A map of urine proteins based on one-dimensional SDS–polyacrylamide gel electrophoresis and Western blotting using one microliter of unconcentrated urine

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Summary

A sensitive one-dimensional SDS–polyacrylamide gel electrophoretic system was devised whereby the proteins in 1 μl of unconcentrated urine could be visualized by silver staining over the range 9 000–900 000 molecular weight. Identification of urine proteins was confirmed by Western blotting using peroxidase labelled antibodies. A map of the major proteins visualized in urine from individuals with renal disease was constructed. We conclude that the information available from the simple analysis of proteins according to their size is limited to general conclusions regarding whether proteinuria is likely to be of tubular or glomerular or mixed origin. More specific identification of individual proteins is not feasible because simple protein staining is not sufficiently reliable to identify individual proteins. The reasons for this conclusion are as follows: (a) many proteins in urine migrate with similar apparent molecular weights, (b) some proteins are not visualized by silver staining, and (c) albumin polymeric complexes and fragments can be present at almost any molecular weight. However, one-dimensional SDS–polyacrylamide gel electrophoresis together with Western blotting does provide reliable information which might be clinically and experimentally useful.

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Introduction

As was demonstrated by Bright in 1836, renal disease is frequently associated with increased excretion of protein in the urine [1]. Analysis of the proteins appearing in urine has proved to be very useful for the diagnosis and management of renal diseases [2–6]. In particular, quantitation of total protein and individual proteins appearing in urine as a result of a charge or size defect in the glomerular filter or as a result of failure of the proximal tubule to reabsorb filtered protein are clinically useful measurements. Also useful is the identification of proteins from blood which cross the normal glomerular filter in large quantities under some circumstances. These include light chains, myoglobin and hemoglobin. From the above facts it is not surprising that electrophoretic analysis of urine proteins often provides helpful clues about renal dysfunction. However, the electrophoretic method routinely used is based mainly on molecular charge rather than on size and requires prior concentration of urine in order to visualize the protein bands. In contrast, SDS–polyacrylamide gel electrophoresis (PAGE) is a rapid, simple, high resolution technique which is amenable to the use of a sensitive silver stain [7] for the nonspecific identification of protein bands. Therefore we attempted to determine whether this technique could be applied to analyze the proteins in unconcentrated urine samples. This report describes the method adopted and the results of our attempts to map the major proteins in urine as they appear on SDS–PAGE gels stained with silver.

Methods

Urine samples
All urine samples were collected from a renal clinic where they were centrifuged for 3 min at $1000 \times g$ prior to storing at $-30°C$ for subsequent analysis.

SDS–PAGE
Proteins were separated on the basis of size by a sensitive SDS–PAGE system utilizing 1 ml of unconcentrated urine. Polyacrylamide gels (0.4 mm thick) were cast between 8×7 cm glass plates (Eastman Kodak Co., Rochester, NY, USA). A discontinuous polyacrylamide gradient consisting of layers of 18, 15, 11 and 6% acrylamide were poured. By running standard proteins including IgM (900000), myosin (200000), β-galactosidase (116250), phosphorylase B (92500), bovine serum albumin (66200), ovalbumin (45000), carbonic anhydrase (31000), soybean trypsin inhibitor (21500), lysozyme (14400) (Bio-Rad Laboratories, Richmond, CA, USA). This gel was shown to be capable of separating proteins over the range of 9000–900000 mol wt.

Preparation of samples
A micromethod employing the Agarose Drop Technique was used [8]. Briefly, test samples were boiled in 100 g/l SDS, 8 mol/l urea for 4 min. To 1 μl of the test sample was added 3 μl of blue agarose (30 μl Bromophenol Blue, 0.7 mg/ml water,

in 0.5 ml agarose that had been prepared by extensive electrophoretic 'washing' of the agarose [8]). The sample was allowed to solidify on a strip of parafilm. The solidified samples ('blue drops') were placed 2 mm apart on the top surface of the stacking gel. The gel was run using a Laemmli buffer system [9], at a constant current of 5 mA during stacking and 10 mA during separation.

