Measurement of Aspartate Aminotransferase Activity: Effects of Oxamate

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Oxamate, a potent inhibitor of lactate dehydrogenase, is shown also to inhibit aspartate aminotransferase activity, both in human serum and in purified isoenzymes of human origin. The inhibition was competitive with respect to 2-oxoglutarate for both isoenzymes. The apparent $K_i$ was 29 mmol/L for the cytoplasmic enzyme and 17 mmol/L for the mitochondrial enzyme. Noncompetitive inhibition was found between oxamate and aspartate. At saturating concentrations of substrate (2-oxoglutarate $\geq$ 15 mmol/L, L-aspartate $\geq$ 150 mmol/L) oxamate inhibited the mitochondrial enzyme but had less effect on the cytoplasmic isoenzyme. Oxamate at 40 mmol/L inhibited the enzyme in serum by 11 and 9% in assays containing 2-oxoglutarate at 6.7 and 15 mmol/L, respectively. This concentration of oxamate inhibited enzyme activity in serum by 5% more than did the same concentration of Cl$^-$ (itself an inhibitor). Oxamate (\leq30 mmol/L) had no measurable effect on the stability or activity of porcine malate dehydrogenase. Until the effects of its inhibitory properties are considered, addition of oxamate to suppress lactate dehydrogenase-mediated side reactions in the assay of aspartate aminotransferase cannot be recommended.

Additional Keyphrases: enzyme activity • enzyme kinetics • variation, source of

A well-recognized source of error in the estimation of aspartate aminotransferase activity in serum or plasma by the malate dehydrogenase/NADH coupled assay is the oxidation of NADH by endogenous enzymes and substrates present in serum (1-4). Three enzyme-substrate pairs are primarily responsible for such interference: lactate dehydrogenase with keto acids; glutamate dehydrogenase with NH$_4^+$; and malate dehydrogenase with oxalacetate (1, 3, 4). The largest source of error, the reduction of keto acids by lactate dehydrogenase and NADH, can be overcome by preincubating until the reaction comes to equilibrium (1-4). Adding exogenous lactate dehydrogenase to speed this reaction has also been commonly advocated (3, 4, 7-9).

The strong inhibitory properties of certain carboxylic acids—e.g., oxamic and oxalic acids—on the lactate dehydrogenase reaction are similarly well established (10-12). Oxamate is a potent inhibitor of lactate dehydrogenase through the formation of an unreactive enzyme—NADH-oxamate ternary complex (11, 12). Two recent reports have proposed that oxamic acid be added as a reagent for the assay of aspartate aminotransferase in serum, to eliminate interference by lactate dehydrogenase (13, 14).

Many carboxylic acids and other anions, however, influence aspartate aminotransferase activity directly (15). Any such effect of oxamic acid could nullify its advantage in reducing other interferences. We have therefore investigated the effects of oxamate on the steady-state kinetic properties of human aspartate aminotransferase isoenzymes and its effect on the measurement of serum activity.

Materials and Methods

Oxamic acid (sodium salt), L-aspartic acid, 2-oxoglutaric acid, β-NADH (grade III), tris(hydroxymethyl)aminomethane (Tris), oxalacetic acid (grade I), and pyridoxal 5'- phosphate were obtained from Sigma Chemical Co., St. Louis, MO 63178. Malate dehydrogenase of porcine heart origin was obtained as a solution in glycerol from Boehringer Mannheim, Indianapolis, IN 46250. De-ionized water with specific resistivity $\geq$15 M$\Omega$-cm was used throughout. Where possible, reagents were filtered through filters of 0.22-μm pore size (Millipore Corp., Bedford, MA 01730).

Aspartate aminotransferase activity was measured by coupling oxalacetate production with malate dehydrogenase and NADH and measuring the decrease in absorbance at 339 nm. Unless otherwise specified the assay concentrations, in millimoles per liter, were: L-aspartate, 180; 2-oxoglutarate, 15; tris(hydroxymethyl)methylamine buffer (pH 7.8), 89; NADH, 0.16; and pyridoxal phosphate, 0.11. Malate dehydrogenase activity was 0.95 U (25 °C) per milliliter of total reaction mixture. Reactions were initiated by adding 2-oxoglutarate. To elucidate the mechanism of oxamate's effect, we varied substrate concentrations as described in Results.

