Inaccuracy in Measuring Glycated Albumin Concentration by Thiobarbituric Acid Colorimetry and by Boronate Chromatography

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We compared thiobarbituric acid colorimetry and boronate chromatography for measuring glycation of serum proteins. With \(^{14}C\)-glycated human albumin as a test material, both methods were acceptably linear and precise. However, comparable estimates (mmol/L) of albumin glycation ranged from 0.22 for thiobarbituric acid to 0.05 for boronate, representing yields relative to \(^{14}C\)glycosyprotein of 42% and 10%, respectively. The low yield with thiobarbituric acid was corroborated independently on the basis of kinetic differences between the reactions of fructose standards and of glycosylprotein, leading to underestimation of glycosylprotein concentration. The lower estimate of glycosylprotein by boronate chromatography was related to an apparent requirement for two \(^{14}C\)glyco groups per albumin molecule to effect binding.

There currently are many methods for estimating glycation of proteins and protein fragments for use as indices of metabolic control in diabetes (7) and of diabetic complications (2). Each method is standardized in its own way and yields results expressed in its own units. The relationship between values derived from these methods has never been clarified, nor indeed has the basis for certain of them been subjected to scrutiny. In particular, evaluation of glycation of serum proteins by use of thiobarbituric acid (TBA) or boronate chromatography lacks a rigorous experimental basis.

In this paper, we have investigated five different procedures based on TBA assay and boronate chromatography by analyzing purified albumin glycated in vitro to varying extents. The procedures chosen were taken from published papers and were intended to mimic those that are used or could be considered for use in the clinical laboratory. We elected to assay a purified protein rather than serum, to minimize nonspecific effects. We used albumin because it is the major contributor to serum protein glycation as judged by a variety of techniques (3–6) and we used material glycated in vitro with \(^{14}C\)glucose to provide an additional quantitative basis for comparison.

Materials and Methods

Materials

Human albumin was from Commonwealth Serum Laboratories, Melbourne, Australia; d-[U-\(^{14}C\)]glucose was from Amersham International, Amersham, Bucks, U.K.; Sephadex from Pharmacia, Uppsala, Sweden; Glygol (immobilized-m-aminophenylboronate) columns and elution buffers were from Pierce Chemical Co., Rockford, IL. All other reagents were of the highest grade available.

Methods

Preparation of albumin. To minimize reactive contaminants (7), \(^{14}C\)glucose preparations were purified by two-dimensional thin-layer chromatography (8). \(^{14}C\)Glucose of more than 98% purity by enzymic analysis was mixed with unlabeled glucose (50 mmol/L; 20–40 mCi/mol final concentration) and incubated with human albumin (40 g/L) for seven days, exactly as described before (8). Triplicate 10-\(\mu\)L aliquots of incubation material were counted in toluene-based scintillation fluid to define the relationship between \(^{14}C\) and glucose. The bulk material was concentrated (in a "Minicon"; Amicon Corp., Danvers, MA 01923) and applied to a 400 \(\times\) 26 mm column of Sephadex G-25 that was both equilibrated with and eluted with 0.15 mol/L NaCl solution. The major albumin-containing fractions were pooled, lyophilized, and dialyzed, then diluted to 42 g/L as determined by absorbance at 279 nm (9).

To assess incorporation of \(^{14}C\), we precipitated 100-\(\mu\)L aliquots, in triplicate, with trichloroacetic acid (0.5 mol/L) and counted radioactivity as described previously (8). Glycated material prepared this way is referred to here as "HI." Albumin not incubated with glucose was similarly chromatographed and reconstituted to give a final concentration of 42 g/L. This material is referred to as "LO." Albumin solutions were stored at \(-20^\circ\)C until use.

Before analysis, albumin was thawed and mixed thoroughly. To provide a linearly related series for analysis, equal volumes of HI and LO were mixed to give a specimen intermediate in value, termed "MD."

Thiobarbituric acid method. The method of Kennedy and Merime (10) as modified by Elder and Kennedy (11) was used. Results were expressed as fructose equivalents (12).

Boronate chromatography. (a) Protein. The procedure, based on that of Gould et al. (13), was performed with about 8 mg of protein applied to commercial boronate columns according to the manufacturer's instructions (Pierce Chemical Co.). Protein was assayed by measuring the absorbance of the effluent at 280 nm. The technique was also repeated with about 2 mg per column and protein was assayed with Coomassie Blue (13, 14). Mean analytical recovery of protein from these columns was 104%.

