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ORIGINAL ARTICLE

Standardization of Cystatin C: Development of primary and secondary reference preparations

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A Primary Reference Preparation has been produced using pure, recombinant, Cystatin C in a solvent of 0.1 mol/L KCl. Dry mass determination of the Primary Reference Preparation resulted in a Cystatin C concentration of 5.20 g/L. Agarose-electrophoresis and SDS-electrophoresis, as well as N-terminal sequencing, verified the purity, homogeneity and identity of Cystatin C in the Primary Reference Preparation. For the Secondary Reference Preparation, a serum pool was collected and stabilized. A pilot batch was made to verify the selected procedure and spiking with the pure, recombinant Cystatin C. The final Secondary Reference Preparation is now produced (4468 vials) and ready for value assignment and further characterization.

Keywords: Cystatin C; dry mass determination; Primary Reference Preparation; Secondary Reference Preparation; standardization of Cystatin C

Introduction

Serum Cystatin C has been shown to be an excellent marker for glomerular filtration rate [1,2]. However, in the clinical routine using different methods, results vary owing to a lack of standardization. The goal of the IFCC Working Group on Standardization of Cystatin C is to produce and characterize both a primary and a secondary reference preparation for Cystatin C.

Primary Reference Preparation

Recombinant human Cystatin C was produced by expression in *E. coli* according to [3]. Cystatin C was purified from the cell extract using dialysis, anion exchange and cation exchange chromatography and gelfiltration. The purified Cystatin C was filled into bottles and lyophilized. The lyophilized Cystatin C was reconstituted using 0.1 mol/L KCl, then dialyzed against 0.1 mol/L KCl for 4 days with repeated replacements of the solvent; finally, it was adjusted to a concentration of approximately 5.2 g/L measured by refractometry. This solution was aliquoted in vials of 0.2 mL and labelled (see Figure 1): Cystatin C – Primary Reference Preparation IFCC Working Group. Lot 11082006 – Store at −80 °C.

Dry mass determination

Dry mass determination was started immediately and performed according to [4]. All weighing was carried out on an analytical balance with five decimals. Pyrex glass vials with fitted lids were filled with 3 mL of either protein solution or solvent. The sequence of the weighing vials (Empty – Protein Solution – Solvent) was repeated four times.

Drying took place in an oven at 90°C with a slight vacuum of 27 kPa over 7 days. Figure 2 shows the weight of the protein solution and the solvent as recorded over the 7 days. The concentration of the Primary Reference Preparation for Cystatin C was measured to be: $C_p = 5.197 \pm 0.0078$ g/L with a CV of 0.15 % and an uncertainty (k=1) of 0.088 g/L.

Agarose screen electrophoresis

In order to characterize the Primary Reference Preparation, agarose screen electrophoresis was performed according to [5]. Figure 3 shows that Cystatin C is monomeric, pure, and has the expected mobility.

SDS-PAGE

Likewise, SDS polyacrylamide gel electrophoresis of the Primary Reference Preparation was performed according to [6]. Figure 4 shows that Cystatin C is monomeric, pure, and has the expected molecular mass compared to a marker.

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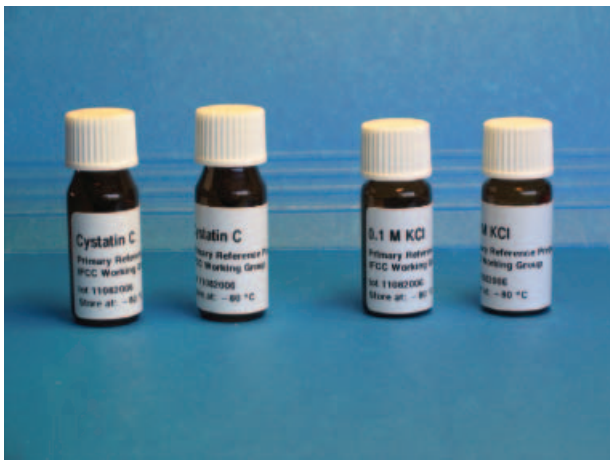

Figure 1. Primary Reference Preparation for Cystatin C and the solvent.

N-terminal sequencing

For verification of purity, homogeneity and identity, an automated Edmann protein sequence analysis was conducted and the following sequence was found: N-SSPGKPPRLVGGP. This sequence is identical with the first 13 amino acids of intact human Cystatin C [7]. No truncated Cystatin C or other non-Cystatin C sequences were detected, which demonstrates the high purity, homogeneity and identity of the intact recombinant Cystatin C used for the Primary Reference Preparation.

