ELSEVIER

Journal of Pharmaceutical Biomedical Analysis
14 (1996) 1157–1162

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Selective catalytic detection of dopamine[1]

Fidel Ortega, Elena Domínguez*

Department of Analytical Chemistry, Faculty of Pharmacy, University of Alcalá de Henares, E-28871 Alcalá de Henares, Madrid, Spain

Received for review 13 September 1995; revised manuscript received 20 November 1995

Abstract

Catalytic detection of dopamine in a flow-injection system based on a packed bed reactor containing immobilized tyrosinase is demonstrated. Electrochemical or photometric transduction of enzyme reaction products results in limits of detection ($S/N = 3$) of 3.5 ± 1.4 μM (16.6 ± 6.6 ng), and 8.4 ± 3.5 μM (40 ± 16.6 ng) respectively. The high catalytic efficiency of the reactor yields a diffusion-controlled system with a sample frequency of 450 h^{-1} when electrochemical transduction, 25 μl injection volume and 0.9 ml min^{-1} flow rate are used. The catalytic system can be used in liquid chromatography post-column mode for the selective detection of dopamine in a complex mixture of drugs.

Keywords: Dopamine; Electrochemical detection; Immobilized enzyme reactor; Liquid chromatography; Tyrosinase

1. Introduction

Dopamine (3,4-dihydroxyphenethylamine) is one of the most commonly analyzed catecholamines. For routine screening tests in complex matrices, detection of dopamine requires selectivity to avoid false-negative results and short time of analysis to achieve high sample frequency [1]. Liquid chromatography with electrochemical detection is currently used as a selective and sensitive method of analysis for catecholamines [2]. However, direct oxidation of catecholamines requires large overvoltages which jeopardise selective detection by simultaneous oxidation of electroactive species [3]. A recent approach to avoid this inherent problem is based on the use of a previous solid-phase extraction step resulting in a clean chromatogram free of interferences from 25 of the most common precursors, metabolites and drugs [4].

Biochemical recognition of analytes is being extensively used as a selective means of detection, particularly with immobilization of the biological component and operating in flow systems to allow continuous operation and high sample frequency [5]. Commercially available polyphenol oxidase, tyrosinase (EC 1.14.18.1), catalyses the oxidation of dopamine to dopaminequinone which readily polymerizes to form coloured polyaromatic compounds. This quinone can also be reduced at low potentials, allowing an optimum working potential range where unselective electrooxidation and/or electroreduction is avoided [6].

* Corresponding author. Fax: (+34) 1885-46-66.

[1] Presented at the Fifth International Symposium on Drug Analysis, September 1995, Leuven, Belgium.

PII S0731-7085(96)01720-7

In a previous work the feasibility of the above catalytic principle for the detection of catecholamines in biological samples using a tyrosinase electrode was demonstrated [7]. A similar detection principle has also been applied to environmental matrices for the screening of phenolic compounds [8,9]. The aim of this work is the characterization and optimization of immobilized tyrosinase in a packed bed reactor incorporating a flow system for fast and selective detection of dopamine.

2. Experimental

2.1. Reagents

Tyrosinase (polyphenol oxidase, EC 1.14.18.1) from mushroom was purchased as a lyophilized powder (Sigma cat. no. T-7755) and was used as received. Glutaraldehyde was purchased as a 25% aqueous solution (Sigma cat. no. G-5882) and the polymerized aldehyde was removed prior to use by addition of activated carbon. The mixture was centrifuged at 4°C and the supernatant was stored at −18°C. Paracetamol, chlorpheniramine maleate, caffeine, acetyl salicylic acid, and dimenhydrinate were purchased from Barcia. Dopamine was provided by Sigma. Acetonitrile was HPLC grade from Scharlau. Ultrapure reagent-grade water was obtained by means of a Milli-Q system (Millipore).

2.2. Preparation of the immobilized tyrosinase reactor (IMER)

The enzyme was immobilized on controlled-pore glass (CPG-10, pore diameter 51.5 nm, particle size 37–74 μm; Serva, cat. no. 44762) after silanization of the glass with 3-aminopropyltriethoxysilane, activation with glutaraldehyde, and following a previously described procedure [7]. 60 465 U of tyrosinase were charged per gram of activated support. These units are expressed as ΔA min^{-1} at 475 nm in 0.1 M phosphate buffer pH 6.0 containing 15 nM dopamine. The coupling yield was 86%, estimated by activity measurements from the unbound enzyme of the clear enzyme solution before and after immobilization. The enzyme-charged CPG was packed into plexiglas reactors with polypropylene nets at the ends. 25 μl reactors were used in this work with an inner diameter of 1.0 mm. When not in use, the charged CPG was stored in 0.1 M phosphate buffer (pH 6.0) at 4°C.

