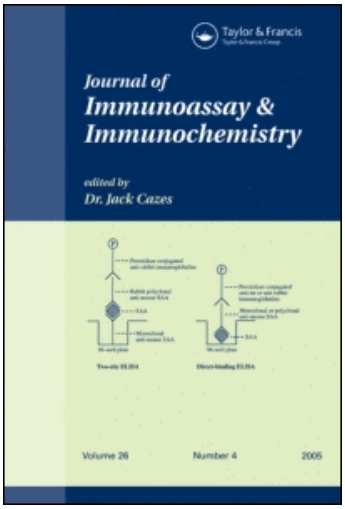

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Direct Radioimmunoassay of Rat Cystatin C: Increased Urinary Excretion of This Cysteine Proteases Inhibitor During Chromate Nephropathy

Allain Collé[a]; Catherine Tavera[a]; Patricia Laurent[a]; Jeanne Leung-tack[a]; Jean Pierre Girolami[a]
[a] INSERM U 133, TOULOUSE Cedex, France

To cite this Article Collé, Allain , Tavera, Catherine , Laurent, Patricia , Leung-tack, Jeanne and Girolami, Jean Pierre(1990) 'Direct Radioimmunoassay of Rat Cystatin C: Increased Urinary Excretion of This Cysteine Proteases Inhibitor During Chromate Nephropathy', Journal of Immunoassay and Immunochemistry, 11: 2, 199 — 214
To link to this Article: DOI: 10.1080/01971529008053269
URL: http://dx.doi.org/10.1080/01971529008053269

JOURNAL OF IMMUNOASSAY, 11(2), 199-214 (1990)

DIRECT RADIOIMMUNOASSAY OF RAT CYSTATIN C: INCREASED URINARY EXCRETION OF THIS CYSTEINE PROTEASES INHIBITOR DURING CHROMATE NEPHROPATHY.

Allain Collé, Catherine Tavera, Patricia Laurent,
Jeanne Leung-Tack and Jean Pierre Girolami.
INSERM U 133, 133 route de Narbonne, 31062 TOULOUSE Cedex,France.

ABSTRACT

Rat cystatin C was purified to apparent homogeneity from rat urine after induction of a tubular dysfunction with sodium chromate.The two-steps purification procedure included a Carboxymethyl-papain affinity chromatography and anion exchange chromatography. The purified protein was identified as rat cystatin C by the following criteria: firstly retained on a Cm-papain affinity column, secondly an apparent molecular weight of 15 kDa and pI of 10.2. Antisera raised in rabbits against our purified rat cystatin C did not cross-react with other urinary proteins such as rat albumin and rat kallikrein, but partially cross-reacted with human cystatin C. A direct radioimmunoassay was developed and it enabled 8.32 fmol/ml of rat cystatin C to be detected. The detection range was between 0.125 and 62.5 ng/ml, with 10% intra-assay variation and 14% inter-assay variation. Physiological rat cystatin C excretion (40 ± 18 µg/24h) was found by the direct assay. In the chromate-intoxicated rat, urinary excretion increased twenty-fivefold (1017 ± 391 µg/24h) and returned to normal level one week after intoxication.This RIA will allow the study of rat cystatin C metabolism particularly during renal dysfunction.

KEY WORDS: Cystatin C. Cysteine proteinases inhibitor. Radioimmunoassay. Nephropathy.

INTRODUCTION

Cystatin C is a major cysteine proteinases inhibitor (1,2) previously known as gamma-trace or post-gamma globulin. Recently, a classification system has

been proposed, dividing the cystatin superfamily into three families (3) : Family 1 cystatins, mainly intracellular (cystatins A and B), Family 2 cystatins, essentially extracellular (cystatins C, S and SU) and Family 3 cystatins, or kininogens. Human cystatin C is an alkaline low molecular weight protein (13 kDa) described for the first time in 1961, in cerebrospinal fluid (4,5).Under normal physiological conditions, cystatin C is present in all tested human biological fluids (6-9) and is particularly abundant in neuroendocrine cells (10,11). Large amounts of cystatin C are found in urine of patients with tubular disorders (12) and in sera of patients with autoimmune diseases (1). Deposits of a cystatin C variant are observed during the course of hereditary cystatin C amyloid angiopathy (13,14) with reduced levels of cystatin C in cerebrospinal fluid (15). In animals, "cystatin C-like" has been detected in "tubular urine" of monkeys (16), dogs (17), mice (18), rats (19) and in bovine colostrum (20). Interestingly, high levels of cystatin C have been detected in urine of chromate treated animals.

