Urol Res (1999) 27:250–254

ORIGINAL PAPER

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Isoelectric focusing of native urinary uromodulin (Tamm-Horsfall protein) shows no physicochemical differences between stone formers and non-stone formers

Received: 5 May 1998 / Accepted: 28 October 1998

Abstract Recent studies have suggested the occurrence of an abnormal form of uromodulin in stone formers which may be related to a reduced sialic acid content of the protein in these patients. Previous attempts to demonstrate these differences have required extensive sample processing prior to analysis, which may in itself alter the nature and behaviour of uromodulin. By employing a sensitive detection system of enhanced chemiluminescence on Western blots following isoelectric focusing we have been able to study the physicochemical properties of uromodulin in whole unprocessed urine from 50 idiopathic calcium stone formers and 15 non-stone formers. Uromodulin, desialated in vitro with either acid or neuraminidase was also analysed using the same system. All urine samples analysed from stone formers and non-stone formers showed a single band of pI 3.5 after isoelectric focusing. Desialated uromodulin showed a series of bands ranging from pI 4.0 to 5.1 reflecting different amounts of sialic acid removed. We conclude there are no charge-related differences in native uromodulin between stone formers and non-stone formers, in particular none relating to the sialic acid content of the protein.

Key words Uromodulin · Idiopathic calcium stones · Enhanced chemiluminescence

Introduction

Renal stone disease affects up to 10% of people in the developed world with calcium stones predominating. Established protocols [12] are able to identify several risk factors in patients leading to renal stone formation yet many cases remain idiopathic with no discernible biochemical or anatomical abnormality. The incidence of recurrence is high (up to 70% after 10 years) [18] even in idiopathic stone formers, increasing the risk of permanent kidney damage, despite modern techniques of stone removal. Understanding the origin of such idiopathic stones will aid the development and administration of preventative treatment regimes.

There are thought to be three main factors relevant to renal stone formation: supersaturation of urine with stone constituents and the concentration of inhibitors and promoters of crystal nucleation, growth and aggregation. All people will at some time achieve urinary supersaturation [19], although only some will form stones. The role of promoters and inhibitors must therefore be vital to predisposing those who form stones to do so, or protecting those who do not.

A number of inhibitors of calcium stone formation have been described. These include citrate and pyrophosphate as inhibitors of crystal growth, and macromolecular factors such as chondroitin sulphate and heparin as inhibitors of crystal aggregation [19]. However, the mode of action of promoters of stone formation is far less clear cut. A number of organic molecules have been investigated as potential promoters, one of which is uromodulin.

Uromodulin, the most abundant protein in normal urine, is an 85-kDa glycoprotein containing 25%–30% carbohydrate, up to 50% of which may be sialated. It is secreted by the thick ascending limb of the loop of Henle [1, 15] where it may act to render the nephron wall impermeable to water. However it has also been implicated as both an inhibitor [6, 9] and a promoter [4, 11] of stone formation. Its inhibitory properties are thought to arise by coating crystals and thus preventing crystal growth and/or aggregation. However, self-aggregation and polymerisation of uromodulin is caused by high calcium concentrations, high ionic strength and low pH [16], which may allow the protein to act as a promoter by forming a mesh to which crystals adhere, thus initiating crystal growth.

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Qualitative analyses of uromodulin from idiopathic calcium stone formers and normal controls have suggested differences in physicochemical properties [8, 13] and sialic acid content [10, 17] between the two groups. The occurrence of an abnormally glycosylated uromodulin could be the explanation for its apparent dual nature in vitro. However, most studies to date have relied upon extensive sample pretreatment prior to analysis such as salt fractionation and lyophyllisation [9, 11, 13] or sample concentration up to 200-fold [8], and these processes may have had a direct effect on the nature of uromodulin and its behaviour in analytical systems.

We have investigated the nature of the differences in the electrophoretic characteristics of uromodulin using an isoelectric focusing (IEF) method which requires no sample processing, and is therefore free from potential sample processing artifacts. We also investigated the effect of urine composition on the IEF characteristics of uromodulin.