(b) Amino acids. Protein was analyzed for glycated amino acids by the methods of Vlassara et al. (15) and Yue et al. (16). Protein (4 mg) was treated with 0.1 mg of NaBH\(_4\) in phosphate buffer (50 mmol/L, pH 7.0), dialyzed against isotonic saline, and lyophilized. This material was hydrolyzed with HCl (6 mol/L) at 110°C for 20 h and dried under reduced pressure. The residue was taken up in EWB buffer (Pierce Chemical Co.) and the pH adjusted to about 9.0 with
NaOH. A portion was taken for amino acid quantitation by using Pluram (Roche) reagent with glycine as standard. The remainder was applied to commercial boronate columns (Pierce Chemical Co.) and eluted successively with EWB buffer as one fraction and HCl (25 mmol/L) as a second. Both fractions were mixed with toluene–Triton X-100 scintillation fluid and the \(^{14}\text{C}\) radioactivity was counted. That eluted with HCl was considered to represent glycated material.

Results and Discussion

Table 1 summarizes the results of analyzing the albumin solutions for glycated protein. Values are in traditional units for each of the assays. Boronate chromatography was tested with different amounts of protein applied to the columns; the lower values found with the higher amount of protein are consistent with earlier reports suggesting overloading of the columns with more than 2 to 3 mg of protein (13, 17).

The imprecision of each of the procedures was low except for boronate chromatography of LO and MID specimens. It should be noted, however, that the LO values for this technique were very low, so that even small errors had a substantial effect. Nonlinearity was acceptably small throughout (Table 1).

To make clear the relationship between the quantities assayed, we expressed each in millimoles (of fructose equivalents) per liter of albumin solution (Table 2). Values from the LO specimens ranged from \(<0.01\) to 0.09 mmol/L, the highest value possibly reflecting lack of specificity of the TBA assay in the absence of a suitable blank.

Comparison of the increments obtained upon incubation with glucose (Table 2) might be expected to minimize any contribution from nonspecific interfering. However, when \(^{14}\text{C}\) was included, the range of values from 0.05 to 0.52 mmol/L was almost as great as that seen for the LO specimens. To gain insight into possible reasons for this discrepancy, we further investigated the assays as follows.

TBA assay is nonstochiometric, mainly because of destruction of hydroxymethylfurfural at elevated temperature (11, 12). The apparent yield of hydroxymethylfurfural was increased about threefold when fructose standards rather than molar absorptivity were used (not shown). However, the experimental results shown in Figure 1 demonstrate that, as with hemoglobin (11), an underestimation of protein specimens remains because of the different kinetics exhibited by standards and specimens. Taking into account the decay of hydroxymethylfurfural (Figure 1), it seems that the true values for the samples should be at least 60% higher than those observed, increasing the yield relative to \(^{14}\text{C}\) (Table 2) at least to 70%.

Boronate chromatography should not be expected to give values strictly related to glyco groups, because it is their proportion in protein that is measured. However, the proportions of glycated albumin that we obtained here, which are representative of those found in routine use (5, 13, 17), lead to surprisingly low estimates of glyco-group concentration in comparison with other indices (Table 2).

To analyze this result further, we examined the fate of \(^{14}\text{C}\) with this technique. We found with the lower amount of protein that the 11.5% of albumin regarded as glycated (Table 1) contained only 31% of the \(^{14}\text{C}\), so that the major portion of \(^{14}\text{C}\) was eluted with the non-glycated fraction. The glycated fraction contained 2.2 mol of \(^{14}\text{C}\)glucose per mole of protein, whereas the non-glycated fraction contained only 0.6 mol/mol. Assuming that \(^{14}\text{C}\)glucose acts chemically in identical fashion to non-radio labeled glucose, this suggests a requirement for at least two glyco groups per mole for binding of albumin to occur. We do not know whether this represents steric hindrance of glyco-group binding to boronate or inadequate affinity for boronate of only a single protein glyco group. Conceivably, these observations relate solely to albumin glycated in vitro, and glycated albumin in patients' specimens may behave quite differently. However, such an outcome is highly improbable because the structure of albumin glycated in vitro (18) is strikingly similar if not identical to that glycated in vivo (19, 20). Moreover, values for patients' specimens assayed by TBA and boronate are similarly discrepant (compare refs. 5, 13, 17 with 10).

Although steric hindrance might preclude binding of certain glyco groups in protein, it seems unlikely that such a phenomenon could explain why 84% of \(^{14}\text{C}\)labeled amino acids escaped binding by boronate in this study. If, on the other hand, there is inadequate affinity for boronate of a

<table>
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<th>Table 1. Comparison of Five Procedures for Measuring Glycoalbumin Concentration*</th>
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<tr>
<td><strong>Units</strong></td>
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<tr>
<td><strong>LO</strong></td>
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<tr>
<td>TBA (i) fructose, no blank</td>
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<tr>
<td>(ii) fructose, blanked</td>
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<tr>
<td>Boronate (i) 8 mg protein</td>
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<tr>
<td>(ii) 2 mg protein</td>
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<td>(iii) amino acids</td>
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* Each specimen was analyzed at least six times by each of the procedures listed. * Nonlinearity was calculated from (2 MID[LO + HI] – 1) × 100%. * Values derived from fructose standards. * Borohydride treatment was used to obtain a blank value (11).
single glyco group per mole, it is tempting to speculate that the species bound is derived from a difructose-amino acid (21).

\[ ^{14}C \text{ incorporation} \] data can be misleading because of reactive contaminants in \[^{14}C\]glucose preparations (7). Despite our purifying the labeled material (see Materials and Methods), the possibility of contamination remains, with the result that our estimates based on \(^{14}C\) may overstate the concentration of glycosylprotein.