Until now, the presence of large amounts of rat cystatin C was assessed by its physico-chemical properties (mainly its electrophoretical mobility) and papain inhibiting assay. In human, cystatin C can be quantified with a radioimmunoassay (using monoclonal antibody) (21), an enzyme amplified single radial immunodiffusion (22) and a time-resolved fluoroimmunoassay (23). Recently two human cystatin C ELISA have been also reported (24,25).

The aim of this investigation was to develop a radioimmunoassay for a direct detection of rat urinary cystatin C. Using this method, we found that urinary excretion of cystatin C is transiently increased during acute renal failure induced by sodium chromate administration in the rat.

MATERIALS AND METHODS

Intoxication protocol.

Male Wistar rats weighing 350-400 g were used. All rats were kept for three consecutive days in individual metabolic cages and received normal diet (UAR A-4-0) with tap water *ad libitum* and 24 h urine was collected. After this control period, the animals were divided into experimental and control groups. The experimental group (16 rats) received a single subcutaneous injection of sodium chromate diluted in sterile saline solution (20 mg/kg body weight), the control group (6 rats) was vehicle-treated. For all the rats, 24 h urinary excretion was collected during 10 consecutive days.

Purification procedure and antibody production.

Purification and identification of rat cystatin C have been described in detail (26). Briefly, urine from chromate-injected rat was collected daily on sodium azide from day 1 to day 6 after intoxication and used as starting material. Urine was pooled daily, centrifuged and concentrated to 50 mg protein/ml , using an Amicon YM5 membrane and stored at -20°C until used.

Samples (5 ml) were dialysed against 0.1 M Tris-HCl buffer, pH 8, containing 0.5 M NaCl and chromatographed on a Carboxymethyl-papain (Cm-papain) Sepharose column (1x10 cm) equilibrated in the same buffer (27). The column was washed with the same buffer until the absorbance at 280 nm approached zero. Bound proteins were eluted with 0.01 M NaOH solution. The fractions containing the single peak of proteins eluted by the alkaline solution were immediately adjusted to pH 7.4 with 1 M sodium phosphate buffer. The protein solution was transferred to a 0.02 M Tris-HCl buffer, pH 9.2, concentrated to 1ml by ultrafiltration and applied to a Mono Q HR 5/5 column (anion exchange

chromatography) in Pharmacia's FPLC system previously equilibrated in the same buffer. Proteins were eluted with a NaCl linear gradient from 0 to 0.3 M , followed by step elution with 1 M NaCl in the Tris-HCl buffer .

Rat cystatin C antibodies were raised in New Zealand rabbits after three injections, at 3 weeks intervals, intra-dermal injections of 50 μg of pure proteins, emulsioned with complete Freund adjuvent according to a technique already described (28,29). Sera were heated for 3 hours at 56°C and tested for their binding capacity with iodinated cystatin C.

Analytical methods.

Protein concentrations were determined according to the Bradford method (30). Polyacrylamide-agarose Coomassie Blue-stained gel electrophoresis was performed in Tris-Glycine buffer, pH 8.7, as previously described (31). Molecular weight was determined by SDS silver-stained gel electrophoresis on 10-15 gradient Phastgel (Pharmacia) run on a Phast system separation unit (Pharmacia) according to the manufacturer's recommendations. Isoelectric points were determined in homogenous polyacrylamide silver-stained gels containing Pharmalyte 8-10.5 carrier ampholytes (Pharmacia). The isoelectric focusing calibration kit was from Pharmacia. Immunoelectrophoresis was performed according to Scheidegger (32).

Antiserum to rat IgG was purchased from Miles Laboratory (Naperville, Ill). Mouse and human cystatin C antibodies were obtained from rabbits as previously reported (18,29).

Preparation of labelled antigen.

The chloramine T method (33) was used for iodination of cystatin C with ^{125}I (from Amersham). In brief, 2μg of purified cystatin was allowed to react with

200 µCi of Na ^{125}I for one minute in the presence of 5 µg chloramine T (5 µl) in 20 µl 0.5 M phosphate buffer, pH 7.4; the reaction was stopped with 20 µg of sodium metabisulfite (20 µl). This reaction mixture was further purified at room temperature on Sephadex G25 medium (Pharmacia) (1 cm x 30 cm), elution was performed with phosphate buffer containing 0.1% lysozyme (Sigma) and 1ml fractions were collected. Fractions showing the highest specific binding were aliquoted and stored frozen at -70°C until used and remained stable for up to two months.

