Inhibitory Effects of Fidarestat on Aldose Reductase and Aldehyde Reductase Activity Evaluated by a New Method Using HPLC with Post-column Spectrophotometric Detection

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A new method to assay the activity of aldose reductase (AR) and aldehyde reductase (AHR) by high-performance liquid chromatography is described. The separation of AR and AHR from tissue extracts using an anion-exchange column was followed by chromatographic measurement of the activity in the elute. AR and AHR activity were expressed as the area under the peak obtained by post-column spectrophotometric detection of the decrease of coenzyme (NADPH) in each enzyme reaction. In the enzyme preparation from rat or human tissues obtained by this method, two active peaks were identified as AR and AHR. The correlation coefficient between the injection volume of the enzyme preparation from each tissue and each peak area was 0.998 or greater. In addition, the within-day preservation rate of AR or AHR activity from each tissue was over 95%.

In a comparative study of fidarestat with other AR inhibitors using this method, it was confirmed that the inhibitory effect of fidarestat on AR activity from each rat tissue was more potent than that produced by sorbinil and equipotent to that of epalrestat and zenithstat. Fidarestat was also found to inhibit AR activity more potently than AHR activity in human erythrocytes. Therefore, this method is applicable to studies of the selective inhibition of AR or AHR by test compounds.

Key words fidarestat; aldose reductase; aldehyde reductase; post-column method

Aldose reductase (AR) [EC1.1.1.21], which converts glucose to sorbitol using NADPH as a cofactor, is a rate-limiting enzyme in the polyol pathway, a sub-pathway for glycolysis, and is found in insulin-independent tissues such as lens, retina, peripheral nerve and kidney, where diabetic complications occur. Consequently, the increase in polyol pathway flux produced by hyperglycemia is considered to be one of the pathogenic factors for diabetic complications, and AR inhibitors are expected to be useful in treating diabetic complications. On the other hand, aldehyde reductase (AHR) [EC1.1.1.20], an enzyme closely related to AR, plays an important role in converting aliphatic and aromatic aldehydes to their respective alcohols using NADPH as a cofactor. The true activities of AR or AHR cannot be obtained from crude extracts containing enzymes that change the amount of NADPH other than AR or AHR, although these enzyme activities have been assayed previously using crude extracts. Thus, assays of the activity of AR or AHR are currently performed after purification by the method of Das and Srivastava and Shiono et al. However, the activity of purified AR and AHR is susceptible to a breakdown which is time-dependent, so the activity of these purified enzymes cannot be assayed repeatedly. Consequently, to investigate the effect of test compounds on AR or AHR activity, a method for multiple assays is required. In addition, in the case of tissues with a very low AR or AHR content like erythrocytes, these enzyme activities need to be assayed without any loss.

In this report, we describe a post-column method to solve the above-mentioned problems. This new method also shortens the time and simplifies the procedure required to assay the enzyme activity.

In addition, using this method, we investigated the effects of a potent AR inhibitor, fidarestat (SNK-860, (+)-(2S,4S)-6-fluoro-2',5'-dioxospino[chroman-4,4'-imidazolinediene]-2-carboxamide, Sanwa Kagaku Kenkyusho, Fig. 1), on the AR and AHR activities of various tissues. These results are also compared with those of other AR inhibitors.

MATERIALS AND METHODS

Chemicals D,L-Glyceraldehyde, D-glucose, D-glucuronic acid and NADPH were purchased from Wako Pure Chemicals Co. (Osaka, Japan). Deionized and distilled water purified with a MilliQ Purification System (Millipore, Milford, MA, U.S.A.), was used for all aqueous solutions. All AR inhibitors, including fidarestat, were prepared at Sanwa Kagaku Kenkyusho Co., Ltd. (Mie, Japan).

