An Automated Procedure for Measuring Biotinidase Activity in Serum

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In this automated procedure for quantifying biotinidase activity in human serum, a manual colorimetric method that measures conversion of the enzyme's artificial substrate N-biotinyl p-aminobenzoate was modified for use with a Technicon AutoAnalyzer II. The intra-run replicate precision (CV) was 2.1% and the day-to-day CV was 4.6% for quality-control sera. Results were linearly related to biotinidase activity in serum over the complete range of clinically relevant values, 0.2 to 11.0 U/L. Moreover, results of the automated assay were not significantly different from those of the manual assay. Because the automated procedure is faster and more precise, we recommend it for population-based studies and some screening studies.

Additional Keyphrases: continuous-flow analysis · heritable disorders · screening

Biotinidase (EC 3.5.1.12) activity is usually measured in human serum by a modification of the procedure of Knappe et al. (1). The quantity of p-aminobenzoic acid (PABA) released from the artificial substrate, N-biotinyl-p-aminobenzoate (BPAB), is determined colorimetrically after coupling an azo compound to the diazonium salt of the PABA produced. Although this manual procedure is reliable and reproducible (2), one can assay only a few specimens with it simultaneously. Because we sought a more rapid and precise method that could be applied to population and screening studies in which many serum samples are to be examined, we adapted the manual method to a continuous-flow procedure. Here we describe the method, and evaluate its speed, precision, accuracy, and applicability to genetic and epidemiological studies.

Materials and Methods

Chemicals and reagents. BPAB and human serum albumin were obtained from Sigma Chemical Co., St. Louis, MO; N-1-naphthylethylenediamine dihydrochloride, ammonium sulfamate, and PABA were from J. T. Baker Chemical Co., Phillipsburg, NJ 08865; trypan blue stain (4 g/L) was from Gibco Laboratories, Life Technologies, Inc., Grand Island, NY 14072; and "Wetting Agent NWA" was from Technicon Instruments Corp., Tarrytown, NY. All other reagents were analytical grade, from standard suppliers, and all solutions were prepared in de-ionized, glass-distilled water.

Specimens. Unless otherwise stated, serum was separated from freshly drawn whole blood, collected from ostensibly healthy, adult volunteers and stored at −70 °C until analysis.

We also obtained 14 specimens from two children with biotinidase deficiency and from some of their family members. Serum quality-control material was prepared by pooling freshly prepared sera from nine healthy adults, and stored at −70 °C. The biotinidase activity of this pooled sample did not differ significantly from the mean activity of normal control samples. Lyophilized pooled serum (Ciba Corning Diagnostics Corp., Irvine, CA) was used as a low-activity quality-control specimen.

Assays for biotinidase activity. Biotinidase activity was determined manually as described previously (2). For a continuous-flow quantification of biotinidase activity in serum we used the components of an AutoAnalyzer II system (Technicon) in the flow diagram shown in Figure 1. In the automated method we used the same concentrations of reagents as in the manual method except that we added 5 mL of the wetting agent to each liter of buffer and 0.5 mL of Triton X-100 (Sigma) to each liter of the other reagents. We also used HCl (1 mol/L) instead of trichloroacetic acid to inactivate the enzyme and to decrease the pH to 1.8–2.0, which is necessary for development of color.

In the automated procedure, serum was incubated at 37 °C with potassium phosphate buffer (50 mmol/L, pH 6.0) containing EDTA (5 mmol/L) and BPAB (50 μmol/L). The incubation time (approximately 19 min) was determined at the start of each run by sampling 133 μL of trypan blue solution and timing its passage through the system. The solution was dialyzed for six minutes against the potassium phosphate buffer to separate the reaction product (PABA) from other components.

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4 Nonstandard abbreviations: PABA, p-aminobenzoic acid; BPAB, N-biotinyl-p-aminobenzoate.

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Fig. 1. Flow diagram of the automated procedure for determining biotinidase activity

Internal tubing diameter (in inches), reagent, and flow rate (mL/min) are labeled for each pump tube: e.g., phosphate buffer flows through 0.03-in.-diameter tubing at 0.32 mL/min. Transmission tubing (Tygon) and glass coils were 1.8 mm i.d. 51 and 52 are stock injection blocks. The 5-turn and 10-turn coils facilitate mixing. The 20-turn and 40-turn coils provide 3- and 10-min delays, respectively. The flow cell (FC) has a 15-mm light path. The substrate buffer consists of 50 mmol of BPAB per liter of potassium phosphate buffer (50 mmol/L, pH 6.0). The potassium phosphate buffer is used for the sample wash and for the recipient stream of the dialyzer. The sampling rate (for 133-μL samples) is 30 specimens per hour, with a sample-to-wash ratio of 2:1.
from high-molecular-mass substances that could interfere with the colorimetric analysis.

Color was developed by the same procedure used in the manual assay. Absorbance at 550 nm was measured colorimetrically, and recorded continuously on a strip-chart recorder at a chart speed of 2.5 cm/min.