Radioimmunoassay protocol

Assay, made in duplicate, was performed in 0.1 M phosphate buffer, pH 7, containing 0.1% lysozyme in order to reduce unspecific adsorbtion. The reactive mixture containing 100 µl of unlabelled rat cystatin C (0.125 to 62.5 ng/ml) or 100 µl of the unknown samples, 200 µl of antiserun ($2/10^4$ final dilution) and 100 µl of radiolabelled rat cystatin C (10,000 cpm), was incubated for 24 h at 4°C. The antigen-antibody complex was separated using the polyethylene glycol method : 1ml of a 20% polyethylene glycol (mol.wt. 6000 Sigma) solution and 200 µl of 1% gamma-globulins (Sigma) as carrier were added in each tube. Each tube was centrifuged (20 min at 4000 x g), the supernatant was aspirated and the unwashed pellet was counted on a computerized gamma counter (Multi Cristal RIA system Packard). Results are expressed using the Logit-Log linearisation of the standard curve where B is the radioactivity of the pellet in the presence of unlabelled purified antigen or unknown samples and B° is the radioactivity bound only in the presence of the radiolabelled antigen.

Reproducibility.

The intra-assay and inter-assay variances were estimated by calculating the variation of 8 determinations of the same serum run in the same assay and in 8 different assays.

Statistical analysis.

All data are expressed as mean ± SEM. Student's *t* test was used for statistical analysis.

RESULTS

Purification of rat cystatin C and characterization of the antiserum.

The purification and characterization of rat cystatin C has been recently described in detail (26). In brief, a typical run consisted of 5 ml of concentrated urine fractionated on a column of Cm-papain-Sepharose that yielded a single peak of eluted proteins. All of the material from the affinity column was loaded onto the Mono Q column of the FPLC apparatus and three main peaks were obtained (Fig. 1). The first part of peak 1, not retained on the Mono Q column, displayed a single band on acrylamide agarose gel electrophoresis (Fig.1A). In sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), rat cystatin C showed an apparent M_r of 15 kDa (Fig.1B). Rat cystatin C subjected to isoelectric focusing in polyacrylamide gel displayed similar mobility to that of cytochrome C, pI= 10.2 (Fig. 1C).

Immunoelectrophoresis showed an antigenic similarity between mouse, rat and human cystatin C (Fig. 2A).

The antibody was raised in rabbit, the antisera obtained after the third injection, was used at a final dilution of 1/40 000 and reach 30% specific binding of freshly iodinated rat cystatin C (Fig. 2B).

