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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND SEPARATION OF PEPTIDE HORMONES IN HUMAN TISSUES AND PLASMA WITH REFERENCE TO CHOLECYSTOKININS

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SUMMARY

A reversed-phase high-performance liquid chromatographic method for the separation of peptide hormones has been applied to the measurement of large and small molecular forms of cholecystokinins in biological tissues. The method was validated for samples of human plasma and intestinal tissues, and for porcine gut extracts The two-stage chromatographic process used semi-preparative reversed-phase packing for initial sample preparation, followed by gradient elution on a Hypersil ODS-5 column. Peptides in the fmol—nmol range were separated reproducibly, and recovered quantitatively. The method has been applied to the purification of a novel biologically active CCK peptide from a porcine gut extract.

INTRODUCTION

Separation methods applicable to peptides include molecular exclusion, electrophoresis and ion-exchange chromatography [1]. Recently, reversed-phase high-performance liquid chromatography (HPLC) has been shown to produce good separation of various standard peptides [2—4]. The potential for high resolution coupled with rapid and reproducible separations using HPLC suggested that this technique should be suitable for the preparation and analysis of peptides from biological samples.

HPLC should have several advantages over conventional chromatography of peptides because, in addition to its high-resolving power, recoveries are very high and samples retain both immuno- and bioactivity [3]. Furthermore, the conditions used for analytical separation may be adapted to a preliminary preparative step permitting clean-up of plasma or tissue samples, removing most of the protein, salts and lipids, without resorting to conventional solvent

extraction or protein precipitation steps when recoveries are often low due to problems with partition or co-precipitation.

We have investigated this approach for a family of peptide hormones found in gut and brain, the cholecystokinins (CCKs). The name cholecystokinin was given to the active principle in gut extracts that caused gallbladder contraction when injected into a second animal. This agent was first discovered in the 1920s and subsequently isolated and sequenced in the 1960s as a 33-amino acid peptide [5]. Subsequent studies have identified a series of CCK peptides in gut and brain of 4, 8, 12, 39 and 58 amino acids [6—10], all sharing a common carboxyl terminal amino acid sequence. Cholecystokinins have been difficult to assay reliably using conventional radioimmunoassays in both blood and tissues firstly because of this molecular heterogeneity which requires knowledge of the proportions of the various molecular forms to determine the biological significance of the levels and secondly because of the similarities of CCKs to a series of related peptides sharing the same C-terminal pentapeptide sequence, but with different biological effects — the gastrins [11].

Extraction and separation of the CCKs from each other and from gastrins, by HPLC, prior to radioimmunoassay of fractions using an antibody common to the C-terminal portion of these molecules, offer the potential of assaying each form of the peptide individually.

HPLC coupled to bio- and immunoassay procedures has been used in this study analytically to assay fmol amounts of CCK peptides in plasma and in intestinal tissues, and preparatively to prepare nmol amounts of pure peptides from a commercial gut extract.

EXPERIMENTAL

Reagents and materials

Acetonitrile (HPLC 'S' grade 50% transmission at 205 nm) was obtained from Rathburn Chemicals (Walkerburn, U.K.), sodium chloride and hydrochloric acid (AristaR grade) from BDH (Enfield, U.K.). Hypersil ODS, 5-μm packing (Shandon Southern, Runcorn, U.K.) and Lichroprep RP-18 (25—40 μm, Merck, Darmstadt, F.R.G.) were supplied by HPLC Technology (Cheshire, U.K.). Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Northwich, U.K.). All glassware was siliconised before use, with trimethylsilane (Repelcote, Hopkin and Williams, Romford, U.K.). Distilled water was prepared and stored in glass containers. Two stock solutions were used. Solution A was 0.155 mol/l sodium chloride, adjusted to pH 2.1 with hydrochloric acid. Solution B was acetonitrile—water (3:2). All solvents were filtered before use using a 0.45-μm Millipore filter (HAWP-aqueous; FHLP-organic, Millipore, Harrow, U.K.). Solvents were regularly degassed with helium, through 25-μm solvent inlet filters).

Radiolabelled $Na^{125}I$ was purchased from Amersham Radiochemicals (Amersham, U.K.). Chloramine T and sodium metabisulphite were obtained from BDH; Norit GSX charcoal and dextran C from Hopkin and Williams.

