Quantitation of Human Pancreatic Beta-cell Function by Immunoassay of C-Peptide in Urine

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SUMMARY

Human proinsulin connecting peptide (C-peptide) was measured by immunoassay in urine from 25 normal subjects, 18 patients with diabetes mellitus, and 34 patients with various degrees of renal insufficiency. Assay validation studies showed that pancreatic C-peptide was quantitatively recovered when added to urine. Fractionation of urine by gel filtration indicated that most endogenous C-peptide eluted in fractions that corresponded to the C-peptide standard.

In 34 nondiabetic subjects with normal kidney function or various renal diseases, C-peptide clearance was independent of creatinine clearance over a range of 6 to 190 ml./min. Urine C-peptide clearance (5.1 ± 0.6 ml./min.) is greater than that of insulin (1.1 ± 0.2 ml./min.), and the total quantity of C-peptide excreted in the urine per day represents 5 per cent of pancreatic secretion, as against only 0.1 per cent of secreted insulin.

Healthy subjects excreted 36 ± 4 μg. C-peptide per 24 hours, while this value in juvenile-onset diabetics was only 1.1 ± 0.5 μg. Adult-onset diabetics excreted 24 ± 7 μg./24 hr., the range overlapping the excretory rates of both normal subjects and juvenile-onset diabetics. Two insulin-requiring adult-onset diabetics showed significant beta-cell reserve during the course of acute infections.

These results suggest that urine C-peptide provides a useful means of assessing beta-cell secretory capacity over a period of time and is especially advantageous when frequent blood sampling is not feasible.

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It is now well established that insulin is synthesized as a precursor molecule, proinsulin, which is converted within the pancreatic beta cell to insulin with release of the connecting peptide and several basic amino acids.[1] In man, the connecting peptide, or "C-peptide," consists of 31 amino acids.[2] It is released from beta cells in equimolar amounts with insulin[3] and can be detected in the peripheral circulation by radioimmunoassay.[4] Since insulin and C-peptide are metabolized at different rates, their peripheral concentrations are generally not equimolar. Nevertheless, serum C-peptide and insulin concentrations are highly correlated in both portal and peripheral veins.[5]

Although peptide hormone concentrations in blood reflect most accurately the minute-to-minute changes in their secretion, measurement of urine levels may be advantageous in reflecting average serum values over a period of time. Furthermore, this approach may also be useful in situations where repeated blood sampling is difficult, such as in small children. While assay of insulin in urine has been shown to be feasible,[6] its value is diminished because only a small fraction of the secreted insulin appears in the urine. Because the urinary clearance of C-peptide is much higher than that of insulin in animals,[7] we have investigated the utility of measuring urine C-peptide in man.

METHODS

Urine samples were collected in plastic containers coated with 10 per cent bovine serum albumin and refrigerated at 4° C. during the collection period. Following the completion of the collection, an aliquot of urine was adjusted to pH 7 to 8 with 1 N NaOH and then frozen until assayed. Any sediment appearing on

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neutralization was removed by centrifugation prior to freezing.

C-peptide immunoreactivity was measured by immunoassay[4] with a rabbit C-peptide antiserum prepared against synthetic human C-peptide (kindly supplied by Dr. Noburu Yanaihara, of Shizuoko College of Pharmacy, Shizuoko-Shi, Japan) and the double-antibody method of Morgan and Lazarow.[8] Proinsulin cross-reacts in this assay about 1/15 as well as C-peptide on a weight basis. Serum and urine creatinine were measured by the alkaline picrate method.[9] Gel filtration of selected urine samples was accomplished by adding 0.2 ml. of urine to 0.8 ml. of 3 M acetic acid and applying this mixture to a 50-×-1-cm. Biogel P-30 column equilibrated in 3 M acetic acid. Columns were calibrated with ^{125}I-labeled proinsulin and unlabeled human C-peptide. 1.0-ml fractions were collected, dried under vacuum, and redissolved in borate-albumin buffer, pH 8.0, prior to immunoassay.

Experimental subjects. Control subjects were recruited from healthy hospital staff personnel who were not taking any medications and were within 15 per cent of ideal body weight. For long-term studies, subjects were admitted to the Clinical Research Center and maintained on an isocaloric diet but remained ambulatory. Except where indicated, diabetic subjects were in stable control on their prescribed diet and free of infectious complications when urine samples were obtained. Patients with a variety of renal diseases (excluding diabetic nephropathy), as well as subjects with normal renal functions, collected a two-hour urine specimen for clearance studies; blood samples for creatinine and C-peptide determination were drawn at the beginning and end of the collection period. Clearances were calculated by the standard method, using the mean of the two serum concentrations. Informed consent was obtained from all subjects, and the studies were approved by the hospital committee on human investigation.