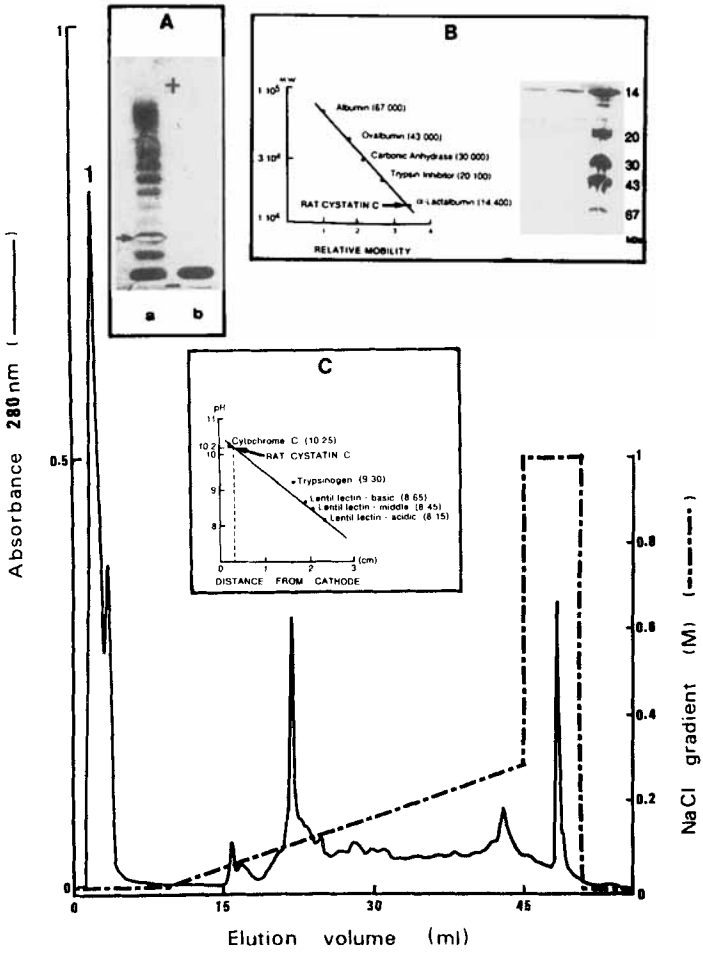

Figure 1.Anion exchange chromatography.
The peak eluted from Cm-papain Sepharose is resolved from several peaks. The peak 1 is not retained on the Mono Q HR 5/5 column.

A -Electrophoresis in 5% polyacrylamide agarose gel: a) proteins eluted from Cm- papain Sepharose (5 mg/ml), b) peak 1 of anion exchange chromatography (1 mg/ml); the arrow indicates inclusions.

B -PAGE-SDS silver-stained gel electrophoresis.Calibration curve established using LMW calibration kit (Pharmacia). Purified urinary rat cystatin C, lane 1 and 2 and molecular weight markers (in kDa), lane 3. Purified rat cystatin C displayed apparent M_r of 15 kDa.

C -Isoelectric focusing. Rat cystatin C isoelectric point is determined in polyacrylamide silver-stained gels containing Pharmalyte 8-10.5. Determination of pH gradient profile using the calibration kit (Pharmacia). The purified rat cystatin C displayed one band with pI of 10.2.

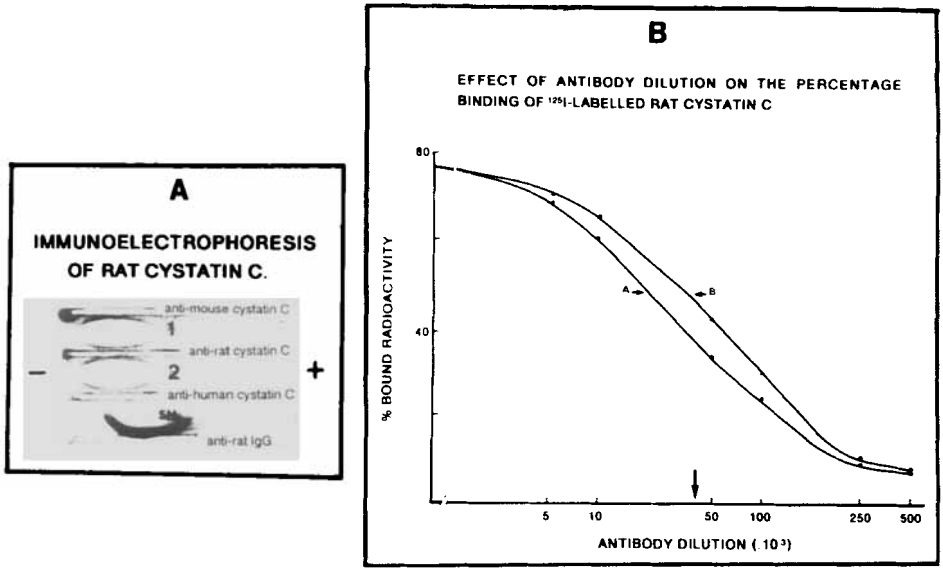

Figure 2. Antiserum characterization.

A -Immunoelectrophoresis. SN, rat normal serum, 1 and 2 rat cystatin C. Rat cystatin C patterns are identical with three polyclonal antibodies (anti-mouse, anti-rat and anti-human cystatin C). Immunoelectrophoresis performed on rat normal serum showed that rat cystatin C is more cathodic than rat IgG.

B -Titration curve of rabbit anti-rat cystatin C antiserum. Curve A, with ^{125}I-cystatin C alone. Curve B in the presence of 1 ng of unlabelled standard rat cystatin C. The arrow indicates the optimal dilution of antiserum used for final RIA conditions, this dilution corresponds to the maximum deviation between curves A and B (1/40000).

