-A as described in the results. Effluents were assayed for eluted peptide.

Plasma extraction procedures

Four methods of extracting CCK from human plasma were compared. Fasting EDTA plasma was collected from healthy volunteers. In one series of experiments, [^{125}I]CCK-8 (approx. 10 000 cpm/ml) was used to determine recovery from plasma. In method I, 1 ml of plasma which contained the tracer dose of radioiodinated CCK was applied directly to an activated ODS cartridge and the peptide was eluted as described above. Method II involved the extraction of 1 ml of plasma by adding an equal volume of acetonitrile, followed by centrifugation at 8000 *g* for 5 min. The resultant pellet was also washed a second time with acetonitrile. Method III involved diluting the 1 ml of plasma with 1 ml 0.5 *M* TEA-A prior to its application to the cartridge. In method IV, 1 ml of plasma was diluted with 200 μl of acetonitrile and then 800 μl of 0.25 *M* TEA-A prior to application to the cartridge. In all methods, all fractions and the cartridge were counted in a gamma spectrometer (Packard Multi-Prias 4, Packard, Downers Grove, IL, U.S.A.). In a second series of experiments, this was repeated, using native CCK peptides (approx. 1 ng/ml) added to fasting human EDTA plasma, and with recoveries determined by RIA.

HPLC separation of peptides

Healthy volunteers donated 100 ml venous blood 20 min after ingesting a standard mixed nutrient meal (700 cal; 20% protein, 40% lipid and 40% carbohydrate). This was collected into EDTA-containing tubes on ice, and the plasma was separated after immediate centrifugation at 4°C. Peptides were extracted from the plasma using method III described above.

The effluent from the ODS cartridge was diluted with 0.1% trifluoroacetic acid (TFA) (Pierce), pH 2.0 to yield an organic solvent concentration of 20%. This was then injected directly onto a Beckman Model 344 chromatograph (Beckman Instruments, Berkeley, CA, U.S.A.) equipped with a 0.5-μm prefilter and a Rainin Short One column (10 cm × 4.6 mm, packed with 3-μm octadecylsilane-coated silica with trimethylsilane end-capping). Starting conditions were acetonitrile—0.1% TFA, pH 2.0 (10:90) at a flow-rate of 1.0 ml/min. Elution conditions included a linear gradient, increasing at 1% acetonitrile per min up to 30% organic solvent, followed by 20-min isocratic elution and another linear gradient of 2% acetonitrile per min up to 60% organic solvent. TFA concentration was maintained at 0.1%. Fractions (30 s, 0.5 ml) were collected using a Foxy fraction collector (Isco, Lincoln, NE, U.S.A.), and aliquots were quantified by RIA.

Quantification of peptides

Peptides were quantified by one of the three methods, depending on the quantity of peptide utilized in that experiment. When using large amounts of

peptide in aqueous solutions which did not contain other proteins, peptides were quantified by measuring the UV absorbance of the solution (at 280 nm) (Beckman spectrophotometer 25, Beckman Instruments, Fullerton, CA, U.S.A.). Another series of experiments utilized radioiodinated CCK-8, CCK-33 and gastrin-17. In this series of experiments, all fractions and the cartridge were directly assessed for radioactivity using a gamma spectrometer.

The most common method of quantitation was RIA. Rabbit antiserum (No. 3438), raised against CCK-8 conjugated to bovine serum albumin with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, was used in the assay. It recognizes the pentapeptide-amide common to CCK and gastrin, and has relative molar immunochemical potencies for the molecular species CCK-33, CCK-8, CCK-8 desulfate, gastrin-17 and tetrapeptide of 0.59:0.59:0.45:1.00:0.87, respectively [14]. In the assay, [^{125}I]gastrin-17 was used as tracer and gastrin-17 was used as standard. Samples were added to the assay after having been diluted with assay buffer (0.02 *M* barbital, pH 8.4 with 0.02% sodium azide, 0.2% human serum albumin and 0.01 *M* EDTA) to adjust the pH, salt, protein and organic solvent concentrations so that they would not interfere with the standard curve. Fig. 1 demonstrates the gastrin-17 standard curve, both in assay buffer as well as when 100 μl of acetonitrile—0.1 *M* TEA-A, pH 5.0 (50:50) (used to elute the cartridges) were added to each tube.

