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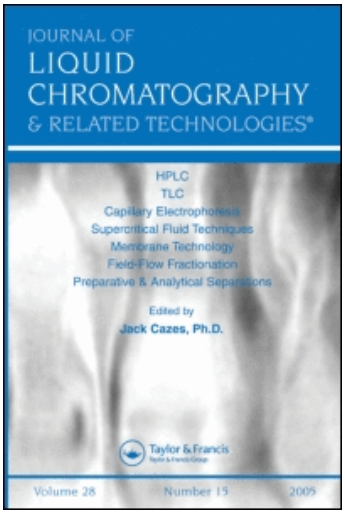

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A HIGH PRESSURE LIQUID CHROMATOGRAPHY-RADIOIMMUNOASSAY METHOD FOR MEASUREMENT OF CHOLECYSTOKININ-8 AND CHOLECYSTOKININ-33/39 IN PLASMA

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ABSTRACT

A new high pressure liquid chromatography-radioimmunoassay method is described for the measurement of cholecystokinin-8 (CCK-8) and CCK-33/39 in plasma. The plasma levels of CCK-8-sulfate, CCK-8-desulfate, and CCK-33/39 were measured during the infusion of intraduodenal fat.

INTRODUCTION

Cholecystokinin (CCK) is a gut peptide hormone that regulates gallbladder contraction and pancreatic exocrine secretion (1,2). Ingestion or intraduodenal (ID) infusion of fat is a potent stimulator for the release of CCK (3,4). Measurement of CCK in plasma is complicated by the variety of CCK forms, the small amounts in plasma, and the similarity of the C-terminal region of CCK with that of gastrin (5,6). The purpose of this study was to develop a high pressure liquid chromatography (HPLC)-radioimmunoassay (RIA) method for the separation and quantitation of CCK-8-sulfate, CCK-8-desulfate and CCK-33/39 in plasma.

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MATERIALS AND METHODS

HPLC-grade acetonitrile and ultrapure water were obtained from Burdick and Jackson Laboratories, Inc, and trifluoroacetic acid (TFA) from Pierce Chemical Company. Sep-Pak C-18 cartridges were purchased from Waters Associates. Radiolabeled Na ^{125}I was supplied by Amersham Corp. Chloramine-T was obtained from Eastman. Pure CCK-8-sulfate and CCK-8-desulfate were obtained from Squibb Institute for Medical Research, and CCK-33 and CCK-39 from the Karolinska Institute.

High Pressure Liquid Chromatography

A Model 342 gradient liquid chromatograph (Beckman Instruments, Inc) equipped with a Model 420 System Controller, two Model 112 Solvent Delivery Modules, a 340 Organizer and a Model 160 selectable-wave length UV detector was used in this study. A Wisp 710B Automatic Sampler (Waters Associates) was used for the injection of samples and standards. A Nova Pak, C-18 analytical column (4 micron particle size) was used for the reverse-phase separation of CCK-8-sulfate, CCK-8-desulfate and CCK-33/39.

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Experimental Design

Lipomul (25% corn oil) (2 g/kg-h in 50% saline) was infused into fasted mongrel dogs (18±3 kg, N = 3) through a duodenal cannula at a rate of 200 ml/h for two hours using a Harvard pump (Harvard Apparatus Co, Inc). Peripheral blood (10 ml) was collected at -15 (basal), 0, 15, 30, 60, and 180 minutes into ice-chilled glass tubes containing 15 U of heparin and 100 kallikrein inhibitory units of aprotinin (Novo Research Institute) per liter of blood. Plasma was separated immediately by centrifugation and stored at -20°C until it was extracted for CCK.

Sample Preparation for HPLC Analysis

Plasma CCK was extracted as follows using a Waters Sep-Pak rack (Waters Associates) (4). C-18 Sep-Paks were prewashed sequentially with 7.5 ml of methanol and 20 ml of water. Plasma was then added (4 ml) and washed with 20 ml of water. CCK was eluted with 2 ml of 100% ethanol:1% TFA in water (4:1 v/v). Extracts were evaporated to dryness under nitrogen gas at 30°C. Residues were reconstituted with 400 μl of water and were filtered through 0.45 μm filters (Rainin Instruments) by centrifugation at 4200 rpm for 30 minutes. Two hundred microliters of the plasma extracts were injected into the chromatograph.