Rat cystatin C radioimmunoassay

The iodination mixture was purified on Sephadex G25. The major radioactive fractions of the first peak (3 ml) showed maximal binding with an excess of antibody. Fractions displaying the major specific binding were stored at -70°C and were stable for 2 months.The specific radioactivity was 60 μCi/μg.

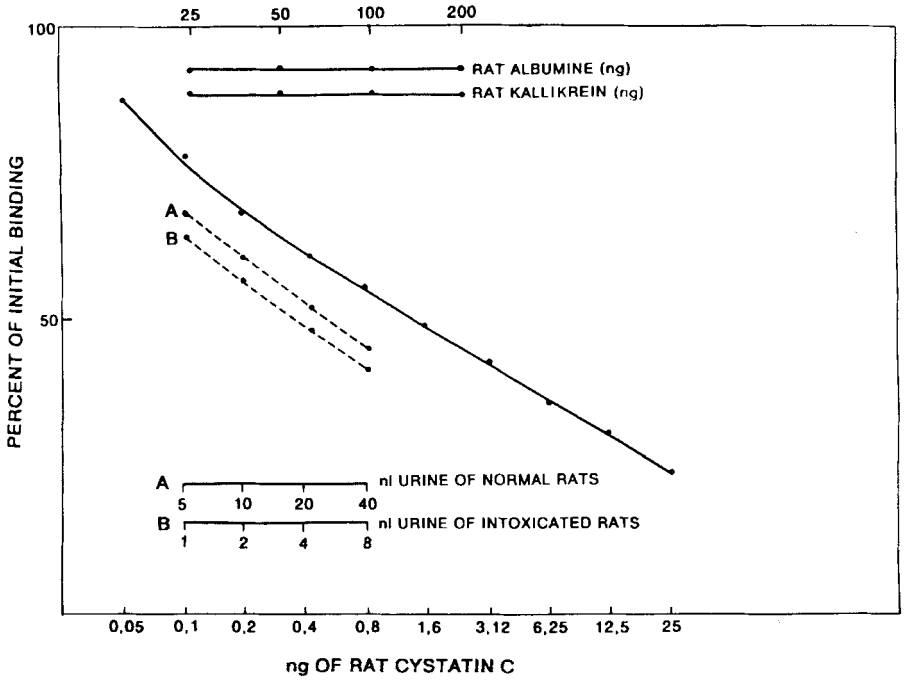

Figure 3. Standard curve for direct radioimmunoassay of rat cystatin C: 0.05 to 25 ng/tube (0.125 to 62.5 ng/ml) and measurement of cystatin C in urine of normal and chromate-intoxicated rats. Addition of purified rat albumin and kallikrein did not induce significant displacement of the standard curve.

The standard radioimmunoassay curve (Fig.3) was obtained when rat ^{125}I-cystatin C was incubated in the presence of various amounts of pure unlabelled cystatin C. The intra-assay and inter-assay coefficients of variation were 10% and 14% respectively. The detectability limit of the assay was 0.125ng/ml equivalent to 8.32 fmol/ml. The detection range was from 0.125 to 62.5ng/ml of purified rat cystatin.