Methods and reagents

Patients and controls

The study protocol was agreed by the UCL Hospitals ethics committee. Informed consent was obtained from all patients. Random mid morning urine samples were collected from 50 idiopathic calcium stone formers (ICF) (39 males, 11 females; age range 28–75 years) attending an outpatient stone clinic at UCL Hospitals. None of the patient group had any risk factors for calcium stone formation that could be detected by conventional means including plasma, and random and 24 h urine biochemistry screens [12] (including urinary oxalate and citrate measurements). A control group of random mid morning urines was collected from 15 healthy volunteers (8 males, 7 females; age range 27–52 years).

Control samples ($n = 15$) and ICF samples ($n = 6$) were analysed on the day of collection, then again after storage for 7 days at 4°C, and up to 16 weeks at −20°C to assess uromodulin stability.

Reagents

All reagents and chemicals were purchased from BDH Ltd (Poole, UK) except where specified. Reagents and buffers were made up in double distilled deionised water (ddH_2O). Precast isoelectric focusing polyacrylamide gels (Ampholine PAGplates pH 3.5–9.5 1 mm thick) were obtained from Pharmacia Biotech, St Albans, UK; protein pI markers (IEF MIX 3.5–9.3), Coomassie blue and neuraminidase from Sigma (Sigma Chemical, Poole, UK) and Protran BA nitrocellulose from Anderman (Kingston-Upon-Thames, UK). Horseradish peroxidase sheep anti-human uromodulin was purchased from The Binding Site (Birmingham, UK) and the enhanced chemiluminescence (ECL) detection system from Amersham Life Sciences (Buckinghamshire, UK).

Methods

Creatinine was measured using an alkaline picrate method (ISP 1000; Monitor Bioscience, Burgess Hill, UK), osmolality by freezing point depression (Roebling Osmometer; Camlab, Cambridge, UK) and calcium and magnesium by atomic absorption spectroscopy (Unicam 929 AA; ATI Unicam, Cambridge, UK). pH was measured with a PHM 82 pH meter (Radiometer, Crawley, UK).

Isoelectric focusing (IEF)

The strategy used for IEF is illustrated in Fig. 1. IEF was performed at 10°C on precast Ampholine PAGplates using the MultiPhor II electrophoresis unit (Pharmacia Biotech). Cathode and anode buffers were 1.0 mol/l sodium hydroxide and 1.0 mol/l phosphoric acid, respectively. Urine samples (15 µl, except where

