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Adapting Homogeneous Enzyme-Linked Competitive Binding Assays to Microtiter Plates[1]

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Recently devised homogeneous enzyme-linked binding assays useful for the rapid detection of carbohydrate structure/content of intact glycoproteins (via use of lectins as binders) and for quantitating given vitamins (e.g., biotin; using soluble binding proteins) are adapted successfully to a microtiter plate reader format. The problem of nonspecific adsorption of the binders and enzyme–saccharide/vitamin conjugates is solved via the addition of Tween 20 to the assay buffer. More convenient and reliable photometric detection of the preferred labeling enzyme, glucose-6-phosphate dehydrogenase (G6PDH), is accomplished by monitoring the rate of generation of reduced thio-NAD (from thio-NAD) at 405 nm instead of NADH (from NAD) at 340 nm. By employing these modifications it is shown that homogeneous enzyme-linked binding assays can be readily adapted to microtiter plates without loss in analytical assay performance. Results further suggest that other homogeneous assays based on G6PDH, including commercial EMIT assays used routinely in clinical chemistry laboratories for detecting drugs of abuse, could, in principle, be run on microtiter plates to significantly enhance sample throughput.

Microtiter plates and associated photometric plate readers have become important tools for the detection of a large number of species in high sample throughput clinical and research laboratories. While, in principle, microtiter plate systems are useful for monitoring the extent of almost any type of solution reaction (1–3), in practice, they are most often employed to perform various solid-phase binding assays in which appropriate biological species (e.g., antibodies, antigens, receptors) are adsorbed/immobilized onto the inner walls of the plastic microtiter plate wells (4,5). These immobilized species can then be used in either competitive or noncompetitive assays with fluorescent, or more commonly, enzyme-labeled molecules (6–8). The widely used enzyme-linked immunosorbant assays (ELISA)[4] generally involve a series of washing steps before determining the amount of bound enzyme label via addition of suitable substrate(s). To date, more rapid single-phase homogeneous enzyme-linked binding assays (ELBAs) (including homogeneous enzyme immunoassays such as the EMIT method (9–11) pioneered by workers at Syva Corp.) have not been adapted to microtiter plate assay arrangements, probably because of problems associated with nonspecific adsorption of the binder and enzyme-labeled reagents to the walls of the plates. In fact, unlike standard ELISAs, in which one reagent is already bound to the walls of the plates, maximum signal modulation in homogeneous ELBAs is highly dependent on both the binder and the enzyme conjugate being completely free in the solution phase. Beyond potential nonspecific adsorption problems, there is also difficulty in reliably assaying certain labeling enzymes with many existing microtiter plate readers. Indeed, dehydrogenase type enzymes often used as labels in homogeneous ELBAs require photometric detection at 340 nm, a wavelength at which many plate readers are not able to monitor absorbances reliably due to lack of the proper filter and/or rather unstable readings owing to back-

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[4] Abbreviations used: ELISA, enzyme-linked immunosorbant assay; ELBA, enzyme-linked binding assay; MDH, malate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; thio-NADH, reduced thionicotinamide adenine dinucleotide; G6PDH, glucose-6-phosphate dehydrogenase; G6P, glucose 6-phosphate; Con A, Conconavalin A; BSA, bovine serum albumin; NHS-activated biotin, *N*-hydroxysuccinimide-activated biotin; rsd, relative standard deviation.

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ground absorbance of the plate material in this region. In this paper, we describe how we have successfully adapted a newly developed lectin-based (12,13) and other binding protein-based homogeneous enzyme-linked competitive binding assays (for vitamins) to a microtiter plate format without loss in assay performance.

The lectin-based homogeneous ELBA developed previously in this laboratory relies on the ability of given lectins to inhibit the catalytic activity of appropriate malate dehydrogenase (MDH)–saccharide conjugates (13). In the presence of glycoproteins or other carbohydrates possessing structures recognized by the given lectin, enzyme activity increases in an amount proportional to the amount of carbohydrate present in the assay mixture (12,13). By using an array of different lectins in conjunction with different MDH–saccharide conjugates (e.g., MDH–mannose, MDH–galactose, etc.), it is possible to rapidly probe the type and relative amount of carbohydrate within a given glycoprotein (13). It is envisioned that this simple homogeneous ELBA method may eventually be useful for assessing relative changes in the glycosylation of intact proteins, particularly those produced by modern recombinant methods.

