Protein Method Influences on Calculation of Tissue Receptor Concentration

PETER J. HOWANITZ, M.D., JOAN H. HOWANITZ, M.D., CAROL A. SKRODZKI, B.S., AND THOMAS WOLOSZYN, B.S.

Many protein methods are used for estimation of tissue receptor concentration. The authors compared performance, analytic variability, and accuracy of six protein methods used in these calculations. They found the Lowry protein procedure standardized with bovine serum albumin (BSA), usually considered the reference method, to be the most imprecise and most time consuming method. When the BSA standards from the Lowry procedure were assayed with the other methods, results ranged from 74 to 141% of expected. For three other protein standards, reactivity among the six methods varied almost twofold. Comparison of Lowry protein concentrations in cytosols from 46 tumors biopsies with other methods indicated best agreement was with an automated turbidometric (TCA) or a Coomassie dyebinding procedure. Use of protein standardization for the two direct spectrophotometric procedures decreased overestimation of receptor protein concentrations. Because receptor concentration is the quotient of receptor quantity and protein concentrations, tissue receptor results are dependent in part on standardization and choice of protein method. (Key words: Receptor assays; Estrogen receptors; Progesterone receptors; Breast cancer; Protein methods; Accuracy; Precision; Reproductibility) Am J Clin Pathol 1986; 85:37-42

QUANTITATION OF CYTOPLASMIC ESTROGEN and progesterone receptors in human breast cancer biopsy specimens is useful in predicting a patient's response to endocrine therapy. In unselected patients with metastatic breast cancer, 30% show objective remission with endocrine therapy, in contrast to patients whose tumors are positive for estrogen receptors who show a 55-60% objective response to endocrine therapy. In general, patients with higher cytoplasmic estrogen receptor levels have a greater chance of response to endocrine therapy. Measurement of progesterone receptor concentration further increases the accuracy of this prediction. Estrogen, progesterone, and other receptor determinations may prove useful in managing patients with a variety of other disorders.

Materials and Methods

Specimens and Reagents

Excess human tumor tissue, free of fat, from more than 50 biopsies on which estrogen and progesterone receptor assays had been performed were pooled and stored at −70 °C until used. Frozen tissue, pulverized with a freezer mill (Spex Industries Inc., Metuchen, NJ), was homogenized at 4 °C with three volumes of TED buffer (TRIS, 10 mmol/L; EDTA, 1.5 mmol/L; dithiothreitol, 1.0 mmol/L), pH 7.4, with five intermittent 10-second bursts at 30-second intervals using a Sorvall Omni-mixer® (Ivan Sorvall Inc., Newtown, CT). The homogenates were centrifuged (1 hour, 10,000 × g) in an ultracentrifuge (Model L5-65®, Beckman Instruments, Palo Alto, CA) and the of cytosol. Binding results usually are analyzed using Scatchard plots to estimate affinity and quantity of receptor sites. The concentration of receptor sites in a tissue specimen is expressed in terms of tissue weight, milligram of DNA content, or milligram of protein. Most commonly, the number of sites is expressed in terms of milligram of cytosol protein. Many methods have been used to determine cytosol protein values. These methods vary not only in the underlying principle of measurement but also in the technic of method standardization. Cytoplasmic estrogen and progesterone receptor concentrations usually are considered positive when there are greater than 3 and 15 fmol of steroid binding per milligram of protein, respectively. Because receptor concentrations are calculated from the quotient of receptor site number and protein concentration, reproducibility is dependent on analytic variability and accuracy of these measurements. In this study, we show that systematic differences and varying imprecision between six protein methods may influence receptor quantitation.

Materials and Methods

Specimens and Reagents

Excess human tumor tissue, free of fat, from more than 50 biopsies on which estrogen and progesterone receptor assays had been performed were pooled and stored at −70 °C until used. Frozen tissue, pulverized with a freezer mill (Spex Industries Inc., Metuchen, NJ), was homogenized at 4 °C with three volumes of TED buffer (TRIS, 10 mmol/L; EDTA, 1.5 mmol/L; dithiothreitol, 1.0 mmol/L), pH 7.4, with five intermittent 10-second bursts at 30-second intervals using a Sorvall Omni-mixer® (Ivan Sorvall Inc., Newtown, CT). The homogenates were centrifuged (1 hour, 10,000 × g) in an ultracentrifuge (Model L5-65®, Beckman Instruments, Palo Alto, CA) and the
quantitation of the commercially available standards. All protein measurements were made in a 1-mL cuvette with a dual beam spectrophotometer (Model 25®, Beckman Instruments, Palo Alto, CA) calibrated weekly for wavelength accuracy, linearity, band pass verification, and stray light with an SS-10 SpectroStandard® kit (Chemetrics Corporation, Burlingame, CA).