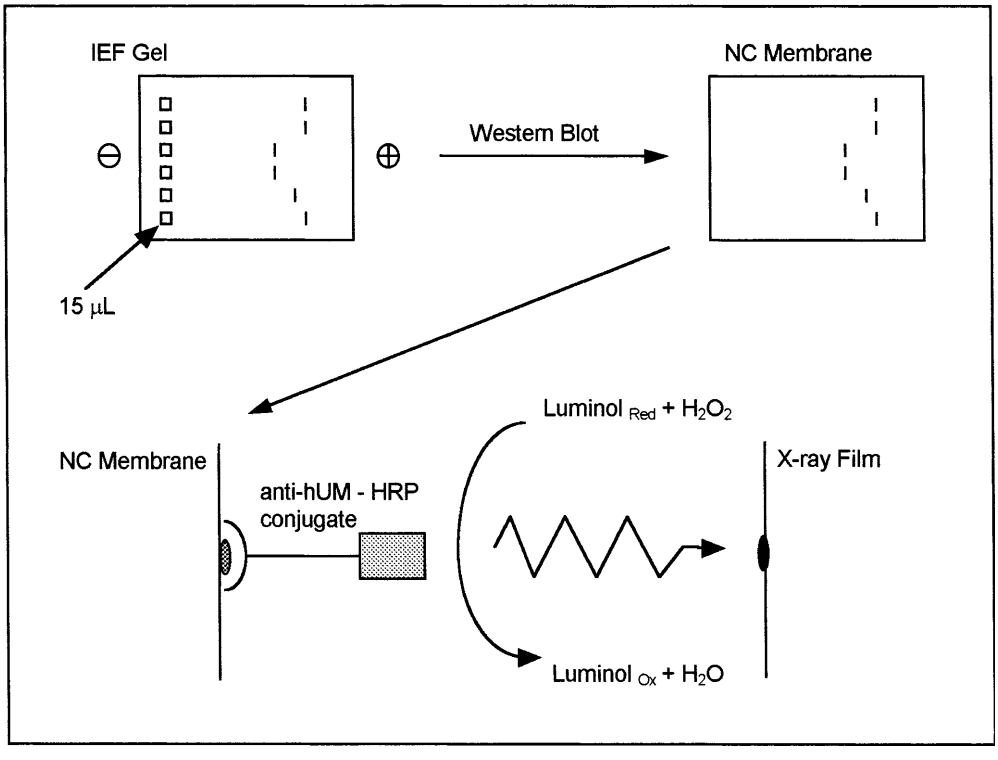

Fig. 1 Urine proteins are separated according to their net charge (pI) by isoelectric focusing (IEF). Proteins are then transferred to nitrocellulose (NC) membrane by electroblotting. Uromodulin is located with polyclonal anti-human uromodulin (hUM) labelled with horseradish peroxidase (HRP) and visualised by enhanced chemiluminescence (ECL). The signal produced is captured on X-ray film

stated) were loaded using plastic applicator wells positioned 10 mm from the cathode wick. For a whole gel (dimensions: 245 × 110 mm) electrophoresis was run at 50 mA, 30 W, 1500 V for approx. 55 min until focusing was complete. pI markers were run at each end of the gel and were cut off before proceeding to the Western blot. The pI markers were stained with Coomassie blue R250 (0.025% Coomassie Blue R250, 40% methanol, 7% acetic acid) and destained in 7% acetic acid, 5% methanol.

Western blot

The gel was equilibrated in transfer buffer (192 mmol/l glycine, 25 mmol/l TRIS base, 20% methanol) for 5 min to remove IEF buffer salts and detergents. The gel was then stripped from its plastic backing and transferred to nitrocellulose pre-wetted with transfer buffer using a Trans-Blot SD electrophoretic transfer cell (BioRad Laboratories, Hemel Hempstead, UK). A double layer of 3MM paper, soaked in transfer buffer, and placed on either side of the gel/membrane sandwich, formed the buffer reservoirs. Transfer took place in the cathode to anode direction at 10 V for 90 min for a whole gel (60 min for half a gel).

Detection

The non-specific binding sites on the nitrocellulose membrane were blocked with 2% (w/v) milk proteins in phosphate-buffered saline-0.05% Tween 20 (PBS-T, 137 mmol/l sodium chloride, 2.65 mmol/l potassium chloride, 8.27 mmol/l anhydrous disodium hydrogen orthophosphate and 1.47 mmol/l potassium dihydrogen orthophosphate, 0.5 ml Tween 20) for 60 min, then washed with three changes of PBS-T for 10 min each. The membrane was incubated for 90 min in antibody solution (dilution 1/5000 in PBS-T) then washed with PBS-T (3 × 15 min). The ECL reagent was prepared immediately before use according to manufacturer's instructions. Excess wash solution was drained off the blot and ECL reagent pipetted evenly over the membrane and incubated for 60 s. Excess ECL reagent was drained off, the membrane wrapped in Saran-Wrap and exposed to X-ray film (HyperFilm, Amersham International Buckinghamshire, UK) for a minimum of 20 s. The film was developed and fixed by standard means.