Silver staining

Following electrophoresis the gel was fixed in 50 ml/100 ml methyl alcohol, 12 ml/100 ml glacial acetic acid overnight. The gel was then placed in 10 ml/100 ml ethyl alcohol, 5 ml/100 ml glacial acetic acid for 1 h followed by 5 min in 0.0034 mol/l $Na_2Cr_2O_7$, 0.0032 mol/l HNO_3. Excess Na-dichromate was removed by four quick washes with deionized water. The gel was then placed in 0.2 g/l $AgNO_3$ for 10 min followed by irradiation with UV light from a Zeiss photomicroscope light source for 40 min in fresh $AgNO_3$. The silver nitrate was then removed. The gel was briefly washed with deionized water and then developed in 32.5 g/l Na_2CO_3 containing 0.5 ml formaldehyde until protein bands were sufficiently dark. The reaction was terminated by soaking the gel in 10 ml/l glacial acetic acid. This technique was capable of detecting 1 ng of protein loaded onto the gel [8].

Western blotting

Proteins were blotted on nitrocellulose paper by electrophoretic transfer using Bio-Rad Transblotting cell (Bio-Rad). The transfer buffer was 25 mmol/l Tris, 192 mmol/l glycine, 20% v/v methyl alcohol, at pH 8.3. The proteins were transferred for 2 h at 100 V in a refrigerated recirculating water bath.

Immunological detection of proteins on nitrocellulose paper

Following transfer, the blots were cut to exactly the same size as the gel, and then soaked in 30 g/l BSA, 100 ml/l goat serum in phosphate-buffered saline (PBS) overnight on a rotating mixer. The blot was then incubated for 1 h with antiserum diluted in 30 g/l BSA, 100 ml/l goat serum in PBS (usually about 1/200 dilution of antiserum). Excess antibody was removed by five rinses with PBS. The blot was then incubated for 1 h with the second antibody (peroxidase conjugated antibody directed against the IgG of the first antiserum) at 1/200 dilution in 30 g/l BSA, 100 ml/l goat serum in PBS. Following a further five washes in PBS to remove unbound antibody the paper was developed with 5,5'-diaminobenzedene (1 mg/5 ml) in 0.1 mol/l Tris, pH 7.4, containing 0.2 ml/l H_2O_2. The reaction was stopped by washing out the reactants with deionized water.

The following antisera were used to identify proteins in urine from Cappel Laboratories, Cooper Biomedical Inc., Molvern, PA, USA: goat anti human uromucoid, goat anti human transferrin, goat anti human light chain, rabbit anti human myoglobin, rabbit anti human hemoglobin, peroxidase conjugated goat anti rabbit IgG, peroxidase conjugated rabbit anti goat IgG; from Miles Laboratories Inc., Elkhart, IN, USA: rabbit anti human retinol binding protein, rabbit anti human β_2 microglobulin; from Boehringer Mannheim Biochemicals, Indianapolis IN, USA: goat anti human Fab frag. of IgG. Goat anti human anti α_1 microglobu-

lin was kindly provided by Dr. Bo Akerstrom, University of Lund, Lund, Sweden. Mouse monoclonal anti human apoprotein A_1 was kindly provided by Dr. Linda Curtis, Scripps Clinic and Research Foundation, La Jolla, CA, USA.

Time to prepare and run samples

To prepare a gel, load the sample and run the gel (10 samples/run) takes approximately 1.5–2 h. We routinely leave the gels in fixing solution overnight and silver stain the next morning. The staining procedure takes about 2 h. Western blotting of the gel takes 0.5 h preparation and 2 h blotting time. We routinely leave the nitrocellulose blot overnight in buffer containing non immune serum. The addition of antibodies, washing step, and development with peroxidase substrate takes about 3 h. It should be noted (1) that much of this time is waiting rather than 'hands-on' and could therefore be used for other gels. (2) That a result could be obtained within one working day if required, although we routinely use an afternoon and the following morning. (3) If particular proteins were being looked for then a mixture of antibodies could be used for the first antibody step.

Results

For the visualization of proteins over a wide range of molecular weight a gradient polyacrylamide gel was required. A simple discontinuous gradient in four layers consisting of 18, 15, 11 and 6% polyacrylamide was adapted. This system separated proteins between 9 000 and 900 000 mol wt. A Laemmli buffer system [9] was used to obtain high resolution separation of proteins. A silver stain was used to obtain maximum sensitivity (see 'Methods'). To further enhance sensitivity (by allowing all the protein in a band to come into contact with the silver) thin (0.4 mm) small (5 cm × 7 cm) polyacrylamide gels were used. To load these thin gels a new technique was developed which has previously been reported [8]. This 'agarose drop technique' requires the sample to be mixed with molten agarose in the presence of SDS and bromophenol blue. Once solidified the drop is placed on the top of the stacking gel and the protein attracted out of the 'agarose drop' into the stacking gel by the electrical potential difference across the gel. As shown in Fig. 1 when unconcentrated urine samples from a renal clinic were analyzed by this method protein bands could be visualized over the range 9 000–900 000 mol wt.

