Evaluation of a Spectrophotometric Method for Measurement of Activity of Diamine Oxidase in Newborn Infants*

STEVEN C. KAZMIERCZAK, Ph.D.
and ALEX F. ROBERTSON, M.D.

Departments of Pathology and Laboratory Medicine and Pediatrics,
East Carolina University School of Medicine,
Greenville, NC 27858-4354

ABSTRACT

Diamine oxidase (DAO) is an enzyme synthesized primarily in the gastrointestinal mucosal cells. Serum levels of DAO have been used as an indicator of the integrity and/or functional mass of the intestinal mucosa. The enzyme is also produced by the placenta and is elevated in newborn serum. Previous radiometric methods for DAO used tritiated putrescine or cadaverine as substrate. A simple and rapid spectrophotometric procedure for DAO with use of histamine as substrate was developed, and this assay was utilized to evaluate the developmental pattern of activity of DAO in umbilical cord blood of newborn full-term and premature infants, in sequential samples from premature infants, and in samples from infants with necrotizing enterocolitis. The spectrophotometric assay was linear to 200 U per L and was also precise with total imprecision (CV) of 11.9 percent and 3.7 percent at DAO activities of 25.6 U per L and 126.1 U per L, respectively. Triglycerides above 275 mg per dL caused a significant reduction in measured activity of DAO; however, this effect could be eliminated by use of ultracentrifugation to remove lipemia. Plasma samples with heparin or ethylenediamine tetraacetic acid (EDTA) as anticoagulant were unsuitable for analysis since DAO activity showed a 24 percent and 32 percent decrease in activity at concentrations of 20 U per mL (heparin) and two mg per mL (EDTA), respectively. Serum samples are the specimen of choice. In infants it was found that the serum activity declined to adult levels by day 12 of life and that this decline is not affected by necrotizing enterocolitis.

Introduction

Diamine oxidase (DAO) is an enzyme synthesized primarily by the gastrointestinal mucosal cells. Mucosal activity of DAO decreases following intestinal ischemia, while activities of the enzyme in the peripheral blood increase. Measurement of activity of DAO in tissue or serum has been used as an indicator of the integrity and/or functional mass of the intestinal mucosa. Diamine oxide is also produced during pregnancy by the
maternal decidua, and this additional source results in increased activity in both maternal and newborn infant blood. Limited studies with a tedious radiometric method using tritiated histamine or putrescine as substrate have suggested that enzyme activity varies with gestational age and, in the full-term infant, does not decline to baseline levels until after a week of life. A rapid and sensitive automated spectrophotometric assay was developed by us for measurement of DAO in serum, and the utility of changes in DAO was evaluated in umbilical cord blood of newborn full-term and premature infants, in sequential samples from premature infants, and in samples from infants with necrotizing enterocolitis (NEC).

Methods

Residual serum was collected from samples submitted to the clinical laboratories for other testing. The serum was separated and frozen at -70°C until analysis.

A kinetic method was developed for measurement of DAO activity and adapted it to a Cobas Fara centrifugal analyzer. Activity of DAO was measured by using histamine as the substrate and by monitoring the rate of ammonia formation following the cleavage of histamine. The assay for ammonia was adapted to the Cobas Fara using a commercially available reagent for determination of ammonia. The histamine substrate (0.75 M) was prepared by dissolving histamine free base in phosphate buffer, pH 7.4. The assay is based on the following sequence of reactions:

\[
\text{DAO} \\
\begin{align*}
1. \text{Histamine} & \rightarrow \text{NH}_3 \\
+ \text{Deaminated Histamine} \\
2. 2\text{-Oxoglutarate} + \text{NH}_3 & \\
\text{GLDH} \\
+ \text{NADH} & \rightarrow \text{Glutamate} \div \text{NAD}
\end{align*}
\]

The rate of decrease in absorbance at 340 nm, owing to oxidation of reduced nicotinamide-adenine dinucleotide (NADH), is proportional to activity of DAO. The effect of endogenous ammonia present in the serum sample was eliminated by performing a 10 minute incubation of the sample with the reagents listed in reaction 2. Histamine was added to the assay system at the end of the incubation period. Instrument settings for the assay are given in table I. One unit of activity is defined as one mmol of ammonia formed per minute per mL of serum at 37°C.

The linearity and precision of our automated DAO assay was determined as described in guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) EP6 and EP5, respectively.