Neuraminidase digest

A buffered enzyme solution was prepared containing 0.31 mol/l sodium chloride, 0.018 mol/l calcium chloride, 6.2 mmol/l sodium azide and 7 U/l neuraminidase in acetate buffer (19.2 mmol/l acetic acid and 180.8 mmol/l sodium acetate, pH 5.5). An equal volume of the buffered enzyme solution was added to a known volume of urine. The mixture was incubated at 37°C for 24 h then dialysed at 4°C for 48 h against three changes of ddH_2O. Controls were included which contained all reagents except the neuraminidase.

Acid digest

Equal volumes of urine and 50 mmol/l sulphuric acid were mixed and incubated at 80°C for 60 min. After hydrolysis the mixture was dialysed at 4°C for 48 h against three changes of ddH_2O.

Results

There was no significant difference in the concentration of any of the physiological variables (creatinine, calcium and magnesium concentrations, pH and osmolality) measured between the two groups (Table 1).

Uromodulin was stable for 7 days when stored at 4°C or for up to 8 weeks when stored at −20°C. No protein degradation or denaturation as evidenced by changes in IEF mobility was seen during these times. There was some evidence of uromodulin degradation in samples stored for 12 weeks or more at −20°C, which manifested as loss of band focusing ability after IEF. In subsequent experiments samples from ICF were stored at 4°C and analysed within 7 days of sample collection.

Fresh control samples all showed a poorly focused band at pI 3.5 (Fig. 2a) which occasionally appeared to resolve into two bands. The intensity of the uromodulin band varied between samples and reflected the concentration of the samples as indicated by the creatinine concentration. The pI of uromodulin band was not affected by the concentration of any of the parameters measured in the sample, despite the wide range seen (Table 1). Analysis of 50 samples from ICF showed the same band pattern as observed in the controls (Fig. 2b). No other bands were seen in any samples.

Enzymatic desialation of uromodulin in control and ICF samples produced multiple bands with pI ranging from 4.0 to 5.1. Similar patterns were seen in ICF and control urines alike (Fig. 3). The change in band pattern was only seen when neuraminidase was present in the incubation buffer.

Acid hydrolysis of the samples resulted in multiple bands stretching from pI 4.0 to 6.0 showing that this process removed more carbohydrate residues than neuraminidase desialation. Acid hydrolysis also revealed a distinct band at pI 6.6 in all samples corresponding to the carbohydrate-free protein. This experiment confirms that the epitopes targeted by the antibody do not rely on carbohydrate residues alone and therefore if asialouromodulin was present then it should be detected by this method. The effect of acid hydrolysis was the same for ICF and control samples.

Discussion

Differences in electrophoretic mobilities of uromodulin have often been attributed to differences in the sialic acid

Table 1 Composition of urine samples. *P* value is probability (*t*-test) of idiopathic calcium stone formers (ICF) against control urines, ($P > 0.05$ not significant)

	Controls ($n = 15$)		ICF ($n = 50$)		
	Range	Mean	Range	Mean	*P*
pH	5.0–7.2	6.2	5.0–7.8	6.2	0.91
Osmolality (mmol/kg)	232–1043	636	92–1023	604	0.79
Calcium (mmol/l)	0.56–9.46	3.95	0.37–10.0	4.1	0.89
Magnesium (mmol/l)	0.6–7.8	3.0	0.0–6.2	2.3	0.34
Creatinine (mmol/l)	2.49–20.6	9.79	1.25–23.8	9.74	0.99