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HPLC Analysis of CCK Variants

The HPLC analysis was carried out at a flow rate of 1 ml/min and monitored by a UV detector at 214 nm. A stepped gradient consisting of 0.05% TFA in water (solvent A) and 9:1 acetonitrile:solvent A (solvent B) was used for the separation of CCK forms (9-13% solvent B from 0-6 minutes, 13-25% solvent B from 6-12.5 minutes, and 26-41% of solvent B from 12.5-45 minutes). The retention times for the CCK variants were characterized by injecting pure standards (CCK-8-sulfate, CCK-8-desulfate and CCK-33, and CCK-39). One milliliter (1 minute) fractions of the HPLC eluant were collected using an LKB 2111 multirac fraction collector in tubes containing 40 μl of 5% sodium plasmanate (Travenol Laboratories, Inc). Fractions were dried under nitrogen gas and analyzed by RIA for CCK variants after reconstitution in CCK assay buffer (0.01 M potassium phosphate, 0.15 M sodium chloride, 0.1% sodium azide, and 1.5% normal rabbit serum).

CCK Radioimmunoassay

The radioimmunoassay procedures for CCK have been described in detail previously (7,8). Standard curves of CCK-8-sulfate,

CCK-8-desulfate and CCK-39 were obtained using pure standards. The plasma levels of CCK-33/39 and CCK-8 were calculated using the respective curves.

Recovery Studies

Recovery studies were done by adding 200 pg each of CCK-8-sulfate, CCK-8-desulfate, and CCK-39 to 3 ml aliquots of dog plasma which contained negligible endogenous CCK-like immunoreactivity. CCK was then extracted as described above. The residues were reconstituted in 400 μl of water, and 200 μl of the filtered extract was injected through the chromatograph. The recoveries of CCK-8-sulfate, CCK-8-desulfate and CCK-39 were quantitated by RIA.

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RESULTS

The efficiency of the extraction of CCK forms (CCK-8-sulfate, CCK-8-desulfate, and CCK-39) from plasma at the Sep-Pak extraction and at the HPLC separation steps are shown in Table 1. The average recovery of CCK-8-sulfate, CCK-8-desulfate and CCK-39 was

TABLE 1

Recovery of CCK by Sep-Pak Extraction and HPLC.

CCK Form	Added to Plasma (pg/ml)	Subjected to Sep-Pak Extraction (%±SEM)	Subjected to HPLC after Sep-Pak Extraction (%±SEM)
CCK-8-sulfate	66.7	71.5±5.3	78.1±12.0
CCK-8-desulfate	66.7	91.6±10.1	67.3±5.2
CCK-39	66.7	98.0±6.6	47.8±4.8

(N = 6)

87% at the Sep-Pak extraction step. The average recovery through the reverse-phase column was 64%. The mean retention times (± SEM, N = 5) of CCK-8-sulfate, CCK-8-desulfate and CCK-33/39 were characterized as 22.9±0.3, 28.0±0.3, and 31.1±0.6 minutes, respectively. Figure 1 shows the reverse-phase HPLC separation of a standard mixture of CCK forms.

Plasma levels of CCK-8-sulfate, CCK-8-desulfate, and CCK-33/39 levels increased during ID infusion of fat. The levels of CCK-8-desulfate and CCK-33/39 increased to maximum levels at approximately 60 minutes and the levels of CCK-8-sulfate reached a plateau at 60-180 minutes (Fig 2).

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DISCUSSION

In the present report, we have described a new procedure for the separation and quantitation of CCK-8-sulfate, CCK-8-desulfate and CCK-33/39. Molecular forms of CCK in extracts of the gastrointestinal tract of the dog, pig, and monkey have been characterized previously by gel filtration (9).