Triplicate aliquots of PABA standard (0.2 mmol/L) were assayed at the beginning and end of each run. The within-run mean peak height of these samples was used for calculating biotinidase activity, as follows:

$$\text{PABA in serum} = \frac{\text{peak height of serum sample/mean peak height of PABA}}{200}$$

One unit of biotinidase activity (U) is equivalent to the release of 1 µmol of PABA from BPAB per minute at 37 °C.

**Evaluation of the automated procedure.** To determine the range of activities over which the response of the colorimeter remained linear, we diluted pooled control serum with 0.15 mol/L sodium chloride solution containing human serum albumin (4 g/L) and assayed the resulting solutions.

We measured the percentage of carryover of activity by sampling and assaying three low-activity quality-control specimens after three high-activity quality-control specimens on four separate occasions.

To evaluate precision, we used samples of the high- and low-activity quality-control specimens. Each sample was assayed in duplicate on each of 16 days spanning an eight-month period. Precision was also evaluated by calculating the sources of variation for data obtained from the subjects' specimens. Sera from groups of five different individuals were tested on each of 16 days.

To estimate the variation within subjects, blood was drawn from each of four healthy volunteers three times per week for six weeks. Serum was prepared and stored until the end of the sampling period, and all specimens were thawed and assayed on a single day. Analysis of variance was used to estimate the proportions of total variance attributable to analytical error, variation within subjects, and variation among subjects (3).

**Comparison of methods.** Biotinidase activity was measured in 111 serum specimens by both the manual and the automated assay procedures, on a total of 20 different days. Ninety-seven specimens were selected without conscious bias from a healthy adult population, and 14 specimens from two families with biotinidase-deficient children also were included. The mean activity of duplicate determinations on each sample was used for the method-comparison analysis. The differences between the values obtained by each method were evaluated using a paired t-test and the median values obtained for each method were compared by the Wilcoxon Rank Sum Test. The Deming regression of the estimates of biotinidase activities generated by the automated method on the estimates generated with the manual method was determined (4) and a Pearson correlation coefficient was calculated.

**Results**

**Performance of the automated assay.** Absorbance was linear ($r = 1.00; P < 0.0001$) for pooled control serum, undiluted or diluted to give concentrations down to 62.5 nM/L for which the corresponding biotinidase activities were 7.5 to 0.48 U/L. The lower detection limit of the assay corresponds to 0.2 U/L. The upper limit, determined with PABA, is approximately 11.0 U/L. The mean carryover was 2.1%.

**Precision and reproducibility.** The overall CVs for the high- and low-activity quality-control were 4.6% (n = 138) and 9.0% (n = 64). The mean intra-run CVs were 2.1% and 5.2%. There was no significant decrease in the biotinidase activity of the control specimens during the eight-month study.

The within-run CV for the subjects' duplicate samples was 4.2%; the overall CV was 16.3%. Differences among subjects accounted for 93.8% of the total variance, and within-run analytical error among replicates accounted for the remaining 6.2%. There was no significant error associated with between-run variation.

**Variation between and among subjects.** The estimated biological variance, i.e., the sum of intra- and inter-individual variance components, was 0.76 (0.60 + 0.16). The highest estimate of analytical variance from the analysis was 0.12. Thus, the ratio of inter-individual variation to intra-individual plus analytical variation was 2.14 (0.60/0.28). If we excluded the analytical variation, then the ratio was 3.75. Hence, the variation between individuals is almost fourfold that within an individual.

**Comparison of methods.** The distributions of enzyme activities for each method were significantly skewed toward low-activity values (Kolmogorov–Smirnov test: D = 0.102, P > 0.01 for the manual method, and D = 0.150, P > 0.01 for the automated assay), because we included specimens from activities in the heterozygous range. The differences between the paired values obtained by each assay were distributed normally (D = 0.08, P > 0.06).

The mean biotinidase activities for the manual and automated procedures, 6.27 (SD 1.7) U/L and 6.11 (SD 1.6) U/L, were not significantly different ($t = 0.62, P = 0.50; n = 111$). The median activities for the manual and automated procedures were 6.59 U/L and 6.64 U/L, and they also were not significantly different (Z = 1.43, P = 0.15; n = 111). The overall CVs for the manual and automated methods were 27.1% and 26.4%. The activities for individual specimens by each method were significantly positively correlated ($r = 0.87, P < 0.0001$). Deming regression analysis yielded the following relationship between activities from the automated procedure (y) and the manual assay (x): $y = 0.23 + 0.94x$. The standard error ($S_{xy}$) of the regression coefficient was 0.04 U/L and the standard deviation of the y-intercept was 0.06 U/L.

**Discussion**

Biotinidase activities measured by the present procedure compared well with those from the manual assay, but the automated method is more precise. Within-run precision of assays on high-activity quality-control material and subjects' specimens was similar for both methods. For the low-activity quality-control specimens, the within-run variation was lower with the automated assay. Total variance of the samples analyzed by the automated assay was lower than that for the manual method.

