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Biological Variability of Albumin Excretion Rate and Albumin-to-Creatinine Ratio in Hypertensive Type 2 Diabetic Patients

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The importance of measuring microalbuminuria is well established. However, only scanty data are available concerning the biological variability of albumin excretion in type 2 diabetic subjects. We report our experience from a large clinical trial of a new antihypertensive drug (Lercanidipine) designed to reduce albumin excretion and blood pressure in type 2 diabetic patients with hypertension and microalbuminuria.

Eighty seven patients with persistent microalbuminuria were studied within 1 year of the clinical trial. The measurements were performed on blood and timed urine samples frozen at –80 °C and shipped to a central laboratory unit. Preliminary experiments were performed to assess albumin stability in urine under various conditions (4 °C, –20 °C and –80 °C), particularly with regard to the albumin/creatinine ratio. Urine samples can be stored up to 3 weeks at 4 °C or up to 2 months at –80 °C. The biological variability of the albumin excretion rate was 25.7%, while that of the albumin/creatinine ratio was 13.4%. These data are useful in defining the analytical goals of imprecision for microalbuminuria (CV = 13% for albumin, and CV = 6% for albumin/creatinine ratio). No correlation between albumin/creatinine ratio and HbA_{1c} was found in the cohort of 61 microalbuminuric patients who completed the trial.

The results of this study confirm that the albumin/creatinine ratio is much more suitable for monitoring albumin excretion in longitudinal studies than the albumin excretion rate. Clin Chem Lab Med 2003; 41(9): 1229–1233

Key words: Albuminuria; Biological variability; Diabetes mellitus; Diabetic nephropathy; Glycated hemoglobin; Specimen handling.

Abbreviations: A/C, albumin/creatinine; AER, albumin excretion rate; HbA_{1c}, hemoglobin A_{1c}.

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Introduction

Glomerular nephropathy is one of the most common and serious long-term complications of diabetes: 30 to 40% of type 1 diabetic patients are likely to develop renal failure as a result of progressive nephropathy (1, 2). Therapeutic interventions aimed at the reversal, or at least the delay, of the progression of nephropathy are known to be effective only if they are initiated at a very early stage of the impairment in renal function (3, 4). As a consequence, early identification of renal disease is considered essential for diabetes care.

With this goal in mind, the quantitative measurement of albumin in urine has assumed an increasing clinical role in the follow-up of diabetic patients, in addition to the measurement of hemoglobin A_{1c} (HbA_{1c}), to assess the glycemic control. In fact, the presence of persistent microalbuminuria has been widely proven, in both type 1 and type 2 diabetic patients, to be an early strong predictor of an increased risk of developing clinical nephropathy (5–8). Apart from its use in monitoring renal functional loss, microalbuminuria is considered a potential cardiovascular risk marker and a general indicator of endothelial dysfunction (9).

Despite widely recognized prognostic and therapeutic implications of microalbuminuria, some analytical aspects are still controversial and no unequivocal agreement in the literature as to the most appropriate procedure by which albumin excretion in urine should be monitored. The conflicting points concern mainly the choice of the urine specimen and the mode of expression of the results (albumin excretion rate (AER), albumin concentration or albumin-to-creatinine (A/C) ratio).

Historically, microalbuminuria has been detected by measuring the AER in 24-hour or overnight urine specimens. Although AER is believed to be the most reliable test to assess the presence of microalbuminuria, its use is hampered by the need for timed urine collection, a procedure cumbersome for patients which may introduce errors due to timing and collection inaccuracies (10, 11). The measurement of albumin concentration alone is certainly more practical, but this parameter may vary markedly depending on the degree of dilution of the urine samples. Many authors, however, have stated that its determination in the first morning urine may be suitable for the diagnosis of microalbuminuria, especially for screening purposes, thereby leaving a smaller number of patients with the need to measure AER (5, 12). Variations in urine flow rate may be corrected by relating albumin to creatinine excretion. The A/C ratio has been found to be closely correlated to AER (11, 13, 14), and its determination has

been proposed as a valid alternative to timed collection procedures. On the other hand, the calculation of A/C requires the measurement of urine creatinine, and this may introduce further variability (15).