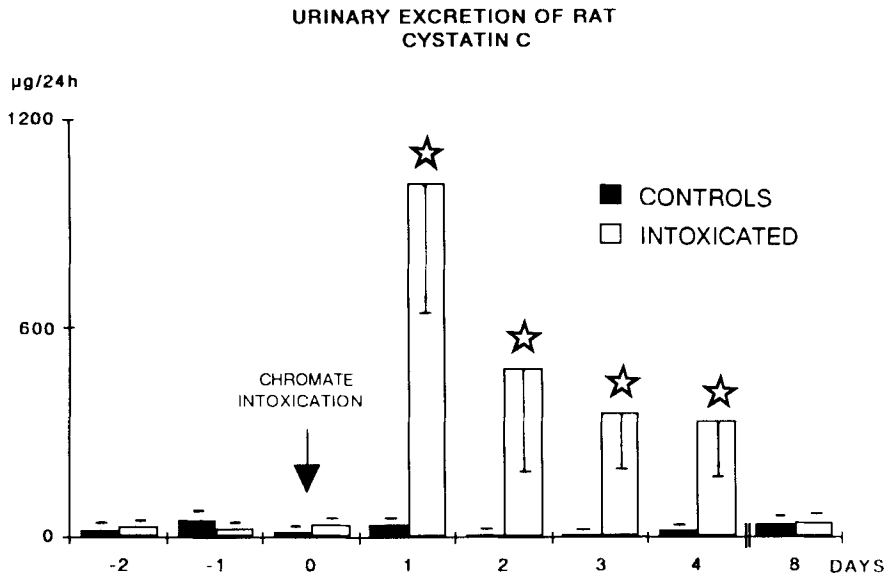

Figure 4. Increase of urinary excretion of rat cystatin C caused by subcutaneous injection of sodium chromate. Blocks are mean values ± SEM. Asterisk indicates statistically significant difference (* $p < 0.001$) compared with values in controls.

Displacement curves obtained with normal and chromate-treated rat urine were parallel with the standard curve, indicating the immunological identity of these rat cystatin C of normal and intoxicated rats. No cross-reaction was found when rat cystatin C antiserum was tested with up to 200 ng of rat albumin and rat kallikrein purified in the laboratory (34).

Cystatin C in normal and chromate-intoxicated rat urines.

Cystatin C was measured by direct RIA in twenty rats before intoxication then after intoxication in the 6 control rats and the 16 chromate-intoxicated rats. The day immediately after intoxication, urinary cystatin C concentration showed a

tenfold increase from 3.10 ± 1.44 to 34.2 ± 12 µg/ml whereas 24h urinary cystatin C excretion was increased twenty-fivefold from 40 ± 18 to 1018 ± 391 µg/24h (values are mean ± SEM, n=16, $p < 0.001$). Diuresis was increased 2.5 fold: 32.3 ± 10 ml/24h versus control 12.7 ± 6 ml/24h. This transiently enhanced excretion of rat cystatin C returned to control values 8 days after intoxication (Fig. 4).

DISCUSSION

The main objective of this study is to describe the first RIA of rat cystatin C. The isolation and purification of rat cystatin C recently described (26), was performed using urine of rats intoxicated with sodium chromate according to an intoxication protocol analogous to the one used on mice (18). Taking advantage of the specific properties of cystatin C: cysteine proteases inhibition and very basic pI, we develop a two-step purification. Our preparation is therefore identified as rat cystatin C by the following criteria, 1) binding with a the cysteine protease papain, 2) single apparent Mr in SDS-PAGE of 15 kDa, 3) basic pI of 10.2, 4) partial sequence, carried out on eight 30 amino acid N terminals (26) identical to partial sequence a rat "cystatin C-like" (glycosylated form) recently isolated (19). Neither our purified urinary rat cystatin C nor the other descriptions of cystatin C purifications did not relate any significant amount of carbohydrate (26).

At to day no assay of rat cystatin C has been described. Enzyme assay by papain inhibition cannot be used with biological fluids, since interference phenomena could occur with the other cysteine proteases inhibitors present. Above all, this technique required the various steps of purification of cysteine proteases inhibitors. Five assays of human cystatin C have been described.

Enzyme-amplified single radial immunodiffusion has enabled to define the concentration of cystatin C in different biological liquids (22). It is a relatively lengthy technique with a sensitivity of only 0.3 mg/ml. A RIA using monoclonal antibodies has given a sensitivity of 2.5 ng/ml (21), but only a small number of applications of this assay have been published. Lastly, there is a time-resolved fluoroimmunoaasy with a sensitivity of 1 ng/ml (23), but it has only been used in man for analytical purposes. It allowed cystatin A, B and C differentiation, but this assay has not been validated on a large number of biological samples.The two ELISA techniques for human cystatin C have given a sensitivity of 0.8 and 1.9 ng/ml respectively (24,25).

The rat cystatin C RIA we have developed has a higher sensitivity when compared with the similar assays described in man, since the detection sensitivity was 0.125 ng/ml (8.3 fmol/ml), and it was highly reproductive.

This assay has enabled us to determine a normal urinary excretion of cystatin C of 40 ± 18 µg/24h in the rat, which is comparable to urinary excretion in man 95 ± 57 µg/24h (22) .

