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FLUORIMETRIC DETECTION OF OCTOPAMINE IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS APPLICATION TO THE ASSAY OF DOPAMINE β-MONOOXYGENASE IN HUMAN SERUM

TORGEIR FLATMARK, TORE SKOTLAND, TORBJØRN LJONES and OLE C. INGEBRETSEN

Department of Biochemistry, University of Bergen, Årstadvollen, N-5000 Bergen (Norway)

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SUMMARY

A high-performance liquid chromatographic procedure is described for the determination of octopamine. The method, which is based on the separation on a microparticulate bonded strong cation-exchange resin and measurement of the native fluorescence, has been applied to give a sensitive assay of dopamine β-monooxygenase (EC 1.14.17.1) activity in human serum with tyramine as the substrate. The procedure, which has been designed for use with an automatic sampler, has a detection limit of about 50 pmoles of octopamine, and the analysis time is approximately 10 min per sample.

INTRODUCTION

A number of high-performance liquid chromatographic (HPLC) systems have recently been found to offer convenient methods for the microanalysis of catecholamines [1—4], using different detectors. So far, three principles of detection have been used: an electrochemical detector [1], a UV detector [2—4] and, more recently, fluorimetric detection following derivatization with *o*-phthalaldehyde [5]. Due to the relatively low sensitivity of the UV detector and the experimental complexity of the other two methods of detection, an alternative method has been developed in the present study.

The native fluorescence of catecholamines is considered to be weak [6], but has nevertheless been used for a simple and rapid assay of total catecholamines in tissues such as the adrenal gland [7]. The application of this method to crude extracts of biological materials is, however, complicated by considerable interference from various endogenous constituents which exhibit native fluorescence, at the same or similar wavelengths as the catecholamines, or by compounds which quench the catecholamine fluorescence [8]. These problems

are eliminated when HPLC is used. In the present report we describe the detection of octopamine (and tyramine), based on its native fluorescence, which gives a sensitivity comparable to the electrochemical detector [1] and the fluorescence derivative method [5]. However, due to its simpler experimental approach, native fluorescence can also be more easily applied to automated analysis to meet the special requirements of multiple analyses. We have found the method very useful in the assay of the dopamine β-monooxygenase (dopamine β-hydroxylase) catalysed hydroxylation of tyramine to octopamine in, for example, human serum.

EXPERIMENTAL

Materials

Tyramine HCl was obtained from Koch-Light (Colnbrook, Great Britain), DL-octopamine HCl (*p*-hydroxyphenyl ethanolamine) and catalase (crystalline, thymol free) from the Sigma Chemical Co. (St. Louis, U.S.A.), and fusaric acid from ICN, (U.S.A.). All other chemicals were obtained from various commercial sources and were of analytical grade quality.

Dopamine β-monooxygenase was purified from bovine adrenal medulla as described earlier [9].

Serum samples were collected from healthy adults and stored at 0° until used.

HPLC analysis

A constant-volume HPLC pump (Model Constametric II G from Laboratory Data Control, Riviera Beach, Fla., U.S.A.) supplied with a Rheodyne valve-loop injector (20- and 100-μl loops) was used. The chromatographic components were detected using a spectrofluorometer (Model SFM 22 from Kontron, Zürich, Switzerland) supplied with a 150-W Xenon lamp (XBO 150 W/1 from Osram, München, G.F.R.) and a 20-μl flow-through cell. The instrument was operated at the highest range of the gain setting ("High HV var"). Fluorescence excitation and emission spectra were monitored by an X-Y recorder (Model 7035 from Hewlett-Packard). No corrections were made for photomultiplier response, monochromator sensitivity or Xenon arc-lamp emission. The fluorescence intensity was expressed on a linear scale.

The chromatographic separation was achieved at 20° on a microparticulate bonded strong cation exchanger (Partisil-10 SCX, pre-packed from Whatman in a 25.9 cm × 4.6 mm I.D. stainless-steel tube) with a theoretical plate number of 21,5000/m. A short pre-column (40 × 2 mm I.D. stainless-steel tube) packed with pellicular silica (HC Pellosil from Whatman) was used to protect the cation-exchange column. The mobile phase, consisting of 50 m*M* acetate buffer (pH 4.2), was pumped at a flow-rate of 2 ml/min (1400 p.s.i.)

Assay of dopamine β-monooxygenase activity

The assay of dopamine β-monooxygenase activity in human serum was performed as described by Fujita et al. [5], but with the following modifications. The pH was 6.0 and the reaction mixture also contained 50 m*M* 2-(N-morpholino)-ethanesulphonic acid (see Results section). The reaction was

quenched by adding an equal volume of ethanol containing 1.0 m*M* fusaric acid, and the mixture was left at 0° for 30 min before centrifugation at 10,000 rpm for 10 min (Eppendorf Model 5412 Microfuge). The supernatant fluid was diluted (4—20 times, depending on the enzymic activity of the serum analysed) with 50 m*M* acetate buffer (pH 4.2), and 20—100-μl aliquots were injected into the liquid chromatograph. The concentrations of the unknown samples were determined from standard curves.