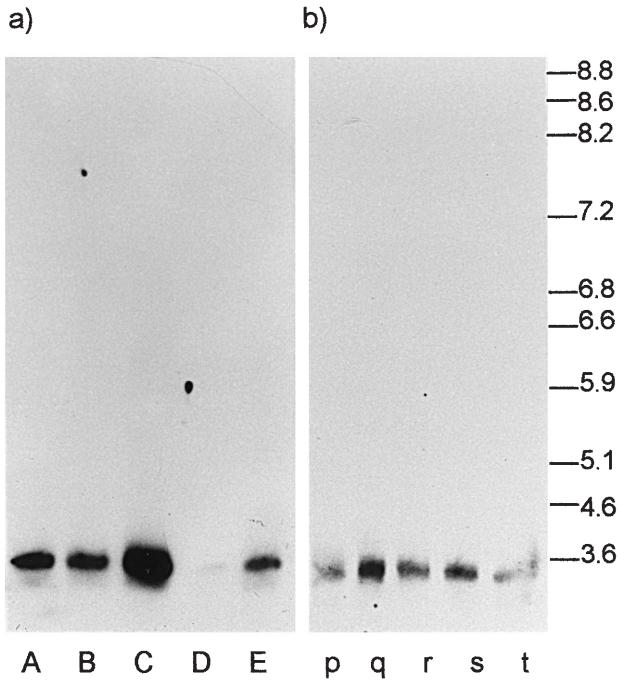

Fig. 2 IEF of fresh urine from **a** normal controls and **b** idiopathic calcium stone formers; pI shown on the right. Creatinine concentrations (mmol/l) are: A = 9.8, B = 13.4, C = 16.4, D = 2.7, E = 7.8, p = 7.7, q = 8.8, r = 8.1, s = 8.0, t = 4.7

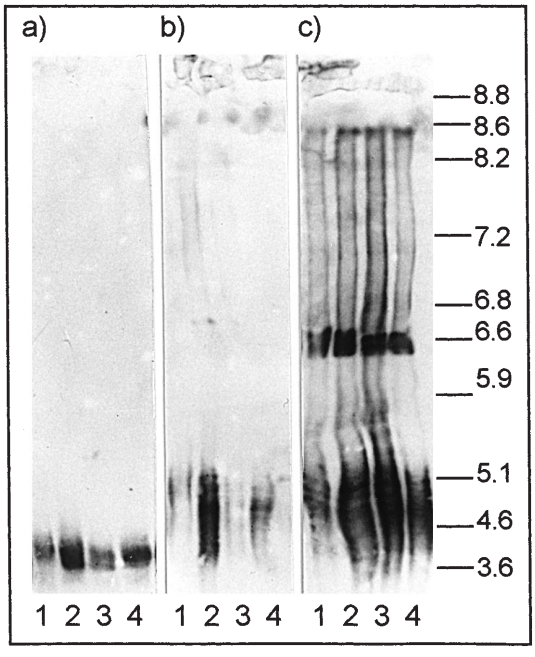

Fig. 3 IEF of urine from ICFs (samples 1–3) and normal control (sample 4) assayed **a** fresh **b** after neuraminidase treatment and **c** after acid hydrolysis; pI shown on the right

content. In support of this argument, the total and bound sialic acid content has been reported to be lower in fresh urine samples from ICFs compared with controls when using a sialidase method [17]. In testing the hypothesis that differences observed in physicochemical properties of uromodulin were related to the degree of sialation of the protein, we have used IEF, which separates molecules according to their net charge or pI. In the current study a specific immunodetection system was used to detect uromodulin which was found, in accordance with previously published data, to have a pI of 3.5 [13]. The appearance of the uromodulin band as a poorly focused doublet was also reported by Schnierle et al. [13] and is probably the result of heterogeneity of the carbohydrate residues. Unlike some previous studies however, this study did not demonstrate any differences in uromodulin from the ICF and control groups, despite this being one of the largest groups of stone formers studied in this way. Schnierle et al. [13] reported an abnormal IEF pattern of uromodulin in the salt precipitable fraction of urine in 71% of ICF studied (n = 14) showing multiple bands in the pI range 4.5–6.0. Jefferson et al. [8], analysing concentrated (200-fold) urine by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis, demonstrated an abnormal form of uromodulin in 84% of their ICF group (n = 37). Asialouromodulin in the salt precipitable urine fraction in ICFs has also been demonstrated by Fourier-transform infrared spectroscopy [10].