Secondary Reference Preparation

As matrix for the Secondary Reference Preparation, it was decided to use a human serum pool collected and stabilized in the same way as used for the production of CRM 470 (Certified Reference Material for immunochemical measurement of human serum proteins, now ERM-DA 470) [8]. This pool would be spiked with pure, recombinant Cystatin C to a target concentration of 5–6 mg/L.

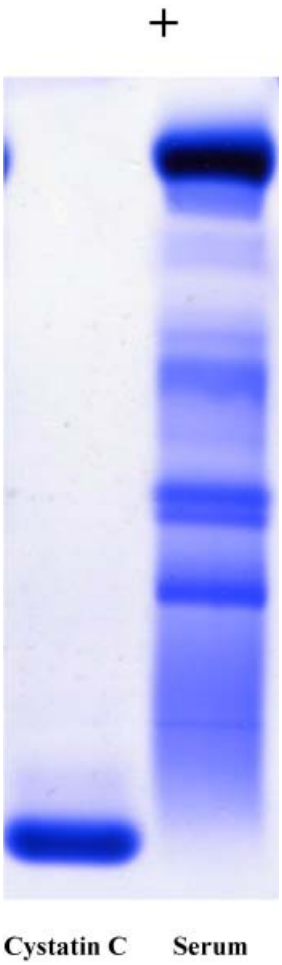

Figure 3. Agarose screen electrophoresis.

Blood collection

Serum from healthy donors was collected in Lund, Sweden and Marburg, Germany using the same protocol at both sites. The collections were preserved by the addition of sodium azide and then frozen. In total, serum from 44 donors (29 from Lund, 15 from

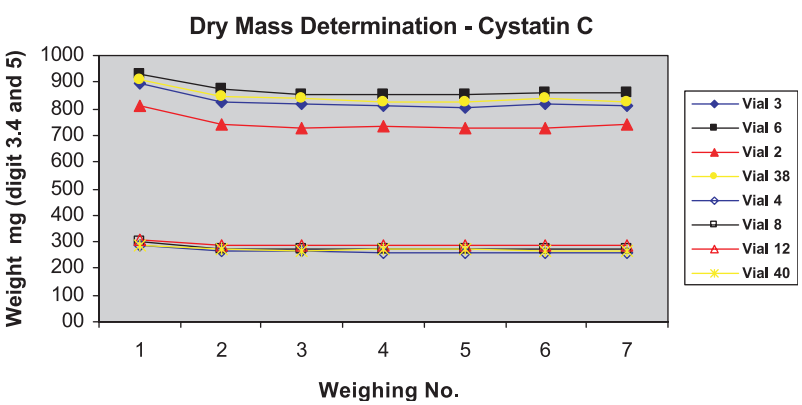

Figure 2. Weight of protein solution and solvent recorded over 7 days.

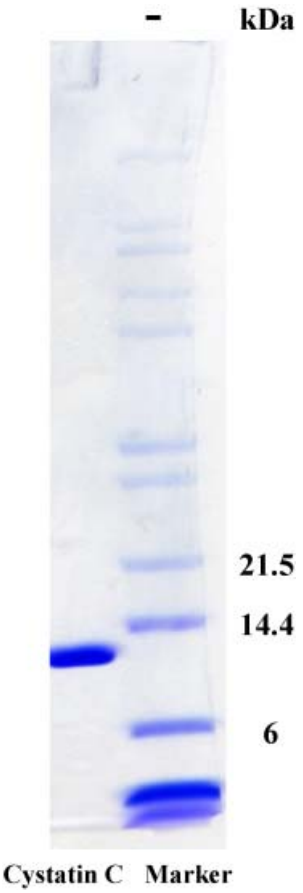

Figure 4. SDS-PAGE.

Marburg) was used for the final pool. The requirements and the results for the single donor are given in Table I.

Stabilization procedure

The individual collections were thawed and pooled to a final volume of 7.880 mL. The pool was delipidated using fumed microparticulated silicon dioxide and then stabilized with the addition of sodium azide, aprotinin and benzamidine. The material was adjusted to pH 7.2 and subjected to sterile filtration, resulting in a final stabilized matrix of 6.220 mL. Total protein concentration of the pool was measured as 64.4 g/L and the intrinsic Cystatin C concentration was determined to be 0.24 mg/L. The final stabilized matrix was stored in portions of 500 mL at −70°C.

