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# Modified high-performance liquid chromatographic determination of diamine oxidase activity in plasma

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## SUMMARY

A previous high-performance liquid chromatographic determination of diamine oxidase activity suitable for tissue homogenates was modified in order to adapt it to plasma samples. Simple additional steps were introduced after the enzyme reaction and before the chromatographic separation, both according to the previous method. In this way the sensitivity and the reproducibility of the overall procedure was suitable for routine plasma diamine oxidase estimations.

## INTRODUCTION

Diamine oxidase [diamine:oxygen oxidoreductase (deaminating), E.C.1.4.3.6] (DAO), catalyses the oxidation of diamines, such as putrescine and cadaverine, to the corresponding aminoaldehydes, which are in equilibrium with $\Delta^1$-pyrroline and $\Delta^1$-piperideine, respectively. DAO is present in high concentration in the intestinal mucosa of man and other mammalian species[1]. The determination of DAO in this tissue has been considered to be a marker of its integrity in different pathological conditions[2–4]. Further, the determination of DAO activity in plasma has been used to monitor by non-invasive means the severity of injury of the intestinal mucosa in rat[5,6] and man[7].

In order to set up a high-performance liquid chromatographic (HPLC) procedure as an alternative to radiochemical measurements[8], we followed the classical method of Holmstedt *et al.*[9]. The aminoaldehyde obtained from enzymatic oxidation of cadaverine was allowed to react with *o*-aminobenzaldehyde (OAB) to give the 2,3-tetramethylene-1,2-dihydroquinazolinium ion ($4Q^+$); pyrroline was used as an internal standard, giving 2,3-trimethylene-1,2-dihydroquinazolinium ion ($3Q^+$)[10]. Recently, we modified the original method in order to speed it up and make it suitable for routine DAO assays in dog tissue homogenates[11]. $4Q^+$ was analysed directly on

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a reversed-phase column without any other post-enzymatic reaction. When we attempted to apply the modified procedure to plasma sample, some problems arose with regard to terminating the enzyme incubation and sensitivity.

This paper describes the modifications introduced to adapt the procedure to the evaluation of DAO activity in plasma. The modified method was applied to dog plasma.

## EXPERIMENTAL

### *Materials*

Dog plasma samples were obtained in heparinized tubes by routine venous puncture from normal healty adult animals and stored frozen until analysis. Other enzyme preparations, reagents and standard solutions were as described previously[11].

### *Apparatus and chromatographic conditions*

The UV spectrophotometer, HPLC system and operating conditions were as described previously[11].

### *Methods*

To 0.5 ml of plasma sample, 0.5 ml of 0.1 *M* phosphate buffer (pH 7.0), 100 $\mu$l of saturated aqueous OAB solution and 50 $\mu$l of 1 m*M* $\Delta^1$-pyrroline solution were added. After a 10-min preincubation at 37°C, the enzyme reaction was started by the addition of 50 $\mu$l of 0.1 *M* cadaverine, buffered at pH 7.0. After 20 min, the incubation was stopped by diluting the mixture with 1 ml of acetonitrile. The suspension was centrifuged, the precipitate was discarded and the supernatant was tranferred into a screw-capped vial; acetonitrile was then evaporated at 37°C under a stream of nitrogen. To the resulting aqueous solution, 1 ml of 0.1 *M* glycine buffer (pH 12) was added; 4 ml diethyl ether were then added and the mixture was shaken well. The organic phase was separated, filtered through anhydrous sodium sulphate and evaporated to dryness under a stream of nitrogen. The residue was finally dissolved in 50 $\mu$l of the HPLC eluent and injected.

### *Quantitative analysis*

Different amounts (1–100 nmol) of $\Delta^1$-piperideine in 1 ml of 0.1 *M* phoshate buffer (pH 7.0) were obtained by oxidation of cadaverine with DAO from pea seedlings. These samples were treated according to the procedure described above. The height ratios between the $4Q^+$ and $3Q^+$ peak were calculated in the final chromatograms and plotted against $\Delta^1$-piperideine amounts to prepare a calibration graph. The DAO activity in mU/ml in a plasma sample was obtained from the corresponding amount of $4Q^+$ formed by enzymatic oxidation of cadaverine; 1 mU is defined as the amount of enzyme that catalyses the oxidation of 1 nmol of cadaverine per minute under the described conditions.

The recovery of $4Q^+$ from post-enzymatic steps was studied by comparing the $4Q^+$ HPLC response before and after addition of glycine buffer, extraction, drying and redissolution in the same original volume.

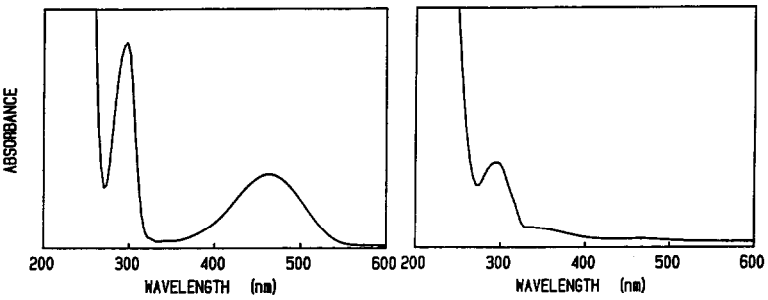


Fig. 1. UV spectra of HPLC eluent mixture of $4Q^+$ (right) and 4QOH (left), obtained from $4Q^+$ purified by HPLC before and after addition of 0.1 *M* glycine buffer (pH 12), respectively.

