Specificity and Clinical Utility of Methods for the Detection of Macroprolactin

Lucille Kavanagh,1,2 T. Joseph McKenna,1,2 Michael N. Fahie-Wilson,3 James Gibney,1 and Thomas P. Smith1*

Background: Increased serum concentrations of macroprolactin are a relatively common cause of misdiagnosis and mismanagement of hyperprolactinemic patients.

Methods: We studied sera from a cohort of 42 patients whose biochemical hyperprolactinemia was explained entirely by macroprolactin. Using 5 pretreatments, polyethylene glycol (PEG), protein A (PA), protein G (PG), anti-human IgG (anti-hIgG), and ultrafiltration (UF), to deplete macroprolactin from sera before immunoassay, we compared residual prolactin concentrations with monomer concentrations obtained by gel-filtration chromatography (GFC). A monomeric prolactin standard was used to assess recovery and specificity of the pretreatment procedures.

Results: Residual prolactin concentrations in all pre-treated sera differed significantly (P <0.001) from monomeric concentrations obtained after GFC. PEG underestimated (mean, 75%), whereas PA, PG, anti-hIgG, and UF overestimated (means, 178%, 151%, 178%, and 112%, respectively) the amount of monomer present. Of the 5 methods examined, PEG correlated best with GFC (r/H11549 0.80) followed by PG (r /H11549 0.78), PA (r = 0.72), anti-hIgG (r = 0.70), and UF (r = 0.61). After UF or pretreatment with anti-hIgG or PEG, recovery of monomeric prolactin standard was low: 60%, 85%, and 77% respectively. In contrast, pretreatment with PA or PG gave almost quantitative recovery.

Conclusions: None of the methods examined yielded results identical to the GFC method. PEG pretreatment yielded results that correlated best and is recommended as the first-choice alternative to GFC.

Circulating prolactin is heterogeneous in nature. The most common form in healthy persons and in most patients with hyperprolactinemia is a monomeric prolactin (Mr 23 000), but higher molecular mass forms such as big prolactin (Mr 60 000) and big-big prolactin, or macroprolactin (Mr 150 000), sometimes predominate (1–4). Macroprolactinemia, characterized by macroprolactin concentrations that are increased while monomeric prolactin concentrations are within reference values, is rare in the general population but, as reviewed recently (5), may account for up to 26% of all reported cases of hyperprolactinemia. Unlike monomeric prolactin, macroprolactin is considered biologically inactive in vivo (2, 6, 7), but it retains immunoreactivity and is detected to various degrees by all prolactin immunoassays (8–11), commonly leading to misdiagnosis.

Macroprolactinemic patients lack the classic signs and symptoms of the hyperprolactinemia syndrome but may show nonspecific symptoms of hyperprolactinemia, making it difficult to differentiate between the apparently benign clinical condition of macroprolactinemia, in which hyperprolactinemia is entirely explained by the presence of macroprolactin, and true hyperprolactinemia, which requires therapy (5, 12). The frequency of macroprolactin detection by immunoassays, together with the failure to screen hyperprolactinemic sera for macroprolactin, can lead to misdiagnosis and unnecessary medical and surgical intervention (13–17). Thus, screening for macroprolactin is indicated in the routine investigation of all hyperprolactinemic patients (18, 19).

Gel-filtration chromatography (GFC)4, the gold stan-
standard for quantifying bioactive monomeric prolactin in sera, is costly and labor-intensive. Where screening takes place, laboratories generally use polyethylene glycol (PEG) precipitation to differentiate macroprolactinemia from true hyperprolactinemia. This method is simple and inexpensive and has been extensively validated against GFC (4, 20, 21). However, PEG interferes with some immunoassays. Possible alternatives include pretreatment of sera with protein A (PA), protein G (PG), anti-human IgG (anti-hIgG), or ultrafiltration (UF) (22–25). Because manufacturers have been slow to incorporate interference data or protocols in their assay literature (26) and laboratories have been reluctant to modify analytical protocols by introducing serum pretreatment steps that do not have national regulatory body approval, the introduction of procedures to eliminate macroprolactin interference continues to lag behind the clinical and scientific evidence.