Table I. Requirements and results for the single donor serum.

Item		Requirement	Result
1	Source	Serum (healthy)	OK
2	Sex	Both sexes	8 ♀♀, 36 ♂♂
3	Age	20–70 years	20–64 years
4	Blood group	Known	OK
5	RF	<30 IU mL	OK
6	Monoclonal components	Absent (checked by zone electrophoresis)	Absent
7	Bilirubin	Visually normal	OK
8	Haemoglobin	Visually absent	OK
9	Lipaemia	Visually absent	OK
10	Infectious agents	Hepatitis B and C, HIV 1+2	Absent

Secondary Reference Preparation (SRP) pilot batch lot PB001

In order to verify that the selected procedure would result in a suitable Secondary Reference Preparation, it was decided to produce a pilot batch. Therefore 194.5 mg Primary Reference Preparation (5.20 g/L Cystatin C) was added to 140 mL of the stabilized serum matrix. The solution was filtered (0.22 μm) and 1.00 mL was dispensed into siliconized bottles and subsequently lyophilized. During the different stages of processing, the Cystatin C concentration was measured using nephelometry (N Latex Cystatin C, Dade Behring) and turbidimetry (Cystatin C immunoassay, Dako). The results are given in Table II.

Production of the final Secondary Reference Preparation

Production of the final Secondary Reference Preparation followed the same procedure as used for the pilot production. Per 1000 mL stabilized serum matrix, 1.090.6 mg Primary Reference Preparation (5.20 g/L Cystatin C) was added. The solution was filtered (0.22 μm) and aliquots of 1.00 mL were dispensed into siliconized bottles and lyophilized. The bottles were labelled and numbered in the order of filling and lyophilization. A total of 4468 vials were produced and sent to the Institute for Reference Materials and Measurements (IRMM), Geel, Belgium. Homogeneity of the filling was determined by weighing 10 bottles taken every

Table 2. Cystatin C concentration at different stages of the Secondary Reference Preparation pilot batch production.

Stage	Dade Behring	Dako
Theoretical conc.	7.46 mg/L	7.46 mg/L
Stabilized serum matrix	0.24 mg/L	0.24 mg/L
Before lyophilization	5.52 mg/L	7.14 mg/L
After lyophilization	5.32 mg/L	6.42 mg/L
Reconstituted with 1 mL water		
CV between bottles (n=10)	1.18 %	1.73 %

10 min during the filling process (mean=1.007.2 mg; SD 1.229; CV 0.12 %).

Turbidity of the Secondary Reference Preparation after reconstitution was determined using nephelometry on a Dade Behring Nepehlometer Analyzer (BNA) and spectrophotometry at 750 nm (6 bottles with 4 determinations each). The BNA gave: Mean=136 bit; SD 18.48; CV 13.56 %; and the spectrophotometer at 750 nm gave: Mean=0.013; SD 0.0047; CV 35.82 %), which demonstrates the low turbidity of the solution. The initial Cystatin C concentration of 10 vials was determined (10 determinations per vial) using turbidimetry and nephelometry:

Initial concentration, turbidimetry (Dako):	6.09 mg/L ±0.02 mg/L	CV 0.2 %
Initial concentration, nephelometry (Dade Behring):	5.02 mg/L ±0.03 mg/L	CV 0.57 %

Discussion

The next step will be further characterization of the Secondary Reference Preparation, including stability studies. The Cystatin C concentration of the Secondary Reference Preparation will be determined using the Primary Reference Preparation with a measured value of 5.20 g/L. Since the Primary Reference Preparation contains pure Cystatin C in 0.1 mol/L KCl, it will be necessary to use a carrier serum to ensure the same matrix in both preparations. The value assignment will be carried out using single radial immunodiffusion, turbidimetry and nephelometry according to the principles in [9] and [10]. The Secondary Reference Preparation is expected to be released in the last part of 2008, when the commercial calibrators can be adjusted accordingly.

Key point from the discussion

- The treatment stabilizing the serum decreases the concentration of Cystatin C in the reference material, which is a lyophilized preparation. Stability studies will be ongoing. Log-logit is the recommended curve-fit for the calibration, but this may be instrument-dependent. Sometimes an exponential fit is possible, but a spline fit is not recommended.

Contributing to the discussion

E. Lamb, J. Coresh.

Declaration of interest: The authors are members of IFCC Working Group on Standardization of Cystatin C. No conflicts of interest have been reported. The authors alone are responsible for the content and writing of the paper.

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