Enzyme Preparation Lens, sciatic nerve and kidney were rapidly removed after rats (male, Sprague–Dawley) were exsanguinated from the abdominal aorta under ether anesthesia, and stored at −80°C until use. Each tissue was minced using scissors or knives and homogenized in approximately 2–20 volumes of 10 mM sodium phosphate buffer (pH 7.0) containing 5 mM 2-mercaptoethanol (A) using a Polytron homogenizer. The enzyme extract was prepared by centrifugation of the homogenate at 5000 × g for 10 min at 4°C and dialysis of the supernatant, or by centrifugation at 100000 × g for 60 min at 4°C and concentration of the supernatant using an Amicon Centricon-10 (Millipore, Bedford, MA, U.S.A.). Then, the enzyme extract was stored at −80°C

Fig. 1. Chemical Structure of Fidarestat ((+)-(2S,4S)-6-Fluoro-2',5'-dioxospino[chroman-4,4'-imidazoline]2-carboxamide)

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until assay.

Human and rat erythrocytes were obtained by centrifugation at 1700×g for 10 min at 4°C following collection of blood samples with heparinized syringes from healthy volunteers, and from rat abdominal aorta under ether anesthesia. The erythrocytes were washed thoroughly with cold physiological saline, and hemolyzed by adding three volumes of 10 mM potassium phosphate buffer containing 5 mM 2-mercaptoethanol (pH 7.0). The hemolysates of human and rat erythrocytes were partially purified by the method of Das and Srivastava\(^9\) and Shiono et al.\(^10\) respectively.

**Chromatographic System** Chromatography was performed with an LC-10 high-performance liquid chromatograph (Shimadzu, Kyoto, Japan). To separate AR from rat tissue extract, the extract was injected into a DEAE-5PW column (75×7.5 mm i.d.; Tosoh, Tokyo, Japan) which had been equilibrated with buffer A, and the column was washed with the same buffer for 10 min. AR was then eluted with a linear gradient of buffer A from 0 to 250 mM NaCl for 25 min at room temperature. The flow rate of the mobile phase was 0.5 mL/min.

To separate AHR from kidney extract, the extract was injected into the same column which had been equilibrated with 20 mM Tris-HCl buffer (pH 7.2) containing 5 mM 2-mercaptoethanol (B) and the column was washed with the same buffer for 10 min. AHR was then eluted with a linear gradient of buffer B containing 0.5 M NaCl from 0 to 500 mM NaCl for 25 min at room temperature. The flow rate of the mobile phase was 0.5 mL/min.

To separate AR or AHR from human erythrocyte extract, the extract was injected into the same column which had been equilibrated with buffer A containing 0.025 M NaCl, and AR or AHR was eluted with the same buffer for 50 min at room temperature. The flow rate of the mobile phase was 0.4 mL/min.

**Post-column Detection** The column eluate was mixed with 0.25 M potassium phosphate buffer (reaction buffer) containing 12.5 mM 2-mercaptoethanol, the substrate and cofactor (pH 6.0 or 7.0 for the AR or AHR reaction, respectively), were delivered by LC-10 Ai pumps (Shimadzu, Kyoto, Japan) at a flow rate of 0.4 mL/min. The final concentration of D-glucose or D,L-glyceraldehyde as a substrate for AR, or D-glucuronic acid as a substrate for AHR, was 0.40—0.44, 0.01 or 0.011—0.015 M, respectively. NADPH, the cofactor for both enzyme reactions, was first dissolved in distilled water to give a final concentration of 0.1 mM.

This mixture was passed through a reaction coil to allow the enzyme reaction to proceed for 1—5 min at 37°C, and the absorbance of NADPH in the final eluate was monitored at 340 nm using a Shimadzu SPD-6AV spectrophotometer (Kyoto, Japan). The enzymatic activity was expressed as the area under the peak of the upside-down chromatogram, determined with a Shimadzu Workstation CBM-10A analyzer. To investigate the effects of AR inhibitors on AR or AHR activity, various concentrations of each inhibitor, dissolved in distilled water or 1% methanol, were added to each reaction buffer, and the enzyme activity was assayed under the conditions described above.

**Calculation of 50% Inhibitory Concentration (IC\(_{50}\))** The concentration of inhibitor added to the assay system and the activity of AR or AHR (the peak area obtained by the

DEAE post-column method) were plotted using the Dixon plot method,\(^{44}\) and a regression analysis was conducted by the least squares method.