Pure peptides were used for column calibration: 99% pure CCK 33 and 39 were generous gifts from Dr. V. Mutt (Karolinska Institute, Stockholm, Sweden); sulphated and non-sulphated CCK 8 were gifts from Squibb, Wirral,

U.K. The synthetic decapeptide caerulein was generously supplied by Farmitalia, Milan, Italy. Gastrin 17-1 was obtained from Serono (London, U.K.) and gastrin 34 was a gift from Dr S. Bloom. The CCK 8 antiserum was a gift from Dr. V. Go (Mayo Clinic, Rochester, NY, U.S.A.).

Semi-preparative apparatus

LiChroprep RP-18 (25—40 μm) was slurry packed in 10 × 0.46 cm stainless-steel columns using 20-μm frits, according to the manufacturer's recommended procedure. After slurrying the silica in isopropanol the column was packed in methanol at 200 bar. Columns were equilibrated as follows: 50 ml acetonitrile; 50 ml of solution B; 25 ml each of solutions A and B (1:1), and finally 50 ml of solution A. Each column was used for four plasma samples (total 40 ml plasma). After elution of peptides from each sample, columns were washed with 50 ml solution of B and then equilibrated as above. An Eldex (Owens Polyscience, Macclesfield, U.K.) high-pressure single-piston pump was used to load plasma samples at 2 ml/min and elute peptides at 1 ml/min from these semi-preparative columns. Sep-Pak C_{18} cartridges were washed with 20 ml each of methanol, acetonitrile, solution B, 10 mmol/l hydrochloric acid and solution A before use. Each cartridge was used once only.

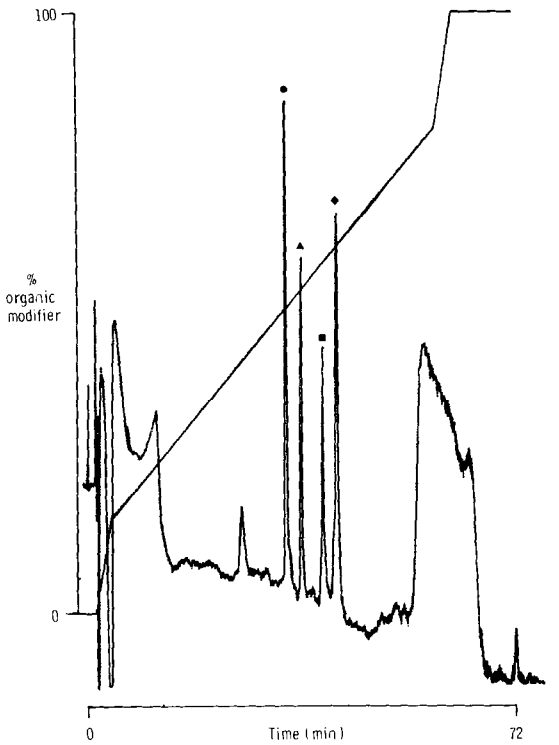


Fig. 1. Separation of mixture of CCK standards. (•) CCK 4 (Tetrin), (▲) caerulein (Ceruletide), (■) CCK 8 and (♦) CCK 33. 0.05 a.u.f.s.; 225 nm; chart-speed 2 mm/min; column, Hypersil ODS. Gradient betweeen 0.155 mol/l sodium chloride, pH 2.1 and acetonitrile—water (3:2).

Analytical apparatus

The high-performance liquid chromatograph (Applied Chromatography Systems, Luton, U.K.) was equipped with a microprocessor gradient controller, together with a Rheodyne sample injector (Model 7215) with a 2-ml loop, and a UV monitor (Cecil Instruments, Cambridge, U.K.). A dual-pen recorder (J.J. Lloyd, Southampton, U.K.) provided both a UV trace and a gradient profile. Data were collected and analysed on a Trilab microprocessor and printer–plotter (Trivector Systems, Sandy, U.K.). A reversed-phase octadecylsilane 5-μm support (Hypersil ODS) was packed in methanol at 350 bar using a slurry packer (Magnus 6050) in the recommended manner. Columns were stainless steel (316 grade), 10 × 0.46 cm and fitted with zero dead volume end fittings and 2-μm frits.