CCK-8 and CCK-33/39 levels were found in approximately equimolar amounts in the plasma of humans following oral ingestion of fat as characterized by HPLC (10). Recently, however, a change in the ratio of CCK-8 and CCK-33/39 along the intestine was reported in HPLC studies of human intestinal extracts (11). These changes along the intestine may explain the varying amounts of CCK forms found in the circulation during ingestion of a fat meal. Using our HPLC-RIA procedure, we have separated CCK-8-sulfate, CCK-8-desulfate, and CCK-33/39 from plasma collected during ID fat stimulation. Reverse-phase HPLC separation provides high resolution and fast, reproducible results compared to molecular exclusion chromatography. In the present study, we have found that the CCK-33/39 levels are almost three times as high as the CCK-8-sulfate level on a molar basis in peripheral fat-stimulated dog plasma. These findings are in agreement with the

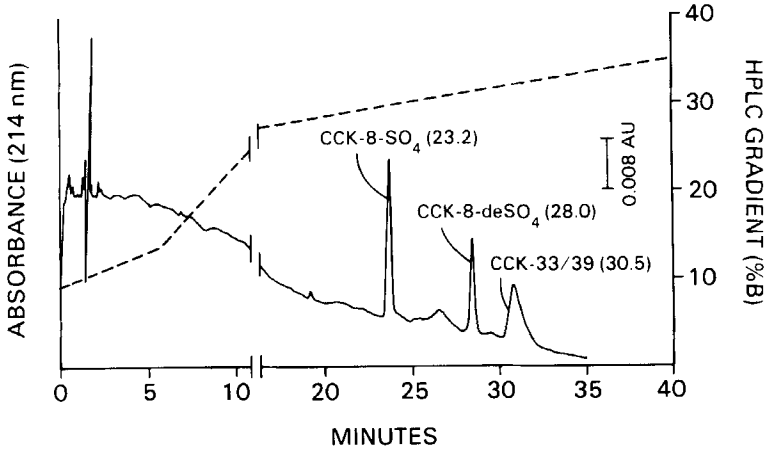

Figure 1. HPLC separation of a standard mixture of CCK-8-sulfate (200 ng), CCK-8- desulfate (200 ng), and CCK-33/39 (2 μg).

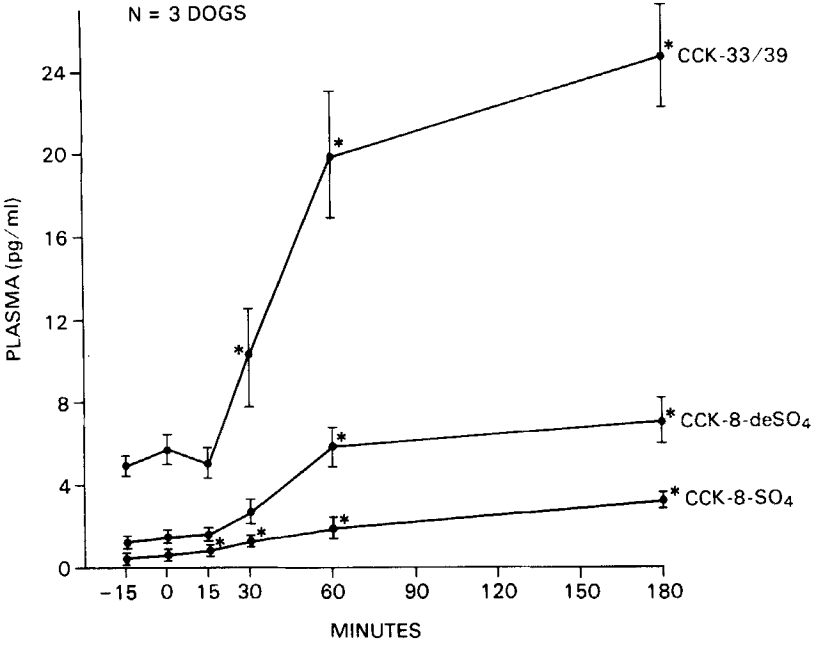

Figure 2. Release of CCK forms in canine plasma during intraduodenal infusion of Lipomul.

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observation by Sakamoto and colleagues (12) that CCK-8 is inactivated on hepatic transit while CCK-33 is not affected. Interestingly, we have also found significant levels of the biologically inactive form of CCK-8.

We have demonstrated that we can separate and quantitate low levels of CCK forms in biological samples using our HPLC-RIA procedure. This method can be applied to plasma samples as well as tissue extracts from dogs and other animals.

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