We compared the available macroprolactin screening methods and analyzed their relative accuracy, consistency, and cost.

**Patients and Methods**

**STUDY PATIENTS**

We obtained blood from 42 patients whose sera indicated the presence of biochemical hyperprolactinemia before, but not after, the sera were treated with 250 g/L PEG. After PEG treatment, prolactin concentrations in all sera decreased to <403 mIU/L. We identified patient macroprolactinemia on the basis of normalization of prolactin concentrations after PEG treatment (17). The cohort was compiled at random from laboratory records and consisted of 39 females and 3 males. The pattern of clinical features was similar to those described previously (17). Approval for this study was obtained from the Research Ethics Committee, St. Vincent’s University Hospital.

**ASSAY METHODS**

We measured prolactin concentrations with the AutoDELFIA immunoassay (Perkin-Elmer) with an imprecision (as the CV) of 5.2% at 108 mIU/L, 4.0% at 633 mIU/L, and 3.6% at 2177 mIU/L. The ADVIA Centaur prolactin immunoassay (Bayer Diagnostics) was used as indicated, with an imprecision of 3.8% at 103 mIU/L and 6.5% at 2950 mIU/L. The data are given as the mean (SD) and are based on blinded samples unless otherwise indicated. We divided prolactin values given in mIU/L by 36 to convert to μg/L. We measured IgG concentrations in depleted sera with a sensitive in-house ELISA for total IgG with goat anti-hIgG capture antibody and a peroxidase-labeled rabbit anti-hIgG detection antibody (Dako).

**SEPARATION METHODS**

**GFC.** All sera were subjected to GFC over Sephacryl S-200HR (60 × 1.6 cm) in phosphate-buffered saline (PBS; 137 mmol/L sodium chloride, 27 mmol/L potassium chloride, 10 mmol/L phosphate), pH 7.4, with an AKTA protein purification system (Pharmacia Biotech) with quantification of monomeric prolactin as previously described (17). To facilitate more precise quantification of M, 60 000 big prolactin, we chromatographed 4 sera over Sephacryl S-100 (40 × 1.6 cm). Estimates of GFC imprecision (CV) were 6.3% with S-100 (n = 6) and 6.2% with S-200HR (n = 5) at monomeric prolactin concentrations of 606 and 405 mIU/L, respectively.

**PEG treatment.** Serum samples were treated with PEG as outlined previously (17). Briefly, 250 μL of serum, mixed with an equal volume of 250 g/L PEG 8000 (Sigma) in PBS (pH 7.4), was incubated for 10 min at room temperature. The suspension was clarified by centrifugation at 14 000g for 5 min before analysis. We used sera with different concentrations of macroprolactin and monomeric prolactin to evaluate the imprecision of the PEG precipitation procedure. For a sample with a total prolactin of 633 mIU/L and a corresponding prolactin concentration of 242 mIU/L after treatment with PEG, the interassay CV for the PEG-treated sample was 7.9% (n = 39); for a sample with a total prolactin of 2177 mIU/L and a prolactin of 820 mIU/L after treatment with PEG, the CV was 6.9% (n = 33). Solubilized PEG precipitates were resuspended and chromatographed over S-100 to examine the composition.

**Immunoadsorption with PA-Sepharose, PG-Sepharose, or anti-hIgG–agarose.** Before use, PA- and PG-Sepharose 4B and Fc-specific goat anti-hIgG agarose (Sigma) were washed in 10 volumes of PBS. We incubated 150 μL of sera with 200 μL of PA- or PG-Sepharose (IgG-binding capacity, 6 and 5 mg, respectively) or 25 μL of sera with 475 μL of anti-hIgG–agarose (IgG-binding capacity 1.5 mg) with agitation for 90 min at room temperature and then clarified the suspension by centrifugation (14 000g for 5 min) before analysis. Testing confirmed that sufficient immunoadsorbant was added to deplete serum IgG concentrations to <0.02 mg/L.