Similarly, we have also shown previously that it is possible to devise very rapid homogeneous ELBAs to detect various vitamins via the use of suitable vitamin binding proteins and enzyme–vitamin conjugates (14–16). The vitamin assays are potentially useful for quality control of tablet formulations, infant formula, foods, etc. However, in all of this earlier work, each data point for the respective competitive binding dose–response curves (for vitamins and glycoproteins) was acquired one point at a time using a single beam spectrophotometer to monitor MDH enzyme activity. Because of throughput considerations, it would be highly desirable to adapt these and other assay schemes to a microtiter plate arrangement.

Our initial attempts to use existing MDH-based conjugates in a microtiter plate format were unsuccessful owing to large and unstable absorbance values of the reduced nicotinamide adenine dinucleotide (NADH) substrate at 340 nm within our Dynatech microtiter plate reader. Since MDH catalyzes the oxidation of NADH to NAD in the presence of oxalacetate, with appropriate initial concentrations of NADH substrate (i.e., 1.6×10^{-4} M), it appeared that the amount of light reaching the detector was too low, causing large fluctuations in the measured absorbance values and subsequent rates of the MDH reaction. As described below, this problem can be solved by employing newly synthesized G6PDH–saccharide (or vitamin) conjugates. When G6PDH is used as the labeling enzyme, either NAD or thio-NAD can be used as the cosubstrate along with glucose 6-phosphate (G6P). The thio-derivative of NAD differs from NAD by a single substitution of sulfur for oxygen on the nicotinamide ring. This substitution shifts the absorption spectrum of the reduced species so that the production of thio-NADH can be monitored at 405 nm (17). Although the production of nonderivatized NADH can also be monitored at 340 nm, thio-NAD was used since many commercially available microplate readers are not equipped with a 340-nm filter. Indeed, it is demonstrated here that G6PDH-based homogeneous competitive binding assays for carbohydrates and the vitamin biotin can be adapted to a microplate format. It is also shown that optimum performance of the homogeneous microtiter plate assays requires careful control of nonspecific adsorption of the binding proteins and enzyme–analyte conjugates to the walls of plastic plates through the addition of surfactants to the working assay buffer.

MATERIALS AND METHODS

The rate of absorbance change from the enzymatic production of thio-NADH in the microtiter plate was measured with a Dynatech MR 5000 microtiter plate reader (Chantilly, VA) using dual wavelength settings of 405 and 630 nm for test and reference filters, respectively. Low-binding polystyrene round-bottomed microtiter plates from Corning Laboratory Sciences Co. (New York, NY) were used for the assays.

G6PDH from *Leuconostoc mesenteroides,* isothiocyanato-derivatives of phenyl pyranosides (mannose and galactose), G6P, NAD, thio-NAD, Con A from jack bean, avidin, BSA, and model glycoprotein analytes (glucose oxidase, β-fructofuranosidase, α-amylase, and α_1-acid glycoprotein) were obtained from Sigma Chemical Co. (St. Louis, MO). Jacalin lectin, NHS-activated biotin, and Tween 20 (10%) surfactant were from Pierce (Rockford, IL). The G6PDH–mannose and –galactose conjugates were prepared as reported previously (12). The conjugates used in this microtiter plate work had high degrees of saccharide substitution (as determined by the phenol–sulfuric acid method (18)): 35.9 mannose and 33.8 galactose per G6PDH molecule. BSA–saccharide conjugates with varying degrees of saccharide substitution were synthesized as previously described (13) and used for glycoconjugate dose–response curves. The G6PDH–biotin conjugate was prepared according to the procedure described by Daunert *et al.* (14) in which a 500:1 NHS-activated biotin to G6PDH ratio was used. The working assay buffer for G6PDH and G6PDH–saccharide/biotin conjugates was 0.05 M Tris–HCl buffer, pH 7.8, containing 0.10 M NaCl, 0.01% (w/v) NaN_3, 0.3% (w/v) gelatin, and 1 or 2% (w/v) Tween 20.