The turbidimetric (TCA) procedure used a DuPont aca® (DuPont, Inc., Wilmington, DE), DuPont® CSF reagent packs, and lyophilized human serum (VERSATOL®, Automated Hi®, Automated Lo®, and Automated Lock-in®, General Diagnostics) for calibration. All specimens were diluted in TED buffer. Cytosol pools made from human breast tumors and pregnant rabbit uterus (Pel-Freez Biologicals, Rodgers, AK 72756) were concentrated 80-fold (Minicon®, Amicon, Lexington, MA) and subjected to electrophoresis (Panagel®, Worthington Diagnostics, Freehold, NJ), and protein patterns were compared with control sera by visual inspection.

Results

Salient features of the compared methods are listed in Table 1. The Lowry method required considerable more time to perform than other methods because of two incubation steps. Interassay precision for the cytosol pool and a representative serum control is shown in Table 2. In each pool, the Lowry procedure gave the most imprecise results, and mean values that were consistently higher than methods using dye binding, turbidity (TCA), or differences in A at 215 and 225 nm.

The BSA standards from the Lowry procedure were made in TED buffer except for procedures using differential absorbance (A) at 215 and 225 nm (215/225) and 230 and 260 nm (230/260), for which dilutions were made in water. The Kjeldahl method was used for protein determination.

Table 2. Interassay Precision

<table>
<thead>
<tr>
<th>Protein Method</th>
<th>Cytosol Pool</th>
<th>Control*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method</td>
<td>Mean (g/L)</td>
<td>CV</td>
</tr>
<tr>
<td>Lowry</td>
<td>6.3</td>
<td>6.6</td>
</tr>
<tr>
<td>TCA</td>
<td>5.9</td>
<td>4.4</td>
</tr>
<tr>
<td>Dye binding</td>
<td>5.6</td>
<td>6.6</td>
</tr>
<tr>
<td>215/225 (Waddell)</td>
<td>6.0</td>
<td>4.8</td>
</tr>
<tr>
<td>230/260</td>
<td>6.4</td>
<td>4.5</td>
</tr>
<tr>
<td>280/260</td>
<td>6.3</td>
<td>3.9</td>
</tr>
</tbody>
</table>

* Precision data on Automated Lo® human serum control. Automated Hi® and Automated Lock-in® gave similar results.
human serum. Electrophoretic patterns of cytosols approximated human serum. The measured to the assigned protein concentrations for the three reference standards—
(1) bovine serum albumin (National Bureau of Standards SRM); (2) human albumin: globulin (70:30 by weight); and (3) human serum estimated by six methods—is seen in Figure 3. The BSA standardized Lowry procedure overestimated, whereas the 215/225 procedure underestimated protein concentrations in all three standards. The TCA procedure overestimated National Bureau of Standards SRM bovine serum albumin by 55%; with increasing proportions of globulin in standards, decreasing TCA reactivity occurred such that with human sera a 9% underestimation occurred. In contrast, the 280/260 method gave the least reactivity (70%) with the NBS BSA and the greatest reactivity (130%) with the human albumin: globulin standard.

Comparison of protein concentrations measured by six methods using 46 tumor cytosols is seen in Figures 4A–E. In the range tested, only the spectrophotometric procedures at 280/260 and 230/260 consistently gave values higher than the Lowry. Methods based on turbidity (TCA), dye binding, or A at 215/225 nm all overestimated receptor protein concentrations at low levels and underestimated them at higher levels when compared with the Lowry method.

Discussion

Many assay methods have been developed for receptor determinations. However, at least for the estrogen receptor, the dextran-coated charcoal assay has become the "reference" method against which all other methods are compared. With this assay, receptor values of less than 3 fmol/mg cytosol protein are equivalent to zero and are considered a negative assay. Accurate receptor determinations are dependent not only on the random and fixed errors of the receptor assay procedure but also upon the choice, standardization, and analytic variability of the protein method. The protein concentrations used in this computation are estimated by a variety of methods, including the method of Lowry; dye binding of Coomassie brilliant blue; turbidity following trichloroacetic acid (TCA) precipitation; absorbance of ultraviolet light comparing wavelengths of 280 and 260 nm; 215 and 225 nm (Waddell); or 230 and 260 nm; calculation from cytosol protein and albumin measurements; and a biuret method. The Lowry, dye-binding, and the direct spectrophotometric procedure using A at 215/225 apparently are employed most commonly. Our data indicate that, of the protein methods compared, the Lowry, which usually is considered the reference method, was the most imprecise and most time consuming.