HPLC analyses were carried out at ambient temperature and monitored at 225 nm, where applicable. The flow-rate was 1 ml/min. A gradient was established between 0.155 mmol/l sodium chloride, pH 2.1 and acetonitrile–water (3:2) as described previously [4] and shown in Fig. 1.

Blood sample preparation

Human blood (20 ml) was collected into lithium heparin tubes containing 8000 IU aprotinin (trasylol, Bayer, Leverkusen, F.R.G.) to inhibit protease activity. After mixing, the blood was centrifuged at 2000 *g* for 10 min. Plasma was removed and stored in glass at −20°C until required. Prior to semi-preparative HPLC the plasma was diluted and centrifuged at 3000 *g* for 15 min to sediment cryoprecipitate. Plasma (10 ml) was diluted with 90 ml of solution A to render the viscosity suitable for pumping onto the column, and the ionic strength suitable for solute–column interaction.

Tissue sample preparation

Full-thickness sections of human jejunal, ileal and mid intestine were washed and stored at −20°C until required. Mucosal layers were separated from muscle layers by scraping. Two extraction procedures were used; a neutral extraction to preferentially extract the smaller molecular forms of cholecystokinin and an acid extraction which primarily extracts the larger forms [8].

Neutral extraction

Samples (ca. 100 mg) were weighed and plunged into 1 ml of boiling water for 2 min. Tissues were homogenised for 30 sec with a Potter-Elvejham homogeniser. After reboiling for a further 15 sec the samples were centrifuged at 2000 *g* for 10 min, and the resulting supernatant decanted.

Acid extraction

To ca. 100 mg tissue samples 1 ml of water was added and the samples boiled for 2 min. The extracts were adjusted to 0.5 mol/l acetic acid by the addition of 17.5 μl of glacial acetic acid and the tissues homogenised as above. The sample was left at 4°C for 30 min, then centrifuged and the supernatant decanted.

Semi-preparative chromatography

Diluted plasma supernatants were loaded onto a LiChroprep RP-18 column. This reversed-phase column packing has a high loading capacity for peptides in a protein-rich medium. The column was then washed with 10 ml of acidified saline (solution A) before eluting peptides with 10 ml of organic modifier (solution B). The sample was reduced to 50% of its original volume by removal of acetonitrile under nitrogen, 5 ml of solution A were added prior to the analytical HPLC step.

Tissue samples were processed through Sep-Pak C_{18} cartridges (with lower peptide:protein load capacity) in the same manner as described for plasma.

Radioimmunoassay

Column fractions or standards in acetonitrile—saline (400 μl), trasylol 1000 IU, veronal buffer 0.1 mol/l, pH 7.6, and antibody 1/40,000 were incubated in a total volume of 2 ml for three days, followed by addition of [^{125}I]CCK 8 (2000 cpm) and a further incubation of three days. The tracer used was non-sulphated CCK 8 iodinated by the chloramine T method and the mono-iodinated product separated from other reaction products by ion exchange [12]. The chromatography used was a gradient of 0.05 mol/l to 0.5 mol/l ammonium carbonate on a DE52 (1 × 10 cm) column (Whatman, Maidstone, U.K.). Separation of bound from free peptide was achieved using charcoal. Activated charcoal (40 g/l) equilibrated with dextran C (4 g/l) in phosphate-buffered saline and 500 μl of the mixture added to each tube. Tubes were centrifuged at 2000 *g* for 5 min and the supernatants decanted. Samples were counted in a gamma-counter and the percentage of tracer bound calculated for standards and samples. Initial binding was 50%, non-specific binding less than 3% and reproducibility within- and between-assay 10.1% and 14.2%, respectively. The detection limits for the assay were 12 fmol per tube for CCK 8, 24 fmol per tube for CCK 33 and 39 fmol per tube for gastrin 17, and for gastrin 34. The antibody had less than 3% molar cross-reactivity with CCK 4 and less than 0.1% cross-reactivity with other gut peptides.