2.3. Equipment

The flow-injection manifold used for this work is presented in Fig. 1. The immobilized tyrosinase reactor was studied in a flow-injection system containing a switch valve after the immobilized enzyme reactor (IMER) to alternate the flow to the photometric or electrochemical transducer (see section 3). An ultramicro flow-through cell from Teknokroma was used for photometric detection at 475 nm. The electrochemical transducer consisted of a wall-jet flow-through amperometric cell connected to a three-electrode potentiostat (Zäta Electronic) with a saturated calomel (SCE) reference electrode, and a Pt wire counter electrode. Rods of spectrographic graphite (RW001, Ringsdorff-Werke GmbH) of 3.1 mm diameter, polished on wet, fine emery paper and thoroughly washed with deionized water were used as working electrodes. The applied potential was kept at −50 mV. Unless otherwise stated, 25 μl samples were injected with a pneumatically-operated valve

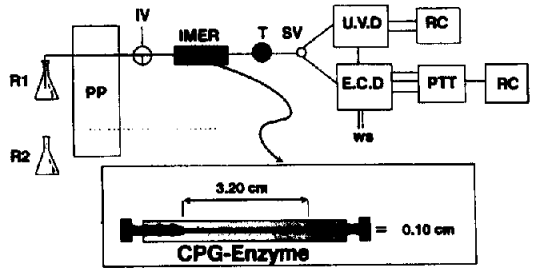

Fig. 1. Flow-injection manifold used in this work: PP, peristaltic pump; IV, injection valve; IMER, immobilized enzyme reactor; T, Mixing; SV, switch valve; U.V.D., photometric detector; E.C.D., electrochemical detector, PTT, potentiostat; RC, recorder; ws, waste. The operating conditions are: R1, 0.1 M phosphate buffer, pH 7.0, and 25 μl injection volume (unless otherwise stated), 25 μl reactor volume; R2, make-up flow to adjust pH (see Section 3). Photometric detection at 475 nm and electrochemical detection at −50 mV vs SCE.

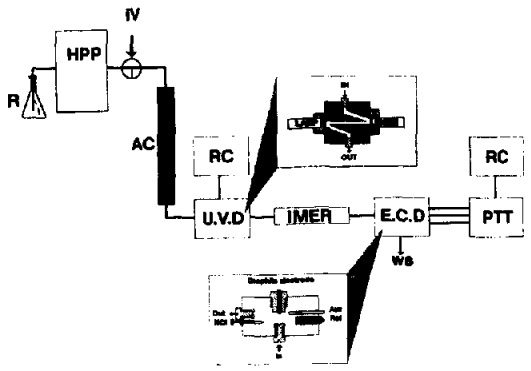

Fig. 2. LC system with photometric detection and on-line catalytic–electrochemical detection for comparison of chromatograms. AC, analytical column. For more details, see Section 2.

(Cheminert type SVA) into the carrier stream which was delivered by a Gilson Minipuls 2 peristaltic pump and consisted of a 0.1 M phosphate buffer (pH 7.0).

Chromatographic analyses were performed using an HPLC system HP1050 (Hewlett-Packard) with a 20 μl injection loop and a photodiode array detector HP 1040 M coupled with a HP 9000/300 personal computer (Hewlett-Packard) and HP 9153 C disk drive. A stainless-steel column (150 mm × 3.9 mm i.d.) packed with Nova-Pak® C18 material (pore size 60 Å, particle size 5 μm; Waters) was used. The mobile phase was pumped in the isocratic mode at 1 ml min^{-1} and consisted of acetonitrile–phosphate buffer (0.1 M; 5:95, v/v; pH* 7.0). Detection was effected at 270 nm. The effluent of the photodiode array detector was connected to the inlet of the IMER and the electrochemical cell (Fig. 2).

3. Results and Discussion

3.1. Characterization of the IMER

Under optimum conditions, the performance of the IMER in a flow system will be controlled by the external mass transfer (diffusive and convective transport from the bulk carrier to the surface of the support) and, in the case of CPG, by the internal mass transfer (diffusion within the support matrix) rather than by the kinetics of the tyrosinase-catalyzed reaction [5,10]. Depending on the reactor volume the reaction becomes diffusion controlled or kinetically controlled in such a way that one can expect the former for large reactor volumes and the latter for small reactors. The kinetic properties of the IMER were determined by measurements of the fractional conversion for dopamine at different flow rates, i.e. various substrate concentrations or residence times. Fig. 3A shows the influence of flow rate on the catalytic conversion with photometric detection and 25 μl injections of 1 mM dopamine into the carrier stream. The degree of conversion was calculated considering as 100% the absorbance value ob-