**TABLE I**

| Cobas-Fara Centrifugal Analyzer Settings for Assay of Diamine Oxidase Activity |
|---|---|
| Measurement mode | Absorb |
| Reaction mode | T-P-I-SR1-AO |
| Reagent blank | STD LIN |
| Wave length | 340 nm |
| Temperature | 37.0°C |
| Decimal | 2 |
| Units | µg/mL |
| Sample volume | 12 µL |
| Diluent | 10 µL |
| Reagent volume | 140 µL |
| Incubation time | 600 s |
| Start reagent volume | 10 µL |
| Time of first reading | 0.5 s |
| Time interval | 300 s |
| Number of readings | 2 |

* Roche Diagnostic Systems, Nutley, NJ 07110.  
† Sigma Chemical Co., St. Louis, MO 63178.
tively. Interference studies were also performed to assess the effect of lipemia and icterus. To evaluate the most appropriate specimen type (plasma vs serum), the effects were compared of varying concentrations of heparin and EDTA (sodium salt) on activity of DAO. The Michaelis constant (Km) of DAO for histamine was determined by use of the Michaelis-Menten curve relating velocity of the DAO-catalyzed reaction to substrate concentration.

Reference intervals were determined by using the REFVAL (reference value) protocol. Reference value groups were compared by use of the Mann-Whitney U test. The 95 percent confidence limit was used for statistical significance.

RESULTS

The automated procedure exhibited a linear response with increasing activity of DAO up to 200 U per L. The lower detection limit of the method was evaluated by analysis of replicate reagent blanks. The enzyme activity, corresponding to an absorbance change that exceeded by three standard deviations (SD) the mean absorbance change of reagent alone, was 2.9 U/L.

The precision of the procedure was evaluated by analyzing two separate pools of patient’s sera with slightly increased and markedly increased DAO activities. The patient-serum pools were aliquoted into individual tubes and stored frozen at -70°C; aliquots were analyzed in duplicate once daily for 20 consecutive days. In table II are summarized the results of the precision studies.

The method for DAO was evaluated for its response to interference by bilirubin and lipemia. A series of five test samples, varying only in the concentration of interferent, was prepared by making mixtures of two pools, one at the highest concentration of interferent to be tested and the other at the lowest. All samples were analyzed together in random order. Interference studies showed no significant effect by bilirubin up to 20.3 mg per dL. However, triglycerides in excess of 275 mg per dL decreased activities of DAO by 33.7 percent. Centrifugation of lipemic specimens in order to remove triglycerides resulted in measurement of expected activities of DAO. The results of the interference studies are shown in table III.

Analysis of serum specimens and plasma specimens with heparin or EDTA as anticoagulant showed that both of these anticoagulants markedly decreased DAO activities. The inhibition of activity
of DAO by both heparin and EDTA was linear with respect to concentration of anticoagulant tested. In figure 1 is shown the effect of increasing concentrations of heparin and EDTA on activity of DAO.

The affinity of the enzyme for histamine was determined using a serum sample with a measured activity of 30 U per L. The Km of DAO for histamine was found to be $9.0 \times 10^{-3}$ mol per L.

Activity of DAO was measured in serum from 40 apparently normal individuals (20 men and 20 women without evidence of disease) who underwent routine health examinations. The reference interval, defined as the central 95 percentile interval was, found to be 4.6 to 9.8 U per L. Analysis of the data by the Mann Whitney U test revealed no significant gender-related differences.

One hundred and fifteen samples were assayed from the umbilical cord serum of infants who were 38 to 42 weeks gestational age, had appropriate weight for gestational age, and had a normal physical exam. The mean (±SD) of activity of DAO was 26.6 ± 11.0 U per L. There was no significant correlation between the activity of DAO in cord serum and birth weight ($r = 0.02$, $p = 0.82$).

One hundred and three samples were assayed from the cord serum of infants who were less than 38 weeks gestational age. The activity of DAO was 32.5 ± 20.6 U per L. There was a low, but nonsignificant, inverse correlation between the activity of DAO and the birth weight ($r = -0.24$, $p = 0.015$) and a nonsignificant inverse correlation with gestational age ($r = -0.14$, $p = 0.15$).

Sequential blood samples were obtained on 15 premature infants whose weight range was 600 to 1250 g and who had no signs of gastrointestinal disease. In figure 2 is shown the decline in activity in serum DAO oxidase. The activity of DAO in serum decreased to less than 10 U per L by day 12 in all infants studied. In one infant followed to day 104, the activity consistently remained below 10 U per L.