As a result, the recently published guidelines of the National Academy of Clinical Biochemistry (16) propose an analytical goal of CV = 18% for urine albumin concentration in the first morning specimen and a CV = 15% for the A/C ratio, both being calculated on the basis of data on the biological variability in healthy volunteers.

In the present work we report our experience in the detection of microalbuminuria in type 2 diabetic patients enroled in a double-blind, randomized clinical trial of Lercanidipine, a new long-acting calcium channel blocker. Because pre-analytical sample handling is frequently required in these multicenter studies, and urine specimens are often stored for long periods before being analyzed, we have also evaluated the stability of albumin concentration in urine samples stored at different temperatures.

Materials and Methods

Patients and samples

Eighty seven type 2 diabetic patients presenting with mild to moderate arterial hypertension and stable microalbuminuria were studied. They were enroled in a double-blind, randomized clinical trial of Lercanidipine promoted by Recordati spa, Milan, Italy (REC 15/2375 SVIC 0007). Patients were asked to provide a timed overnight collection of urine for three consecutive days. Urine specimens were collected in polystyrene test tubes without preservative or additive. After measuring urine volume, aliquots of each sample were stored at −70 °C until they were sent on dry ice to the central laboratory where albumin and creatinine concentrations were measured. Before analysis, urine and blood samples were thawed at room temperature, thoroughly mixed by manual inversion and centrifuged at 1000 *g* for 5 min to remove debris.

Analytical methods

Urinary albumin and creatinine were measured on a DCA 2000 plus system (Bayer, Milan, Italy) in which albumin is detected immunoturbidimetrically. Creatinine was measured colorimetrically by the Benedict-Behre reaction. Microalbuminuria was defined as AER in the range 20–200 µg/min. Glycated hemoglobin was measured by HPLC (Menarini HA 8140, A. Menarini Diagnostics, Florence, Italy). Between-run CV for HbA_{1c} was 1.9% (assessment based on the participation in an international external quality assessment scheme (www.glicata.org)), while the mean relative bias against the DCCT (Diabetes Control and Complications Trial) reference system was −0.64.

Stability test

Spot urine samples were collected without preservative from 28 diabetic outpatients attending the laboratory for routine urinalysis. Each sample was divided into three aliquots, which were then stored at 4 °C, −20 °C and −80 °C. Baseline urine samples were assayed for albumin within 8 hours after storage at 4 °C (median: 27.1 mg/l; range 4.0 mg/l–506 mg/l; 25–75% CI: 13.0–54.3 mg/l; n = 28). Time points for determination were fixed at 3 weeks, 2, 3, 4 and 6 months. All samples were thoroughly vortexed and centrifuged at 1000 *g* for 5 min immediately before assay. The stability data were analyzed by means of Wilcoxon signed rank test.

Calculated parameters

The biological variability (CV_{biol}) was calculated according to the formula $CV_{biol} = (CV^2_{tot} - CV^2_a)^{1/2}$ where CV_{tot} and CV_a are total and analytical variability, respectively (17). With regard to AER and A/C ratio for each patient, a between-day CV was estimated from the results obtained on separate urine samples collected over three consecutive days. CV_{tot} was calculated as the mean of individual between-day CVs. With regard to the DCA 2000 measurements, CV_a was calculated from the reproducibility tests performed using patient samples collected on separate days, by the method of differences of duplicate.

Results

The imprecision of the albumin, creatinine and A/C ratio measurements on the DCA system is shown in Table 1. The analytical CV of A/C ratio was found to be slightly higher compared to albumin and creatinine, and a slight increase in the CVs was observed when thawed urine samples instead of fresh urine were analyzed.