RESULTS

Validation of C-peptide assay in urine. Four urine samples from normal subjects were assayed in volumes ranging from 5 to 20 μl. (table 1). Variation between values assayed at different dilutions did not exceed 7 per cent within the working range of the assay. Also, there was no tendency for values to increase or decrease with changing dilution, indicating that urine C-peptide dilutions lay parallel to the C-peptide standard curve.

TABLE 1

Effect of dilution on urine C-peptide measurement

Sample	Volume Assayed (μl.) 5	10	20	Mean	C.V.
1	76.2	74.1	70.5	73.6	4%
2	178	170	—	174	3%
3	50.0	47.3	54.5	50.6	7%
4	70.0	74.0	74.5	72.8	3%

Values in table show C-peptide concentrations (ng./ml.) in original undiluted urine, obtained by multiplying assayed values by appropriate dilution factor. C.V. = coefficient of variation.

In another study, 0.1, 0.5, or 1.0 ng. of C-peptide standard was added to 0.2-ml. aliquots of urine from juvenile diabetics with low endogenous C-peptide concentrations. The results are shown in figure 1 and, by chi-squared test, did not differ significantly from the line indicating 100 per cent recovery.

To confirm that we were measuring C-peptide rather than a mixture of C-peptide and proinsulin, urine samples were gel-filtered on Biogel columns equilibrated in acetic acid and the eluates measured in the C-peptide assay. A typical result is shown in figure 2. All C-peptide immunoreactivity in this urine sample was found in fractions corresponding to the C-peptide marker. Some trailing of the peak was seen,

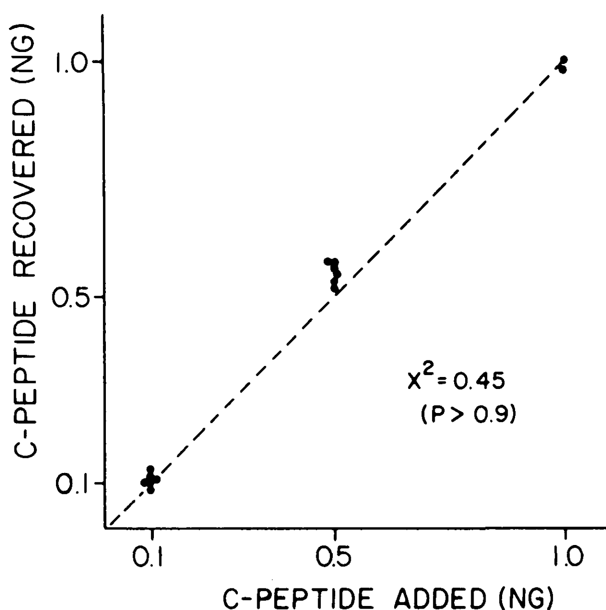

FIG. 1. Recovery of C-peptide added to 0.2-ml. aliquots of urines obtained from juvenile-onset diabetics and having unmeasurable endogenous C-peptide. The dashed line indicates complete recovery, and the value of chi-square indicates that there is no significant deviation from this line.

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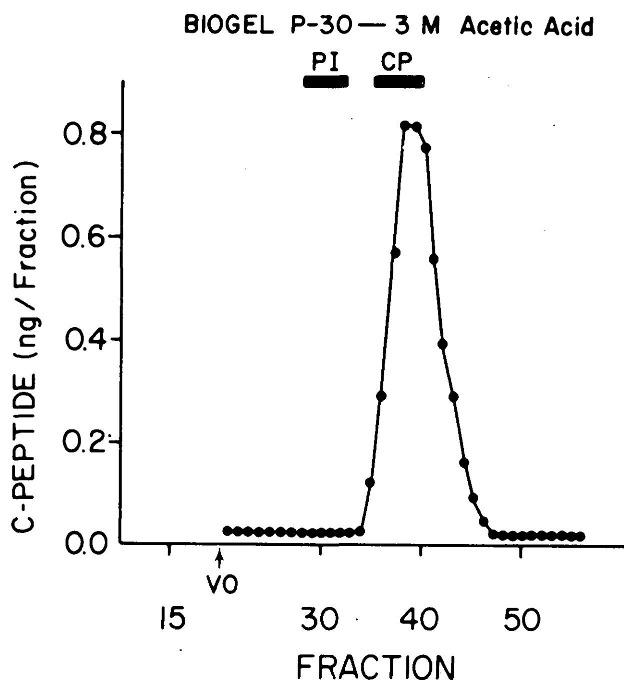