Bioassay

CCK bioactivity was assayed using an in vivo guinea pig gallbladder contraction assay modified from that of Ljunberg [13]. Fasting guinea pigs (ca. 300 g) were anaesthetised with Hypnorm (1 ml/kg intramuscular, Janssen Pharmaceuticals, Lamberhurst, U.K.) and valium (0.5 ml/kg intra-peritoneal, Roche, Welwyn Garden City, U.K.), the abdomen opened, the gallbladder exposed and a silk ligature attached to its apex after the gallbladder bile had been removed by aspiration. The silk thread was attached to a counter-weighted beam, the other end of which was connected to a linearsyn pressure transducer. The transducer was in turn connected to an amplifier, containing smoothing circuits to damp out respiratory movements. By stabilising the baseline this modification allowed greater amplification of the gallbladder contraction signal and made the assay sensitive to less than 0.2 pmol CCK 8 or CCK 33 — a much greater sensitivity than previously described in in vivo bioassays. Standard peptides and samples lyophilised to remove acetonitrile and then reconstituted were assayed by measuring the amplitude of gallbladder contraction induced following

administration of samples through a jugular vein. The gallbladder contraction produced by a particular sample was independent of the intravenous sample size between 0.05 and 1.0 ml.

Validation

Estimation of peptide recovery. Extraction procedures for both plasma and tissue samples were assessed for recovery at various stages, using both fmol of radiolabelled non-sulphated CCK 8 and/or pmol amounts of sulphated CCK 8, non-sulphated CCK 8 and CCK 33. Recovery of iodinated tracer was assessed using gamma-counting and recovery of unlabelled peptides estimated by radioimmunoassay.

Semi-preparative stage. Approximately 15,000 cpm (0.5 fmol) of iodinated CCK 8 were added to 10 ml of human plasma. A portion of the sample (1 ml) was counted both before and after loading onto the Lichroprep RP-18 column. Likewise, 1 ml of the saline wash solution A and 1 ml of eluent solution B were retained for estimation of radioactivity. Similar studies were performed with iodinated CCK 8 in tissue samples. Peptides (4 pmol CCK 8 SO4 and 13 pmol CCK 33) were added to 10 ml of human plasma and treated as above. CCK was estimated using radioimmunoassay; stock solutions were used for comparison of pre- and post-extraction samples.

Analytical stage. Radiolabelled or standard unlabelled cholecystokinins were applied to the analytical HPLC. Fractions (1 ml) were collected for the duration of the chromatography and analysed using the gamma-counter or radioimmunoassay as appropriate.

Reproducibility. Microgram quantities of a single batch of HPLC-purified caerulein (Ceruletide, Farmitalia) were repeatedly chromatographed to determine the reproducibility of the analytical system.

RESULTS AND DISCUSSION

Previous studies [3] have shown the capabilities of HPLC in the separation of a wide variety of purified hormonal polypeptides. Recently other groups have used reversed-phase HPLC for the isolation and characterization of peptides from several biological matrices, viz. brain [14] neurosecretory system of the carp [15], leukaemia viruses [16], human [17] and bovine [18] pituitary tissues and salivary glands [19]. In each case there are preliminary extraction or purification steps unrelated to HPLC. In this study, in contrast, we have modified our analytical HPLC technique to provide a simple preparative step. Using this technique small forms of CCK peptide hormones have been assayed in biological fluids and tissues. We have shown the HPLC has several advantages over conventional chromatography including high resolution, virtually complete recovery of peptides (> 90%) which retained both bio- and immunoactivity, and a chromatography time of minutes rather than hours. Various detection systems could be used after HPLC; UV spectra where microgram quantities of peptides were available; bioassay for pmol amounts and radioimmunoassay where a sensitivity to fmol of peptide was required.

CCK 8 SO4 and CCK 33/39 in plasma and tissues were separated reproducibly on this system as shown by the radioimmunoassay profiles of