In normal urine, under nonreducing conditions, a single band at 60 000 mol wt (which comigrated with purified human albumin) was routinely seen. In some normal urine samples, particularly in concentrated urine, a second band at 80 000 mol wt (shown below to be Tamm Horsfall protein) was also visualized (Fig. 1, lane A). For comparison, when 0.03 μl of plasma was run on the same gel numerous protein bands were seen as illustrated in Fig. 1, lane H. Protein bands in urine samples from a renal clinic varied widely from the presence of a few low molecular weight proteins (Fig. 1, lanes B and C) to numerous low and high molecular weight proteins (Fig. 1, lanes D and E) to mainly high molecular weight proteins (Fig. 1, lanes F and G). From these data, it can be seen that proteins in urine can easily be separated into high and low molecular weight species by this approach. It is also
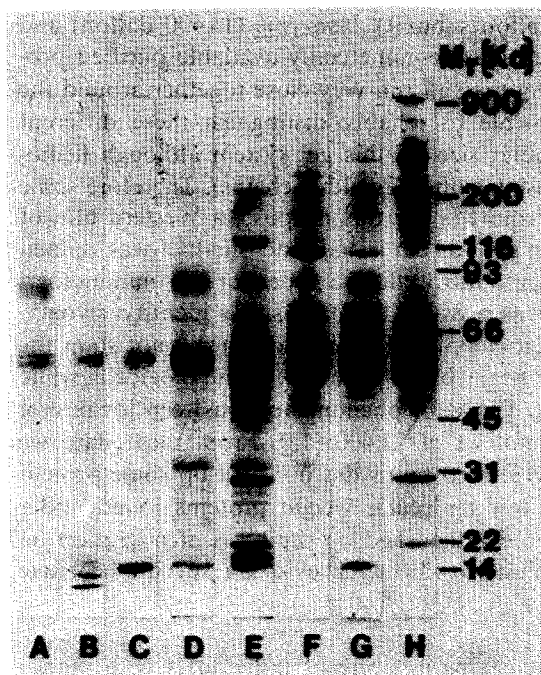
Fig. 1. Silver stained SDS–PAGE gel showing protein bands visualized in 1 microliter of unconcentrated normal urine (lane A) and 0.03 microliter of normal plasma (lane H). Lanes B to G show urine samples (1 ml) from individuals with various forms of renal disease. B, Infantile polycystic kidney disease; C, early Alport's syndrome; D, Familial proteinuria; E, Lowe's oculocerebrorenal syndrome; F, IgM glomerulonephropathy; G, late Alport's syndrome.

apparent that other information about renal function might be available from these protein patterns if they could be interpreted correctly. For example, there appeared to be many bands visible in urine samples that could not be visualized either in plasma or in normal urine. We therefore initiated studies to determine what these bands might be and to map the major proteins in urine as they appeared under nonreducing conditions on this SDS–PAGE system.

Albumin and its polymeric complexes and fragments As has previously been described [6,10–12], albumin exists as both polymeric complexes and fragments in urine. From analysis of urine from nephrotic individuals we conclude that a protein band almost anywhere on the gel could be an albumin band, and that it would be hazardous to identify a band simply on the basis of its molecular weight as defined on the SDS–gel. This conclusion is particularly true when large amounts of albumin are present in the urine sample. For further studies we therefore identified proteins by Western blotting.

The 9–22 kDa range Several low molecular weight proteins found in large quantity in some urine samples are in this size range. These include myoglobin