**Centrifugal UF.** We diluted 25 μL of sera to 500 μL with PBS, and then subjected the diluted sera to centrifugal UF (1000g for 45 min) through a Micron YM-100 ultrafilter (Amicon) before analysis of the ultrafiltrate.

**Costs of separation methods.** The costs of the various separation procedures examined, including reagent and labor costs, were estimated. We assumed that in routine use sera for macroprolactin screening would be batched.

**ANALYTE RECOVERY**

We measured recovery of a purified human pituitary prolactin standard ([WHO 3rd International Standard for Prolactin, code 84/500], National Institute for Biological Standards and Controls (NIBSC), Hertfordshire, UK). The standard was reconstituted in PBS to a concentration of ~1000 mIU/L before being subjected to the various pretreatment procedures.
METHOD IMPRECISION
We assessed the imprecision of the 5 pretreatment methods by subjecting the NIBSC standard to each procedure in quadruplicate on 4 separate occasions. In addition, 12 replicates of 1 macroprolactinemic serum with a total prolactin concentration of 1858 mIU/L and a monomeric concentration of 405 mIU/L were subjected to each procedure on one occasion.

STATISTICAL ANALYSIS
We used the Student unpaired t-test to evaluate statistical significance and linear regression analysis to calculate Spearman correlation coefficients between variables (GB-STAT, Ver. 9; Dynamic Microsystems). SPSS, Ver. 11 (SPSS Inc.), was used to calculate intraclass correlation coefficients.

Results
CHARACTERIZATION OF MACROPROLACTINEMIC SERA BY GFC
Macroprolactin concentrations in the 42 sera subjected to GFC followed by DELFIA immunoassay were 418-5490 mIU/L [mean (SD), 1527 (1180) mIU/L] with monomeric concentrations of 126–628 mIU/L [290 (108) mIU/L]. Of the 42 sera examined by GFC, 38 exhibited almost identical chromatographic elution profiles, with the bulk of the immunoreactive prolactin eluting as a single high-molecular-mass (Mr ~170 000) macro form (Fig. 1a). However, 4 sera (specimens 7, 18, 25, and 39) contained a substantial amount of additional immunoreactive material that eluted between macroprolactin and monomeric prolactin. Rechromatography over Sephacryl S-100 improved resolution (Fig. 1b), allowing more precise quantification of macroprolactin, big prolactin, and monomeric prolactin.

CORRELATION OF PROLACTIN CONCENTRATIONS IN SERA BY IMMUNOASSAY AND GFC
The prolactin concentrations measured in each specimen by the DELFIA and Centaur immunoassays, together with the corresponding monomeric prolactin concentration determined after GFC, are shown in Fig. 2. Total prolactin concentrations in the 42 macroprolactinemic sera measured by DELFIA immunoassay, a so-called high-reacting assay, were 750-5747 mIU/L [1849 (1176) mIU/L]. Analysis of the same sera with the Centaur immunoassay, a low-reacting assay, yielded prolactin concentrations of 197-1459 mIU/L [509 (305) mIU/L]. Reactivity of the Centaur immunoassay toward macroprolactin varied considerably from sample to sample (Fig. 2). In specimens 3, 24, and 36, the Centaur immunoassay detected little or no macroprolactin, and the results agreed with GFC. However, for specimens 18, 29, and 42, more than one half of the macroprolactin present was detected by the Centaur immunoassay. When we used the Centaur immunoassay, we observed clinically significant hyperprolactinemia in 8 sera (19%) with prolactin concentrations of 754–1459 mIU/L.

COMPARISON OF RESIDUAL SERUM PROLACTIN CONCENTRATIONS AFTER VARIOUS PRETREATMENTS WITH THE GFC MONOMERIC PROLACTIN REFERENCE CONCENTRATION
The monomeric prolactin reference concentrations obtained by GFC and the residual prolactin concentrations obtained with the 5 pretreatment methods used for each of the 42 sera are shown in Fig. 3. PEG treatment was generally associated with a lower concentration of prolactin, on average 75% of that obtained for monomeric prolactin after GFC (Table 1). However, individual residual prolactin concentrations varied after PEG: 1 serum exhibited a concentration identical to the GFC target; 6 sera ranged above target (maximum deviation, 1.3-fold), and the remaining 35 sera were below target (minimum deviation, 0.4-fold).