FIG. 2. Typical gel-filtration pattern of urine on Biogel P-30 eluted with 3 M acetic acid. Elution volumes of ^{125}I-labeled proinsulin (PI) and unlabeled C-peptide (CP) standards are indicated by the horizontal bars.

suggesting that smaller immunoreactive fragments of C-peptide may be present. A few urine samples showed, in addition to the major peak in the C-peptide region (centering on fraction 38), a minor peak of higher-molecular-weight immunoreactive material. This eluted in fraction 27, about three fractions before a proinsulin standard (fraction 30), but well after the void volume (fraction 20). Previous studies had shown that proinsulin cannot be detected in urine by insulin immunoassay unless it is concentrated approximately 100-fold. Taken together, these findings indicate that C-peptide immunoreactivity in urine represents C-peptide exclusively but that some of the C-peptide may be present in large aggregates and some in lower-molecular-weight species.

Studies in normal and diabetic subjects (figure 3). Twenty-five healthy subjects excreted 36 ± 4 μg. C-peptide in 24 hours. The urine C-peptide in 12 stable insulin-requiring adult-onset diabetic patients maintained on their usual diet and exogenous insulin was 24 ± 7 μg./24 hours, but the range was large. A group of six juvenile-onset diabetics who had reached adulthood and who had creatinine clearances greater than 60 ml./min. excreted 1.1 ± 0.5 μg. C-peptide/24 hours.

To assess the constancy of 24-hour urine C-peptide excretion, two healthy subjects resided in the Clinical Research Center for four weeks. They were given an isocaloric diet but continued their daily activities. Twenty-four-hour urine C-peptide values showed a coefficient of variation of 22 per cent in one subject and 32 per cent in the other. Expressing results as micrograms C-peptide per gram creatinine gave coefficients of variation of 13 per cent and 35 per cent, respectively. All samples from each subject were measured in the same assay; the intra-assay coefficient of variation was 7 per cent.

Two diabetic patients who had been well controlled on small doses of insulin were studied after they developed foot infections that eventually required amputation. Sequential 24-hour urine C-peptide measurements are shown in figure 4. L.J. (lower panel) had previously been included in our group of stable adult-onset diabetics, giving us the advantage of having measured baseline urine C-peptide levels. In both subjects, urine C-peptide rose to very high levels during the septic period, exceeding those of unstressed

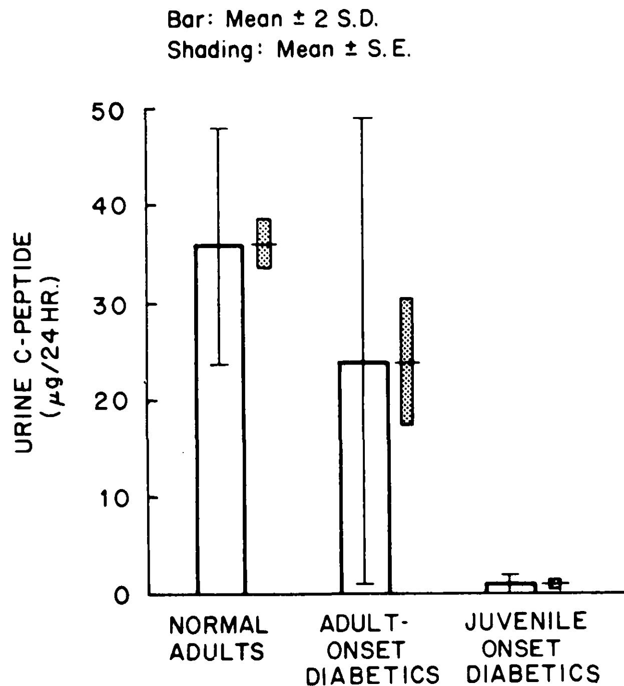

FIG. 3. Twenty-four-hour urinary C-peptide excretion in normal adults, adult-onset diabetic patients, and juvenile-onset diabetics who had attained adult age. The lines above and below the open bars give the means ± 2 S.D., to give an indication of the range of values, whereas the shaded bars give mean ± S.E.