24 hours after intoxication with sodium chromate, a tenfold increase in rat urinary cystatin C concentration was found, 34.2 ± 12 µg/ml versus control 3.10 ± 1.44 µg/ml, whereas a twenty-fivefold increase was found, when the result were normalized per 24h, 1018 ± 391 µg /24h versus control 40 ± 18 µg /24h. The tubular disorders induced by sodium chromate are reversible and values return to normal level one week after intoxication. Whether urinary excretion of rat cystatin C can be used as marker of glomerular and tubular function is already suggested (36).

In conclusion, the direct RIA described in this article enables rapid assaying of small quantities of rat cystatin C. It allowed us to demonstrate that the distribution found in dog organs (37) is also found in the rat (38). It could be an useful sensitive method in order to investigate the metabolism of this still poorly known protein, and to quantify its secretion at the cellular level.

ACKNOWLEDGMENTS

We thank Ms Catherine Blanpied and Mr Christian Mora for technical assistance.

REFERENCES

1. Brzin J, Popovic T, Turk V, Borchart U, Machleidt W. Human cystatin, a new protein inhibitor of cysteine proteinases. Biochem Biophys Res Commun 1984; 118: 103-9.

2. Barrett A J, Davies M E, Grubb A. The place of human γ-trace (cystatin C) amongst the cysteine proteinase inhibitors. Biochem Biophys Res Commun 1984; 120: 631-6.

3. Barrett A J, Fritz H, Grubb A et al. Nomenclature and classification of the homologous with the cysteine proteinase chicken cystatin. Biochem J 1986; 236: 312.

4. Clausen J. Proteins in normal cerebrospinal fluid not found in serum. Proc Soc Exp Biol Med 1961; 107: 170-2.

5. Mac Pherson C F C, Cosgrove J B R. Immunochemical evidence for a gammaglobulin peculiar to cerebrospinal fluid. Can J Biochem 1961; 39: 1567-74.

6. Hochwald G M, Thorbecke G J. Use of an antiserum against cerebrospinal fluid in demonstration of trace proteins in biological fluids. Proc Soc Exp Biol Med. 1962; 109: 91-5.

7. Hochwald G M, Thorbecke G J. Trace proteins in cerebrospinal fluid and other biologocal fluids. 1. Effect of various fractionation procedures on ß-trace and γ-trace proteins and methods for isolation of both proteins. Arch Biochem Biophys 1963; 101: 325-34.

8. Cejka J, Fleischmann L E. Post -γ globulin: isolation and physicochemical characterization. Arch Biochem Biophys 1973; 157:168-76.

9. Collé A, Guinet R, Leclercq M, Manuel Y. Occurence of $ß_2$-microglobulin and post-γ-globulin in human semen. Clin Chim Acta 1976; 67: 93-7.

10. Grubb A, Löfberg H. Human γ-trace, a basic microprotein: amino acid sequence and presence in the adenohypophysis. Proc Natl Acad Sci USA 1982; 79: 3024-27.

11. Möller C A, Löfberg H, Grubb A, Olsson S O, Davies M E, Barrett A J. Distribution of cystatin C (γ-trace), an inhibitor of lysosomal cysteine proteinases, in the anterior lobe of simian and human pituitary glands. Neuroendocrinology 1985; 41: 400-4.

12. Butler E A, Flynn F V. The occurence of post-γ protein in urine: a new protein abnormality. J Clin Pathol 1961; 14: 172-8.

13. Cohen D H, Feiner H, Jensson O, Frangione B. Amyloid fibril in hereditary cerebral hemorrhage with amyloidosis (HCHWA) is related to the gastroenteropancreatic neuroendocrine protein, gamma trace. J Exp Med 1983; 158: 623-8.

14. Ghiso J, Jensson O, Frangione B. Amyloid fibril in hereditary cerebral hemorrhage with amyloidosis of Icelandic type is variant of γ-trace basic protein (cystatin C). Proc Natl Acad Sci USA 1986; 83: 2974-8.

15. Grubb A, Jensson O, Gudmundsson G, Arnason A, Löfberg H, Malm J. Abnormal metabolism of the γ-trace alkaline microprotein: the basic defect in hereditary cerebral hemorrhage with amyloidosis. N Engl J Med 1984; 311: 1547-9.

16. Collé A, Sire J, Jeannin M, Manuel Y. Néphropathies experimentales et $ß_2$-microglobuline chez le singe. Pathol Biol 1978; 26: 325-8.

17. Poulik M D, Shinnick C S, Smithies O. Partial amino acid sequences of human and dog post-gamma globulins. Mol Immunol 1981;18: 569-72.