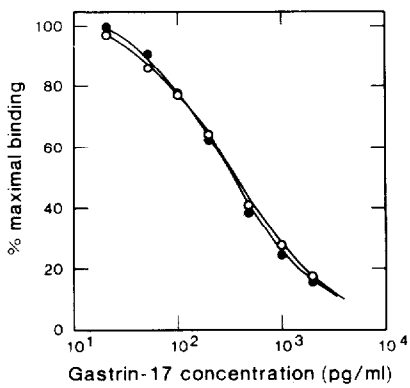


Fig. 1. Gastrin-17 standard curve in RIA utilizing antiserum No. 3438 and [^{125}I]gastrin-17 tracer. Standard curves are compared (•) when set up directly in assay buffer and (○) when 100 μl of acetonitrile—0.1 *M* TEA-A, pH 5.0 (50:50) was added to each tube. This demonstrates that CCK peptides eluted from ODS cartridges could be quantified using the standard curve.

RESULTS

Effect of sample characteristics on peptide extraction

Since homogenates and biological fluids may vary in pH, protein concentration and salt concentration, the effects of these variables were assessed in a controlled manner on the ability of the ODS cartridge to adsorb each of the CCK and gastrin peptides (Table I).

The effect of pH was evaluated using 0.1 *M* sodium phosphate buffers between pH 3 and 7. When loading each of the peptides in amounts ranging from 1 to 10 μg, no peptide was ever detected in the effluent or TEA-A wash by

TABLE I
PEPTIDE ADSORPTION TO SEP-PAK C_{18}

Peptide	Peptide adsorption* (%)									
	pH Variation			Protein concentration			Sodium chloride concentration			
	pH 3	pH 5	pH 7	1% BSA	5% BSA	10% BSA	0.5%	1.0%	1.5%	2.0%
CCK-4	100	100	100	99.6 ± 0.1	99.5 ± 0.2	98.6 ± 0.2	100	100	100	100
CCK-8	100	100	100	100	99.6 ± 0.1	95.9 ± 0.3	—	—	—	—
CCK-8 desulfate	100	100	100	99.2 ± 0.2	98.5 ± 0.5	97.0 ± 0.7	—	—	—	—
CCK-33	100	100	100	100	98.7 ± 0.3	99.9 ± 0.1	—	—	—	—
Gastrin-17	100	100	100	99.3 ± 0.4	95.4 ± 1.5	92.7 ± 0.9	—	—	—	—

*Peptide adsorption expressed as percentage of peptide applied.

RIA (detection limit, 20 pg/ml gastrin-17 equivalents). Each condition was repeated at least five times.

Protein concentration was evaluated by adding bovine serum albumin (Miles Labs., Elkhart, IN, U.S.A.) to 0.1 *M* sodium phosphate, pH 7.0. There was a trend toward reduced adsorption of each peptide to the cartridge with increasing protein concentration. However, more than 95% of all CCK peptides were adsorbed even in the presence of 10 g bovine serum albumin per 100 ml.

The effect of salt concentration was tested by adding sodium chloride to 0.1 *M* sodium phosphate, pH 7.0. This was tested only with tetrapeptide, but, again, no peptide was detected in the effluent with sodium chloride concentrations as high as 2 g per 100 ml.