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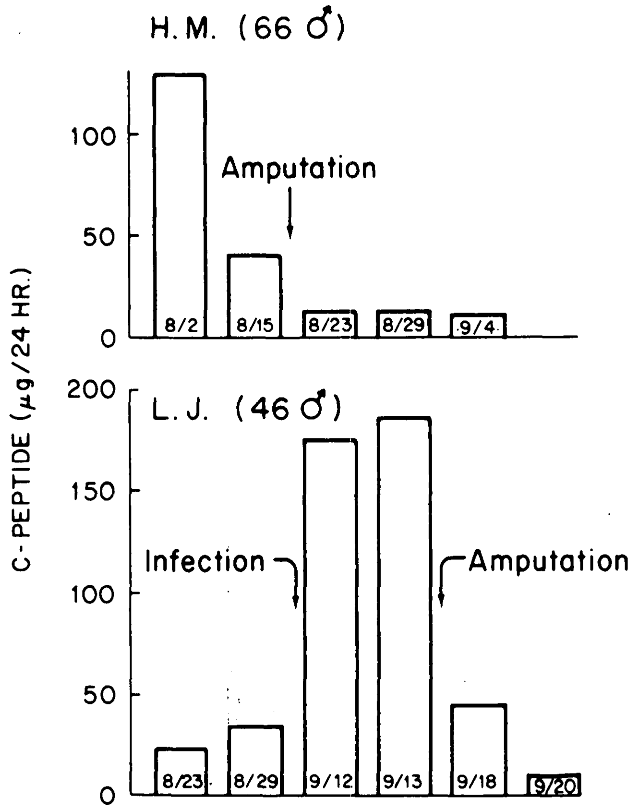


FIG. 4. Sequential 24-hour urine C-peptides on two diabetic patients suffering foot infections ultimately requiring amputation. Patient L. J. had also been studied prior to his developing septic complications on dates 8/23 and 8/29.

normal subjects, and subsequently fell markedly following amputation. In H.M. (upper panel), a decrease in urine C-peptide was seen when large amounts of exogenous insulin were given before surgery in order to improve diabetic control (second bar, 8/15).

Relationship of urine C-peptide to renal function. To ascertain the relationship of urinary C-peptide excretion to renal function, creatinine and C-peptide clearances were measured simultaneously in 34 nondiabetic subjects whose creatinine clearance ranged from 6 to 190 ml./min. Mean C-peptide clearance was 5.1 ± 0.6 ml./min. and was independent of the creatinine clearance (figure 5). Urinary C-peptide excretion (figure 6) was also unrelated to creatinine clearance. Those patients with proteinuria had no increased C-peptide excretion.

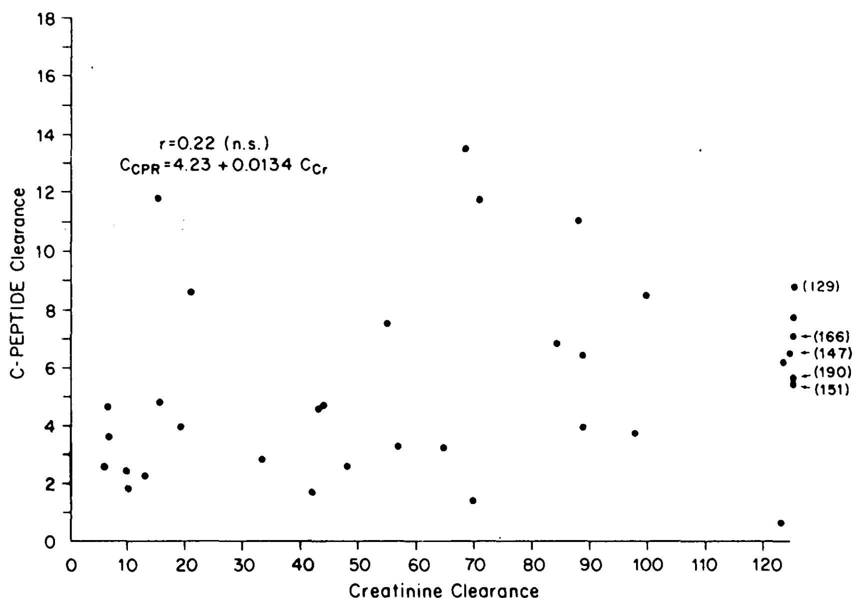
DISCUSSION

The present results indicate the feasibility of using the C-peptide content of urine as a means of monitoring beta-cell secretion over a period of time. The data indicate an absolute deficiency of insulin in established juvenile-onset diabetes, while the wide range of values for adult-onset diabetes is consistent with varying degrees of beta-cell loss. Urine C-peptide measurements also suggest that certain insulin-requiring adult-onset diabetic patients may show significant beta-cell reserve when confronted by an acute stressful situation.

