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Note

Improved high-performance liquid chromatographic determination of diamine oxidase activity

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Diamine oxidase [diamine:oxygen oxidoreductase (deaminating), EC 1.4.3.6.] (DAO) catalyses the oxidation of various diamines, such as putrescine and cadaverine, to the corresponding aminoaldehydes, which are in equilibrium with their cyclic condensation products, Δ^1-pyrroline and Δ^1-piperideine, respectively.

Since determination of DAO activity has been proposed as a marker of intestinal mucosa integrity [1] its importance in clinical studies has increased [2,3]. As an useful alternative to the generally used radiometric measurements [4,5], we recently developed a high-performance liquid chromatographic (HPLC) method that was applied to plant extracts and plasma samples [6].

In order to study DAO distribution in the intestinal tract of domestic animals, we propose in this note a modification of our method, which makes it more rapid, simple and suitable for routine analysis of tissue homogenates. The classical method of Holmsted et al. [7], using *o*-aminobenzaldehyde (OAB) to trap aminoaldehydes obtained from enzymic oxidation of short-chain aliphatic diamines, was followed. However, the resulting 1,2-dihydroquinazolinium ions have been directly analysed by HPLC, without the need for chemical oxidation to 4-quinazolones and extraction with organic solvents employed in our previous method. Moreover, the final HPLC analysis was not performed by the normal-phase technique, but on reversed-phase columns. In order to demonstrate the suitability of this new procedure, DAO activity was measured in dog

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intestinal mucosa samples; the optimal assay conditions, the specificity and reproducibility of the revised analytical method were studied.

EXPERIMENTAL

Materials

OAB was prepared by reducing *o*-nitrobenzaldehyde [8]. A solution of Δ^1-pyrroline, used as internal standard, was obtained by treating 4-aminobutanal diethylacetal (Janssen, Beerse, Belgium) with 50 m*M* hydrochloric acid. Tissue homogenates were prepared from mucosa scraped from dog small intestine and treated in an Ultraturrax 10 N homogenizer with 10 volumes of ice-cold 0.1 *M* phosphate buffer (pH 7.0) and centrifuged for 60 min at 25 000 *g* in a refrigerated Beckmann J2-21 centrifuge. DAO from pea seedlings was prepared according to the method of Hill [9] up to step 4.

Apparatus

A Jasco (Tokyo, Japan) HPLC system equipped with a Familic 300 S pump and an Uvidec 100-V detector was used. Preliminary kinetic experiments were performed with the same Jasco Uvidec 610 spectrophotometer used to obtain UV spectra.

Chromatographic conditions

Separations were carried out on a cartridge (25 cm×4 mm I.D.) filled with LiChrosorb RP-8 (7 μm) mounted on a Manu-Fix holder. A mixture of 0.1 *M* phosphoric acid adjusted to pH 3 with triethylamine–acetonitrile (85:15, v/v) was used as eluent at a flow-rate of 1.0 ml/min. The absorbance was measured at 465 nm.

DAO assay

Mucosal extracts (0.5 ml) were preincubated at 37 °C with 1 m*M* Δ^1-pyrroline solution (50 μl) and OAB-saturated aqueous solution (100 μl). The enzymic reaction was started by adding 0.1 *M* cadaverine solution in 0.1 *M* phosphate buffer, pH 7.0 (50 μl). After 20 min the tube containing the assay mixture was placed on a sand-bath maintained at 155 °C for 2 min. After centrifugation, aliquots (10 μl) were analysed by HPLC.

In order to verify the specificity of the assay, samples obtained from the same mucosa specimen were preincubated either as described above or after the addition of a 10 m*M* solution (100 μl) of aminoguanidine, chloral hydrate or acetaldehyde.

