Methods for Concentration of Urinary Immunoreactive Insulin

Bao-Heng Du M.D.*, John Eng M.D. and Rosalyn S. Yalow M.D.

Solomon A. Berson Research Laboratory Veterans Administration Medical Center,
Bronx, New York 10468, U.S.A.

Insulin is readily concentrated from 10 to 50 ml of urine with better than 75% recovery using octadecylsilyl (ODS) silica columns (C₁₈ Sep-Pak cartridge) and can then be measured by radioimmunoassay. Fractionation on Sephadex G50 gel filtration reveals that the apparent immunoreactivity corresponds for the most part to 6000 dalton insulin. Renal clearance of insulin in 5 normal subjects does not appear to differ in the fasted or fed state and ranged from 0.34 to 0.58 ml/min with an average of 0.44 ± 0.10 (S.D.) ml/min. Increased urinary insulin output was observed following feeding and fell during prolonged fasting. Insulin output in urine from 7 non-diabetic subjects ranged from 11 to 39 mU/24 hr, averaging 25 ± 10 mU/24 hr. In normal subjects without renal disease a single determination of renal insulin clearance and a timed urinary insulin output appear to be sufficient for determination of mean plasma insulin during that time period. Concentration of urine using this methodology could provide the material for HPLC screening for abnormal insulins and for their subsequent purification to determine the site of change in amino acid sequence.

Key Words: Concentration of Urinary Insulin

Abbreviation: Insulin-dependent diabetic (IDD); octadecylsilyl (ODS)

INTRODUCTION

Attempts to determine urinary excretion of insulin date back a half-century. (Lawrence et al., 1930; Cutting, 1942). A major effort at concentrating insulin report of Mirsky et al. (1948) in which it was concluded that the daily excretion averaged 170 mU/day in normal subjects and 70 mU/day in noninsulin dependent diabetics. These concentrations have since proven to be too high. When radioimmunoassay procedures (RIA) were introduced, insulin in urine was determined directly although the sensitivities of the assays were so low

that the urine had to be assayed virtually undiluted. Jorgensen (1966) first reported 24 hour urinary insulin excretion in 10 normal subjects to range from 8 to 30 mU/day, considerably lower than the bioassay results (Mirsky et al., 1948). Several other reports confirmed these findings and determined also the effects of renal disease, pregnancy and other conditions on the renal handling of insulin (Rubenstein et al., 1967a; Chamberlain et al., 1967; Rubenstein et al., 1967b; Trayner, 1967; Rabkin et al., 1970).

The measurement of urinary insulin appears to have many advantages including, among others, the ability to study diurnal changes in plasma insulin without the need for multiple blood sampling and to determine plasma free insulin concentrations in insulin-treated subjects having circulating insulin antibodies but no proteinuria. However, since urinary excretion of insulin in normal subjects is generally less than 1 μ g/day and that of C-peptide is in excess of 30 μ g/day, (Horwitz

Address for Correspondence: Dr. Rosalyn S. Yalow
Solomon A. Berson Research Laboratory VAMC
130 West Kingsbridge Road Bronx, New York 10468
(212) 584-9000, Ext. 1711

*Visiting physician, Tianjin, China

et al., 1979) measurement of C-peptide has become more common. In this report we describe a simple method for extraction and concentration of insulin from urine using C_{18} Sep-Pak cartridges and its potential application to clinical and biochemical studies.

METHODS

Subjects for the study included male and female non-diabetic controls ranging in age from 20 to 60 years and one insulin-dependent diabetic (IDD). Informed consent was obtained for these studies which had been approved by the Institutional Review Board of Montefiore Hospital and Medical Center and the Committee on Human Subjects of the Bronx Veterans Administration Medical Center.

