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Short communication

Determination of monoamine oxidase B activity by high-performance liquid chromatography with electrochemical detection

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Abstract

A sensitive high-performance liquid chromatography method with electrochemical detection for measuring monoamine oxidase B activity in blood platelets is described. Dopamine is used as substrate and is incubated with isolated platelets and aldehyde dehydrogenase to convert dihydroxyphenylacetaldehyde to dihydroxyphenylacetic acid (DOPAC). The acid and the added internal standard hydrocaffeic acid are separated from dopamine and the incubation mixture by extraction with 5 ml of ethyl acetate–toluene (5:1, v/v). The organic phase is evaporated under nitrogen stream and the residue dissolved in 0.1 *M* citric acid. Dihydroxyphenylacetic acid and the internal standard dihydrocaffeic acid are then separated on a Eurosphere 100-C_{18} 5 μm column. The mobile phase used was a mixture of sodium acetate, citric acid, and acetonitrile at pH 2.5. The standard curve was linear from 125 pg to 10 ng. Absolute recovery of DOPAC was $85 \pm 3.8\%$ and of hydrocaffeic acid $87 \pm 4.1\%$. The method presented is sensitive (detection limit 8.0 pg of DOPAC injected) and reproducible (coefficient of variation 0.4–1%) with good accuracy (94–98%).

1. Introduction

Monoamine oxidase (MAO; monoamine O_2:oxidoreductase, EC 1.4.3.4) catalyzes the oxidative deamination of monoamines and neurotransmitters [1]. It is a membrane-bound mitochondrial enzyme [2] that exists in two forms: MAO A and MAO B. MAO A preferentially catalyzes the oxidative deamination of norepinephrine and serotonin and is selectively inhibited by clorgyline [3]. MAO B preferentially catalyzes the oxidative deamination of benzylamine and β-phenylethylamine and is sensitive to inhibition by deprenyl and pargyline [4]. Both forms of the enzyme are found in most human tissues: in kidney [5], renal medulla, liver, and in platelets [6–8]. Human blood platelets contain only MAO B. Therefore it is suggested and often used as indicator for MAO B in the central nervous system, in the brain, and in liver. Kevin et al. [9] reported that the deduced amino acid sequences of MAO B from frontal cortex, platelets, and liver are identical. These findings prove the validity of using platelet MAO B as marker for MAO B in many human organs, representing a successful tool in the study of MAO B in humans. Furthermore, MAO activity is a very important parameter in the study of Parkinson's disease [10,11].

The most frequently used method for the

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determination of MAO activity is the radiochemical assay, because of its specificity and high sensitivity [12–14]. As substrate ^{3}H- and ^{14}C-marked compounds are used, such as (^{3}H)5-hydroxytryptamine, (^{14}C) β-phenylethylamine, (^{14}C) benzylamine, and (^{14}C) dopamine [15,16]. HPLC is less frequently used for detection of MAO activity, although it is selective and highly sensitive. An HPLC method with fluorescence detection for the assay of MAO A activity has been reported [17]. MAO B activity in brains was measured by HPLC using benzylamine as a substrate and ultraviolet detection of the product, benzaldehyde, at 254 nm [18].

In our laboratory, we developed a selective and sensitive HPLC method for the determination of MAO B activity with dopamine as substrate. The end product dihydroxyphenylacetic acid (DOPAC) is measured by electrochemical detection (ED), ensuring a high sensitivity of the method.

2. Experimental

2.1. Chemicals and reagents

Sodium dihydrogen phosphate, sodium acetate, citric acid, pentasulfonic acid, phosphoric acid, ethyl acetate, toluol, methanol, and acetonitrile were purchased from E. Merck (Darmstadt, Germany).

Dopamine (DA), dihydroxyphenylacetic acid (DOPAC), hydrocaffeic acid (HCA), and aldehyde dehydrogenase (ALDH) were obtained from Sigma (Steinheim, Germany). β-nicotinamide adenine dinucleotide, oxidized from (β-NAD), and dithiothreitol (DTT) were purchased from Serva (Heidelberg, Germany), nicotinamide from Aldrich Chemie (Steinheim, Germany).

