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NEW APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR ANALYSIS OF URINARY C-PEPTIDE

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SUMMARY

A successful application of high-performance liquid chromatography for analysis of urinary C-peptide is described. Samples (1.0 ml of human urine) were first subjected to gel chromatography to remove interfering substances, and then applied to a reversed-phase column (LiChrosorb RP-18, 7 μm). The detection of C-peptide was performed using a highly specific radioimmunoassay.

With the newly developed techniques, at least four forms of immunoreactive C-peptide were detected in human urine. One of these peptides was indistinguishable from authentic C-peptide. The present study has clearly demonstrated the heterogeneity of urinary C-peptide.

INTRODUCTION

It is now well known that insulin is synthesized as a precursor peptide, proinsulin, which is converted within the pancreatic β-cell to insulin and C-peptide [1]. C-peptide (31 amino acids, molecular weight approximately 3000) is subsequently secreted in equimolar amounts with insulin into the circulation [2], and is largely metabolized by the kidney [3]. Since a fraction of the total C-peptide produced is excreted in the urine, the measurement of urinary C-peptide immunoreactivity (CPR) is useful in assessing β-cell secretory capacity [4, 5]. However, very little information is available at present on the nature of urinary CPR.

High-performance liquid chromatography (HPLC) coupled with radioimmunoassay (RIA) has recently emerged as an excellent analytical tool for many bioactive peptides. This is the first study reporting the successful application of the HPLC—RIA technique for the analysis of urinary C-peptide.

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EXPERIMENTAL

Reagents and standard

All reagents were of analytical grade purity. Acetonitrile (E. Merck, Darmstadt, F.R.G.) was chromatographic quality; water was deionized and double-distilled.

Synthetic human C-peptide (position 33—63 of proinsulin) was kindly provided by Dr. Inoue (Shionogi Research Labs., Fukushima-Ku, Osaka, Japan) [6].

Sample collection

Urine samples were collected for 2 h after meals from healthy volunteers ranging in age from 22 to 72 years. The urine was stored at −20° C; samples were thawed and centrifuged before analysis.

High-performance liquid chromatography

HPLC was performed using a Waters HPLC apparatus (Waters Assoc., Milford, MA, U.S.A.) consisting of two pumps (Model 6000 A and Model M-45), a Model 660 solvent flow programmer, a Model U6K injector, a Model 441 UV detector operating at 214 nm, and a Unicord dual-channel chart recorder (Nippon Denshi Kagaku, Kyoto, Japan). The column (250 × 4 mm) consisted of reversed-phase LiChrosorb RP-18 (7 μm) packing supplied by Kanto Kagaku (Tokyo, Japan). The method of separation is based on the earlier report of Igano et al. [6]. The HPLC mobile phase, consisting of 0.1 *M* sodium phosphate pH 7.0—acetonitrile (81.6:18.4), was eluted isocratically for 20 min followed by a linear gradient of acetonitrile from 18.4% to 80% in 15 min at a flow-rate of 1.0 ml/min.

Radioimmunoassay of human C-peptide

The RIA of human C-peptide was performed using a kit provided by Daiichi Radioisotope Labs. (Tokyo, Japan). The detection limit of this kit is 0.1 ng of C-peptide. This assay system is highly specific for human C-peptide (and its derivatives), and, except for human proinsulin, no other cross-reactive materials are known in human urine [7, 8].

Major CPR peptides (a, b, c and d in Fig. 2) after HPLC were serially diluted and measured by RIA, and the dilution slopes were compared with that of synthetic human C-peptide.

Procedure

All urine samples were subjected to gel chromatography first and then to HPLC. A 1-ml volume of clarified urine was applied to a Bio-Gel P-10 column (100—200 mesh, 40 × 1.5 cm) and eluted with 1 *M* acetic acid at a flow-rate of 0.4 ml/min. Aliquots of each 1.0 ml fraction were lyophilized, reconstituted in water, and assayed for C-peptide. The fractions containing CPR were pooled and lyophilized. The residues were reconstituted in 120 μl of the mobile phase and filtered under centrifugal force through a 0.45-μm filter (Bioanalytical Systems, West Lafayette, IN, U.S.A.). A 90-μl aliquot of the solution was injected into the HPLC column and 1-min fractions (1.0 ml) were collected. A

0.1-ml aliquot of each fraction was lyophilized, reconstituted in water, and assayed for C-peptide.