18. Collé A, Tonnelle C, Jarry Th, Coirre C, Manuel Y. Isolation and characterization of post gamma globulin in mouse. Biochem Biophys Res Commun 1984; 122: 111-5.

19. Esnard A, Esnard F, Faucher D, Gauthier F. Two rat homologues of cystatin C. FEBS Lett 1988; 236: 475-8.

20. Hirado M, Tsunasawa S, Sakiyama F, Niinobe M, Fujii S. Complete amino acid sequence of bovine colostrum low-M_r cysteine proteinase inhibitor. FEBS Lett 1985; 186: 41-5.

21. Poulik M D, Perry D J, Vokac E, Sekine T. Post-gamma globulin. II.Radioimmunoassay determination of levels of post-gamma globulin and β_2-microglobulin. Clin Chem Acta 1983; 128: 249-60.

22. Löfberg H, Grubb A. Quantitation of γ-trace in human biological fluids: indications for production in the central nervous system. Scand J Clin Lab Invest 1979; 39: 619-26.

23. Joronen I, Hopsu-Havu V K, Manninen M, Rinne A, Järvinen M, Halonen P. Detection of low molecular weight cysteine proteinase inhibitors by time-resolved fluoroimmunoassay. J Immunol Methods 1986; 86: 243-7.

24. Olafsson I, Löfberg H, Abrahamson M, Grubb A. Production, characterization and use of monoclonal antibodies against the major extracellular human cysteine proteinase inhibitors cystatin C and kininogen. Scand J Clin Lab Invest 1988; 48: 573-82.

25. Ishiguro H, Ohkubo I, Mizokami M, Titani K, Sasaki M. The use of monoclonal antibodies to define levels of Cystatin C in normal human serum. Hybridoma 1989; 8: 303-13.

26. Tavera C, Guillemot J C, Capdevielle J, Ferrara P, Leung-Tack J, Collé A. A rapid'two-step purification of rat cystatin C, one major inhibitor of cysteine proteinases. Prep Biochem (in press).

27. Anastasi A, Brown M, Kembhavi A, Nicklin M, Sayers C, Sunter D, Barrett A. Cystatin, a protein inhibitor of cysteine proteinases. Improved purification from egg white, characterization, and detection in chicken serum. Biochem J 1983; 211: 129-38.

28. Vaitukaitis J, Robbins J B, Nieschlag E, Ross GT J. A method for producing specific antisera with small doses of immunogen. Clin Endocrinol Metabol 1971; 33: 988-91.

29. Tonnelle C, Collé A, Leclercq M, Sire J, Manuel Y. The different electrophoretic forms of post γ-globulin their antigenic identity and their structural variability. Biochem Biophys Acta 1977; 490: 35-43.

30. Bradford M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-54.

31. Uriel J. Méthode d'électrophorèse dans des gels d'acrylamide-agarose. Bull Soc Chim Biol 1966; 48: 969-82.

32. Scheidegger J J. Une microméthode de l'immunoélectrophorèse. Int Arch Allergy Appl Immunol 1955; 7: 103-10.

33. Greenwood F C, Hunter W C, Glover J S. The preparation of ^{131}I-Labeled human growth hormone of high specific activity. Biochem J 1963; 89: 114-23.

34. Girolami J P, Bascands J L, Pecher C, Suc J M. Direct radioimmunoassay of rat urinary kallikrein: its application to the determination of active and inactive kallikrein concentration after HPLC analysis. J Immunoassay 1987; 8: 115-30.

35. Esnard A, Esnard F, Gauthier F. Purification of the Cystatin C-like inhibitors from urine of nephropatic rats. Biol Chem Hoppe-Seyler 1988; 369 Suppl: 219-22.

36. Simonsen O, Grubb A, Thysell H. The blood serum concentration of Cystatin C (γ-trace) as a measure of the glomerular filtration rate. Scand J Lab Invest 1985; 45: 97-101.

37. Sekine T, Poulik M D. Post-gamma globulin. 1. Tissue distribution and physicochemical characteristics of dog post-gamma globulin. Clin Chem Acta. 1982;120: 225-35.

38. Tavera C, Prévot D, Girolami J P, Leung-Tack J, Collé A. Tissue and biological fluid distribution of cysteine proteinases inhibitor: cystatin C. Biol Chem Hoppe-Seyler (in press)