Studies in controls included: urinary insulin during a 24-hour period of normal feeding; urinary and, in some cases, plasma insulin in response to a standard test meal consisting of 2 eggs, 1 slice dry toast, 4 oz orange juice (16g protein, 13g fat, 36g carbohydrate, 325 Kcal); urinary insulin during a period of fasting. The diurnal pattern of urinary insulin in an IDD was also determined.

Blood samples were collected in heparinized syringes, placed in plastic tubes and centrifuged at 3000 rpm for 15 minutes at 4°C. The plasma was then removed and stored at -20°C until assayed.

Urine was collected in plastic containers. After measuring the volume and pH the urine was centrifuged at 3000 rpm for 20 min at 40°C and portions of the supernatant were stored at -20°C. The insulin content of some urines was determined by direct RIA. Insulin was also concentrated from urine before assay using octadecylsilyl (ODS) silica columns (C_{18} Sep-Pak cartridges, Waters Associates). The basic method for concentration of insulin using C_{18} cartridge has been described previously. Cartridges are prepared by washing with 10 ml 75% ethanol in 0.01 HCl followed by 10 ml distilled water. Urine is passed through

the cartridge under pressure at a rate of 10 ml/min. The cartridge is washed with 10 ml of distilled water and eluted with 75% ethanol in 0.01M HCl. The optimal elution volume was determined and subsequently the first 0.7 ml was discarded and the next 1 ml saved for assay. Recoveries following elution with 96% ethanol and 96% ethanol in 0.01M HCl were also determined. To determine the nature of urinary immunoreactive insulin some concentrated samples in the fasted and fed state were subjected to Sephadex G50 gel filtration.

Insulin in plasma, urine, C_{18} cartridge concentrated urine and Sephadex gel eluates was determined using guinea pig antisera 102-1-15 or 11-8-14 according to methods established in the laboratory. Minimal detectability for insulin with 102-1-15 or 11-8-14 is 10 pg/ml or 2 pg/ml, respectively.

RESULTS

The stability of insulin in refrigerated and unrefrigerated urine is shown in Table 1. At a pH of 5.5 very little insulin is lost during storage for 6 hours at room temperature and only 1/3 is lost by 24 hours. Insulin is less stable in urine at pH 7. However, even at this pH, storage in a refrigerator permits recovery of almost 60% of the insulin after 24 hours.

Over 75% of labeled insulin in volumes up to 50 ml could be adsorbed to C_{18} Sep-Pak cartridges at any pH between 2 and 8. Following washing with distilled water, the insulin was eluted with 96% ethanol or 96% ethanol in 0.01N HCl or 75% ethanol in 0.01N HCl. The first 0.7 ml contained 4%, 8% and less than 1%, respectively. The next 1 ml contained 33%, 79% and 94%, respectively, of the 125 I-insulin absorbed to the column. Therefore, elution with 75% ethanol in 0.01N HCl was employed in subsequent studies.

Concentrated urine obtained from a subject in the fasted and fed state was fractionated on Sephadex G50 (Fig. 1). In the fasted state proinsulin accounted

Table 1. Stability of Insulin in Urine as a Function of Time % of original concentration

		pH 7							
	Fresh Sample (620 pg/ml)	2 hr	4 hr	6 hr	8 hr	24 hr	48 hr	72 hr	
room temp	100	83	68	42	35	26	24	8	
4°C		99	94	95	84	58	55	48	
		pH 5.5							
	Fresh Sample (2325 pg/ml)	2 hr	4 hr	6 hr	8 hr	24 hr	48 hr	72 hr	
room temp	100	97	91	88	69	65	42	24	
4°C		110	104	90	88	85	80	69

Fig. 1. Sephadex G50 fractionation of immunoreactive insulin concentrated by C_{18} from morning urines after an overnight fast (top) and after feeding (bottom).

for about one-third the immunoreactivity in the urine; in the stimulated state 6000 dalton insulin predominated.