We observed considerable divergence of results from
the GFC target when we examined specific methods to deplete serum IgG; e.g., PA, PG, and anti-hIgG. In almost every case, pretreatment of serum with PA, PG, or anti-hIgG led to higher prolactin concentrations than those measured by GFC (Fig. 3). All 3 reagents only partially removed high–molecular-mass prolactin immunoreactivity from sera, leading to significant overestimations of monomeric prolactin (mean, 178% for PA and anti-hIgG and 151% for PG; Table 1). Specifically, PA removed 39%–102% [mean (SD), 80% (16%)], PG removed 55%–112% [88% (12%)], and anti-hIgG removed 46%–101% [82% (12%)] of the high–molecular-mass prolactin immunoreactivity. After either anti-hIgG or PA treatment, 11 of 42 sera exhibited apparent monomeric prolactin concentrations >2-fold the GFC target, with 2 and 3 sera, respectively, exhibiting concentrations at least 3-fold higher than the target. Residual prolactin concentrations after PG treatment were considerably lower, with 5 of 42 sera exhibiting apparent monomeric prolactin greater than twice the GFC target (Fig. 3). However, despite the general discordance from GFC, there was overall concor-
dance between sera subjected to the 3 IgG-depleting reagents. Agreement was closest when PA- and PG-treated sera were compared, although similar general trends were also seen with anti-hIgG. Residual prolactin concentrations in 26 sera treated with PA were within 10% of those obtained with PG. Residual prolactin concentrations in sera treated with PA compared with those treated with anti-hIgG were within 10% in 17 cases. However, significant divergence in residual prolactin concentrations were seen with certain sera treated with PA, PG, or anti-hIgG (e.g., specimens 4, 13, 16, and 39; Fig. 3).

The prolactin concentrations recorded after UF compared with those after GFC varied considerably from sample to sample, although the mean (112%) was close to the GFC target. For individual prolactin values after UF, 20 were lower with respect to GFC, with a minimum deviation of 0.4-fold, 1 was almost identical, and 21 were higher, with a maximum deviation of 2.4-fold. Residual prolactin concentrations after all pretreatments differed significantly \((P < 0.001)\) from GFC monomeric concentrations (Table 1).
S-100 chromatography.

Peaks precipitate (Fig. 4). Comparison of prolactin forms present in a solubilized PEG concentrations yielded Correlation of residual prolactin concentrations for each macroprolactin pretreatment procedures used to remove concentrations with residual prolactin after correlation of monomeric prolactin that eluted in the 16%, and 36%, respectively) of immunoreactive prolactin and 39; Fig. 3) contained significant amounts (11%, 42%, and 39) of macroprolactinemic sera subjected to a variety of separation procedures.

<table>
<thead>
<tr>
<th>Separation method</th>
<th>Monomeric prolactin, mIU/L</th>
<th>Residual prolactin mIU/L</th>
<th>Correlation coefficient vs GFC</th>
<th>Cost per specimen, US $</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFC</td>
<td>290 (108)</td>
<td>100</td>
<td>275.00</td>
<td></td>
</tr>
<tr>
<td>PEG</td>
<td>218 (90)</td>
<td>75</td>
<td>11.00</td>
<td></td>
</tr>
<tr>
<td>UF</td>
<td>324 (194)</td>
<td>112</td>
<td>16.00</td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>517 (283)</td>
<td>178</td>
<td>26.00</td>
<td></td>
</tr>
<tr>
<td>PG</td>
<td>438 (194)</td>
<td>151</td>
<td>28.00</td>
<td></td>
</tr>
<tr>
<td>Anti-hIgG</td>
<td>516 (190)</td>
<td>178</td>
<td>20.00</td>
<td></td>
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</table>

Correlation of monomeric prolactin concentrations with residual prolactin after pretreatment procedures used to remove macroprolactin