Malate dehydrogenase activity was measured by the method of Rej and Vanderlinde (16) but without adding lactate dehydrogenase or adjusting for specimen blank activity. Absorbance changes were measured with either a Cary Model
Fig. 1. Effect of oxamate concentration on activity of cytoplasmic aspartate aminotransferase
The ordinate is the ratio of initial velocity (V) obtained in the absence of oxamate to that obtained with various concentrations of oxamate (V_i). 2-Oxoglutarate concentration was varied as indicated, L-aspartate was at 120 mmol/L.

![Graph](image1)

219 spectrophotometer (Varian Instrument Div., Palo Alto, CA 94303) or LKB 2086 and 8600 reaction rate analyzers (Bio Dynamics/bmc, Indianapolis, IN 46250). All measurements were performed at 30.0 ± 0.1 °C. The temperature was verified with either a YSI Model 45 CU cuvette thermometer (Yellow Springs Instruments, Yellow Springs, OH 45387) or a Cary 219 temperature readout accessory, each calibrated against a YSI Gallium Cell (6N110).

Purified cytoplasmic and mitochondrial aspartate aminotransferases were prepared from human erythrocytes and liver as described previously (17, 18). The specific activity of each preparation was ≥150 kU/g (30 °C), and each was free from contaminant isoenzyme by the criteria of polyacrylamide and cellulose acetate electrophoresis (18). Sera from patients were selected without conscious bias from specimens collected for a hospital clinical laboratory, stored at 4 °C, and assayed within 24 h of collection.

Steady-state kinetic parameters were calculated graphically by the double-reciprocal plot method of Lineweaver and Burk (19) and were verified independently with the iterative-fit FORTRAN program described by Cleland (20).

Fig. 2. Effect of oxamate concentration on activity of mitochondrial aspartate aminotransferase
For definition of V/V_i and assay conditions, see Figure 1

![Graph](image2)

The ability of oxamate to inhibit the aspartate aminotransferase activity of human serum was investigated with sera from patients, ranging in activity from 7 to 75 U/L. Replicates of each serum were assayed as described. In one series NaCl was added at an equimolar concentration to oxamate. NaCl was selected as a control because aspartate aminotransferase activity is sensitive to anion concentration (15). Specimen blank reaction rates (in the absence of L-aspartate) were measured for each serum in the presence or absence of oxamate or NaCl. No exogenous lactate dehydrogenase was added to the test or blank mixtures. Each serum was incubated in the assay or blank mixture, lacking only 2-oxoglutarate, for 20 min at 30 °C before the assay was initiated.

**Results**

As oxamate concentrations increased up to 72 mmol/L, relative velocities decreased for both the cytoplasmic and mitochondrial isoenzymes (Figures 1 and 2, respectively). However, the inhibiting effect of oxamate was limited by increasing concentrations of 2-oxoglutarate. This phenomenon is typical for competitive inhibition. We therefore examined the effects of oxamate addition on the steady-state kinetic behavior of the isoenzymes. The results (Figures 3 and 4) confirmed that both isoenzymes are inhibited by oxamate and

![Graph](image3)

![Graph](image4)

**Fig. 3. Competitive inhibition of cytoplasmic aspartate aminotransferase for 2-oxoglutarate by oxamate**
L-aspartate concentration was 21 mmol/L. Insert: Replot of data derived from reciprocal plot: apparent K_i for 2-oxoglutarate vs. oxamate concentration. Intercept with abscissa is -K_i.

**Fig. 4. Competitive inhibition of mitochondrial aspartate aminotransferase for 2-oxoglutarate by oxamate**
For explanation, see Figure 3

![Graph](image5)
that this inhibition is competitive with respect to 2-oxoglutarate.

The apparent $K_m$ for 2-oxoglutarate were obtained from their respective primary plots and verified by direct fit of the hyperbolic curve (20). The apparent $K_i$ for oxamate was 29 mmol/L for the cytoplasmic isoenzyme and 17 mmol/L for the mitochondrial isoenzyme (Figures 3 and 4, insets). Similar $K_i$ values were calculated by Dixon's method (21) and by direct calculation of the relationship $K_{m(app)} = K_m (1 + [I]/K_i)$.