In principle, each of the assays examined here has the potential to act as some measure of serum glycosylprotein concentration. However, with albumin preparations designed to mimic the physiological and pathophysiological ranges of glycation, both the TBA and boronate chromatography procedures underestimated glycated albumin concentration. Independently of the \(^{14}C\) data, poor recovery in the TBA method was observed directly (Figure 1), and analytical recovery from boronate chromatography was even lower (Table 2). Underestimation by TBA can probably be expected whatever the protein species (cf. ref. 12). However, this cannot be said of boronate chromatography, because comparable values for glycated hemoglobin (boronate) and hemoglobin \(A_1c\) (cation-exchange) have been reported (22). Bearing in mind our finding here of an apparent requirement for binding to boronate of two glyco groups per mole of protein, it may be significant that formation of hemoglobin \(A_1c\) requires glycation of the N-terminal valine residue of both beta chains (23).

These observations with boronate chromatography may have clinical significance when the object is to detect subtle changes of glycated protein concentration at lower ranges of concentrations, such as those seen in gestational diabetes. Moreover, they may explain why discrepant results are seen in comparisons of methods.

References


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Modified TDx® Assay for Cyclosporine and Metabolites, for Use with Whole-Blood Samples

Wolfgang Vogt and Irene Weisch

A recently introduced fluorescence polarization immunoassay (FPIA) for determination of cyclosporine A in serum and plasma is discussed with regard to its use for whole-blood samples, with and without hemolysis before the assay. The performance characteristics of the modified FPIA method are highly satisfactory (within-run CVs 2.06 to 5.50% and 1.99 to 3.39%, respectively; long-term between-run CVs under routine conditions 5.73 to 8.95%). The limit of detection is 30 μg/L. Results agree well with those obtained with the RIAs compared, but the modified FPIA is more convenient and faster.

Additional Keyphrases: fluorescence polarization immunoassay • transplantation • immunosuppression • therapeutic drug monitoring

Cyclosporine A (CyA) is widely used as an immunosuppressant to combat tissue rejection after organ transplants. Its clinical usefulness in the treatment of patients with autoimmune diseases is also under investigation. Given the poor dose–response relationship and the considerable toxicity of the agent, therapeutic drug monitoring of CyA concentrations is indispensable. Radioimmunoassays (RIA) and high-performance liquid chromatography (HPLC) methods have to date been the only commercially available techniques for this purpose. These methods are cumbersome and time consuming, though the 125I assay is comparatively easy to perform. A fluorescence polarization immunoassay (FPIA) has recently been described (1) and now has also become available commercially. However, this method is designed for use on plasma specimens only.

Given the clearly temperature-related distribution of CyA among the various blood components, whole blood is obviously the preferred material for analysis (2, 3). We therefore sought to adapt this new FPIA to allow tests on whole-blood samples. For comparison we used a tritiated RIA and an 125I-labeled RIA. Because we receive both unprocessed whole-blood samples and deep-frozen hemolysates for testing, we studied the usefulness of the modified FPIA for both these materials.

Materials and Methods

Instrumentation

The FPIA was performed in a TDx Analyzer (Abbott, Irving, TX). Radioactivity was measured with a 1261 Multigamma γ-scintillation counter and a 1219 Betarack β-scintillation counter (KLB-Wallac, Turku, Finland). Dilutions were made with a Microlab 1000 dilutor (Hamilton, Bonaduz, Switzerland).

To prepare hemolysates, we subjected blood cells to −20 °C for at least 1 h or used a Soniprep 150 ultrasonic disintegrator (MSE Scientific Instruments, Crawley, Sussex, U.K.), three 20-s bursts.

Reagents

We used test kits from Sandoz Ltd., Basle, Switzerland (Cyclosporin RIA-Kit); INC-DRG-Instruments, Marburg, F.R.G. (CYCLO-TRAC); and Abbott Laboratories, Chicago, IL (TDx Cyclosporine and Metabolites Assay).

Specimens

Blood anticoagulated with EDTA (1 mg of K3EDTA per milliliter of whole blood) was used for all assays. All patients were being treated with CyA after heart transplants. EDTA-treated blood from healthy blood donors was used to prepare the calibration standards and to dilute samples with high CyA concentrations for checking linearity.