Most of the difference in total variance between the manual and automated procedures is attributable to the difference in magnitude of the between-run variance which, for the later method, was negligible.

The activity determined by the automated procedure was linear for the complete range of clinically relevant activities (0.2 to 11.0 U/L). Our range of "normal" activity is 4.4 to 10.0 U/L (mean = 7.1 U/L; n = 521). Carryover of a specimen is unlikely to affect a measurement except when a specimen from a subject with normal activity precedes a
biotinidase-deficient specimen in the autosampler. A sample cup containing phosphate buffer should be inserted between each sample suspected of having deficient or very low activity.

Interfering substances such as sulfonamides, if present in a sample, can cause color development, such that a biotini-
dase-deficient specimen could exhibit color corresponding
to normal activity and a normal specimen could exhibit color
corresponding to activity exceeding the upper limit of nor-
mal. Therefore, samples tested by either method also should be
tested in the absence of substrate for the presence of other
cromogenic compounds.

The applicability of the automated method to clinical,
genetic, and epidemiological studies depends on the relative
contributions of analytical and biological variation to total
variation in activity, and on the relative contributions of
intra- and inter-individual variation to total biological vari-
ation. Although there is no clear definition of "acceptable"
analytical variation, Tonks (5) suggested that total analyti-
cal variation should not exceed one-fourth of the total
biological variation. The analytical variation of the auto-
mated assay is sufficiently low (less than one-sixth of
biological variation) to assure that true differences between
subjects will not be obscured and that abnormal laboratory
results will be identified reliably. The differences among
individuals accounted for two to four times more variation
than did variation within a single individual and, therefore,
the automated system is acceptable for use in genetic and
epidemiological studies.

This automated assay provides a reliable means for
coveniently determining biotinidase activity in a large
number of serum specimens, and results are more precise
than with the manual assay. Thirty samples can be ana-
lyzed in an hour, whereas about 3 h is required to analyze an
equivalent number of samples by the manual method.
Furthermore, because handling of the samples and reagents
is minimized, there is less possibility of human error. The
speed, precision, and reliability of the present procedure
make it the preferred method for large population-based
studies of biotinidase activity in human serum.

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References
1. Knappe J, Brommer W, Brederbick K. Reinigung und Eigen-
schaften der Biotinidase aus Schweinenieren und Lactobacillus
2. Wolf B, Grier RE, Allen JD, et al. Biotinidase deficiency:
the enzymatic defect in late-onset multiple carboxylase deficiency.
3. Snedecor GW, CoRnan WG. Statistical methods, 7th ed. Ames,
4. Parvin CA. A direct comparison of two slope-estimating tech-
niques used in method-comparison studies. Clin Chem
5. Tonks A. A study of the accuracy and precision of clinical
chemistry determinations in 170 Canadian laboratories. Clin Chem

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Statistical Interpretation of Concentrations of Magnesium, Zinc, Calcium, Potassium,
Cholesterols, and Creatine Kinase Isoenzymes in Men at Different Stages of Ischemic Heart
Disease

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We present a statistical interpretation of plasma (Pl) and (or)
erythrocyte (Erc) concentrations of magnesium, zinc, calci-
um, potassium, and total high-density lipoprotein (HDL)
cholesterol, as well as of the activity of total creatine kinase
(CK) and its CK-MB isoenzyme, in 28 men with pre-infarction
syndrome (PIS) and 34 men with acute myocardial infarction
(MI). Discriminant analysis allowed overall comparison of
both groups and determination of the most significant vari-
ables: CK and Pl-Zn. By non-hierarchical cluster analysis we
defined three homogeneous subgroups among MI men, with
CK, CK-MB, and Pl-Zn differing significantly between the
groups. In PIS men, Pl-Zn was correlated with Pl-Ca, where-
as in MI men Pl-Zn was correlated with Pl-Mg. Stepwise
regression indicated that Pl-Zn was the most significant regres-
sor of CK in PIS men and of CK-MB in MI men. All
these statistical interpretations support a special role of Pl-Zn
in diagnosis and perhaps prognosis. After MI, interleukin-1
release may possibly mediate observed hypozincemia via
formation of a heart Zn-metallothionein.

In previous papers, we studied the concentrations of
various trace elements and biological variables at day 1 in
two groups of men: one with pre-infarction syndrome (PIS)
and the other with acute myocardial infarction (MI) (1, 2).3
We now present a statistical study involving cluster analy-
sis and stepwise regression to show possible relationships
among the variables investigated and changes according to
the severity of ischemic heart diseases. We studied the
following plasma (Pl) and erythrocyte (Erc) variables: mag-
nesium (Pl-Mg, Erc-Mg), zinc (Pl-Zn, Erc-Zn), calcium (Pl-
Ca), potassium (Erc-K), and total and high-density-lipopro-

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3 Nonstandard abbreviations: Pl, plasma; Erc, erythrocytes; HDL,
high-density lipoproteins; CK, creatine kinase (EC 2.7.3.2); PIS,
pre-infarction syndrome; and MI, myocardial infarction.