A study of the effects of oxamate on steady-state behavior with respect to L-aspartate showed noncompetitive inhibition of both isoenzymes (Figures 5 and 6). This pattern was observed at all 2-oxoglutarate concentrations from 0.2 to 10 mmol/L.

In the study of inhibition by oxamate of aspartate aminotransferase activities in 51 human sera, the results with oxamate were 95.1% of those with NaCl (Table 1). Blank reaction rates were insignificantly different ($p = 0.32$) with NaCl (1.34 ± 0.39 U/L) and oxamate (1.29 ± 0.38 U/L).

To further verify the applicability of the kinetic studies in predicting the behavior of patients' sera, we examined the effect of oxamate on the activity of 50 additional serum specimens. Two different assays were selected to study the competitive effects of 2-oxoglutarate: conditions approximating those recommended by the International Federation of Clinical Chemistry (IFCC) and some national organizations (3, 7, 8), with 2-oxoglutarate at 15 mmol/L, and conditions recommended by Henry et al. (2), with 2-oxoglutarate at 6.7 mmol/L. The concentration of L-aspartate was 180 mmol/L.

The results showed that oxamate (40 mmol/L) is inhibitory at both concentrations of 2-oxoglutarate commonly used in clinical laboratories and that the inhibition is more pronounced at the lower concentration. The inhibition was 9% for 2-oxoglutarate at 15 mmol/L and 11% at 6.7 mmol/L (Table 1). With oxamate at 10 mmol/L, a 4% inhibition was observed (Table 1).

Oxamate at concentrations up to 31 mmol/L did not affect the measurement of porcine malate dehydrogenase activity (16) and had no effect on the stability of this enzyme at 30°C during 30 min.

Discussion

Assessment of blank activity to correct measurements of aspartate aminotransferase activity by the malate dehydrogenase/NADH coupled technique has been the subject of some controversy (3, 4). Several reports suggest as an alternative that excess lactate dehydrogenase be added to equilibrate quickly the reduction of keto acids by NADH (4, 7, 8). Difficulties may be encountered, however, due to the presence of contaminant aspartate aminotransferase in some auxiliary enzyme preparations (3, 4, 22, 23). At least one recent national recommendation (24) allows for sufficient preincubation so that exogenous lactate dehydrogenase need not be added.

Because of the potential disadvantages of including exogenous lactate dehydrogenase, the suggestion that oxamate be added to the assay mixture to eliminate or reduce its side reactions (13, 14) is appealing. However, our results show that oxamate also inhibits human aspartate aminotransferases. For both isoenzymes the inhibition is competitive with respect to 2-oxoglutarate (Figures 1–4) and noncompetitive with respect to L-aspartate (Figures 5 and 6).

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### Table 1. Effect of Oxamate on Aspartate Aminotransferase Activity of Patients’ Sera

<table>
<thead>
<tr>
<th>Oxamate</th>
<th>2-Oxoglutarate</th>
<th>NaCl</th>
<th>Average inhibition, % ± 1 SEM</th>
<th>Regression equation</th>
<th>$r^2$</th>
<th>$p$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>40, 0</td>
<td>6.7</td>
<td>0</td>
<td>11 ± 1</td>
<td>$y = 0.885x + 0.06$</td>
<td>0.999</td>
<td>&lt;0.0001</td>
<td>50</td>
</tr>
<tr>
<td>40, 0</td>
<td>15.0</td>
<td>0</td>
<td>9 ± 1</td>
<td>$y = 0.933x - 0.27$</td>
<td>0.999</td>
<td>&lt;0.0001</td>
<td>50</td>
</tr>
<tr>
<td>40, 0</td>
<td>15.0</td>
<td>40</td>
<td>5 ± 1</td>
<td>$y = 0.950x + 0.90$</td>
<td>0.893</td>
<td>0.010</td>
<td>51</td>
</tr>
<tr>
<td>10, 0</td>
<td>15.0</td>
<td>0</td>
<td>4 ± 1</td>
<td>$y = 0.963x + 1.37$</td>
<td>0.964</td>
<td>&lt;0.0001</td>
<td>65</td>
</tr>
</tbody>
</table>

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*a Other experimental conditions as described in Materials and Methods; L-aspartate was present at 180 mmol/L.