Determination of enzymatic activity and maximum percentage inhibition. The activity of G6PDH and G6PDH–saccharide/biotin conjugates was determined by measuring the rate of increase of thio-NADH con-

centration at 405 nm after mixing 50 μl of thio-NAD (7.4×10^{-4} M) and 50 μl of G6P (7.1×10^{-3} M) with 50 μl of appropriately diluted G6PDH or G6PDH conjugate in wells containing 100 μl of assay buffer. All solutions were kept at 0°C until the addition of the reagents. For each assay, after mixing reagents and subsequent shaking for 5 s, the absorbance of the reaction mixture in each well was read over a 10-min period for the G6PDH–saccharide conjugates and 21 min for the G6PDH–biotin conjugate. To determine the percentage inhibition value induced by the lectin or avidin, 50 μl of the assay buffer was replaced by 50 μl of lectin or avidin solution prepared in assay buffer. In addition, the conjugates were first incubated with the lectin or avidin for 10 min before subsequent addition of substrate solutions. Typical absorbance changes (at 405 nm) in the microtiter plate for the uninhibited G6PDH–saccharide conjugates were 0.15 *A* units, while in the presence of the lectin, absorbance changes were typically about 0.04 *A* units over the 10-min incubation period. In the case of the G6PDH–biotin conjugate, the completely active enzyme exhibited ΔA values of 1.05, while in the presence of avidin, the ΔA values were typically 0.125 *A* units over the 21-min assay period.

Effect of surfactant on the binding protein dilution curves. A Jacalin solution in Tris–HCl–gel, or Tris–HCl–gel–Tween 20 (1 or 2%) was incubated with 50 μl of 1.6×10^{-9} M G6PDH–galactose conjugate for 10 min. The substrate solution was then added and the enzymatic activity was measured as described above. A binding protein dilution curve was prepared by plotting percent inhibition vs amount of lectin added.

Dose–response curves for BSA–saccharide conjugates and glycoproteins with G6PDH–galactose/Jacalin and G6PDH–mannose/Con A systems. Standard solutions of different concentrations of synthetic BSA–saccharide conjugates and glycoproteins were prepared in Tris–HCl–gel–Tween 20 (2%) assay buffer. In the final assay protocol, 50 μl of standard solution and 50 μl of G6PDH conjugate solution were added to wells containing 50 μl of Jacalin solution (20 μg/ml) or Con A (23 μg/ml) and the mixtures were incubated for 10 min on a shaker. The resulting enzymatic activity was measured as described above. Dose–response curves were prepared by plotting percentage inhibition vs logarithm of the concentration of each analyte in the 50 μl of standard solutions added to the assay mixture. ED_{50} values (effective doses of BSA–saccharide conjugates or glycoproteins that results in 50% of the maximum or zero dose inhibition) were determined graphically from the dose–response curves.

Detection of biotin in vitamin tablets. For vitamin tablet preparation, 10 tablets were ground and the amount equivalent to 1 tablet was dissolved in 50 ml deionized water in a 50-ml centrifuge tube. The solution was vigorously shaken for 30 min at room temperature. The suspension was then centrifuged at 3200 rpm for 15 min. The supernatant was saved and the solid was again washed with 50 ml of deionized water and 50 ml 0.04 M NaOH with centrifugation between washes and pooling supernatant solutions. The solution was brought to a final volume of 200 ml with deionized water, 0.01% (w/v) NaN_3 was added to prevent bacterial growth, and the solution was stored at 4°C in the dark. Standard biotin solutions were prepared in Tris–HCl–gel–Tween 20 (2%) assay buffer. In the final assay protocol, 50 μl of biotin standard or sample solution and 50 μl G6PDH–biotin conjugate (1.2×10^{-8} M) were added to wells containing 50 μl of avidin solution (2.5 μg/ml) and the mixtures were incubated for 10 min on a shaker. The resulting enzymatic activity was measured as described above. Dose–response curves were prepared by plotting percentage inhibition vs logarithm of the concentration of biotin in the 50 μl standard solution added to the assay mixture. The amount of biotin in the vitamin tablets was determined by performing a logit analysis of the data (19).

RESULTS AND DISCUSSION

In our original lectin-based homogeneous ELBA studies, we found that MDH-based conjugates could be used successfully with lectins to devise a relatively rapid test for estimating carbohydrate content/structure of intact glycoproteins. As described above, to perform the same type of homogeneous binding assays in microtiter plates, it became necessary to switch to G6PDH–saccharide conjugates. Indeed, Florini (20) had demonstrated previously that G6PDH from *Leuconostoc mesenteroides* could be assayed at 405 nm in a microtiter plate by using thio-NAD as a substrate. The only potential disadvantages of using thio-NAD vs NAD is that both the V_{max} and the K_m of the G6PDH enzyme are lowered moderately (by 20 and 10%, respectively). Further, thio-NAD is less stable than NAD in light, and therefore more care must be taken when storing the reagents. While Florini's efforts were aimed at using the thio-NAD indicator reaction in a coupled enzyme sequence for measuring creatine kinase activity, it seemed reasonable to assume that activity of G6PDH–saccharide as well as other conjugates (e.g., G6PDH–vitamin) could be assayed in a similar manner for implementing new homogeneous ELBAs in a microtiter plate format.

