Centriprep Ultrafiltration for Fractionation of Serum and Urinary Proteins before Electrophoresis

To the Editor:

Centriprep concentrators (Amicon Ltd., Stonehouse, UK) offer a simple and rapid method for ultrafiltration of relatively large sample volumes (15 mL) and exploit low adsorption membranes (cutoffs of 3000, 10 000, 30 000, or 100 000 Da) to optimize protein recovery. We have investigated their use for concentration/fractionation of human body fluids before electrophoresis. The results indicate successful fractionation of urinary proteins with the potential to recover either Tamm–Horsfall mucoprotein (by using Centriprep C100) or low-Mr proteins (by using Centriprep C30) in a concentrated and relatively pure form.

Sample pools consisting of equal volumes of either normal serum (stored at −70°C) or urine (fresh, early morning, midstream samples) were filtered through Centriprep C100 or Centriprep C30 and the respective filtrates concentrated in Centriprep C10. The serum was first diluted with four volumes of 62.5 mmol/L Tris HCl, pH 6.8, and the urine was first clarified by centrifugation (1500 × g, 5 min). The samples were subsequently processed according to the manufacturer’s instructions. Protein recovery was monitored with the Coomassie Blue protein assay (Pierce Chemical Co., Rockford, IL), and the protein profile of the fractions was investigated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions (1). Briefly, we mixed the fractions with a sufficient volume of sample denaturing solution (per liter, 20 g of SDS and 200 g of glycerol in 62.5 mmol/L Tris HCl, pH 6.8) and heated them to 95°C for 10 min. The samples were loaded (2 μg of serum protein, 0.3 μg of urinary protein) onto 60 to 200 g/L polyacrylamide gradient gels (75 × 75 × 3 mm) and electrophoresed (1 h; 200 V, 50 mA/gel) in precooled (4°C) electrophoresis buffer (per liter, 1 g of SDS, 25 mmol of Tris, and 200 mmol of glycerine) by using a Micrograd Electrophoresis Unit and a Consort E455 Power Supply Unit (Flowgen Instruments Ltd., Sittingbourne, UK). The proteins were detected by silver staining (2).

The fractionation of serum proteins was poor (Figure 1, tracks 2–6). Both filtrates contained <2% of the original protein, and SDS-PAGE revealed the presence of high-Mr proteins (tracks 4 and 6). Poor recovery may be due to the high protein concentration of serum (leading to adsorption and blockage of the filters), but more extensive dilution would be counterproductive.

The fractionation of urinary proteins was good (tracks 7–11). Both the C100 and C30 filtrates contained substantial amounts of protein (70% and 30% recovery, respectively), and SDS-PAGE indicated depletion of high-Mr proteins (particularly Tamm–Horsfall mucoprotein) and enhanced detection of the low-Mr proteins (tracks 9 and 11). Comparison of equal protein amounts revealed the C100 retentate contained Tamm–Horsfall mucoprotein in relatively pure form (track 8).

Centriprep fractionation may be a useful prerequisite to clinical evaluation of urinary proteins by SDS-PAGE (3, 4). Prefractionation of high- and low-Mr urinary proteins before polypeptide mapping by high-resolution two-dimensional electrophoresis should differentiate low-Mr monomeric proteins from the polypeptide subunits of oligomeric proteins of higher molecular mass. This may enhance detection of tissue-specific proteins and more clearly distinguish glomerular and tubular proteinuria.

References
1. Marshall T, Williams KM. Simplified automated assay of urinary oxalate with the Cobas Mira Analyzer

To the Editor:

Oxalate may be measured by use of oxalate decarboxylase (OXDEC; EC 4.1.1.2) to produce formate, which is measured by a NAD+–dependent reaction catalyzed by formate dehydrogenase (FDH; EC 1.2.1.2) (1). We describe an adaptation of this method to a Cobas Mira analyzer, software 8735 (Roche, Basel, Switzerland), which allows a fully automated assay. Compared with other applications on the Cobas-Bio centrifugal analyzer (Roche) (2, 3), our assay is rapid (e.g., 17 vs 30 min (2)) and avoids time-consuming pretreatment of the sample (3).

We collected 24-h urine specimens in plastic vessels containing 10 mL of concentrated HCl, and stored them at −20°C until analysis. For the assay, the urine was thawed, mixed, filtered (0.45 μm pore-size nylon filter unit), and put into a single sample cup without any other treatment.

The advantage of using the Cobas Mira for this assay lies in the versatility of its software: the option RATIO allows several reactions on the same sample.

We measured the sample reaction without an internal standard (SAMPLE); the sample reaction with an internal standard (IS); and the sample blank (BLANK). These three reactions
are carried out in separate cuvettes. The reaction with internal standard is intended to minimize the influence of interfering substances present in urine, e.g., ascorbate, divalent cations, some anions (1, 4). The sample blank is measured to obviate problems related to the reaction with FDH in the presence of endogenous formate or NAD*-consuming substances (2).