*b In the form: $V_{aspartate} = a + b; correlation coefficient*.

*c Each assay system was examined in the absence and presence of oxamate as shown.

*d NaCl was included only in the control assay without oxamate; see Results.
The mitochondrial isoenzyme is particularly influenced. At a saturating concentration of 2-oxoglutarate (15.2 mmol/L) it is inhibited 15% by oxamate at 50 mmol/L (Figure 2), while the cytoplasmic isoenzyme is inhibited about 2% (Figure 1). This is consistent with the finding by Cheng et al. (15) of a more pronounced anion competition with substrate by the mitochondrial enzyme of porcine heart origin.

In human serum the enzyme was also inhibited by 9% by oxamate at 40 mmol/L and otherwise optimal concentrations of substrate (l-aspartate at 180 mmol/L and 2-oxoglutarate at 15 mmol/L). At the concentration of 2-oxoglutarate most popularly used by clinical laboratories for this assay (6.7 mmol/L), oxamate at 40 mmol/L produced an 11% inhibition. The magnitude of this inhibition is influenced by such factors as oxamate concentration, substrate concentrations, and the isoenzyme composition of the serum specimen. Inhibition should be maximum when substrate concentrations are low, when oxamate exceeds 50 mmol/L, and when serum specimens contain largely mitochondrial isoenzyme. Inhibition should be minimum in the assay of cytoplasmic isoenzyme with saturating concentrations of substrate and with oxamate concentration less than 50 mmol/L.

Minimum inhibition is reflected in the experiments with serum. Most human sera with normal aspartate aminotransferase activity contain largely the cytoplasmic isoenzyme (18, 25, 26), and the assay procedure included optimal concentrations of substrate, with conditions similar to those recommended by the IFCC (27) and other national societies (3, 7–9, 24). Since many anions affect the activity of aspartate aminotransferase (15), Cl− was included in the control assay at a concentration equal to that of oxamate. We have previously demonstrated that Cl− inhibits both the cytoplasmic and mitochondrial enzymes; the apparent K1 is 137 and 41 mmol/L, respectively (28). This inhibition has recently been confirmed by Bergmeyer et al. (29). The human enzyme was inhibited by 5% more by oxamate than by Cl− at equimolar concentrations. This is consistent with the lower K1 values for oxamate (Figures 3 and 4) in comparison with Cl−.

Although the inhibition of aspartate aminotransferase by oxamate is competitive, it could be demonstrated at saturating concentrations of 2-oxoglutarate (Figures 1 and 2).

Because oxamate is capable of inhibiting aspartate aminotransferase—in particular of mitochondrial origin—even in the presence of optimal concentrations of substrate, the benefits of including it in the assay must be carefully considered. Lustig and Redman (14) found that oxamate at 10 mmol/L successfully inhibits lactate dehydrogenase, and they reported that serum aspartate aminotransferase activities obtained in the presence of oxamate were equal to results obtained by preincubation in the absence of oxamate. Our data suggest that oxamate at this low concentration would have a small effect on the cytoplasmic enzyme (Figure 1). The small (4%) but statistically significant decrease in activity found with serum at this oxamate concentration (Table 1) is consistent with this observation.

The low blank activities found after a 20-min preincubation and the insignificant effect of oxamate on this blank suggest that the recommendation (24) for preincubation without exogenous lactate dehydrogenase is valid. Such an approach appears desirable for a reference method for aspartate aminotransferase activity.

The conditions recommended by the IFCC for measurement of aspartate aminotransferase (27) have been based in part on kinetic theory and the affinity of the enzyme for each substrate, including inhibition effects (29). Addition of oxamate to such an assay without complete reevaluation of all calculated optima would be inappropriate and inadvisable.

I am pleased to acknowledge the excellent technical assistance of Mrs. Catherine Nelli.

References