In the earlier homogeneous ELBAs using conventional spectrophotometer detection (12–16), plastic tubes (polypropylene) used for reaction mixture incubations were precoated with gelatin prior to the addition of solutions containing the enzyme conjugates and/or binding proteins. This helped reduce nonspecific adsorption of these assay reagents to the walls of the tubes (e.g., enzyme conjugate, binding protein, and glycopro-

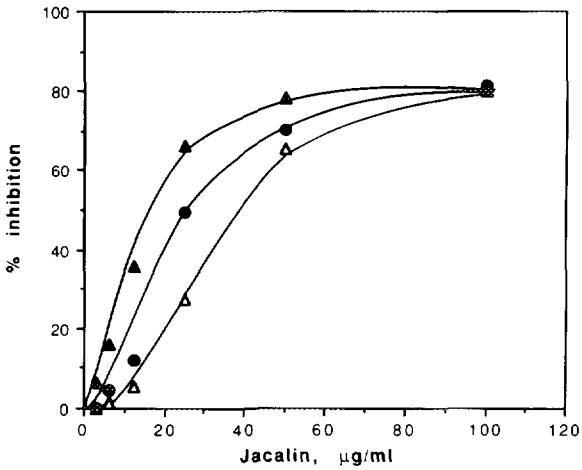

FIG. 1. Effect of adding Tween 20 to assay buffer on Jacalin inhibition of G6PDH-galactose conjugate (1.62×10^{-9} M in 50-μl aliquot) within wells of microtiter plate: (△) Tris-gel; (●) Tris-gel-Tween 20 (1%); (▲) Tris-gel-Tween 20 (2%).

tein analyte). In the present work, however, we found that prior adsorption of the gelatin alone to the plates was uneven and not adequate to completely eliminate nonspecific adsorption of the reagents. This may be due to the fact that the microtiter plates were made of polystyrene, not polypropylene, and the adsorptive behavior of these two materials are likely to be somewhat different. Nonspecific adsorption of either the binding protein or the enzyme–analyte conjugate can cause a significant change in the degree of enzyme inhibition observed. This is due to the fact that the adsorbed species, while perhaps still capable of interacting with its target molecule (i.e., the G6PDH–analyte conjugate or binding protein), may do so in a conformation or steric arrangement which does not yield a significant modulation in enzyme activity. It is known that for homogeneous ELBAs involving MDH or G6PDH enzymes, substantial inhibition of catalytic activity by biological binders (antibodies, binding proteins, lectins, etc.) can only be observed when a high ratio of binder to conjugate is used, presumably enough binder to fully surround the enzyme–ligand conjugate in solution (11). Indeed, the stoichiometry of the binder/conjugate reactions is probably considerably higher than 1:1 based on the fact that relatively high ligand substitution is required on the enzyme to achieve a significant level of homogeneous inhibition.

A variety of surfactants and proteins (including casein) have been used previously to reduce nonspecific adsorption in microtiter plate based ELISAs. However, since it has been reported that nonionic surfactants do not alter the ability of lectins to bind glycoproteins in affinity chromatography systems (21,22), Tween 20, a nonionic surfactant, was chosen and added in the assay buffer in an effort to overcome these initial nonspecific adsorption problems. As shown in Fig. 1, the amount of Tween 20 added to the assay buffer influences significantly the degree of inhibition of G6PDH-galactose conjugate by varying levels of Jacalin lectin. At low concentrations of lectin, the degree of conjugate inhibition is greatly enhanced by increasing amounts of surfactant, yet in the region of excess lectin (100 μg/ml), there is essentially no difference in the degree of enzyme modulation. The maximum degree of inhibition by excess lectin is dependent on the degree of saccharide substitution on the enzyme (12); however, even conjugates with very high degrees of substitution (greater than the 36 mannose/enzyme and 34 galactose/enzyme used here) cannot be inhibited 100% (12), and are essentially useless for binding assay purposes because residual enzyme catalytic activity is almost completely lost. From the results shown in Fig. 1, it is inferred that in the absence of surfactant, a certain portion of Jacalin may be adsorbed on the surface of microwells in a conformation not capable of binding the conjugate, or in a state where such binding does not induce large changes in enzyme activity. In all subsequent experiments, Tris-gel assay buffer containing 2% (w/v) of Tween 20 was used for the microplate homogeneous binding assays.