**Dixon plot**

X-axis: the inhibitor concentration
Y-axis: 1/V (= the reciprocal of the peak area of AR or AHR activity)
Equation for IC\(_{50}=(\text{the reciprocal of the peak area without the inhibitor})\times2—Y\text{ intercept}/X\text{ coefficient}

**RESULTS AND DISCUSSION**

**Identification of AR and AHR** In the analysis of the enzyme preparation from rat kidney, two active peaks were found (Fig. 2A). Peak 2 was identified as AR because it increased in the presence of SO\(_4^2-\) ion.\(^{15}\) This was also confirmed in the analysis of the enzyme preparation from rat lens, sciatic nerve and erythrocytes (Table 1). Peak 1 was difficult to evaluate correctly because it was close to the column void volume, and although other anion contaminants interfered with detection of the peak, it was assumed to be AHR activity. Therefore, AHR was separated from the rat enzyme preparation by altering the method to obtain Fig. 2A, which gave two peaks far from the column void volume (Fig. 2B). Peak 1 obtained with D-glucuronic acid was much larger than peak 2. In addition, it has been reported that the elution of AHR from a DEAE-cellulose column is faster than that of
AR in the analysis of a rat enzyme preparation. Consequently, peak 1 was found to be AR activity.

In the analysis of the enzyme preparation from human erythrocytes, two active peaks were also observed (Fig. 3). AR and AHR from human erythrocytes were defined as follows according to Das and Srivastava.1) The peaks obtained by DEAE-cellulose column chromatography. 2) The peaks obtained by reaction with glyceraldehyde or glucuronide as a substrate using NADPH as a cofactor (AR and AHR are more reactive to glyceraldehyde and glucuronide, respectively). 3) The large and small peak in the presence of SO_{2}^{2-} ion indicate AR activity and AHR activity, respectively. Peak 1 on DEAE-cellulose column chromatography obtained with glyceraldehyde was larger than that with glucuronide. On the other hand, peak 2 obtained with glucuronide was smaller than peak 2 with glyceraldehyde (Table 2). In addition, the presence of SO_{4}^{2-} ion increased peak 1, while peak 2 was reduced (Table 3). Therefore, peak 1 and 2 were identified as AR and AHR, respectively.

**Linearity and Precision** Linearity was estimated by investigating the relationship between the injection volume of the enzyme preparation and the peak area of AR or AHR on the chromatogram. The injection volume of the enzyme preparation from rat kidney correlated well with each peak area within the range of 5–20 μl (y=24450x+2001 r=0.999 for AR; y=70105x+31282, r=0.998 for AHR). In the enzyme preparation from rat tissues, the correlation coefficient was 0.999 or greater. In addition, this close relationship was also found in enzyme preparations from human erythrocytes at the volume between 50 and 150 μl (y=358x–3964, r=0.999 for AR; y=1879x–3002, r=1.000 for AHR). These results confirm the high degree of linearity for assays of AR or AHR activity.

Each enzyme preparation was preserved at 4°C for 6 h to perform all assays, so the within-day preservation rate of enzyme activity was investigated over 6 h, after keeping each enzyme preparation at 4°C, as the index of precision for assays of AR or AHR activity. The preservation rate of AR or AHR activity from each tissue was over 95%, which also assures high assay precision.

### Table 2. Effects of Substrates on Peak 1 and 2 Areas Obtained in the Analysis of Enzyme Preparations from Human Erythrocytes

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Peak 1 area (%)</th>
<th>Peak 2 area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D,L-Glyceraldehyde</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>D-Glucuronic acid</td>
<td>66</td>
<td>135</td>
</tr>
</tbody>
</table>

### Table 3. Effects of Sodium Sulfate on Peak 1 and 2 Areas Obtained in the Analysis of Enzyme Preparations from Human Erythrocytes

<table>
<thead>
<tr>
<th>Na_{2}SO_{4} (0.4 mM)</th>
<th>Peak 1 area (%)</th>
<th>Peak 2 area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>+</td>
<td>206</td>
<td>50</td>
</tr>
</tbody>
</table>

a) D,L-Glyceraldehyde was used as substrate. b) D-Glucuronic acid was used as substrate.