(17 000 dalton), hemoglobin (17 000 dalton subunit), lysozyme (14 400 dalton) and β_2-microglobulin (12 000 dalton). When these commercially available purified proteins are run on the SDS–PAGE system they all run very close together around the 14 000 mol wt marker. It is not possible to reliably distinguish these different proteins purely on the basis of molecular size on this gel system although hemoglobin usually is seen as two bands close together (α and β chains). Therefore some additional means of identification is required. For this purpose, a Western blot of the gels was used (see 'Methods'). Each gel was cut in half and one-half was stained for protein and the other half blotted onto nitrocellulose paper for the immunological detection. Using this approach it was possible to conclusively identify myoglobin, hemoglobin, lysozyme and β_2-microglobulin. The silver stain did not stain the bands well so that the blot might show heavy bands detected immunologically while little was seen on the silver stained gel. The same phenomenon was seen for retinol binding protein (RBP) which has a mol wt of 21 400 [13]. From these data we conclude (a) that the silver stain as performed by us did not pick up some protein bands (see 'Discussion'); (b) that the low molecular weight proteins found under some circumstances in urine cannot be distinguished on the basis of migration on the SDS–PAGE gel alone, and (c) Western blotting provides a specific sensitive method for identifying these proteins.

Light chains (25 kDa range)   Light chains in urine of individuals with monoclonal gammopathy or renal disease were often not easily seen with the silver stain. However, Western blotting demonstrated that light chain was present in several different forms. In particular light chain dimers [17] were commonly seen in urine of individuals with myeloma and were commonly the predominant form of light chain in urine migrating with an apparent M_r of about 45 kDa. No light chain was detected in normal urine samples by Western blotting. It is not possible to distinguish between light chains of monoclonal or of polyclonal origin by this approach, since separation is on the basis of size rather than charge.

31 kDa range   Two proteins were commonly seen at the 31 kDa marker position. They were apoprotein A_1 of the HDL complex and α_1-microglobulin. Apoprotein A_1 was also shown present in plasma as well as in the urine samples of individuals with nephrotic syndrome. In the case of α_1-microglobulin, on more than ten analyses performed, the blot was positive and corresponded to a band just above apoprotein A_1 and was not present in normal human plasma. In each case the α_1-microglobulin was seen in urine only in individuals with significant renal failure (GFR < 50 ml/min). Carbonic anhydrase (31 000 mol wt) would also be expected to run at this molecular weight but it has not been identified immunologically. An albumin fragment also commonly runs at this position.

45 kDa range   No proteins have been identified immunologically at this position with the exception of albumin fragments and light chain dimer. However, α_1 antiproteinase should migrate here. We have not been able to detect this protein in plasma or in urine by Western blotting using anti α_1 antiproteinase serum presuma-

bly because the denaturing conditions used changed the antigenicity of the molecule.

60–70 kDa range   Albumin is the major protein at this position where it obscures other proteins on silver stained gels.

80–110 kDa range   The two major proteins seen in this range are transferrin and Tamm Horsfall protein. Albumin polymeric complexes may also be present in this range.

160–200 kDa range   The major protein seen in the 160–200 kDa range is IgG. However other proteins which include albumin polymers, IgA monomer and C_3 (which runs just above the IgG band) run in this range.

300–400 kDa range   The major proteins in plasma in this range are fibrinogen and fibronectin. Fibronectin is commonly seen in urine samples containing high molecular weight proteins; however, fibrinogen at native molecular size is not seen in urine since fibrinogen undergoes proteolysis into plasmin-generated fragments similar to those seen when urokinase is added to plasma. Albumin polymeric complexes also occur in this range of molecular weight.

Greater than 400 kDa   IgM is the major protein at this molecular weight range.

Discussion

The purpose of this study was to determine whether simple one-dimensional analysis of urine proteins on the basis of their size in unconcentrated urine was technically feasible, and, if so whether this might be clinically useful. This method would be in contrast to the routine electrophoretic analysis of urine proteins based on charge [2], or the more complicated two-dimensional ('Iso-dalt') separation which is usually performed under reducing conditions [14] or the relatively low resolution SDS–PAGE systems previously reported for analyzing urine proteins on the basis of their size [15,16]. All of the above methods require prior urine concentration.

By using small thin gels to maximize the sensitivity of the silver stain [7] together with a novel method of loading the gels [8] it was possible to reliably visualize proteins even in normal unconcentrated urine. By using a polyacrylamide gradient and a Laemmli buffer system [9] it was possible to achieve high resolution of proteins over a very wide range of molecular weight (9 000–900 000). Urine analyzed in this way could easily be separated into those containing low molecular weight proteins, those containing high molecular weight proteins, and those with a mixed pattern. This simple, rapid, sensitive, analytical approach might therefore be clinically useful.