Deionized water used in preparing the mobile phase and all other solutions and reagents were Milli-Q water prepared by the Millipore-reagent water system (Eschborn, Germany).

2.2. Instruments

The HPLC system consisted of a Beckman pump (Model 126; Beckman, Munich, Germany), a Perkin-Elmer ISS-100 autosampler with a temperature-controlled sample tray (Perkin Elmer, Bodenseewerk, Überlingen, Germany), an electrochemical detector (ESA Coulochem model 5100 A) with a high-sensitive analytical cell (Model 5100; ESA, Bedford, MA, USA), and the Beckman chromatography software version 5.1 (System Gold Chromatography; Beckman, Berkeley, CA, USA). The chromatograms obtained were plotted either with the Epson LQ-850 printer or with a laser printer (Brother HL-4V). The mobile phase was degassed in the ERC. 3520 degasser (ERC, Alteglofsheim, Germany). The number of platelets per μl was calculated by the Sysmex K100 device (Digitana, Hamburg, Germany). Cell homogenization was done by a cell disrupter (Cell disrupter, Branson B15, Sonic Power Company, Donbury, CT, USA).

2.3. Chromatography

The mobile phase was prepared by dissolving 7.9 g sodium acetate, 4.1 g citric acid, and 150 mg pentasulfonic acid in 945 ml of deionized water. The pH of the solution was adjusted to 2.5, and 55 ml of acetonitrile were added. The solution was vacuum-filtered through a 0.45-μm membrane filter and degassed in the ERC degasser. Chromatographic separation was carried out on the Eurospher 100-C_{18} steel column (120 × 4.0 mm I.D., 5 μm particle size) (H. Knauer, Berlin, Germany). The flow-rate was 1.0 ml/min.

2.4. Isolation of platelets

Venous blood was drawn by using a plastic citrate syringe. The citrate blood was centrifuged in a refrigerated centrifuge for 15 min at 200 *g*. The thus derived platelet-rich plasma (PRP) was separated by using a plastic pipet and transferred into a cone-shaped polypropylene tube. The PRP was centrifuged for 10 min at 3000 *g* and 4°C. The supernatant plasma was aspirated, and 1 ml

of NaCl solution (0.15 *M*, 4°C) was carefully placed over the platelet precipitate. The sample was centrifuged again for 10 min at 3000 *g* (4°C), the NaCl wash solution was aspirated, and the platelet precipitate elutriated in 1.0 ml of 0.09 *M* KCl (4°C). The platelet suspension was thoroughly vortex-mixed to homogeneity. The number of platelets per μl suspension was calculated by electronical cell counting (Thrombocell, Contraves, Zürich, Switzerland), and the platelets thus derived stored at −20°C until further processing.

2.5. Determination of MAO (type B) activity

The sample stored at −20°C was thawed at 4°C and the platelets disrupted on ice by a cell disrupter (Bronson B 15, 1 pulse/s). A 50-μl aliquot of suspension and 80 μl of 100 m*M* KH_2PO_4 buffer, pH 7.4, were enriched with oxygen by thorough vortex-mixing, and the uncapped tube preincubated for 5 min at 37°C. After addition of a 50-μl mixture (26 n*M* DTT, 22 n*M* β-NAD, 2.9 μ*M* nicotinamide, and 0.15 units ALDH) the reaction was started by adding 20 μl of substrate solution and the batch incubated for 30 min at 37°C in a shaking water bath. Total incubation volume was 200 μl (final concentration of DA 200 μM). The reaction was terminated after 30 min by adding 100 μl of 2 *M* citric acid (4°C) which contained the internal standard of hydrocaffeic acid (5.0 ng/ml). The dihydroxyphenylacetic acid obtained and the internal standard were extracted by a 5 ml mixture of ethyl acetate–toluene (5:1, v/v) after 20 min of shaking. The mixture was centrifuged for 10 min at 3000 *g*, and the organic phase separated. The organic phase was then evaporated under nitrogen stream, the precipitate dissolved in 250 μl of 0.1 *M* citric acid, filtered (0.45 μm filter), and 50–150 μl injected into the HPLC device (Fig. 1C).