During the study, five infants developed necrotizing enterocolitis (NEC) diagnosed at surgery or by the clinical picture including pneumatosis intestinalis. The gestational age range was 30 to 40 weeks, and the weight range was 1694 to 3084 g. All the infants were considered healthy at birth and were fed with formula by 12 hours of age. All of these infants developed NEC within the first 48 hours of life. The insert in figure 2 shows the time course of their serum DAO activity. The pattern in infants with NEC is the same as in the premature infants without NEC. Two of these infants died, and three survived. There was no difference in the pattern of decline between the survivors and the non-survivors.

Discussion

Our spectrophotometric method was found to be rapid and precise for the quantitative measurement of activity of DAO. However, increased concentrations of triglyceride significantly reduced measured activities of DAO in serum.
Figure 2. Serum diamine oxidase activity after birth. The larger figure shows the sequential diamine oxidase activity in 15 premature infants. The smaller figure shows the activity in five infants who developed necrotizing enterocolitis in the first two days of life.

owing to the effect of lipemia on the initial absorbance readings of the assay. This effect, however, is easily eliminated by the use of centrifugation to remove triglycerides from the specimen.

One notable finding was the effect of heparin and EDTA on DAO activity. The DAO showed a linear decrease in activity with increasing heparin concentrations (figure 1). The inhibitory effect of heparin in adults in vivo is probably not noticed in studies which measure post-heparin activity of DAO because the dose of heparin usually used in these studies, 5,000 to 15,000 units, results in a final blood concentration of one to three U per L, assuming a total blood volume of five L. At these concentrations, the inhibitory effect of heparin on activity of DAO is at most four percent. However, blood samples drawn into tubes with heparin as anticoagulant usually have approximately 20 units of heparin per mL of blood to prevent clot formation. The DAO activity was found to be inhibited by 24 percent at a heparin concentration of 20 U per L. These results raise serious concerns with regards to those studies which use heparinized plasma as specimen for measurement of activity of DAO.

Similar results were found with use of EDTA as anticoagulant. Ethylenediamine tetraacetic acid is effective as an anticoagulant at a final concentration of one to two mg per mL of blood. Activity of DAO decreased from 15 to 26 percent at EDTA concentrations of one and two mg per mL, respectively (figure 1). One previous report on the affects of EDTA on activity of DAO showed that at a concentration of 0.4 mg per dL, EDTA inhibited activity of DAO by 14 percent. Serum was found to be the specimen of choice for analysis of activity of DAO as heparin and EDTA significantly inhibited enzyme activity at concentrations normally found in tubes used to collect plasma specimens.
The Km of DAO for histamine, $9.0 \times 10^{-3}$ mol per L was similar to that reported by Buffoni et al who used a manometric assay for activity of DAO.

The activity of DAO observed by us in umbilical cord serum was up to 15 times that seen in adult sera. Similar results have been reported by Forget et al who measured activities of DAO in the peripheral serum of neonates on days 1, 4, and 7 of life. Unlike our study, these authors used a radiometric procedure for measuring activity of DAO which makes direct comparison of our results and those of Forget and colleagues difficult. However, our results do agree in that enzyme activity in neonates declines rapidly, reaching adult levels by 12 days of age. In addition, the rate of this decline was not affected by NEC. The half-life of DAO in the circulation is approximately 16 hours.

The cases of infants with NEC illustrate that intestinal tissue necrosis does not alter the decline in the serum activity of DAO in newborns. In human infants, the incidence of NEC is reduced by prenatal treatment with glucocorticoid hormones. These findings suggest that activity of DAO or synthesis of DAO might be induced prenatally and be protective against NEC. Studies in the rat have shown that mucosal and serum DAO follow a parallel increase with postnatal intestinal maturation while studies in dogs have shown that inhibition of DAO leads to increased tissue damage following mesenteric artery occlusion and reperfusion. Intestinal ischemia in the rat causes an elevation of activity of serum diamine oxidase, and one case of acute necrosis of the intestinal mucosa in a human is reported with increased concentration serum diamine oxidase. Diamine oxidase activity was found by us not be useful in newborn infants to diagnose acute gastrointestinal ischemia as seen in NEC. The increased values seen in newborn serum may mask any increase that otherwise might be seen in older children and adults.

It was our hope that abnormal concentrations of DAO might prove to be a marker for the risk of NEC. However, activity of DAO in all of our infant samples decreased to activities seen in normal adults within 12 days of age and stayed at that concentration. In children and adults more information is obtained by the rise in activity of DAO after an injection of heparin. The response of premature infants to heparin induced release of DAO may offer more insight into the integrity of their developing gastrointestinal tract.

Acknowledgments:

Thanks are extended to Edna D. Hodges for the collection of blood samples and the measurement of activity of DAO and of Mike Cruze for the statistical analyses.

References