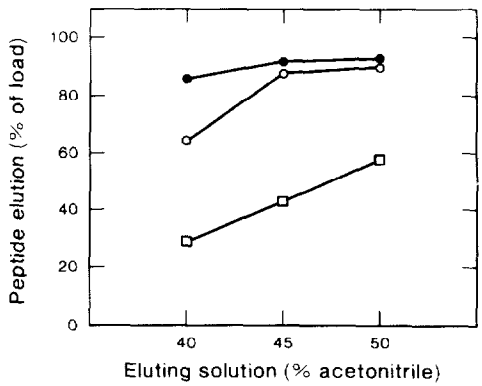
Capacity of the cartridge for peptide extraction

The capacity of the ODS cartridge to adsorb peptides was investigated using tetrapeptide, the least retained molecular form of CCK. Both the total amount of peptide capable of being adsorbed and the rate of presentation of the peptide were evaluated. The capacity of the cartridge was tested up to 5 mg of peptide. Tetrapeptide solutions of 1 μg/ml, 10 μg/ml and 1 mg/ml were injected in volumes of 1, 2, 5 and 10 ml at a rate of 1 ml/min. The 10 μg/ml solution was also tested at injection rates up to 5 ml/min. Each condition was tested at least five times. In every condition, no peptide was detected in the effluent from the cartridge.

Peptide elution conditions

To optimize the conditions necessary to elute the adsorbed peptides from the octadecylsilane, buffer molarity and organic solvent concentration were assessed. Fig. 2 illustrates the elution of the highly retained radioiodinated gastrin-17 (it must be noted that iodinating a peptide increases its binding to ODS supports). Elution was very poor with 0.02 *M* TEA-A, pH 5.0. It was better using 0.05 *M* TEA-A, pH 5.0, and reached 98% with 0.1 *M* TEA-A, pH 5.0. For the native (non-iodinated) peptides, there was no significant difference in elution using concentrations between 0.02 and 0.1 *M* TEA-A (data not shown).

Using this buffer (0.1 *M* TEA-A, pH 5.0), the concentrations of acetonitrile needed to elute each of the molecular species of CCK were assessed (Fig. 3). For each experiment, approx. 0.1 ng of peptide in 0.1 *M* potassium phosphate, pH 7.0 with 0.05 g bovine serum albumin per 100 ml, was injected onto the



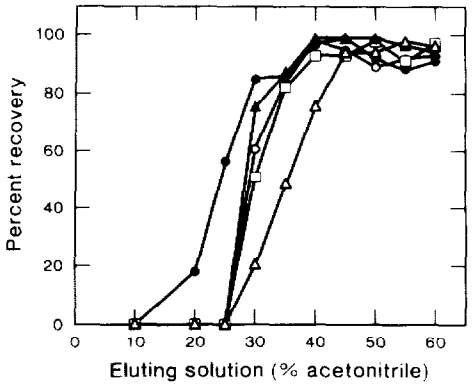


Fig. 2. Dependence on TEA-A, pH 5.0, concentration for elution of [^{125}I]gastrin-17 from ODS cartridge. (•) 0.10 *M* TEA-A; (○) 0.05 *M* TEA-A; (□) 0.02 *M* TEA-A.

Fig. 3. Dependence on acetonitrile concentration for elution of various CCK and gastrin peptides from ODS cartridge. Total recovery is shown after 0.1 ng of peptide has been applied and eluted. (•) Tetrapeptide; (○) CCK-8; (□) CCK-8 desulfate; (▲) CCK-33; (△) gastrin-17.

cartridge, and it was eluted with concentrations of acetonitrile between 20 and 60%. Each experimental condition was tested in triplicate at least two times. Tetrapeptide was eluted with the lowest organic solvent concentration. The other CCK peptides also eluted relatively easily, with greater than 93% recovery with 40% acetonitrile. Gastrin-17 was more difficult to elute efficiently, but using 45% acetonitrile, all species of CCK and gastrin-17 were recovered in excess of 93%.