Blank values for each determination were obtained by addition of pargyline (5 n*M*) into the incubation vessel. Chromatograms of blank values showed no interfering peaks with retention times similar to those of DOPAC (Fig. 1B). Enzyme activity was expressed in nmol of product/10^8 platelet/30 min.

3. Results and discussion

MAO activity with dopamine as substrate was mainly determined by radiometry, using ^{14}C-labeled DA [15]. Hidaka et al. [19] and Nagatsu [20] described a fluorimetric method for determining MAO activity using serotonin as a substrate with measurement of 5-hydroxyindoleacetic acid. Their incubation mixture contained aldehyde dehydrogenase in order to convert 5-hydroxyindoleacetaldehyde into 5-hydroxyindoleacetic acid. The acid was separated from serotonin by a cation-exchange column and assayed fluorimetrically.

It was the aim of our laboratory to develop a highly sensitive method for the determination of MAO activity without radioactive substances. Use of the HPLC method and electrochemical detection allows the determination of low concentrations of neurotransmitters and chatecholamine metabolites. HPLC methods are inexpensive and rapid, and respective devices are found in almost every laboratory.

In this study, blood platelets were used for the determination of MAO B activity. The methodological rationale was based on the fact that dihydroxyphenylacetaldehyde, which is obtained by oxidative deamination of dopamine by MAO B, is oxidized to DOPAC with the aid of another enzyme, i.e., ALDH. HPLC–ED allows the determination of DOPAC at low concentrations. Separation of DOPAC and HCA by ion exchange, as it is used in most radiometric methods, was not successful because dopamine itself was partly eluted, which exerted an adverse effect on the chromatographic separation. Therefore, DOPAC and HCA were isolated from the incubation mixture by extraction with an organic solution mixture (ethyl acetate–toluene).

3.1. Extraction

By extracting the incubation mixture with ethyl acetate–toluene (5:1, v/v), mainly DOPAC