Recovery

Urinary CPR peptides (a, b, c and d in Fig. 2) after HPLC were desalted separately by a Bio-Gel P-2 column (22 × 1.0 cm, 1 *M* acetic acid), lyophilized, and then known quantities (4—10 ng) of these peptides were subjected to HPLC in the same way as described above. A minimum of three chromatographic runs was performed for each peptide and their recoveries were determined by measuring the corresponding peak areas of CPR.

The significance of differences between means was assessed by Student's *t* test.

RESULTS

A representative gel chromatography pattern of immunoreactive C-peptide from urine is shown in Fig. 1. In all cases investigated, CPR in the eluate was demonstrated in a single peak; the apparent molecular weight for this peak was 3000. The mean recovery of CPR from the gel chromatography was 87.6 ± 4.2% (mean ± S.D., $n = 8$).

After partial purification by gel chromatography, the samples were analysed by HPLC. The results of a typical HPLC run are shown in Fig. 2. Four peaks of CPR were identified with retention times of (a) 4.0 min, (b) 9.0 min, (c) 17.5 min, and (d) 28.0 min. Peak c co-eluted with human C-peptide standard. The levels of CPR present in each peak area were determined and are summarized in Table I together with those of an additional seven samples. The relative amounts of the four peaks were 19.1 ± 1.5% (mean ± S.D.) for peak a, 9.7 ± 1.7% for peak b, 52.4 ± 4.2% for peak c and 14.1 ± 3.7% for peak d. The zones between the peaks contained only trace amounts (0.8—3.7%) of activity. The total recovery of CPR from HPLC was 88.5 ± 4.5% (mean ±

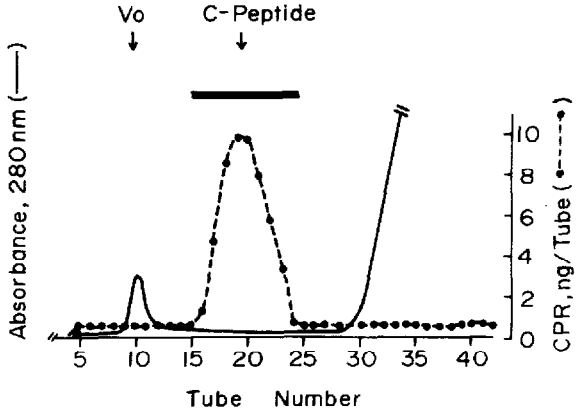

Fig. 1. Representative elution profile of urinary CPR, V_0 = void volume. A 1-ml volume of urine was applied to a Bio-Gel P-10 column (40 × 1.5 cm) and eluted with 1 *M* acetic acid. Aliquots of the fractions were assayed for CPR. The fractions containing CPR were pooled (as marked), lyophilized, and then subjected to HPLC. The column was calibrated with C-peptide standard (molecular weight 3000) as indicated by the arrow.

TABLE I

CPR PEPTIDES IN EIGHT SAMPLES OF HUMAN URINE DETERMINED BY HPLC–RIA

Sample	CPR applied (ng)	CPR recovered [ng (upper) / percentage of total (lower)]								Total HPLC recovery** (%)
		a*	$\overline{ab}$*	b*	$\overline{bc}$*	c*	$\overline{cd}$*	d*	Total	
1	24.3	4.1	0.4	2.3	0	11.4	0	2.6	20.8	85.6
		19.7	1.9	11.1	0	54.8	0	12.5	100	
2	15.7	3.2	0.2	1.5	0	8.7	0	1.9	15.5	98.7
		20.7	1.3	9.7	0	56.1	0	12.3	100	
3	22.6	3.6	1.1	2.1	0.2	11.3	0.1	2.5	20.9	92.5
		17.2	5.3	10.1	1.0	54.1	0.5	12.0	100	
4	21.5	3.3	1.1	1.9	0	10.8	0.1	1.5	18.7	87.0
		17.6	5.9	10.2	0	57.8	0.5	8.0	100	
5	29.4	5.0	0.9	2.5	0.5	12.4	0	3.7	25.0	85.0
		20.0	3.6	10.0	2.0	49.6	0	14.8	100	
6	42.7	7.1	1.6	4.6	0.9	17.5	0.5	5.6	37.8	88.5
		18.8	4.2	12.2	2.4	46.3	1.3	14.8	100	
7	15.7	2.8	0.5	1.0	0.1	6.1	0	2.8	13.3	84.7
		21.1	3.8	7.5	0.8	45.9	0	21.1	100	
8	16.1	2.4	0.5	0.9	0	7.6	0.1	2.4	13.9	86.3
		17.3	3.6	6.5	0	54.7	0.7	17.3	100	
Mean ± S.D. (%)		19.1 ± 1.5	3.7 ± 1.4	9.7 ± 1.7	1.6 ± 0.7	52.4 ± 4.2	0.8 ± 0.3	14.1 ± 3.7		88.5 ± 4.5