Correlation of residual prolactin concentrations for each of the 5 pretreatment procedures with GFC monomer concentrations yielded r values ≤0.8. The highest correlation coefficients [Spearman (intraclass) correlation = 0.80 (0.77)] were obtained with PEG (GFC monomeric prolactin = 0.937 × PEG prolactin + 86 mIU/L; P < 0.0001). The correlations were lower with PA [0.72 (0.46); GFC monomeric prolactin = 0.264 × PA prolactin + 153 mIU/L; P < 0.0001], PG [0.78 (0.64); GFC monomeric prolactin = 0.418 × PG prolactin + 107 mIU/L; P < 0.0001], and anti-hIgG [0.70 (0.54); GFC monomeric prolactin = 0.357 × anti-hIgG prolactin + 106 mIU/L; P < 0.0001]. The lowest correlation was that obtained after UF [0.61 (0.51); GFC monomeric prolactin = 0.334 × UF prolactin + 182 mIU/L; P < 0.0001].

Recovery of monomeric prolactin and method imprecision

GFC of NIBSC 84/500 prolactin standard confirmed that the material consisted exclusively of monomeric prolactin (M₉ ~23 000). After UF, recovery of standard was low (59.9%) and variable (CV = 16%; Table 2). Pretreatment with PEG yielded a mean recovery of 77% with a CV of 4.9%. In contrast, NIBSC standard pretreated with PA or PG exhibited almost quantitative recovery (95.2% and 100.3%, respectively) together with low variability. Recovery of standard after anti-hIgG pretreatment was somewhat lower (84.8%). Imprecision data for macroprolactinemic sera subjected to each of the pretreatment procedures are illustrated in Table 2. Method imprecision (CV) was acceptably low, 1.7%–2.5%, for PEG, PA, PG, and anti-hIgG. Sera subjected to UF exhibited significantly higher imprecision (8.7%).

Discussion

This study represents the first comprehensive evaluation of the specificity and clinical utility of available procedures to remove macroprolactin from sera before immunnoassay. Of the pretreatments examined, all yielded results that diverged considerably from GFC. Although UF yielded a mean prolactin value closest to that obtained with GFC, the correlation coefficient was the lowest of the 5 procedures examined. Moreover, recovery of monomeric NIBSC standard was low, and the method was associated with considerable imprecision. Our findings are comparable to those reported by Prazeres et al. (25), in which sera containing little or no macroprolactin by GFC exhibited apparent macroprolactin concentrations after UF that differed from −21% to +47%. We cannot recommend UF as a suitably precise or reliable method for screening.

Although several groups have examined the utility of IgG-binding reagents (22, 24, 27), the specificity of these reagents is unclear. In one study (22), GFC reference data were not available for comparison, and in another (24), PA adsorption data were correlated with PEG rather than a reference procedure. More recently, Schiettecatte et al. (23) found that the GFC monomeric prolactin concentration agreed closely (r = 0.998) with the residual prolactin concentration after immunoprecipitation with PG-agarose. In this study we observed considerable divergence of results from GFC with PA, PG, and anti-hIgG. Pretreat-

Fig. 4. Comparison of prolactin forms present in a solubilized PEG precipitate (A) relative to those in untreated serum (B) after Sephacryl S-100 chromatography.

Peaks: A, macroprolactin; B, big prolactin; C, monomeric prolactin.
IgG-depleting reagents such as PA, PG, or anti-hIgG, a viewpoint supported by our findings of gross divergence among specimens limits their usefulness for removal of macroprolactin from serum before assay.

Measurement of residual prolactin after treatment of macroprolactinemic sera with PEG provided the highest correlation coefficient. Although a relatively high correlation between GFC and PEG precipitation has been reported previously, quantitatively the monomeric prolactin concentrations were invariably lower than GFC values (20, 28). PEG has been reported to induce precipitation of a significant amount of monomeric prolactin in normal sera (17). Our current findings confirm that this phenomenon also occurs with macroprolactinemic sera.