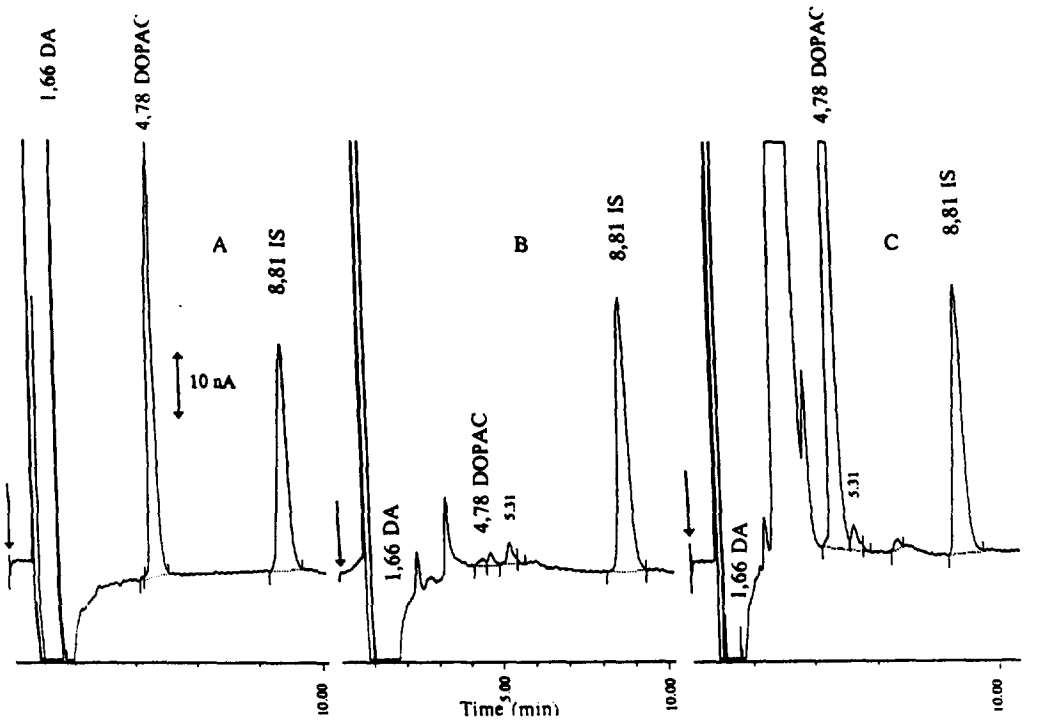

Fig. 1. Chromatograms of: (A) standard mixture of DA, DOPAC, and the internal standard (IS) HCA in 0.1% citric acid each, representing 1.0 ng absolutely; (B) blank: (50 μl) platelet suspension incubated with DA (final concentration 200 μM), and 5 nM pargyline (IS 5.0 ng/ml); (C) 50 μl platelet suspension incubated with DA (final concentration 200 μM) without adding the inhibitor pargyline, IS 5.0 ng/ml, DOPAC 25 ng/50 μl platelet suspension. Chromatographic conditions: Eurospher 100-C_{18} reversed-phase column (120 × 4.0 mm I.D., 5 μm particle size). Eluent: 7.9 g sodium acetate, 4.1 g citric acid, 150 mg pentasulfonic acid in 945 ml deionised water; pH 2.5 and 55 ml acetonitrile. Flow-rate: 1.0 ml/min. Detector: ESA Coulochem EC detector with highly sensitive analytical cell (Model 5011). Electrode 1: +350 mV. Electrode 2: −260 mV.

and the internal standard HCA were isolated. Comparison of the areas of signals obtained after injection of DOPAC and HCA, both directly and after extraction into the HPLC system, showed an absolute recovery of DOPAC of 85 ± 3.8% (mean ± S.D.; $n = 7$), and of HCA of 87 ± 4.1% (mean ± S.D.; $n = 7$).

3.2. Calibration curves

With each analytical run, a calibration curve using concentrations of 125, 500, 1000, 2000, 4000, 8000, and 10 000 pg/ml of DOPAC in 2 M citric acid was prepared, using stock solutions (1 mg/ml methanol stored at −20°C). To each assay, 100 μl (5 ng) of the internal standard were added. These calibration samples were assayed according to the procedure as described in the experimental part. Calibration curves were constructed by plotting the peak area of DOPAC against the internal standard (y) as a function of its concentrations added (x). The experimental points were fitted by least squares regression analysis ($w = y$, w = weight, y = internal standard) to an equation

$$y = ax + b$$

with a representing the slope and b the intercept of the calibration curve at $x = 0$.

3.3. Characteristics of the assay

Linear responses of DOPAC/internal standard peak-area ratios were observed for DOPAC from 0.10–50 ng/ml citric acid mixture. The detection limit was 8 pg of DOPAC injected

Table 1
Reproducibility of the assay

Pool	Dopac (pg/ml)	Intra-assay ($n = 6$)		Inter-assay ($n = 6$)	
		M.C. (pg/ml)	C.V. (%)	M.C. (pg/ml)	C.V. (%)
I	250	260	3.1	258	4.6
II	1500	1370	2.5	1440	3.8
III	7000	7105	4.7	6942	5.7

Pools I, II, and III in 2 *M* citric acid solution were spiked with 250, 1500, and 7000 pg/ml DOPAC. M.C. = mean concentration; C.V. = coefficient of variation. The spiked pools were extracted and analysed according to the incubation mixture described in the experimental part.

(under standard assay conditions the detection limit was 50 pg/ml). The correlation coefficient found was 0.998 to 0.996 for DOPAC. The variation in the slope and in the intercept of the calibration curve was acceptable. The concentration of DOPAC as derived from samples spiked with 250, 1500, and 7000 pg/ml was used for determining intra-assay as well as inter-assay precision (on 6 consecutive days). The results obtained showed good reproducibility (Table 1).

The MAO B activity was measured in isolated blood platelets of patients with congestive heart failure. The activity ($n = 10$) was 0.47 ± 0.24 nmol/10^8 platelet/30 min (mean ± S.D.) with 0.2 m*M* dopamine as substrate. This value is comparable with values obtained by radiochemical assay [10,11].

The HPLC method described proved to be precise, rapid and sensitive.

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