These assays start with a first step, in pH 5.0 buffer, in which the OXDEC decarboxylates oxalic acid into formic acid (OXDEC is not added for sample blank). After about 8 min, a second step is activated in pH 9.5 buffer, in which formate is oxidized to bicarbonate by FDH, in the presence of NAD*, yielding NADH. The first reading takes place at the beginning of the second step, and the last after about 8 min. The change in absorbance (ΔA) of each determination is utilized for the final calculation, set up (as test OX) in the RATIO programs of the instrument. The algorithm used to calculate the concentration of oxalate in the sample is:

\[
OX = \frac{(\Delta A_{\text{SAMPLE}} - \Delta A_{\text{BLANK}})}{(\Delta A_{\text{IS}} - \Delta A_{\text{SAMPLE}})} \times 0.555
\]

where 0.555 is the concentration (mmol/L) of the internal standard. The Cobas Mira settings for the three determinations involved in oxalate analysis are listed in Table 1.

The three series of reactions that take place in analyzer can be summarized as follows:

**SAMPLE:** sample + OXDEC + Reagent → NAD* + FDH  
**IS:** sample + OXDEC + Reagent/IS → NAD* + FDH  
**BLANK:** sample + H2O + Reagent → NAD* + FDH

The quantities of reagents, formulated as below, are sufficient for at least 20 tests:

**Buffer A:** 14.2 g of citric acid·H2O and 30.2 g of K2HPO4·3H2O dissolved in distilled water and made up to 1 L; pH must be about 5.0.

**Buffer B:** 34.2 g of K2HPO4·3H2O and 200 mg of pyrazole diluted in distilled water up to 1 L; pH must be about 9.5.

**Sample diluent:** 150 μL of OXDEC (no. 479856, from Aspergillus species; Boehringer Mannheim, Mannheim, Germany) in 1.2 mL of buffer A; put in a Cobas rack reagent “10.”

**Reagent:** 10 mL of buffer A plus 25 μL of K2EDTA, 0.123 mmol/L.

**Reagent/IS:** 9 mL of buffer A/EDTA solution plus 1 mL of internal standard (0.555 mmol/L of oxalate in buffer A). Both Reagent and Reagent/IS are put in two different sectors of a Cobas rack reagent “5a.”

**Start reagent 1:** 250 mg of NADLi·2H2O (Boehringer Mannheim; no. 223468) in 10 mL of buffer B.

**Start reagent 2:** 84 mg of FDH (Boehringer Mannheim; no. 244678) in 10 mL of buffer B. Start reagents 1 and 2 are placed beside Reagent and Reagent/IS in the Cobas rack reagent “5a.”

We estimated the precision of the method by testing two urine samples (0.277 and 0.777 mmol/L oxalate) for 5 days in sequences of 10 assays per day. Within-series CVs were 4.5% for the first sample and 2.4% for the second. Between-series CVs were, respectively, 6.1% and 4.2%.

We performed a recovery experiment according to Massart et al. (5), adding known amounts of oxalate (up to a maximum expected concentration 5.5 mmol/L) to a pool of 40 urines (pH 1.8; oxalate concentration 0.333 mmol/L). The same quantities were added to H2O. The regression between the results obtained from the pool (y) and the corresponding data from aqueous standards (x) is expressed by the equation \( y = 1.02x + 0.321 (r = 0.98) \), which was linear up to at least 5.0 mmol/L. Regression of the results of the same assays, in absence of internal standard, yielded a lower correlation coefficient \( r = 0.85 \).

The detection limit, evaluated as recommended by Massart et al. (5), by serial twofold dilutions of urine, was 0.03 mmol/L.

To investigate the possible interfering effect of ascorbate, each of three urine pools—containing oxalate at 0.35, 0.69, and 1.42 mmol/L, respectively—were supplemented with ascorbic acid (0.5 and 10 mmol/L, from a fresh concentrated solution made in HCl 0.01 mol/L) and analyzed for oxalate. No variation was observed in the recovery of the analyte for every assay.

Finally, we tested with our method 25 urine samples from a center specializing in the study of nephrolithiasis, previously analyzed by ion-chromatography (6), with oxalate concentrations from 0.06 to 1.91 mmol/L. The correlation between ion-chromatography \( (x) \) and the Cobas Mira \( (y) \) is described by the equation \( y = 0.93x + 0.02 (r = 0.95, S_{xy} = 0.068) \). We estimated the reference values for healthy adults by testing the 24-h urines of 42 normal individuals: range 0.06–0.58 mmol/24 h, mean = 0.27, and SD = 0.09, in accordance with other reports (3, 7).

We conclude that our enzymatic assay of oxalate in untreated urine is
both easy to perform (fully automated) and provides good analytical recovery, acceptable precision, and sufficient sensitivity for clinical specimens. On the whole, a single determination costs about US $4, and no time is required for pretreating samples.

Our method can be performed also without an automated analyzer, by making use of the conditions listed in Table 1. At present, we have adopted this manual procedure experimentally for the assay with serum.

References

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