The normal daily beta-cell output of insulin has been estimated to be in the range of 1.6 mg. (40 U.) insulin and, hence, 0.8 mg. C-peptide (C-peptide having approximately one-half the molecular weight of insulin). The excretion of 36 μg. C-peptide in urine of healthy subjects thus represents 5 per cent of the

FIGURE 5

Relationship between urinary two-hour creatinine and C-peptide clearances. CCPR and CCr refer to the clearances of C-peptide and creatinine, respectively; r is the product-moment correlation coefficient. All values are in ml./min.



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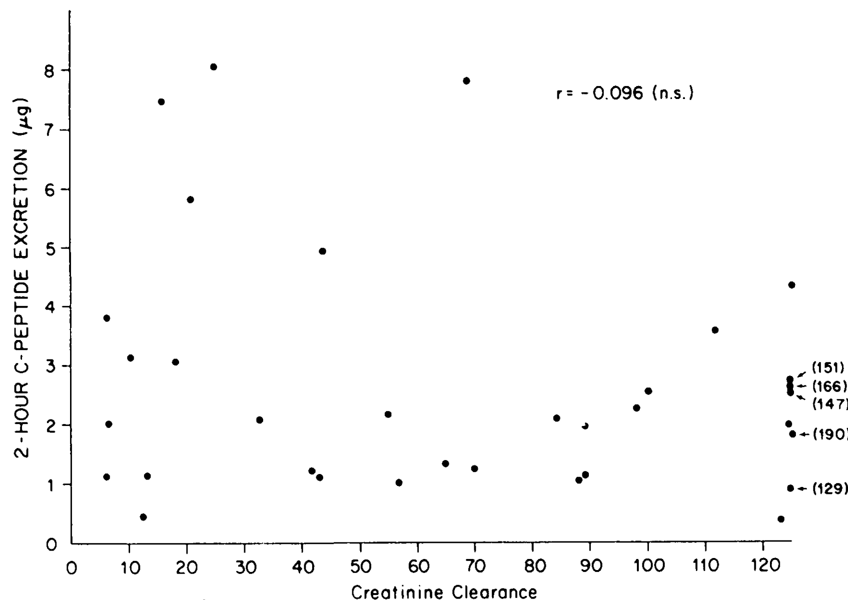

FIGURE 6

Relationship between two-hour C-peptide excretion and creatinine clearance.

secreted C-peptide. On the other hand, less than 0.1 per cent of secreted insulin appears in the urine in an immunoreactive form.[6] The higher percentage of C-peptide in urine can be attributed in part to the lesser hepatic catabolism[10] and higher serum concentrations of C-peptide than insulin and in part to decreased fractional tubular reabsorption and catabolism of C-peptide than insulin.[7,11]

Because of the frequency of coexistent renal disease in diabetic patients, it was necessary to determine the influence of renal function on the urinary C-peptide excretion. The observation that C-peptide clearance is independent of creatinine clearance over a wide range is noteworthy, for it implies that the excretion of C-peptide is mainly dependent on its plasma concentration. The constancy of C-peptide excretion reflects a consistent glomerulotubular balance with regard to the handling of C-peptide: as glomerular filtration decreases, tubular reabsorption of the peptide decreases proportionately. This situation is somewhat different from that of insulin, the clearance of which remains constant when the creatinine clearance exceeds 25 ml./min. but rises disproportionately in patients with creatinine clearances below 25 ml./min.[12]

The substantial day-to-day variability of urinary C-peptide in subjects maintained on isocaloric diets indicates that small changes in this value must be interpreted with caution. Because the coefficient of variation was not substantially changed by relating urinary C-peptide excretion to the 24-hour urine creatinine, incomplete urine collections do not explain this variability. It appears, therefore, that differences in C-peptide excretion reflect day-to-day variations in pancreatic C-peptide secretion, possibly produced by such factors as exercise or emotional stress.

The accuracy of the urinary C-peptide measurements may be affected by the immunologic heterogeneity of C-peptide as demonstrated by gel-filtration samples. Heterogeneity of C-peptide in serum has been suggested by Heding,[13] who noted that serum dilutions did not always lie parallel to the standard curve. She attributed this finding to differences between endogenous C-peptide and the synthetic C-peptide standard or to the presence of fragments of C-peptide. The former explanation is unlikely in our assay, because a natural pancreatic C-peptide standard was used. However, our studies also suggest that immunologically heterogeneous forms of C-peptide are present in urine.

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