In urine samples from some individuals with nephrotic syndrome there were protein bands present which were not visualized either in normal plasma or in normal urine. As has previously been described [6,10–12] most of these bands

URINE PROTEIN MAP

BANDS   **M$_r$(Kd)**   **PROTEIN**

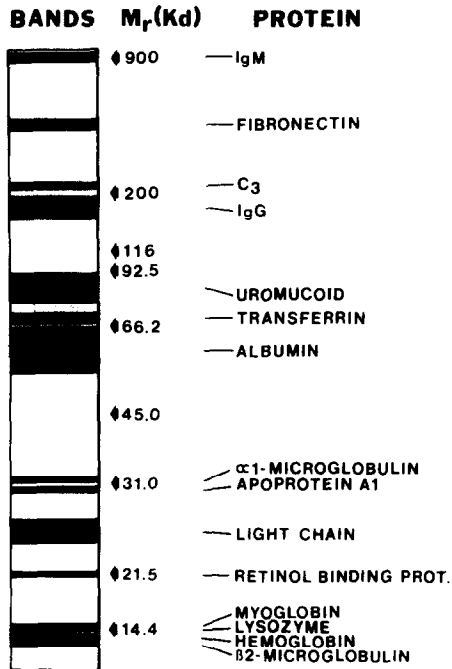|		
	◆ 900	—IgM
		—FIBRONECTIN
	◆ 200	—C$_3$
		—IgG
	◆ 116	
	◆ 92.5	
		＼UROMUCOID
	◆ 66.2	—TRANSFERRIN
		—ALBUMIN
	◆ 45.0	
	◆ 31.0	／ α1-MICROGLOBULIN ＼ APOPROTEIN A1
		—LIGHT CHAIN
	◆ 21.5	— RETINOL BINDING PROT.
	◆ 14.4	／MYOGLOBIN ＜LYSOZYME ＜HEMOGLOBIN ＼B2-MICROGLOBULIN

Fig. 2. Map of major proteins seen in normal and abnormal urine samples identified by Western blotting of one dimensional SDS–PAGE gels. The molecular weights shown are the relative position of marker proteins run on the same gels.

proved to be polymeric complexes or fragments of albumin which are present over a wide range of molecular weights in the urine of some individuals with nephrotic syndrome. From these data it was immediately apparent that it would not be possible to reliably identify proteins in urine purely on the basis of their size on the SDS–PAGE system.

As a further indication that simple SDS–PAGE analysis together with silver staining might not be a reliable technique some urine proteins did not stain well with the silver stain. This was particularly true for retinol binding protein. The reasons for this may be due to isoelectric point of the protein, carbohydrate content, amino and side chain content or protein concentration as has previously been described [18–22]. Therefore, taken together, these findings suggest that it is essential to include Western blotting of the SDS–PAGE gel to obtain reliable information about the identity of individual protein bands.

Using this approach a map of the proteins commonly seen found in urine samples when analyzed by SDS–PAGE was made (Fig. 2). In particular, several protein bands were seen in the 30 000 molecular weight range. Three were identified. These included an albumin fragment, α_1-microglobulin and apoprotein A$_1$ of the

HDL complex. α_1-Microglobulin, an immunosuppressant protein [23], was consistently found the urine of individuals with impaired renal function. α_1-Microglobulin excretion in urine has previously been reported to reflect tubular function [24]. Apoprotein A_1 of the HDL complex normally is associated with the lipid complex which has a molecular radius of about $55°A$ [25]. We can, therefore, conclude that the appearance of apoprotein A_1 in urine is probably associated with a glomerular leak of at least that size. Other high molecular weight proteins were also seen in some urine samples, particularly IgG, fibronectin and IgM. Again these proteins would be helpful for predicting large holes in the glomerular filter.

In summary, proteins in 1 ml of unconcentrated urine could be visualized by SDS–PAGE and silver stain. The high and low molecular weight pattern of proteins visualized could be used to characterize the form of renal disease present. However, we believe that identification of specific proteins on the basis of their size is not reliable unless accompanied by Western blotting to confirm the identity of the protein bands visualized. Although this approach to analysis of urine proteins is probably too complex for routine analyses it is possible that this method could provide more information about different forms of renal dysfunction when further experience with this approach has been gained. These data provide a useful map of the proteins found at various molecular weight positions in urine from individuals with renal diseases as well as showing the feasibility of using unconcentrated urine samples. The sensitivity of the combined SDS–PAGE/Western blot system also makes this a potentially useful method for analyzing proteins in small volumes of fluid such as might be obtained experimentally by micro techniques.

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