Purified dopamine β-monooxygenase was also assayed as described by Wallace et al. [10].

RESULTS

Fluorescence properties of octopamine

The uncorrected fluorescence excitation and emission spectra of octopamine are shown in Fig. 1. The amine (10 nmoles) was injected into the liquid chromatograph, and when the peak height was reached the solvent flow was stopped and the spectra recorded. It is seen that the solvent contributed very little to the spectra of octopamine (λ_{ex} = 280 nm and λ_{em} = 303 nm). The fluorescence properties of tyramine were very similar to those of octopamine.

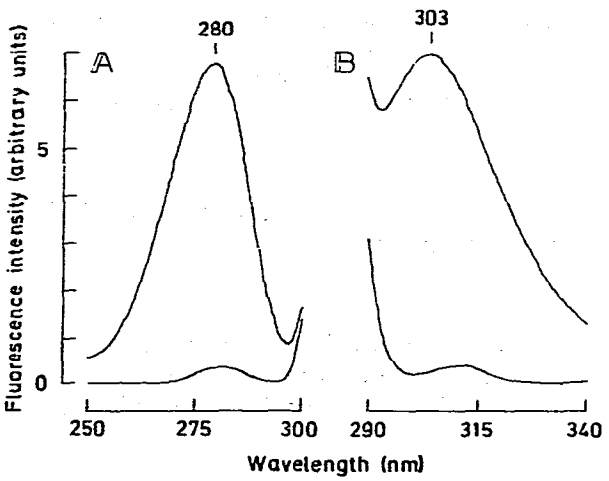

Fig. 1. Uncorrected fluorescence excitation (A) and emission (B) spectra of octopamine at 20° obtained by a Model SFM-22 spectrofluorometer from Kontron, supplied with a 20-μl flow-through cell. The fluorescence spectra of the mobile phase represent the baselines in (A) and (B). λ_{ex} = 280 nm and λ_{em} = 303 nm were selected for the recording of the fluorescence emission and excitation spectra, respectively. For experimental details, see text.

Chromatographic conditions

A microparticulate bonded strong cation-exchange resin was selected for the separation of octopamine from tyramine using a 50 m*M* acetate buffer, (pH 4.2) as the mobile phase. Fig. 2 shows that the selected chromatographic conditions allow the complete separation of octopamine (t_R = 3.65 min) from tyramine (t_R = 4.85 min), and this separation was obtained even in the presence of a 1000-fold concentration of tyramine. The peak height was lowered

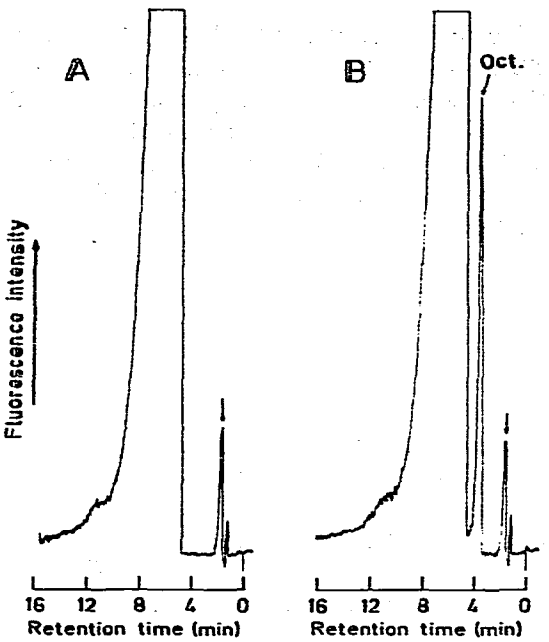

Fig. 2. HPLC fluorescence elution pattern of 100 μl supernatant from an incubation mixture in the assay of dopamine β-monooxygenase activity of a human serum. (A) Zero-time control, and (B) separation of octopamine (Oct., t_R = 3.65 min) from tyramine (t_R = 4.85 min). The arrows indicate the solvent front. 100 μl of the diluted (16 times) incubation mixture were injected into the liquid chromatograph; λ_{ex} = 280 nm and λ_{em} = 303 nm. For experimental details, see text.

by approx. 10% only when the same amount of octopamine was injected in 100 μl compared with 20 μl, with no loss in resolution.

Linearity and sensitivity

A linear relationship was obtained between the injected amount of octopamine and the peak height (r = 0.99) or the integrated peak area (data not shown). The limit of detection was approx. 50 pmoles octopamine (signal-to-noise ratio of 5).

Assay of dopamine β-monooxygenase in human serum

The useful application of the HPLC method to the assay of dopamine β-monooxygenase activity is most clearly shown by the assay of the enzyme in human serum. The hydroxylation of tyramine to octopamine by dopamine β-monooxygenase in human serum of high activity is shown in Fig. 2. None of the endogenous substances of the serum or components of the incubation mixture interfere with the chromatographic separation and the fluorimetric detection of octopamine, which is completely separated from the substrate to be hydroxylated (tyramine). Furthermore, no interference from compounds with high retention times was observed in multiple analyses in sequence.