Quantitative analysis

Amounts of cadaverine varying between 5 and 200 nmol were converted into Δ^1-piperideine by incubation for 30 min with a large excess of pea seedling DAO

in 0.1 *M* phosphate buffer (pH 7.0) containing OAB (total volume 0.65 ml). A 50-μl volume of a 1 m*M* solution of the internal standard Δ^1-pyrroline was finally added, and the mixture was treated in a sand-bath at 155 °C for 2 min. The resulting 2,3-tetramethylene-1,2-dihydroquinazolinium ion (tetraMDHQ), obtained from Δ^1-piperideine, and 2,3-trimethylene-1,2-dihydroquinazolinium ion (triMDHQ), obtained from Δ^1-pyrroline, were analysed by HPLC. The ratio of their peak heights was calculated and plotted against the amounts of treated cadaverine. The DAO activity (in mU/g) in a tissue homogenate sample was obtained from the corresponding amount in nanomoles of tetraMDHQ formed by enzymic oxidation of cadaverine. [One milliunit (mU) is defined as the amount of enzyme that catalyses the oxidation of 1 nmol of cadaverine per minute under the described conditions.]

RESULTS

The quinazolinium ions, purified by HPLC, exhibited absorption spectra with λ_{max} at 295 and 465 nm for tetraMDHQ and at 278 and 434 nm for triMDHQ in the eluent mixture.

Fig. 1 shows a typical chromatographic profile from a sample obtained from dog intestinal mucosa extract. A linear relationship between the ratio of the peak heights (R_h) of tetraMDHQ and triMDHQ and the amounts of oxidized

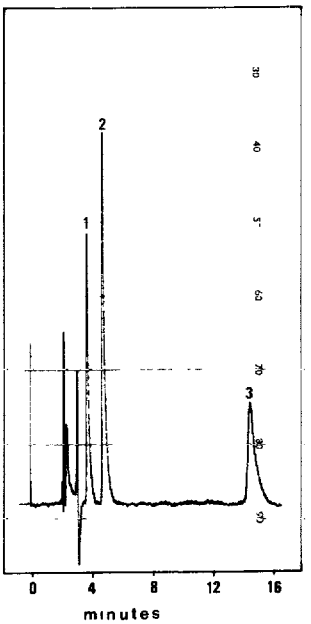

Fig. 1. Typical HPLC profile from a sample of small intestine extract assayed for DAO activity. Peaks: 1 = triMDHQ; 2 = tetraMDHQ; 3 = OAB.

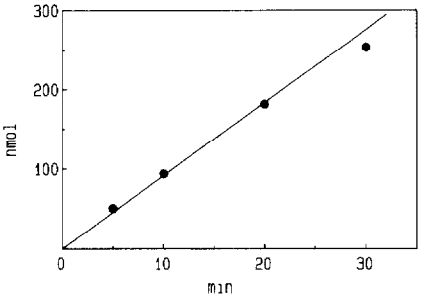

Fig. 2. Time dependence of the appearance of the oxidation product obtained from cadaverine.

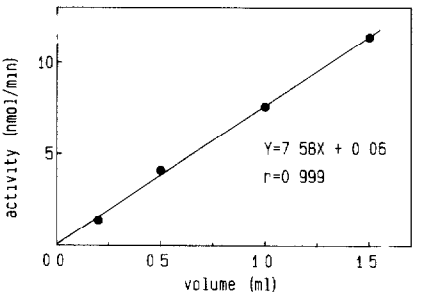

Fig. 3. Relationship between the measured DAO activity and the extract volumes.

cadaverine was found in the range 5–200 nmol, according to the equation $R_h = 0.0134x + 0.010$ ($r = 0.997$) where x is the amount of oxidized cadaverine in nanomoles. By this method tetraMDHQ could be detected in amounts as low as 1 nmol, corresponding to 0.05 mU of DAO activity.

The time dependence of the appearance of the product of enzymic oxidation is shown in Fig. 2. The amount of tetraMDHQ formed was linear with respect to time for at least 20 min. The detected activity was also shown to be linear with different volumes of tissue extract (Fig. 3).

The reproducibility of the method was determined from repeated analyses of the same mucosa extract. DAO activity was found to be 85.4 ± 3.3 mU/g (mean $\pm$ S.D., $n = 5$).

The specificity of the assay was verified by adding in the preincubation step either aminoguanidine as inhibitor of DAO or chloral hydrate or acetaldehyde as inhibitors of aldehyde dehydrogenase. In the first case no peak appeared in the region of the chromatogram corresponding to tetraMDHQ; in the second case no significant difference was detected in tetraMDHQ/triMDHQ ratio between samples treated and untreated with inhibitors.

