On 22 D-Punjab (β121Glu→Gln) heterozygotes, the mean β-chain mass was 15 866.832 Da (0.039 Da SD), which is 0.423 Da lower than that determined for the normal β-chain. This mass difference is readily detected and even allows the mean abundance of the variant β-chain to be estimated as 43.0%, which agrees with the mean abundance determined from the HPLC data associated with each sample (41.0%, 3.2% SD, n = 22). The above SD values imply that mass changes down to between 0.1 and 0.2 Da can be reliably detected. A –0.2 Da mass change would suggest a heterozygous –1 Da mutation (Glu→Lys, Glu→Gln, Asp→Asn, or Asn→Ile) at 20% abundance or heterozygous Lepore-Boston-Washington (normal β –2 Da) at 10% abundance.

Of course, although ESI-MS of the globin chains can detect ±1 Da mutations, precise identification requires enzymatic digestion. Hemoglobins C, D-Punjab, E, and O-Arab can all be positively identified directly from the mass spectra of 30-min digests with trypsin (4). It is still necessary to initially detect zero mass change mutations by electrophoretic or chromatographic means, however. Nevertheless, it is useful to know that if HPLC has detected a >20%abant variant with a significant polarity change and ESI-MS has not detected a mass change, then Gln→Lys mutations should be considered.

We also point out that the minor components, HbA1c and HbA2, are readily quantified by ESI-MS. HbA1c can be determined from the concentrations of glycated α- and β-chains after calibration with standards (5). Furthermore, we routinely determine HbA2 (approximately 3%) and find it useful for distinguishing homozygous HbE and HbE/β-thalassemia (HbA2 and HbE coelute by HPLC).

The methods we employ for analyzing Hb have been described in detail (3–5). To reliably achieve the mass measurement precision mentioned above, however, we stress that the data must be acquired over a limited m/z range (we currently scan m/z 930–1210) with a minimum of 32 data points per m/z unit. Also, each m/z spectrum must be internally calibrated, generally using the multiply protonated α-chain ions present from most samples. In this way, inaccuracies due to uncertainties in the atomic weights of the elements are largely eliminated, since the latter are likely to be the same in all proteins in a given sample. We deconvolute m/z 980–1180 to produce mass spectra using the maximum entropy (MaxEnt)-based software supplied with the mass spectrometer.

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**References**


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**Macromolecular Cystatin C Can Be a Caveat for Estimating Glomerular Filtration Rate in Biliary Obstruction**

**To the Editor:**

Creatinine is the most commonly used marker for the estimation of glomerular filtration rate (GFR) in routine clinical use (1), but serum concentrations of low molecular mass proteins can provide an interesting alternative for estimating renal function. Serum concentrations of low molecular weight proteins are primarily determined by GFR, and an ideal marker should have a constant production rate and not vary in concentration during an acute-phase reaction. Cystatin C (13 kDa) and β-trace protein (23–29 kDa) share these properties (1–3). Cystatin C appears to be superior to creatinine for estimation of GFR, especially in the so-called creatinine-blind range. As a screening test for detecting decreased GFR, cystatin C has fewer inherent limitations than serum creatinine. Moreover, unlike creatinine, cystatin C reflects renal function independent of age, sex, height, and body composition (1). Formulae have been developed allowing estimation of GFR based on cystatin C (1). However, standardization for cystatin C assays is still lacking. Recently, several caveats have been reported:
Cystatin C concentrations appear to be affected by upregulation in certain tumors and drug treatments (e.g., glucocorticoids). In addition, thyroid dysfunction affects cystatin C concentrations, possibly influencing the production rate of the protein.

We were struck by discrepant results between serum creatinine and cystatin C in a 63-year-old female patient (height 1.60 m, weight 49 kg) presenting with biliary obstruction. The patient’s medical history included breast cancer, arterial hypertension, hypercholesterolemia, osteoporosis, and autoimmune hemolytic anemia. During recent treatment with corticoid therapy the patient was observed to have diabetes. At presentation, she was found to have high levels of liver enzyme activity, particularly 5'-glutamyl transferase (596 U/L; reference range, 9–36 U/L) and alkaline phosphatase (403 U/L; reference range, 30–120 U/L). C-reactive protein concentrations were very high (293 mg/L; reference range, <5 mg/L). Serum creatinine was very low (29.2 \( \mu \)mol/L; reference range, 49–85 \( \mu \)mol/L), corresponding to an estimated GFR of >90 mL/min (Modification of Diet in Renal Disease formula) and a creatinine clearance of >120 mL/min (Cockcroft-Gault formula). After 2 months of hospitalization, the patient’s inherent muscle wasting made a creatinine-based GFR estimation less reliable. Serum -trace protein was low (0.393 mg/L; reference range, 0.20–0.72 mg/L), corresponding to an estimated GFR of 101 mL/min (3).

In contrast, serum and EDTA cystatin C, measured using a serum cystatin C assay (Tina-quant cystatin C, Roche), were strongly increased to 2.5 mg/L and 5.3 mg/L, respectively (reference range, 0.63–1.44 mg/L), which corresponded to an estimated GFR of only 30 mL/min (1). Serum electrophoresis of alkaline phosphatase revealed the presence of a macromolecular fraction (4). Further cystatin C analysis using an alternative method [latex particle–enhanced fixed-time immunonephelometry on a BN II analyzer, (Dade Behring)] based on rabbit antihuman cystatin C antibodies (2) revealed a moderately increased serum cystatin C value (1.14 mg/L, reference range, 0.53–0.95 mg/L). Patient serum (30 \( \mu \)L) was injected into a Waters 650E advanced protein purification system (Millipore) and subjected to high-pressure gel permeation chromatography (HPGPC). A 300-mm Protein Pak glass 300SW column (Waters Nihon) was used with a mobile phase of PBS (0.1 mol/L, containing 137 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na2HPO4, 1.47 mmol/L KH2PO4, pH 7.3). The flow rate was 1.0 mL/min. Concentrations of the various cystatin C–containing fractions were confirmed nephelometrically. The chromatogram showed a bimodal distribution: macromolecular cystatin C complexes (36%) were separated from an excess of free cystatin C (47%). The macromolecular cystatin C fraction was characterized by an elution volume very close to the void volume of the HPGPC column. After extraction of the patient’s serum with n-butanol, the macromolecular fraction disappeared on HPGPC. Fig. 1 illustrates the unique molecular mass distribution of the patient’s cystatin C.

The present case demonstrates that when biliary obstruction occurs, serum cystatin C may be present not only as a free 13-kDa molecule but may also be present in a macromolecular form. In biliary obstruction, the presence of triple-layered vesicles (containing plasma membrane–bound enzymes) in serum is a common finding (4). The n-butanol experiment demonstrates that cystatin C is partly associated with these lipid particles, causing an accumulation of cystatin C in serum. In the presence of Ca2+ ions, cystatin C is known to bind brush border membranes (5). This property could explain the observed differences between EDTA and serum samples in

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**Fig. 1.** High-pressure gel permeation chromatography of the patient’s serum (Case) compared with a reference sample (Ref) before and after n-butanol extraction.
the patient we describe. Clinicians should be aware that in these circumstances, estimating GFR using cystatin C–based formulas might be invalid. Methods that do not involve measuring the lipid-associated fractions might underestimate total cystatin C concentrations, but may yield a more reliable GFR measurement.

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References

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