The comparison of the results obtained on urine samples stored at 4 °C, −20 °C and −80 °C for albumin, creatinine and A/C ratio is shown in Table 2. As can be seen, albumin concentration was quite stable even after storage for 3 months at 4 °C, although creatinine concentration clearly decreased with a resulting increase in the A/C ratio. Marked changes in albumin were observed in the specimens stored at −20 °C, while a better stability was found at −80 °C. We conclude from these data that the samples are most stable when urine is stored for up to 3 weeks at 4 °C or for up to 2 months at −80 °C. Since it was not feasible to centralize the tests when samples had been stored at 4 °C, because of difficulties in handling such a temperature during transport, the samples from the clinical trial were stored at −80 °C, shipped on dry ice and analyzed within 2 months from the collection time.

Table 3 shows total and biological variability calculated for AER and A/C ratio from the measurements performed in the urine samples collected over three consecutive days. The total day-to-day variability of AER was found to be quite large in each group of pa-

Table 1 Reproducibility of the measurements in urine samples.

	n	Mean	SD	CV (%)
Albumin (mg/l)				
Fresh urine samples	23	42.4	1.2	2.7
Thawed urine samples	23	42.0	2.0	4.6
Creatinine (mg/dl)				
Fresh urine samples	24	105.3	3.0	2.9
Thawed urine samples	24	102.6	2.9	2.8
A/C ratio (mg/g)				
Fresh urine samples	24	51.9	2.8	5.5
Thawed urine samples	24	50.3	3.2	6.3

Table 2 Stability of urine samples stored under different temperatures.

Temperature	Parameter	Time of storage	n	Median	CI 25–75%	p*
4 °C	Albumin	Baseline	28	27	13–54	0.213
	(mg/l)	3 weeks	12	30	13–67	
		2 months	28	27	13–53	
		3 months	28	28	13–53	
	Creatinine	Baseline	28	109	77–145	<0.001
	(mg/dl)	3 weeks	13	78	64–129	
		2 months	28	102	71–137	
		3 months	28	98	70–132	
	Albumin/creatinine	Baseline	28	22	11–61	<0.001
	(mg/g)	3 weeks	12	31	13–73	
		2 months	28	22	11–67	
		3 months	28	24	12–68	
–20 °C	Albumin	Baseline	28	27	13–54	<0.001
	(mg/l)	3 weeks	13	30	11–70	
		2 months	28	18	8–54	
		4 months	28	15	5–45	
	Creatinine	Baseline	28	109	77–145	0.300
	(mg/dl)	3 weeks	13	79	65–130	
		2 months	28	102	73–137	
		4 months	28	108	78–143	
	Albumin/creatinine	Baseline	28	22	11–61	0.001
	(mg/g)	3 weeks	13	41	10–108	
		2 months	28	14	7–57	
		4 months	28	14	5–50	
–80 °C	Albumin	Baseline	28	27	13–54	0.073
	(mg/l)	3 weeks	13	33	14–71	
		2 months	28	28	14–56	
		6 months	28	27	17–54	
	Creatinine	Baseline	28	109	77–145	0.683
	(mg/dl)	3 weeks	13	83	65–135	
		2 months	28	108	78–142	
		6 months	28	108	80–143	
	Albumin/creatinine	Baseline	28	22	11–61	0.053
	(mg/g)	3 weeks	13	42	13–109	
		2 months	28	21	12–61	
		6 months	28	26	12–61	

* Significance of the differences between median values at the baseline and the longest storage time.

Table 3 Total and biological variability of albumin excretion rate (AER) and albumin/creatinine (A/C) ratio in the entire study group and in patients with different severity of albuminuria.

Patients	n	AER (CV, %)		A/C (CV, %)	
		Total	Biological	Total	Biological
Normoalbuminuria	17	36.4	36.1	28.6	27.8
Microalbuminuria	64	25.2	24.8	12.9	11.3
Macroalbuminuria	6	22.8	22.3	7.7	4.4
Entire study group	87	26.1	25.7	13.4	11.8

tients. When A/C ratio was measured, a lower day-to-day variability was observed in both micro- and macroalbuminuric patients.