With the addition of Tween 20 to the assay buffer, the reproducibility for measuring the inhibition of G6PDH–analyte conjugates by binding proteins within the wells of the microtiter plate is excellent. Figure 2 illustrates the mean and full range of measurement values obtained for the titration of the G6PDH–saccharide conjugates with varying levels of lectins using multiple

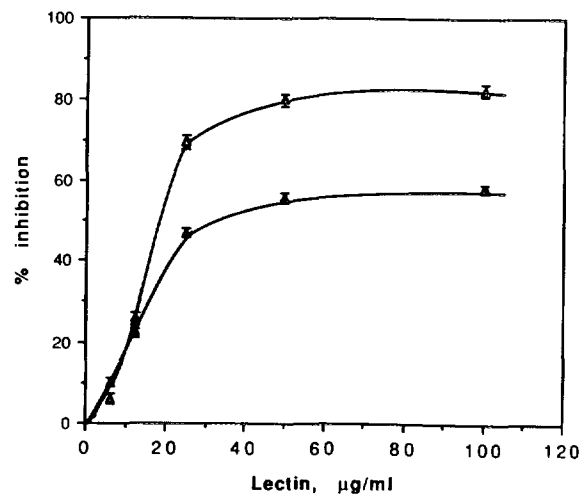

FIG. 2. Reproducibility of lectin inhibition curves for G6PDH–saccharide conjugates (1.6×10^{-9} M in 50-μl aliquot) obtained using thio-NAD as substrate in microtiter plate assay: (△) Jacalin/G6PDH-galactose system, $n = 6$; (▲) Con A/G6PDH-mannose system, $n = 3$; error bars represent full range of inhibition values for n determinations.

TABLE 1

Effect of Monosaccharide Substitution on ED_{50} Values for BSA–Saccharide Conjugates in Two Different Lectin-Based Homogeneous ELBA Performed in Microtiter Plate

	Jacalin/MDH–galactose		ConA/MDH–mannose	
Initial ratios of ligand to BSA	Degree of galactose substitution	ED_{50} (μg/ml)	Degree of mannose substitution	ED_{50} (μg/ml)
100	9.6	12.6	7.9	38.5
250	13.4	6.3	12.5	25.1
500	28.5	2.2	18.9	17.7

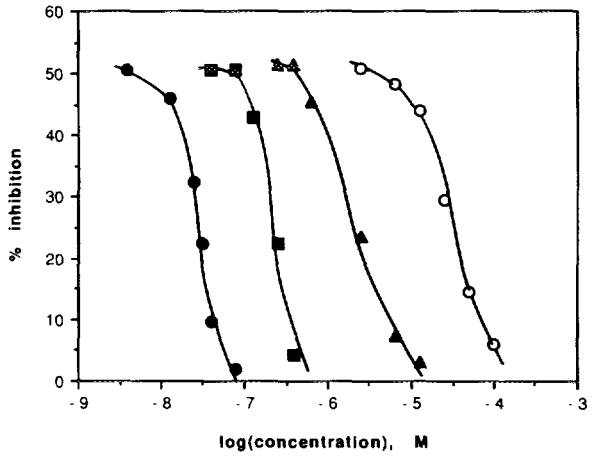

FIG. 3. Glycoprotein dose–response curves obtained in microtiter plate reader with G6PDH–mannose (1.6×10^{-9} M in 50-μl aliquot)/Con A (23 μg/ml): (●) β-fructofuranosidase; (■) glucose oxidase; (▲) α-amylase; (○) α_1-acid glycoprotein. Data points for each curve are average of three wells.

wells for each concentration of lectin. By utilizing the Jacalin/G6PDH–mannose conjugate system, relative standard deviations of the actual enzyme activity determinations (not percentage inhibition) for each data point are $\leq \pm 1.6\%$ ($n = 6$). Similar precision was obtained with the G6PDH–mannose conjugate using Con A as the selective inhibitor ($n = 3$) (Fig. 2) and the G6PDH–biotin conjugate using avidin ($n = 3$) (data not shown).