In the standard assay procedure (pH 6.0) the amount of octopamine formed was proportional to the reaction time up to approx. 30 min (Fig. 3A) and to the amount of enzyme (serum) added (Fig. 3B). This result is in contrast

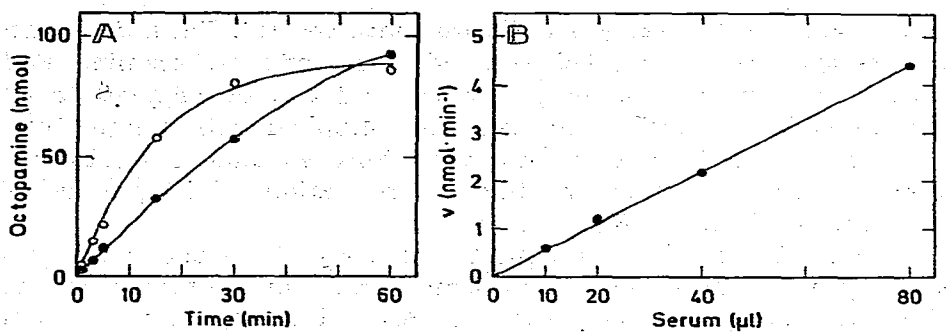

Fig. 3. (A) Time course of tyramine hydroxylation catalysed by dopamine β-monooxygenase of a human serum at pH 6.0 (•) and pH 5.0 (○). The ordinate indicates octopamine formed by 40 µl serum in 400 µl incubation mixture. (B) Effect of increasing amounts of enzyme (serum) on the rate of tyramine hydroxylation. The amount of octopamine formed was measured at pH 6.0 with a fixed incubation time (t = 30 min). The rate (v) was expressed as nmole • min^{-1}. For experimental details, see text. Incubation volumes were 400 µl.

to that obtained at pH 5.0 where the time course (Fig. 3A) is non-linear. It should also be mentioned that under our standard incubation conditions (pH 6.0, serum added) standard octopamine was found to be perfectly stable during an incubation period of 1 h, and the recovery was 100%. The reproducibility of the method was found to be better than ± 1% (S.D.).

A close correlation was found between the enzymatic activity of purified dopamine β-monooxygenase when the HPLC method was compared with the periodate oxidation method [10] (data not shown). When applied to sera from healthy adult subjects, the present HPLC method gave enzymatic activities which were in a range comparable to that obtained by a previously published method [5].

DISCUSSION

A recent trend in the microanalysis of biogenic amines has been the development of advanced HPLC methods for the rapid, specific and sensitive assay of the different amines and their metabolites from the same sample of biological material [1—4]. The improvements in sensitivity, specificity and versatility over previously used methods [for review, see ref. 8] are well-documented [1—4], and at the present stage of development the critical problem of this chromatographic procedure is the sensitivity of detection of the amines and the application as a routine method.

The recent developments of electrochemical detectors [1] as well as of continuous fluorescence monitoring, using *o*-phthalaldehyde as the fluorescence labelling reagent [5], have increased the sensitivity compared to UV detection [2—4]. Under favourable conditions these two methods allow the measurement of picomole amounts of amines eluted from HPLC columns. In the present study, however, detection based on the native fluorescence of octopamine has been developed to give an equivalent sensitivity, i.e. the detection limit is better than 50 pmoles.

The use of native fluorescence has obvious advantages over the two other detection methods. First, it is more specific since the different groups of

biogenic amines have their characteristic fluorescence spectra. Thus, detection by native fluorescence can be applied to a wide range of catecholamines and their biosynthetic precursors. Secondly, the method can be adapted to all commercially available spectrofluorometers now supplied with micro flow-cells. Finally, when used in combination with fully automated equipment (auto-injector), this method allows routine determination of 50 pmoles of octopamine with an efficiency of 6 analyses per h.

The useful application of the technique to the assay of dopamine β-monooxygenase activity in human serum is also demonstrated in the present study, including the assay conditions necessary to ensure linearity as a function of time and serum concentration. The method allows convenient and accurate measurements of low activities of dopamine β-monooxygenase in human serum, and presents advantages compared with other assay methods. The samples after incubation require little preparation, i.e. only precipitation of protein; the time required for a complete chromatogram is less than for the system based on reversed-phase partition chromatography [5], and no derivatization is required before detection.

The method presented in this report, although somewhat less sensitive than current radiochemical assay methods, offers advantages due to its speed, low cost, and avoidance of coupled enzyme reactions (see ref. 11 for references to methods and a discussion of problems in interpretation of dopamine β-monooxygenase assays). The sensitivity of our method, however, can be increased with the use of labelled tyramine. The advantage of the present chromatographic method is the more rapid separation of substrate and product compared with previously published HPLC methods [1—4].

ACKNOWLEDGEMENTS

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