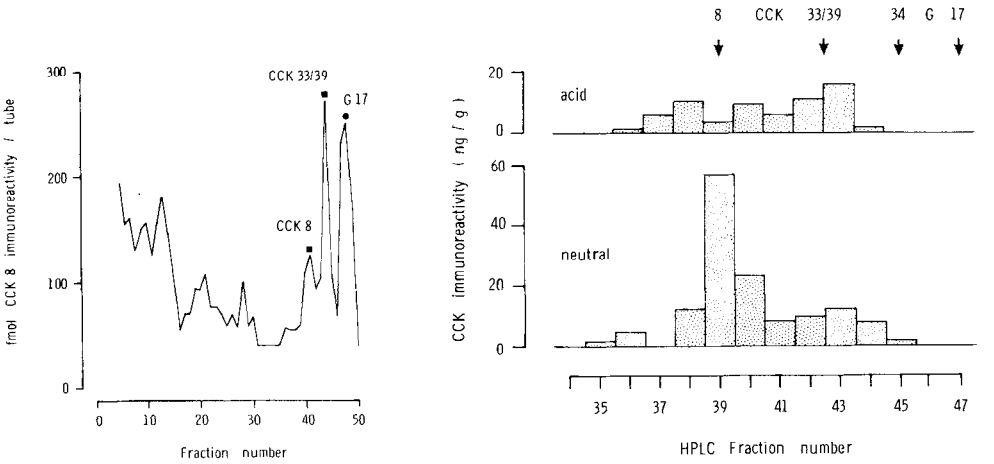


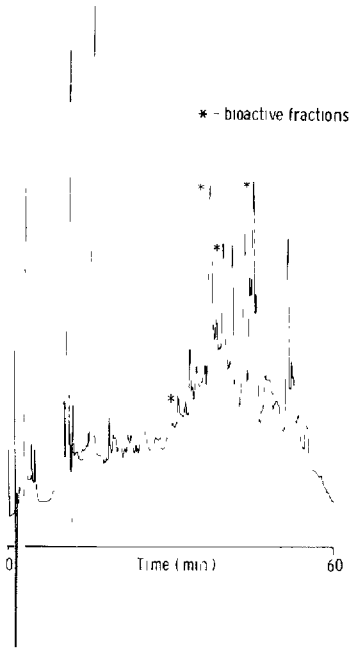
Fig. 2. Radioimmunoassay profile of a plasma sample fractionated into 1-ml fractions by HPLC. For reference the elution times of standard CCK 8, CCK 33/39 and gastrin 17 are shown.

Fig. 3. CCK immunoreactivity in acid and neutral extracts of jejunal mucosa separated by reversed-phase HPLC into 1-ml fractions. The elution times of pure standards CCK 8 and 33/39 peptides and gastrin 34 and gastrin 17 peptides are shown by arrows.

plasma (Fig. 2) and tissue (Fig. 3) sample. Indeed such a separation is a necessity if a non-specific antiserum which cross-reacts with the common C-terminal portion is used to assay forms of CCK. Gastrins were well separated from CCKs and from each other. In addition, other forms of immunoreactivity were present, particularly in plasma which were not biologically active CCKs or gastrins. These results parallel the separation of standard CCK peptides (Fig. 1) [4].

Purification of a novel biologically active CCK peptide was achieved using HPLC. Chromatography of a commercial gut extract (Boots Pancreozymin, Boots Pure Drug, Nottingham, U.K.) showed it to contain numerous peptides detected by UV absorbance (Fig. 4). Only some of these fractions, however, were likely to be CCKs as shown by the fact that only a few fractions contained bioactivity. In a more detailed analysis using column fractions both bio- and immunoactivity were noted in regions corresponding to CCK 8 and CCK 33/39 and in a region which did not co-chromatograph with these standards (Fig. 5). This column fraction (fraction 35) was subjected to gel chromatgraphy of a Sephadex G50SF matrix (Fig. 6) which again showed the CCK to be intermediate in molecular size between CCK 8 and CCK 33. These fractions from the Sephadex were then rechromatographed on reversed-phase HPLC. A single peak was detectable by UV (Fig. 6b) representing considerable purification of the original extract in a form suitable for determination of primary structure.

The HPLC system was validated with respect to recovery of peptides using both labelled and unlabelled peptides added to plasma and tissue samples. The recovery of [^{125}I]CCK 8 from plasma during the initial extraction procedure was 105 ± 10% S.D. (n = 6) and from the analytical column 98.5 ± 4% (n = 5). Recovery of CCK 8 S04 in this system was 95.6% (n = 3, range



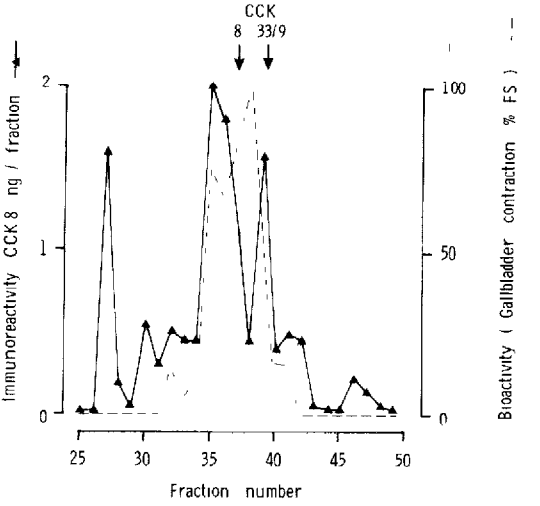


Fig. 4. High-performance liquid chromatogram of Boots Pancreozymin (a commercially available gut extract). Details as in Fig. 1 Asterisks represent bioactive fractions.