Using GFC, we identified significant amounts of big prolactin (M, 60 000) in 4 patients, or 10% of sera examined. Similar to macroprolactin, big prolactin is also reported to lack biological activity in vivo (12, 29, 30). Although the physicochemical nature of big prolactin is unclear, the immunoassay reactivity appears similar to that of macroprolactin. Big prolactin is clearly detected by the DELFIA immunoassay (Fig. 1b), and a recent report from the United Kingdom National External Quality Assessment Schemes (UK NEQAS) has indicated that the Abbott Architect, Bayer Centaur, Beckman Dxi, DPC Immulite 2000, Roche modular 170, and Tosoh AIA all detect big prolactin to the same extent as monomeric prolactin (31). Assessment of bioactive monomeric prolactin concentrations in patient sera with substantial amounts of big prolactin will necessitate depletion of both macroprolactin and big prolactin before immunoassay. This task is likely to be unachievable with specific IgG-depleting reagents such as PA, PG, or anti-hIgG, a viewpoint supported by our findings of gross divergence from the GFC target in the 4 sera containing big prolactin. Although PG treatment of serum yielded a correlation coefficient similar to that for PEG, PG results deviated considerably from the target, particularly when big prolactin was present. In contrast, treatment of sera containing big prolactin with PEG, a less selective reagent, yielded residual prolactin concentration results that corresponded closely to the GFC target, suggesting that PEG precipitated both big prolactin and macroprolactin. Direct examination of a solubilized PEG precipitate by GFC confirmed that PEG precipitated big prolactin (Fig. 4).

In contrast to the DELFIA immunoassay, the Centaur assay is reported to have limited reactivity toward macroprolactin and has traditionally been regarded as a "low-reacting" assay (8). However, in 20% of 42 sera analyzed on the Centaur, we observed apparent clinically significant hyperprolactinemia. Such a degree of misdiagnosis is unacceptable and clearly mandates that laboratories using so-called low-reacting assays implement screening procedures to exclude macroprolactin as a cause of hyperprolactinemia.

The labor-intensive nature of chromatography, together with the requirement to measure prolactin in multiple fractions, renders GFC considerably more expensive than any of the alternative pretreatment options (Table 1). For GFC alternatives, each serum screened requires 2 estimates of the prolactin concentration, the first to ascertain whether the specimen exhibits biochemical hyperprolactinemia and the second to measure the residual prolactin concentration after treatment. The least expensive screening procedure involved PEG pretreatment at US $11.00 per sample. Gibney et al. (32) reported that when a high-reacting assay was used, the introduction of routine macroprolactin screening led to a 30% increase in the cost of prolactin analysis. However, reduced use of computed tomography and magnetic resonance imaging, together with decreased dopamine agonist prescription, yielded a net cost saving for the institution, offsetting the additional cost associated with the introduction of screening.

Routine screening of hyperprolactinemic sera for macroprolactin has been authoritatively recommended (18, 19). On the basis of the findings in this study, the method of choice for the simultaneous removal of both macroprolactin and big prolactin from hyperprolactinemic sera is pretreatment with PEG. Although this method provides the best correlation with GFC, achieves acceptable precision, and is the least expensive method, the results obtained underestimate monomeric prolactin concentrations by ~25%. The divergence in residual prolactin

Table 2. Precision and recovery data for NIBSC prolactin standard (WHO 3rd IS 84/500) and precision data for one macroprolactinemic serum subjected to the 5 pretreatment methods.

<table>
<thead>
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<th>Prolactin recovered after pretreatment with</th>
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<tr>
<td></td>
<td>PEG</td>
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<tr>
<td>NIBSC prolactin standard</td>
<td>Mean, a mIU/L</td>
</tr>
<tr>
<td>(WHO 3rd IS 84/500)</td>
<td>CV, %</td>
</tr>
<tr>
<td>Macroprolactinemic serum</td>
<td>CV, a %</td>
</tr>
</tbody>
</table>

a Mean recoveries represent the average of quadruplicate determinations carried out on 4 separate occasions.

b CVs are based on 12 replicates carried out in 1 assay.
concentration relative to target make it preferable to use a PEG-specific reference interval based on the residual prolactin concentration after identical treatment of normal sera as recommended previously (17).

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References