### Table 4. Effects of AR Inhibitors on AR Activity from Rat Lens and Kidney

<table>
<thead>
<tr>
<th>Fidarestat</th>
<th>Sorbinil</th>
<th>Zenarestat</th>
<th>Epalrest</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC_{50} against lens AR</td>
<td>18 nm</td>
<td>280 nm</td>
<td>11 nm</td>
</tr>
<tr>
<td>IC_{50} against kidney AR</td>
<td>29 nm</td>
<td>440 nm</td>
<td>10 nm</td>
</tr>
</tbody>
</table>

**Effects on AR Activity from Rat Tissues** To compare the effect of fidarestat on AR activity with other AR inhibitors, enzyme preparations from lens and kidney with a high AR content were used to assay the AR activity with D,L-glyceraldehyde as a substrate. The IC_{50} value of each inhibitor was calculated (A, B, C and D in Fig. 4), and the IC_{50} values of the other AR inhibitors were similar to previous results. The inhibitory effect of fidarestat on AR activity from each tissue was more potent than that of sorbinil and equipotent to that of zenarestat and epalrest (Table 4). In addition, the IC_{50} value of fidarestat against AR activity from sciatic nerve was 28 nm, which implies that fidarestat inhibits AR activity to the same extent in all tissues.

**Effects on AHR Activity from Rat Kidney** To compare the effect of fidarestat on AHR with other AHR inhibitors, AHR from kidney with a high AHR content was used with D-glucuronic acid as a substrate, and the IC_{50} value of each inhibitor was calculated. Fidarestat, sorbinil, zenarestat and epalrest inhibited AHR activity with IC_{50} values of 1.3, 1.7, 12.0 and 8.6 μM, respectively. Therefore, the relative selectivity of fidarestat, sorbinil, zenarestat and epalrest for AHR inhibition (IC_{50} value against kidney AHR/IC_{50} value against kidney AR) was 45, 4, 1200, 176, respectively. It has been reported that even sorbinil, with the lowest relative selectivity, did not produce any clinical side-effects related to AHR inhibition, which suggests that fidarestat, with a relative selectivity over 10-fold greater than sorbinil, will also have no clinical side-effects. In fact, fidarestat has been shown to be very safe in diabetic patients so far (unpublished observation).

**Effects on AR and AHR from Human Erythrocytes** To estimate the effects on AR and AHR from human tissues, AR and AHR from human erythrocytes were used to assay the activity with D-glucose and D-glucuronic acid as substrate, respectively. Only the effect of fidarestat on AR and AHR activity was investigated because human erythrocytes...
have very low levels of AR and AHR. Considering that AR inhibitors are expected to be therapeutic drugs for diabetic complications due to hyperglycemia, D-glucose is more suitable, especially in human tissues, than D,L-glyceraldehyde as a substrate for assaying AR activity. The IC$_{50}$ values of fidarestat against AR and AHR were 9 nM (A in Fig. 5) and 1.3 μM, respectively, and the relative selectivity of fidarestat for AR inhibition was 144, which suggests that fidarestat produces highly selective inhibition of human AR. In addition, the effect of fidarestat on AR activity from rat erythrocytes was investigated using D-glucose as a substrate, which gave an IC$_{50}$ value of 57 nM (B in Fig. 5). Therefore, these results indicate that fidarestat is a more potent inhibitor of human AR than rat AR. We will investigate the effects of the other AR inhibitors on human AR and AHR in the future because it is presumed that there will be differences in their IC$_{50}$ values for AR or AHR between human and rat tissues as is the case with fidarestat.

In conclusion, this method allows the selective assay of both AR and AHR from kidney. This method is faster and simpler than previous methods as far as the collection of enzyme fractions and assay procedure are concerned. In addition, the method allows the assay to be performed without loss of AR or AHR activity from human erythrocytes, which have very low levels of AR and AHR. Therefore, this new method is applicable to studies of the selective inhibition of AR or AHR by test compounds using tissues containing both enzymes, or very low levels of either enzyme.

Acknowledgement We would like to thank Dr. N. Tomiya for his help with the evaluation of AR inhibitors.

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Fig. 4. Inhibitory Effects of Fidarestat (A), Sorbinil (B), Zenarestat (C) and Epalrestat (D) on Rat Lens AR Activity Using the Dixon Plot Method D,L-Glyceraldehyde was used as substrate.

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Fig. 5. Inhibitory Effects of Fidarestat on Human (A) or Rat (B) Erythrocyte AR Activity Using the Dixon Plot Method D-Glucose was used as substrate.
REFERENCES