## RESULTS

Fig. 1. shows the UV spectra of $4Q^+$ and its derivative obtained after the pH increase, 2,3-tetramethylene-1,2,3,4-tetrahydro-4-hydroxyquinoline (4QOH). By monitoring the change in the UV spectrum, the progress of the conversion of $4Q^+$ to 4QOH was followed; the absorption at 465 nm disappeared when pH 11 was reached. A similar behaviour was shown by $3Q^+$, obtained from $\Delta^1$-pyrroline used as an internal standard. When the 4QOH solution was again acidified, the original spectrum was obtained, demonstrating the reversibility of hydroxide addition to $4Q^+$. Different amounts of $\Delta^1$-piperideine in the presence of the same amount (50 nmol) of $\Delta^1$-pyrroline were treated according to the described post-enzymatic steps and a linear relationship between $4Q^+/3Q^+$ peak-height ratio ($R_h$) and piperideine amount was found:

$$R_h = 0.0265 \text{ (nmol } \Delta^1\text{-piperideine)} + 0.0176 \ (r = 0.998)$$

Using the described procedure, $4Q^+$ could be detected in amounts as low as 0.5 nmol, corresponding to 0.025 mU of DAO activity; $4Q^+$ (50 nmol), after conversion to 4QOH, extraction with diethyl ether and evaporation, was recovered with yields of 65 $\pm$ 3.1% ($n = 5$).

The reproducibility of the method was determined by repeated analyses of the same plasma sample. The mean DAO content was 1.35 $\pm$ 0.10 mU/ml ($n = 5$). In ten samples from different normal healthy adult dogs the DAO activity was found to be between 0.18 and 1.41 mU/ml with a mean of 0.62 mU/ml.

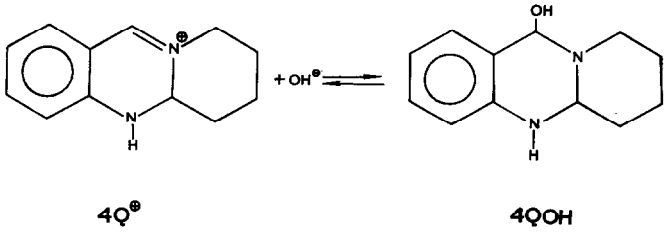


Fig. 2. Equilibrium reaction between $4Q^+$ and 4QOH.

## DISCUSSION

With regard to DAO assay, plasma samples appeared to differ from intestinal mucosa homogenates, mainly in two respects, lower DAO activity and higher protein content. When we attempted to adapt our modified HPLC procedure, suitable for tissue homogenates, to plasma, we encountered two difficulties, low sensitivity and poor reproducibility.

In the previous procedure, the incubation mixture was injected directly onto the HPLC column after the inactivation of DAO by heat treatment. When a low content of DAO was present in the sample, the final chromatographic profile did not allow the determination of small amounts of $4Q^+$. However, with plasma specimens the heat treatment always gave rise to a final mixture appearing as a gel because of its high protein content, and the final withdrawal of the supernatant solution was very difficult. The usual method of increasing the sensitivity of a HPLC method is to extract the compound to be analysed into a volatile solvent, evaporate it to dryness and dissolve the residue in a small volume of eluent. As the condensation product of piperideine and OAB is an ionic compound at pH 7, it was not possible to apply this method directly to the final incubation mixture. Nevertheless, a pH increase is known to convert $4Q^+$ to 4QOH according to the equilibrium shown in Fig. 2. By exploiting this reaction, we modified the procedure with simple additional steps: addition of alkaline buffer to pH between 11 and 12 and extraction of QOH with diethyl ether. After evaporation of the organic phase, a final concentrated solution of the analyte is obtained and a higher sensitivity is achieved. It is remarkable that, when 4QOH is dissolved in an acidic eluent solution, $4Q^+$ is again formed. Hence the subsequent HPLC analysis can be performed according to the previous chromatographic procedure[11].

The operating conditions for the conversion of $4Q^+$ to 4QOH were checked. Satisfactory recovery of $4Q^+$ after its hydroxylation and extraction was achieved only when the pH was carefully adjusted to between 11.0 and 12.0 with glycine buffer and extraction was performed immediately. When pH 12 was exceeded or extraction was delayed, only low yields of $4Q^+$ were obtained, demonstrating the instability of this compound in alkaline medium. Despite the poor recovery of $4Q^+$ after conversion to 4QOH, the reduction of the final volume to be analysed and the good reproducibility made the overall procedure convenient for achieving a suitable sensitivity.

For terminating the enzyme incubation, addition of acetonitrile was chosen instead of heat treatment. In this way, precipitated proteins were discarded after centrifugation and a clear aqueous acetonitrile mixture was obtained. However, as in the presence of acetonitrile the reaction between aminoaldehydes and OAB does not occur to a significant extent, the organic solvent was evaporated. It is noteworthy that at neutral pH the rate of formation of $4Q^+$ is clearly higher than that at acidic or slightly alkaline pH. Hence, at the end of the evaporation step, carried out at pH 7.0 and 37°C, the condensation between aminoaldehydes and OAB reached completeness and, at the same time, the sample volume was decreased, making the subsequent extraction step more efficient.

In conclusion, the DAO content of plasma can be determined by direct HPLC of $4Q^+$, provided that additional steps are introduced in the previously described procedure. The sensitivity and reproducibility of this modified method are suitable for routine plasma DAO determinations in both biochemical and clinical experiments.

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