There was no significant correlation between HbA_{1c} concentration and urinary A/C ratio measured at the same patient visit ($r^2=0.0057$).

Discussion

Despite the recognized importance of the quantitative measurement of albumin in urine to detect microalbuminuria, the data are inconsistent with regard to the pre-analytical factors. Our experience shows that sta-

bility at 4 °C is quite good with respect to the protein, and poor regarding creatinine, the latter probably being the result of the bacterial growth. Indeed, it is common practice in some laboratories to add sodium azide as a preservative to urine specimen for microalbumin detection if samples have to be stored for several days before the analysis.

On the other hand, freezing the samples at –20 °C is deleterious for microalbuminuria testing. This was more pronounced in our study than in that of Innanen *et al.* (18), who reported a decrease of microalbuminuria in a small number of frozen urines, with no improvement with mixing after thawing of samples. Similar findings were also reported by others (19, 20). Apparently, even the addition of detergents such as Triton X-100 or SDS, or the use of different types of containers such as glass, polycarbonate and polystyrene, does not prevent albumin loss during storage at –20 °C (data not shown). Hara *et al.* (21) have reported that the treatment of polystyrene containers with ozone could minimize the adhesion of proteins to the container surface, but we were not able to test this approach. Sorensen (22) also pointed out the underestimated importance of albumin adhesion to the walls of the container, a factor difficult to standardize. The addition of inhibitors of serine proteases and metalloproteinases, and of antimicrobial agents, has also been proposed to prevent protein degradation during storage of urine samples at 4 °C and –20 °C (23, 24).

However, in this work we report that if freezing is performed rapidly at –80 °C, albumin, creatinine and albumin/creatinine ratio are well preserved for at least 2 months. We therefore confirm the previous observations by MacNeil *et al.* (25) who found no decrease in albumin over 160 days at –80 °C but did not report on the stability of the albumin/creatinine ratio. Along the same lines, Hara *et al.* (26) found that –80 °C was the optimal temperature for the long-term storage of samples for the measurement of urinary albumin, but again not reporting on the stability of the albumin/creatinine ratio.

The analytical quality of the measurements performed with the DCA 2000 system is acceptable and is in agreement with the validation performed by Parsons *et al.* (27). The analytical goals that can be established from the data shown in Table 3 are CV = 13% for albumin and CV = 6% for albumin/creatinine ratio. These goals seem more reasonable than those presented in the recent American guidelines (16) where the goal of 15% does not differentiate between the measurements of albumin in urine and albumin/creatinine ratio.

Conflicting evidence is reported in the literature with regard to the correlation between albumin excretion and HbA_{1c}. Our results are in agreement with several observations from other investigators. Twyman *et al.* (28) in a recent study of 361 samples collected from young type 1 diabetic patients found that the presence of microalbuminuria was independent of HbA_{1c} and the duration of diabetes. This was in agreement with previous observations of Watts *et al.* in a cohort of 160 insulin-dependent diabetics (29) and with the earlier observations from Feldt-Rasmussen *et al.* (30). In cases where a positive correlation between albumin excretion and HbA_{1c} had been demonstrated, the limited number of studied cases makes the comparisons with our observations difficult. For instance, a correlation ($r = 0.68$) was observed between HbA_{1c} and 24 h urinary albumin excretion rate in 23 type 2 diabetics (31). In other cases, the observed correlation was weak (32, 33) except when patients with macroalbuminuria were also included (34, 35). Only in long-term studies the correlation between glycemic control and albuminuria becomes evident, as in the 10-year study of Torffvit (36) or in that of Scott *et al.* (37).

In conclusion, our experience suggests that albumin and creatinine measurements in frozen urine samples stored at –80 °C are reliable and can be performed if no other way of storing samples is feasible, as frequently occurs in large multicenter studies.

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