Figure 2 shows the responses to the standard test meal. On the left the glucose, plasma insulin and hourly urinary output are shown for each of 3 subjects and on the right the hourly urinary output for each of 3 other subjects is shown. It is evident that urinary insulin reflects plasma insulin. The renal clearance in 5 normal subjects ranged from 0.34 to 0.58 ml/min and averaged 0.44 ± 0.10 ml/min (Mean $\pm$ Standard Deviation) in the fasting state and averaged 0.47 ± 0.11 ml/min during the four hour period following feeding. Thus the renal clearance of insulin is independent of the fed or fasted state.

Typical diurnal urinary insulin outputs are shown in Figure 3 for an IDD (top, right), during a prolonged fast in two normal subjects (middle and lower right) and in three normal subjects during a usual day (left). What is noteworthy is that endogenous urinary insulin normally reflects the feeding pattern, whereas in the IDD urinary insulin peaked in the late afternoon and fell by the evening meal.

Daily urinary insulin output in 7 non-diabetic subjects ranged from 11 to 39 mU, averaging 25 ± 10 mU/24 hours.

Fig. 2. Response to standard test meal in normal subjects. Plasma glucose and insulin were determined in three subjects (left) and hourly urinary insulin was determined in all 6 subjects.

DISCUSSION

This study confirms the earlier studies employing RIA with respect to the daily urinary insulin output (Jørgensen 1966; Rubenstein et al., 1967a; Chamberlain et al., 1967; Rubenstein et al., 1967b; Trayner et al., 1967; Rabkin et al., 1970). However, in most of these studies insulin was assayed virtually undiluted which not infrequently leads to problems of non-specific interference in the assay system. We have found insulin in urine to be more stable in a slightly acid pH than in an alkaline pH as used by others (Rubenstein et al., 1967a; Rubenstein et al., 1967b).

The ability to concentrate urinary insulin as described in this report permits additional studies that would otherwise not be possible. Thus, one can readily determine urinary insulin in the fasted as well as in the fed state in normals and in overnight specimens of those with insulin dependent diabetes.

Since the renal clearance of insulin seems to remain constant in any individual and has a rather narrow range among subjects without renal disease, the timed urinary excretion reflects integrated plasma levels during that time period. Thus, with a single measurement of renal clearance and total urine collection over a time period, the integrated plasma level during that interval can be determined. This would be of particular value in studying infants and young children in whom multiple venous sampling should be avoided. Since

Fig. 3. Diurnal urinary insulin in 3 normal subjects on their regular diet (B, breakfast; L, lunch; D, dinner; S, snack) (left). Shown in the two lower frames on the right is the urinary insulin during a 24-hour fast in two normal subjects. In the insulin-dependent diabetic shown in the upper right, the use of ultralente made possible an insulin level throughout the night comparable to the insulin levels found in normal subjects. However, insulin peaked in late afternoon and was falling by the time of the evening meal.

antibody-bound insulin is not found in the urine except in proteinuria, urinary insulin in an IDD with circulating insulin antibody reflects the free insulin in plasma. Urinary insulin and therefore the free insulin in plasma peaked in the late afternoon in the IDD studied. Studies such as this could provide a basis for adjusting insulin dosage to peak at more appropriate time intervals.

It is evident that concentration of urinary insulin permits ready determination of the hormonal form (s) in plasma. Thus it was easy to demonstrate that proinsulin is more prominent in the fasted state than in the fed state. Since we did not determine the renal clearance of proinsulin, the proinsulin/insulin ratio in plasma and urine may not be identical.

An additional use of this methodology that until now had not been considered is its application to screening for abnormal insulins. The report of a patient with an abnormal insulin of low biologic activity in which a leucine was substituted for one of the two phenylalanine residues in the C-terminal end of the B chain raises the question as to whether other abnormal insulins may yet be discovered. Since typical daily

urinary outputs in normals average about 150 pmol, concentration of insulin from urine rather than from plasma could easily provide the material for HPLC screening for abnormal insulins and for their subsequent purification and determination of amino acid content and sequence.

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