Fig. 5. CCK immunoreactivity (designated by ▲) and CCK bioactivity (— — —) in HPLC fractions from a gut extract of porcine upper intestinal mucosa. Elution times of standard CCK 8 and CCK 33/39 are designated by vertical arrows.

92—100%) after the initial extraction, and 96.0 ± 5% (n = 4) at the analytical step. Recovery of CCK 33 added to plasma and processed through both the preparative and analytical systems was 103.3 ± 11% (n = 4). Recovery of tracer from tissue homogenates was not significantly different from 100%. These results, although remarkable when compared with conventional chromatography of peptides, are in accordance with other methods for peptides separated by HPLC [3].

Although CCK 8 is insoluble in acid conditions, no losses of CCK 8 were observed during HPLC analysis at pH 2.1. Presumably this is because it is in intimate association with protein prior to column loading, is protected whilst on the column and only elutes when sufficient acetonitrile is present in the eludate to maintain its subsequent solubility.

The radioimmunoassay for carboxyl terminal of CCKs could be performed in the presence of acetonitrile and acid saline if two precautions were followed. Firstly the buffer used was of relatively high osmolality (0.1 mol/l) in order to maintain the pH of the assay tubes > 7.4. Secondly if the samples were not lyophilised to remove acetonitrile prior to assay then appropriate amounts of acetonitrile were added to standards. This is because acetonitrile has a slight non-specific inhibitory effect on antigen—antibody binding.

When acetonitrile was blown-off under nitrogen losses of labelled or unlabelled CCKs were insignificant until the volume was reduced by > 60%. Therefore, the volume was never reduced to less than 50% of the original.

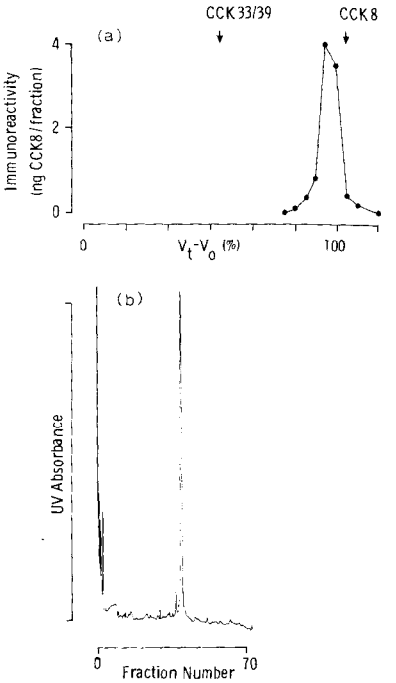


Fig. 6. (a) Gel chromatography (Sephadex G50 SF) of HPLC fraction 35 from a porcine gut extract possessing CCK immuno- and bioactivity of unknown molecular size. Intermediate form of CCK. The elution positions of CCK 33/39 and CCK 8 on Sephadex G50 SF are shown by arrows. (b) Rechromatography by HPLC of immunoreactive material from Sephadex G50 SF (intermediate form on CCK). Details as in Fig. 1.

Before the analytical HPLC the sample was made up to its original volume with solution A, to adjust pH and ionic strength to optimal conditions for interaction of solute with the analytical column packing. The small amounts of acetonitrile remaining did not preclude the peptides chromatographing satisfactorily on the analytical HPLC.

Using a pure synthetic analogue of CCK, caerulein, the coefficient of variation of retention time (min) observed was 0.017 (n = 7).

The method described here for peptide extraction from plasma and tissues has been validated for large and small forms of cholecystokinin using the analytical tool as a basis for semi-preparative conditions. In this system, CCKs are separated from each other and from gastrins, are recovered quantitatively and may thus be assayed. In principle, this system is applicable to extraction of many peptides from biological media providing the analytical step has been optimised for the peptides of interest.

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