The results of the current study showed no evidence of an abnormally charged uromodulin in any of the patients or controls investigated, suggesting that the uromodulin molecules contained similarly charged groups. These results are in agreement with those of Grover and Resnick [2] who failed to demonstrate any abnormality of charge or molecular weight of uromodulin by two-dimensional electrophoresis in freeze-dried desalted urine samples reconstituted in SDS buffer (n = 20), although other proteins unique to ICFs were seen. Hess et al. [5] found that the salt-precipitable protein fraction from ICF urines (n = 15) contained the same amount of sialic acid as controls, whereas the supernatant contained significantly more. The possibility of the occurrence of other sialic acid-containing proteins, which are not precipitated by the method of Tamm and Horsfall but which may be relevant to stone disease, must therefore not be overlooked.

Enzymatic desialation of uromodulin from ICF and control groups resulted in a markedly different IEF pattern from untreated samples clearly demonstrating the effect of removing charged groups from the native protein. This difference was not due to the heating or dialysis procedures as it was not observed in samples subjected to the same conditions but without neuraminidase. A range of pI values from 4.0 to 5.1 was seen after neuraminidase treatment, suggesting varying degrees of desialation had occurred. This method of neuraminidase digestion removes approximately 82% of sialic acid residues [3] accounting for the slight variations in the resulting band patterns. Despite this, and the different band intensities due to differences in concentration, a number of common bands are shared by the digested samples.

Although the neuraminidase digest band pattern was similar to that reported previously for uromodulin in the

salt-precipitable fraction of urine from an ICF group [13] (reportedly asialouromodulin) the pI range was less acidic (4.5–6.0) suggesting more or differently charged groups were missing compared with the neuraminidase-treated samples. The effect of enzymatic desialation was seen in both the ICF and the control groups whereas the proposed asialouromodulin in the earlier study was only seen in the ICF group.

The possibility that salt fractionation can cause loss of sialic acid residues from uromodulin is hinted at by Hession et al. [7] who noted that salt-precipitated uromodulin from the urine of stone formers had a 10-fold lower immunosuppressive activity (known to reside in the sialic acid moieties) than that isolated by lectin adherence columns from pregnancy urine. However, the authors attributed the difference to extra carbohydrate residues added to uromodulin during pregnancy rather than residues lost during salt fractionation.

Asialouromodulin (enzymatically prepared) has been shown to increase the in vitro crystallisation of calcium oxalate and phosphate [3]. The effect of sample concentration on the polymerisation of uromodulin and its subsequent promotional effect on calcium oxalate crystallisation has also been documented [14]. This effect was attributed to a reduction in the negativity of the crystal zeta potential reducing the effectiveness of other polyionic inhibitors present in urine, and not to uromodulin directly.

We have no explanation for the apparent differences in uromodulin seen between ICF and controls in other studies. One could argue that sample processing disrupts the carbohydrate residues of uromodulin and that uromodulin from ICFs may be more susceptible to those effects than uromodulin from non-stone formers.

The method used in this study is capable of detecting uromodulin in urine without the need to concentrate the sample or extract the protein, thus avoiding the possibility of artifacts resulting from sample processing. This method is also capable of detecting asialouromodulin when present. We have shown that native uromodulin from ICFs and normal controls shows no difference in net charge or sialic acid content.

Acknowledgments The authors wish to thank Dr W.G. Robertson for organising sample collection, Mr H. Whitfield for access to patient material, Dr P. Hallson for his useful comments and advice, and the staff of the Department of Chemical Pathology, UCL Hospitals for assistance with the routine biochemical analyses.