*a, b, c, d = peak zones; $\overline{ab}$, $\overline{bc}$, $\overline{cd}$ = zones between the peaks (shown in Fig. 2).
**(Total CPR recovered/applied CPR) × 100.

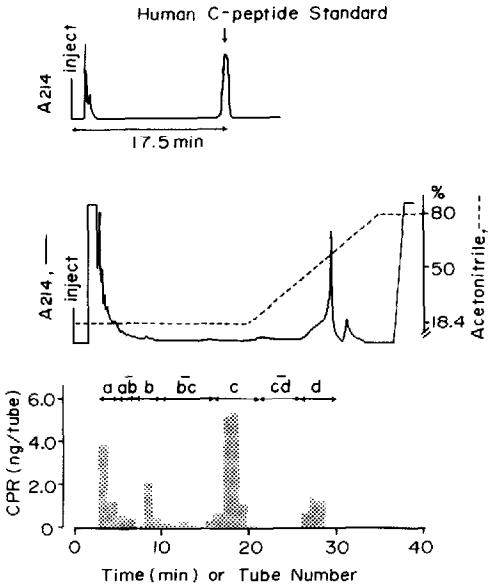

Fig. 2. Representative HPLC of the CPR peptides purified by gel chromatography. Chromatographic conditions: column, LiChrosorb RP-18, 250 × 4 mm; eluents, 18.4% acetonitrile in 0.1 *M* sodium dihydrogen phosphate pH 7.0, isocratic for initial 20 min followed by a linear gradient of acetonitrile from 18.4% to 80% in 15 min and maintained at 80% for an additional 5 min; flow-rate 1.0 ml/min; room temperature. Fractions of 1.0 ml were collected and aliquots used for RIA. The fractions containing CPR (fractions 4—30) were divided into seven zones as indicated: four peak zones (a, b, c and d) and three zones between the peaks ($\overline{ab}$, $\overline{bc}$ and $\overline{cd}$). For comparison, a chromatogram of C-peptide standard is shown in the upper panel.

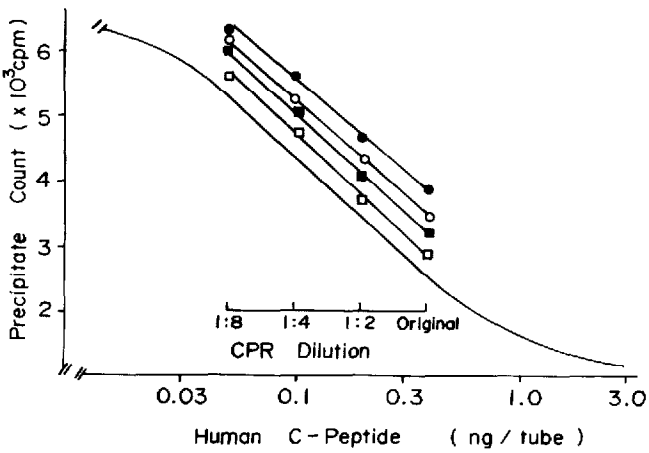

Fig. 3. Standard curves of human C-peptide (—) and dilution slopes of urinary CPR peptides. (○) peak a; (●) peak b; (□) peak c; (■) peak d.

S.D.), and there were no significant differences ($p > 0.05$) in the recoveries of the four CPR peptides from HPLC: 88.4 ± 4.1% (mean ± S.D.), 89.9 ± 2.9%, 86.8 ± 5.6% and 91.7 ± 7.6% for peaks a, b, c and d, respectively.

Serial dilutions of the four CPR peptides of urine were parallel to the displacement curve of synthetic human C-peptide in the RIA (Fig. 3).