Peptide recovery from human plasma

Four different methods to extract [^{125}I]CCK-8 from human EDTA plasma were compared (Fig. 4). Application of the native plasma directly to the ODS cartridge yielded only a 68% recovery, with 29% of the label not adsorbed (method I). This yield could be improved by simply adding an equal volume of acetonitrile to the plasma and centrifuging the sample to remove precipitat-

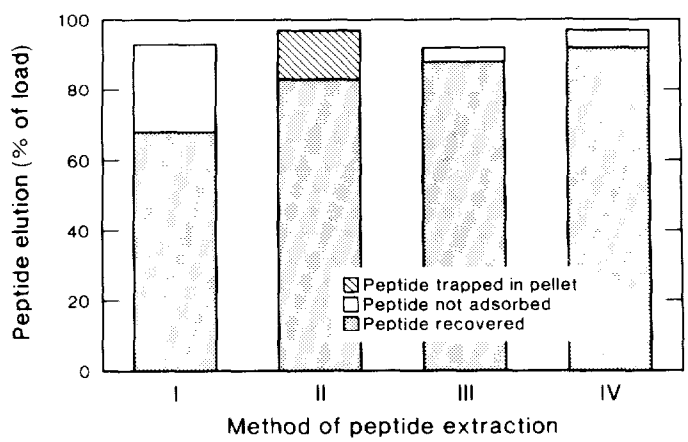


Fig. 4. [^{125}I]CCK-8 recovery from human plasma using the four methods for extraction detailed above.

ed proteins (method II); however, this is not adequate preparation for HPLC as the pellet traps or adsorbs a large amount of [^{125}I]CCK-8. Methods III and IV, which yield the best peptide recoveries, both involve diluting the plasma prior to injection onto the cartridge, with one incorporating TEA-A alone and the other adding 10% acetonitrile to this.

When native CCK peptides were added to fasting human EDTA plasma and Method III was used for extraction, each peptide was recovered with the following yields: CCK-33, 90%; CCK-8, 94%; CCK-8 desulfate, 89%; tetrapeptide, 82%.

DISCUSSION

The physiologic importance of CCK is well recognized, but not well studied, because of the difficulties in quantifying this hormone. Only recently have sensitive and specific RIAs been described [1]. In this study, we have established a simple, rapid technique to extract CCK peptides from biological fluids so that they can be separated by HPLC and quantified by RIA. Since the widely available type of COOH-terminal antiserum which recognizes both CCK and gastrin is useful at that stage, this will give many laboratories the opportunity to quantify each of the molecular species of CCK.

Cartridges packed with octadecylsilane have a strong affinity for CCK peptides. Each of CCK-33, CCK-8, CCK-8 desulfate and tetrapeptide can be adsorbed very efficiently to this cartridge from solutions of varied pH, protein and salt content. After washing through salts and large proteins which might interfere with HPLC, these peptides can be efficiently eluted from the cartridge with 2 ml of acetonitrile—0.1 *M* TEA-A, pH 5.0 (45:55). The cartridges have a capacity for CCK peptides in excess of 5 mg, far greater than any amount of CCK found endogenously in any physiologic situation.

This method has the advantages of being rapid, quite simple and not requiring special equipment. It has been validated for four molecular species of CCK. Although human species are not yet fully characterized, analogy with the porcine peptides is expected and is probably sufficient to use the same methodology. We have indirect evidence that this is so, since no endogenous human peptide was unadsorbed from EDTA plasma after injection through the cartridge, and more than 90% of the CCK immunoreactivity could be recovered.

Plasma offered special problems. Modification of the handling of plasma, however, gave excellent recoveries of the CCK peptides. There were suggestions of a concentration-dependent binding of CCK to plasma proteins which interfered with adsorption to the cartridge. Dilution of the plasma before injection overcame that problem.

This type of procedure is probably quite universal for many peptides. Because each peptide has unique charge and hydrophobic character, conditions must be set up and evaluated specifically for the peptides of interest, but certainly in a manner analogous to this.

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