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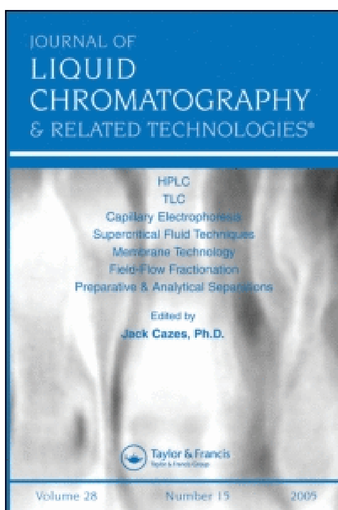

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CHOLECYSTOKININ CHARACTERIZATION IN URINE BY HPLC AND IMMUNOBLOTTING

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ABSTRACT

This paper describes a qualitative immunoreactivity cholecystokinin (CCK) characterization in 24 hours urine specimens. Urine was subjected to ethanol precipitation, followed by ion exchange chromatography, and immunoblotting with 2 antibodies. HPLC was developed to authenticate qualitatively the presence of CCK revealed by immunoblotting.

The results obtained by HPLC showed this method was more specific. HPLC allowed us to distinguish, through anti-CCK reactive fractions, a single peak which reacted only with CCK antibodies and not with gastrin antibodies.

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INTRODUCTION

Cholecystokinin (CCK) is an hormone which has many important functions. The first one concerns gut with regulation of pancreatic growth, enzyme secretion, and contraction of the gallbladder. Moreover, CCK influences intestinal motility and satiety. It is also a widespread transmitter in the nervous system[1] and recently was shown to be involved in pancreatic carcinogenesis.[2] At first, methods were based on biological assays,[3] such as pancreatic secretion or gallbladder contraction. These assays were difficult to interpret because of effects of other hormones or neural influences. To avoid these disadvantages, CCK-like activity in blood was developed. RIA and ELISA techniques in plasma came onto the market over the last 20 years. These methods presented some problems as the presence of different molecular forms of CCK, cross-reaction between CCK and gastrin because of an amino acid sequence similarity, limited peptide availability and low blood concentrations of CCK (in the picomole range), and difficulties with isotope labelling and peptide synthesis.[4] In contrast with CCK, plasma concentrations of gastrin were much higher and can explain non-specific responses with RIA and ELISA methods. For these reasons, HPLC was chosen in the present experiment. This paper is only the first part of a work which aim it is to quantify, subsequently, total CCK secretion, hoping that measurements in the urine would supplant difficulties with measuring CCK in blood. Urine samples were performed to circumvent variability depending on a nyctemeral cycle. Preliminary steps were necessary to optimise CCK detection in human samples.

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EXPERIMENTAL

Materials

Ion exchange chromatography: polypropylene columns (0.8 cm diameter and 4 cm height, BioRad, Ref. 731-1550), Dowex 50 W-X2, 100-200 Mesh H^+ form (Ref. 44460, Fluka).

Immunoblotting: high resolution agarose gel (Ref. 3040, Helena Laboratories), nitrocellulose membrane (Touzart and Matignon, Ref. 944.103.00), primary antibodies (rabbit polyclonal antiCCK-8 antibodies, Ref. CA 1125, TEBU Laboratories, rabbit polyclonal antiCCK-39 antibodies, Ref. CA 1124, TEBU Laboratories, rabbit antigastrin I antibodies, Ref. G07856, Sigma Biosciences), peroxidase labelled secondary antibodies (rabbit anti Ig G (H+L)).

HPLC: C18 5U reverse phase Econosphere column (Ref. 70066, Alltech), 250 mm length, 4.6 mm diameter, two pumps LC T-414 (Kontron Instruments) and an UV 432 detector with variable wavelength.

Methods

Urine samples (24 h), collected from patients in different clinical departments of the Saint-Etienne hospital and also from healthy people, were tested. Samples were treated with 99.5% ethanol according to IMAMURA,[5] centrifugated, and the supernatant was evaporated to dryness. The residue, dissolved in water,[6] was adjusted at pH 3. Ion exchange chromatography of the sample was performed. The fraction, eluted with 2 mol/L NH_4OH containing 20 % methanol, was collected. Immunoblotting was performed with antiCCK-8 antibodies and anti CCK-39 antibodies.

Only urines that were CCK-positive on immunoblotting were tested by HPLC.

The two elution reagents were: buffer A containing pyrogen-free water with 0.05% TFA (trifluoroacetic acid) and buffer B containing 50% acetonitrile, 49.95% pyrogen-free water, and 0.05% TFA. The following gradient was used: 0-5 minutes: buffer A, 5-10 minutes: 0 to 40 % buffer B, 10-80 minutes: 40 to 75 % buffer B, 80-120 minutes: 100 % buffer B (Fig. 1). The flow rate was 1 mL/min. Fractions were collected over 75 minutes at 5 minutes intervals, evaporated to dryness, dissolved in 20 µL pyrogen-free water, and tested by two immunoblottings with anti CCK and antigastrin antibodies.

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RESULTS

Immunoblottings showed that the most positive reaction was obtained when the ethanol residue was dissolved in water and brought to pH 3 before ion exchange chromatography. Immunoblotting with the antiCCK-39 antibody gave a more intense signal than with anti CCK-8 antibody. We also found that ion exchange chromatography was previously necessary before the immunotest to obtain positive reactions. Samples only precipitated with ethanol produced no immunoblotting signal, whatever the antibody.