References

1. Gokhale JA, McKee MD, Khan SR (1996) Immunocytochemical localisation of Tamm-Horsfall protein in the kidneys of normal and nephrolithic rats. Urol Res 24:201
2. Grover PK, Resnick M (1995) Evidence for the presence of abnormal proteins in the urine of recurrent stone formers. J Urol 153:1716
3. Hallson PC, Choong SKS, Kasidas, Samuell CT (1997) Effects of Tamm-Horsfall protein with normal and reduced sialic acid content upon the crystallisation of calcium phosphate and calcium oxalate in human urine. Br J Urol 80:533
4. Hallson PC, Rose GA (1979) Uromucoids and urinary stone formation. Lancet I:1000
5. Hess B, Jaggi M, Zipperle L, Jaeger P. Sialic acid content of salt- and non-salt- precipitable urine fractions as well as of Tamm-Horsfall glycoprotein in recurrent calcium renal stone formers and healthy controls. In: Pak CY, Resnick MI, Preminger GM (eds) Urolithiasis 1996. VIII International Symposium on urolithiasis, Dallas, Sept. 29–Oct. 2 1996, p 193
6. Hess B, Nakagawa Y, Coe F (1989) Inhibition of calcium oxalate crystal aggregation by urine proteins. Am J Physiol 257:F99
7. Hession C, Decker J, Sherblom A, Kumar S, Yue C, Mattaliano R, Tizard R, Kawashima E, Schmeissner U, Heletky S, Chow E, Burne C, Shaw A, Muchmore A (1987) Uromodulin (Tamm-Horsfall glycoprotein): a renal ligand for lymphokines. Science 237:1479
8. Jefferson A, Reynolds T, Elves A, Wierzbicki (1996) Patients with recurrent renal stones have a physico-chemically altered urinary Tamm-Horsfall glycoprotein profile. Ann Clin Biochem 33:540
9. Kitamura T, Pak CYC (1982) Tamm-Horsfall glycoprotein does not promote spontaneous precipitation and crystal growth of calcium oxalate in vitro. J Urol 127:1024
10. Knorle R, Schnierle P, Koch A, Buchholz N-P, Hering F, Seiler H, Ackermann T, Rutishauser G (1994) Tamm-Horsfall glycoprotein: role in inhibition and promotion of renal calcium oxalate stone formation studied with Fourier-transform infrared spectroscopy. Clin Chem 40:1739
11. Rose GA, Sulaiman S (1982) Tamm-Horsfall mucoproteins promote calcium oxalate crystal formation in urine: quantitative studies. J Urol 127:177
12. Samuell CT, Kasidas GP (1995) Biochemical investigations in renal stone formers. Ann Clin Biochem 32:112
13. Schnierle P, Hering F, Seiler H (1996) Isoelectric focusing of Tamm-Horsfall glycoproteins: a simple tool for recognizing recurrent calcium oxalate renal stone formers. Urol Res 24:79
14. Scurr DS, Robertson WG (1986) Modifiers of calcium oxalate crystallisation found in urine. III. Studies on the role of Tamm-Horsfall mucoprotein and of ionic strength. J Urol 136:505
15. Sikri KL, Foster CL, MacHugh N, Marshall RD (1981) Localisation of Tamm-Horsfall glycoprotein in the human kidney using immuno-fluorescence and immuno-electron microscopical techniques. J Anat 132:597
16. Stevenson FK, Cleave AJ, Kent PW (1971) The effect of ions on the viscometric and ultracentrifugal behaviour of Tamm-Horsfall glycoprotein. Biochim Biophys Acta 236:59
17. van Aswegen CH, van der Merwe CA, du Plessis DJ (1990) Sialic acid concentrations in the urine of men with and without renal stones. Urol Res 18:29
18. Watts RWE (1996) Urinary stone disease (urolithiasis). In: Weatherall DJ, Ledingham JGG, Warrell DA (eds) Oxford textbook of medicine, 3rd edn. Oxford Medical Publications, Oxford, pp 3251–3257
19. Wickham JEA, Buck AC (1990) Renal tract stone disease – metabolic basis and clinical practice. Churchill Livingstone, Edinburgh, pp 295–306
20. Yu H, Papa F, Sukhatme V (1994) Bovine and rodent Tamm-Horsfall protein (THP) genes: cloning, structural analysis, and promoter identification. Gene Expr 4:63