Once demonstrating that enzymatic activity of G6PDH–saccharide conjugates and their inhibition by binding proteins could be measured reliably with thio-NAD, the feasibility of conducting competitive binding assays within the microtiter plate wells for estimating carbohydrate content/structure of glycoproteins was examined. Synthetic BSA–galactose and BSA–mannose glycoconjugates were prepared with varying degrees of substitution (13). As shown in Table 1, the ED_{50} values for each lectin/conjugate system tested decrease in an amount proportional to the degree of saccharide substitution on the BSA. The results closely resemble those obtained with previous MDH–saccharide conjugate/lectin systems for the same type of synthetic BSA–saccharide conjugates. Again, very reproducible inhibition data for these competitive binding curves were obtained (typical rsd for competitive binding inhibition points were $\leq \pm 1.2\%$ ($n = 6$), percentage enzyme inhibition).

We further examined dose–response behaviors of several natural glycoproteins with the Con A/G6PDH–mannose system within the microtiter plate arrangement. As shown in Fig. 3, the dose response to each protein is very different. For example, β-fructofuranosidase, which has a mannose content of 50–77 wt%, has the lowest detection limit while glucose oxidase with a mannose content of only 14 wt% has the highest detection limit (23). The order of response within the microtiter plate arrangement correlates well to that observed when a conventional kinetic spectrophotometer was used to monitor Con A/MDH–mannose reaction (10). As noted previously (10), dose response behavior for pure glycoproteins using an array of different lectins and enzyme–saccharide conjugates may be used to rapidly screen for changes in the carbohydrate structure/content of given glycoproteins. However, since lectins have very broad carbohydrate specificity, such assays will not be useful for detecting given glycoproteins in complex samples, where a large number of different glycoproteins and other structures possessing carbohydrate groups can exist.

The typical dose-response for the homogeneous biotin ELBA using G6PDH–biotin and avidin as the reagents is shown in Fig. 4. The precision and selectivity

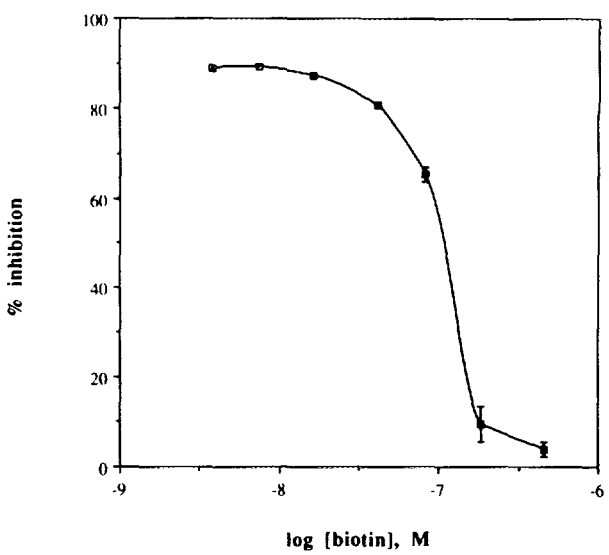

FIG. 4. Typical dose–response curve for homogeneous biotin assay as performed in microtiter plate using MDH–biotin conjugate (1.2×10^{-8} M) and avidin (2.5 μg/ml). Data points are average of three wells.

TABLE 2

Results for the Determination of Biotin in Commercial Vitamin Tablets

Sample	Found[a] (μg/tablet)	Claimed (μg/tablet)
Natalins	29 ± 0.7	30
Kroger multivitamins	25 ± 2	30
Kroger B100	112 ± 3	100

[a] Average of three determinations (±SD).

of the biotin assay was demonstrated by determining the biotin content of commercial vitamin tablet preparations. Results obtained were in good agreement with the manufacturer's claim (see Table 2).

The concept of using G6PDH–ligand conjugates with the thio-NAD substrate described here allows the easy adaptation of existing homogeneous enzyme-linked binding assays to a microtiter plate format. Indeed, these conditions could also be extended to adapt more conventional homogeneous EMIT type drug assays, as well as several analogous binding protein-based homogeneous assays for vitamins (e.g., for folate (15) and B_{12} (16)) to the convenient microtiter plate detection system, particularly for laboratories running large numbers of samples.

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