A few interlaboratory studies of limited nature have indicated that, not only is there wide variability of receptor results among laboratories, but even false positive and false negative results occur. These interlaboratory
discrepancies may relate, in part, to the protein method used. When receptor numbers were expressed in terms of protein concentration, three was increased variability in data from a small interlaboratory quality control program. In contrast, a pilot investigation conducted by the College of American Pathologists suggested that expressing receptor numbers in terms of protein concentration does not alter variability of results significantly. Our precision data indicate that protein concentration (and hence receptor concentrations) in a cytosol pool differ by approximately 10% when six different protein methods standardized in a variety of ways were used. However, when standards such as used for the Lowry protein method were measured by six methods, values differed up to almost 100%, depending on the method.

Protein methods are plagued by lack of an appropriate and reliable standard. There are differences between endorsements by scientific societies for a primary standard for total serum protein measurements, even though the choice may result in patient values that differ by 5.0 g/L. This choice is dependent on chemical similarity and reactivity of standards compared with specimens assayed as well as exigencies of price and supply. In specimens other than serum, standards are even less satisfactory, not only because of above considerations, but also because of methodologic problems leading to inaccuracy and imprecision. In addition, intraindividual variation and alterations in protein levels occurring with disease processes are not completely studied for most types of nonserum specimens.

Although serum proteins are heterogenous, the method of standardization usually involves choice of a single protein as a primary or secondary standard. Bovine serum albumin has been recommended as a primary standard for serum protein assays; this Standard Reference Material calibrated by the Kjeldahl method is available from the National Bureau of Standards (NBS). Lyophilized secondary standards, many of which are not BSA, also are available. Traditionally, some nonserum protein methods express results in relation to standard curves derived from various protein standards, while others express results in relation to a variety of protein calibrations and others use mathematic formulas relating differential absorbance. A survey of 15 laboratories performing receptor measurements revealed five different ways in which assays were standardized.

At least for body fluids other than serum, the choice of a standard is influenced by the relative amounts of protein present. The choice of standards for receptor protein measurements also is based on a similar consideration; however, these methods do not lend themselves easily to standardization because there is no material available that approximates tumor proteins. As seen in Figure 2, both uterus and tumor cytosols mainly contain proteins with electrophoretic properties of serum proteins. Hence, it seems reasonable to recommend use of the serum protein standard, bovine serum albumin, as the standard for receptor protein measurements.

If the compared methods were standardized with the NBS protein standard in which we found measured values of 70–155% of assigned values, depending on the method, the 280/260 procedure would overestimate and the TCA procedure underestimate receptor protein concentration by 143 and 64%, respectively. This would result in receptor concentrations of 70% and 155%, respectively, of those measured using the Lowry protein method.

Similarly, if human albumin-globulin was used, the standard's concentration was overestimated by 131 and 116% for these same two methods, resulting in lower cytosol protein values and high receptor concentrations.
FIG. 4. Comparison between results by the method of Lowry and other protein methods: (A, upper, left) TCA; (B, upper, right) dye binding; (C, center, left) Waddell; (D, center, right) 230/260; (E, lower, left) 280/260.
Hence, it appears differences in assay results are not minimized with the use of a single standard. Even for the same standards, observed protein values are method dependent. For example, BSA and albumin:globulin standards gave 155% and 117% of assigned values, respectively, with the TCA method, while with the 215/225 method the same standards gave results of 80% and 93% of assigned values. Thus differences between protein results can be minimized or maximized by choice of methods as well as standards.

When Lowry protein concentrations in 46 tumor cytosols were compared with those measured by the five compared methods, proportional differences in concentration were seen. When calibrated in the manner reported here, protein concentrations measured by dye-binding and TCA procedures approximated Lowry values at about 3.4 g/L, whereas 215/225 values approximated Lowry values at 5.0 g/L. At lower or high values than these, overestimation or underestimation occurred, respectively. When the 280/260 and 230/260 methods were compared with the Lowry, overestimation of receptor concentrations occurred for all cytosols. Some of these differences might be minimized by alternative standardization procedures. For example, standardization of the 230/260 and 280/260 procedures with human serum would diminish overestimation of cytosol protein concentrations for all cytosols.

Although no one method minimized inaccuracy and imprecision and was technically easy to perform, the TCA, standardized with human serum, and Waddell (215/225) methods best fulfilled these criteria. Implementation of one of these protein methods for receptor quantification is dependent on laboratory equipment availability. Based on our data, we conclude that cytosol protein, and hence receptor concentrations, are dependent on choice of protein method and standardization. Choice of a single standard for all six receptor protein measurements increases intermethod inaccuracies. By standardizing with human serum instead of wavelength calibration for the 230/260 and 280/260 methods, protein measurements better approximate those made by TCA, dye-binding, and 215/225 methods.

References