DISCUSSION

Like the previously described HPLC method, our approach is based on the procedure introduced by Holmsted et al. [7] for the spectrophotometric assay of DAO, which exploits the trapping of Δ^1-pyrroline with OAB. In order to adapt the original spectrophotometric method to the HPLC technique, and to improve the ease and the speed of our previously described HPLC method, preliminary tests were carried out to find the optimal conditions for each step of the analytical procedure.

The availability of 4-aminobutanal diethylacetal, a precursor of Δ^1-pyrroline which is a useful internal standard, confirmed cadaverine as the substrate of choice for the enzymic assay. The dependence of the measured activity on the

ratio of the tetraMDHQ and triMDHQ peak heights rather than on the peak corresponding to the oxidized substrate alone ensures the reliability of the final results.

The time dependence of the appearance of the product was found to be linear for only 20 min. This was not unexpected, because studies carried out by Macholan [10] on 1,2-dihydroquinazolinium salts demonstrated that their stability at neutral pH, although increased by the presence of OAB, was not so high as at low pH values over long periods of incubation at 37°C.

The problem of terminating the enzymic reaction has been the most difficult one to resolve. In preliminary experiments trichloroacetic acid was used, but it caused great interference with the peaks of tetraMDHQ and triMDHQ in the final chromatogram. The same interference was reported in the HPLC assay of ornithine aminotransferase, according to O'Donnell et al. [11], where Δ^1-pyrroline-5-carboxylic acid reacted with OAB.

Furthermore, the rate of the reaction between Δ^1-pyrroline and Δ^1-piperideine with OAB clearly depends on the pH, being higher at neutral than at very low pH values. Thus, in order to exploit both the kinetic features of the post-enzymic reaction and to terminate in a reliable way the enzymic oxidation, the procedure recommended by Rossomando [12] was followed. This consists of brief treatment of the sample at high temperature (2 min at 155°C) which, in our experience, ensures both inactivation of the enzyme and complete formation of the 1,2-dihydroquinazolinium ion. In fact, the tetraMDHQ/triMDHQ peak-height ratio did not increase after a second incubation subsequent to the heat treatment, indicating that complete denaturation of the enzyme was achieved. Conversely, a reaction mixture containing Δ^1-pyrroline or Δ^1-piperideine and OAB reached the maximum absorbance at 434 and 465 nm, respectively, either after the described heat treatment at neutral pH or after longer periods at acidic pH and at lower temperatures, i.e. in the conditions (15 min at 80°C in 5% trichloroacetic acid) chosen in our previous HPLC assay.

With respect to the chromatographic conditions, the eluent composition was chosen to increase the speed of the analysis. We used the maximum ratio of acetonitrile to aqueous phase suitable to separate triMDHQ, the first significant peak, from the immediately foregoing negative peak caused by the sample injection. To decrease the analysis time further earlier elution of OAB could be obtained by programming a higher flow-rate after the appearance of the tetraMDHQ peak. The eluent absorbance was measured at the λ_{max} of tetraMDHQ in the visible region, because interfering peaks from the sample appeared when the detector was set at 295 nm, the other λ_{max} of tetraMDHQ in the UV region. In spite of the lower energy emitted by deuterium lamp in the visible than the in the UV region, a linear response was obtained against different amounts of injected sample also at 465 nm. On the other hand, a significant increase in sensitivity was not achieved using an Uvidec 100-III

detector equipped with a tungsten lamp, which can emit higher energy than the deuterium lamp at 465 nm.

It is noteworthy that the HPLC analysis was more selective than the spectrophotometric assay using putrescine as substrate, because at the λ_{max} of the resulting triMDHQ, excess OAB gives a large absorption background. On the contrary, OAB is completely separated during HPLC.

The specificity of the method has been demonstrated both by complete inactivation of the enzymatic oxidation of cadaverine by aminoguanidine, a known DAO inhibitor, and by the non-dependence of the results on the presence of chloral hydrate and acetaldehyde, inhibitors of aldehyde dehydrogenase, which could oxidize aminoaldehydes produced from diamines, the substrates of DAO [13].

In conclusion, the procedure described offers some advantages over the corresponding spectrophotometric method (sensitivity, specificity of the absorbance response and reproducibility due to the use of an internal standard) or over our previously reported HPLC method (ease of routine handling and the use of stable reversed-phase columns). By this analytical protocol DAO activity in the intestinal tract of domestic animals under normal and pathological status will be studied.

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