Only fraction 9 of HPLC, corresponding to a peak at 54 minutes, contained true CCK (see Fig. 1).

DISCUSSION

It is clear that the pH of the sample used for ion exchange chromatography influences the yield of CCK eluted by NH_4OH; the best yield was obtained with a sample adjusted to pH 3.

The urine samples gave positive results on immunoblotting when they had been previously treated with ethanol, then ion exchange chromatography, but

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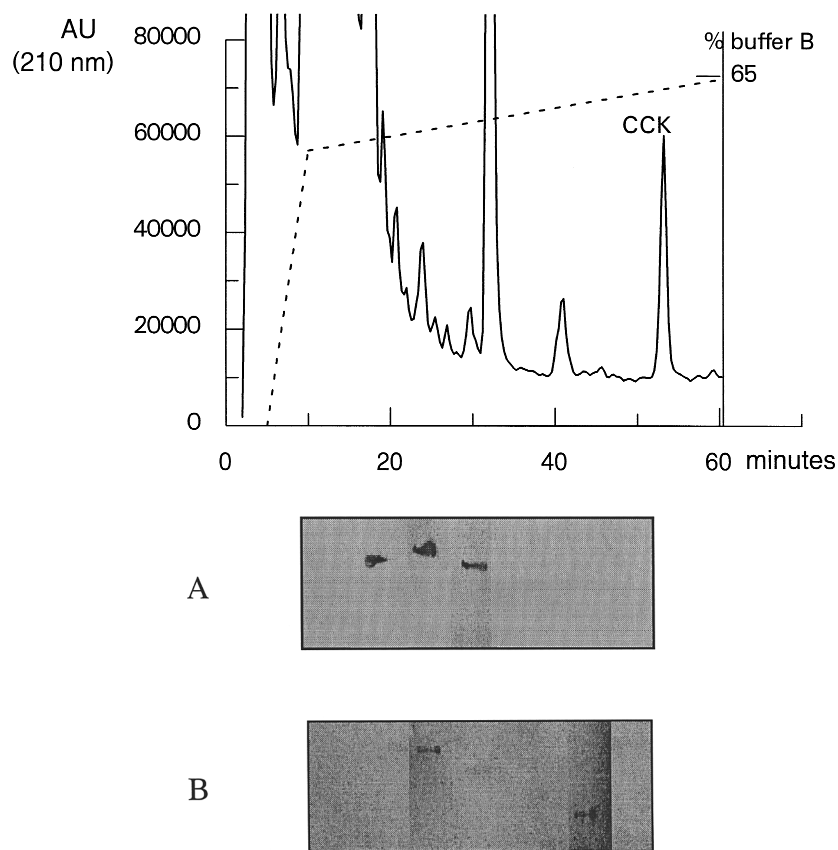

Figure 1. Urine chromatogram, HPLC eluted as the former gradient (—-) with buffer B containing 0.05% TFA, 50% acetonitrile; immunoblottings of fractions collected every 5 minutes and characterized by gastrin antibodies (A), CCK antibodies (B).

negative results when treated with ethanol alone. We concluded that the ion exchange chromatography step is required before immunoblotting. Some components of urine might inhibit the immunoblotting antigen-antibody reaction and they were removed by ion exchange chromatography. Samples were not subjected to ion exchange chromatography alone without ethanol treatment, because urine proteins and mucus must be removed before analysis.

Immunoblotting is a useful technique for detecting positive urine samples because many samples can be screened simultaneously. As the anti CCK-39 antibody gave a more intense signal than the anti CCK-8 antibody, the anti CCK-39 antibody was used for all subsequent screening experiments. However, the

immunoblotting technique can give false positive results because the antibodies used react both with CCK and gastrin.

HPLC analysis was developed to authenticate the presence of CCK revealed by immunoblotting. Fraction 9 was positive with anti CCK-39 antibody and negative with the anti gastrin antibody, and was identified as true cholecystokinin.

In conclusion, unlike the immunoblotting reaction which concerned only a part of the CCK molecule corresponding to the epitope, the HPLC technique separated the complete molecule. So, the results obtained by HPLC showed that this method was more specific, but had the inconvenience of being less sensitive than immunoblotting.

REFERENCES

1. Vanderhaegen, J.J.; Signeau, J.C.; Gepts, W. New Peptide in the Vertebrate CNS Reacting with Antigastrin Antibodies. Nature **1975,** *257*, 604-605.
2. Reubi, J.C.; Shaer, J.C.; Waser, B. Cholecystokinin CCK-A and CCK-B/Gastrin Receptors in Human Tumors. Cancer. Res. **1997**, *57*(7), 1377-1386.
3. Sjodin, L.; Nederman, T.; Olsson, P.A.; Viitanen, E. In Vitro Bioassays for Cholecystokinin. J. Pharm. Pharmacol. **1989**, *41*, 402-406.
4. Liddle, R.A. On the Measurement of Cholecystokinin. Clin. Chem. **1998**, *44*, 903-904.
5. Imamura, M.; Yamauchi, H.; Nakajima, Y.; Shin, S. Improved Radioimmunoassay of Cholecystokinin (CCK) Using OAL-656 by Ethanol Extraction of Human Plasma. Tohoku J. Exp. Med. **1993**, *170*, 53-58.
6. Massoubre, C.; Rascle, F.; Pellet, J.; Frey, F. False Positive Immunoassay Results for Cholecystokinin with a Phosphate Buffer Containing Bovine Serum Albumin. Eur. J. Clin. Chem. Biochem. **1996**, *34*, 65.

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