TABLE II

REPRODUCIBILITY

	Urinary CPR peptides			
	a*	b*	c*	d*
Intra-assay (n = 4)				
Mean (ng)	5.1	2.7	12.9	4.1
S.D. (ng)	±0.43	±0.25	±0.89	±0.45
C.V. (%)	8.4	9.3	6.9	11.0
Inter-assay (n = 4)				
Mean (ng)	6.8	3.8	18.4	4.1
S.D. (ng)	±0.37	±0.51	±2.4	±0.53
C.V. (%)	5.5	13.5	12.8	13.0

*a, b, c, d are shown in Fig. 2.

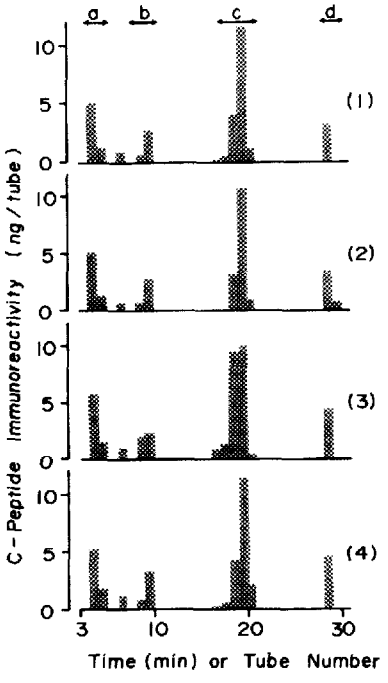

Fig. 4. Effect of sample storage on HPLC pattern of urinary CPR peptides. One urine sample was divided into four equal volumes and analysed on four different occasions: before storage (1) and after storage at −20°C for two days (2), seven days (3) and fourteen days (4). a, b, c, d = peak zones of CPR.

The stability of the HPLC system from day to day was confirmed by checking the retention time of C-peptide standard; the average time was 17.5 ± 0.38 min (mean ± S.D., n = 8). Within-day reproducibility of the method was estimated by carrying out four determinations of one urine sample containing CPR at a concentration of 29.4 ng/ml. Between-day reproducibility was tested by measuring 1.0-ml aliquots of the same sample on four different days over a period of two weeks. These results are shown in Table II and Fig. 4.

DISCUSSION

In the present study, we have described a new application of HPLC coupled with RIA detection for the analysis of urinary C-peptide. Igano et al. [6] reported the successful application of HPLC for the purification of synthetic C-peptide. In their experiment, the retention time of C-peptide standard was about 4 min. Under these conditions, however, the ability of the method to differentiate between authentic C-peptide and potential analogues in urine is questionable. We therefore designed the HPLC conditions to increase the retention time of C-peptide, employing another type of column and lower concentrations of acetonitrile. In this study, the retention time of C-peptide standard was 17.5 min (on average), and the stability of the chromatographic system was extremely high.

For sample purification prior to HPLC analysis, we used gel chromatography. As reported previously by other investigators [4, 9], urinary CPR eluted as a single peak from Bio-Gel P-10 columns in the region corresponding to the C-peptide marker. No immunoreactivity was found in the proinsulin (molecular weight approximately 9000) region of the column. This procedure is simple and rapid in removing proinsulin, if any, and major interfering substances. An additional advantage is the higher recovery of CPR from gel chromatography.

The HPLC system described has demonstrated the excellent separation of urinary CPR peptides. The intra- and inter-assay precision studies have indicated the method to be reliable and reproducible. As shown in Fig. 4, a higher stability for each CPR peptide in urine on storage at -20°C is indicated. Kuzuya et al. [7] reported that there were no changes of CPR concentrations for up to one year.

With the newly developed HPLC method, we have clearly demonstrated the presence of the heterogeneity of urinary C-peptide. In all cases tested, at least four forms of CPR were detected. It was suggested that these four peptides have similar antigenic determinants, but that they are probably not structurally identical because of their different behaviours on HPLC. One of the peptides (peak c in Fig. 2) was chromatographically indistinguishable from authentic C-peptide; the contribution of this peptide to the total CPR was 52.4 ± 4.2%. There were no considerable differences in the recoveries of the CPR peptides from HPLC, suggesting that the relative abundance of the four forms of CPR is independent of the chromatographic procedure.

Gaynor et al. [10] have recently reported the presence of the heterogeneity of urinary C-peptide using polyacrylamide gel electrophoresis. Moreover, Kuzuya and co-workers [11, 12] have indicated heterogeneity of circulating and pancreatic C-peptide. The pathophysiological importance of the heterogeneity of C-peptide remains to be elucidated.

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