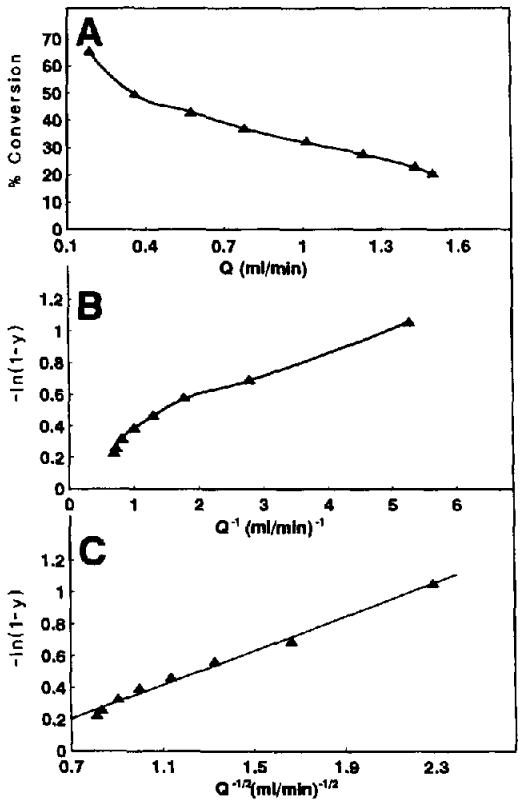

Fig. 3. Effect of the flow rate on the catalytic detection with photometric transduction. (A) Conversion efficiency of the IMER in the flow-injection system at different flow rates. 25 μl of 1 mM dopamine was injected into the carrier stream (0.1 M phosphate buffer, pH 7.0, reactor volume 25 μl). (B) Logarithmic representation of the fractional conversion (y) versus 1/flow rate and (C) versus $(1/\text{flow rate})^{-1/2}$.

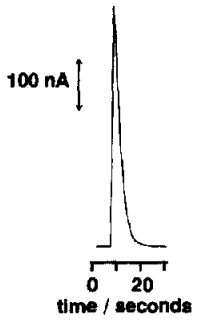

Fig. 4. Flow-injection peak profile obtained with the 25 μl IMER and electrochemical transduction. 25 μl unjection of 1 mM dopamine at 0.9 ml min^{-1}.

tained with a solution containing 0.5 mM dopamine and an excess of tyrosinase (the dispersion in the flow system was estimated as $D = 2$). It has been determined that if the logarithmic representation of the fractional conversion (y) vs. the reciprocal of the volumetric flow rate, Q^{-1}, or the reciprocal of the square root of the volumetric flow rate, $Q^{-1/2}$, results in a straight line, the reactor is controlled by kinetics or diffusion respectively [11]. These representations are shown in Figs. 3B and 3C respectively. Despite the small reactor volume used (25 μl) a rather diffusion-controlled system was observed, indicating the high catalytic efficiency of the immobilized tyrosinase. Determination of the apparent rate constant ($K_{ps}^{app} = 0.21\ s^{-1}$) and the fractional void volume (0.532 μl) allowed one to calculate that a 515 μl reactor volume would give 100% conversion [10]. Nevertheless, this large reactor volume was not used to avoid band broadening and lower sample throughput when working in flow-injection systems. The sample throughput with the 25 μl reactor was determined in the flow-injection mode with electrochemical detection of reaction products at 0.9 ml min^{-1}. The time profile of a dopamine peak with electrochemical detection is seen in Fig. 4. A theoretical maximum sample frequency of 450 h^{-1} was calculated with a $2\sigma_t$ value of 4 s and assuming a carryover of 2%. A practical sample frequency of 240 h^{-1} was achieved with consecutive injections of dopamine and recovery of baseline.

3.2. Effect of pH, temperature and injection volume

The dependence of the dopamine catalytic response, with photometric transduction, on these parameters is presented in Fig. 5. To eliminate the dependence of the absorptivity of the reaction products on the pH, a second carrier of 0.3 M phosphate buffer pH 7 was pumped in R2 (see Fig. 1) and conveniently mixed with the enzyme products. The pH dependence of the immobilized tyrosinase is compared with the free enzyme in Fig. 5A. In both cases the optimum pH is 7.0 but at pH 6.5 the immobilized tyrosinase retains most of its activity while a clear decrease is seen for the free enzyme. The temperature dependence of this detection system is profound (Fig. 5B). Maximum response is obtained at 50°C, but a desirable long operational stability recommends working at 25°C. The injection volume dependence is seen in Fig. 5C. A 25 μl injection volume was used for further analytical characterization.

3.3. Reproducibility, calibration and limit of detection

The relative standard deviation for 10 replicate injections was 1.7% and 0.8% at the 0.5 mM level with electrochemical and photometric transduction respectively. Typical calibration curves showed a linear relation between catalytic detection and dopamine concentration over the range 0.005–1 mM ($r = 0.996$, $n = 9$) with electrochemical transduction, and 0.05–0.75 mM ($r = 0.998$, $n = 5$) with photometric transduction. The limit of detection ($S/N = 3$) was also more favourable for the electrochemical transduction, 3.5 ± 1.4 μM (16.6 ± 6.6 ng), versus 8.4 ± 3.5 μM (40 ± 16.6 ng) achieved with photometric detection.

3.4. Selectivity

The catalytic detection with electrochemical transduction was tested as a selective detection system for LC with the configuration shown in Fig. 2. For this reason, the inlet of the IMER was connected to the outlet of a diode array detector. 20 μl of a mixture containing 2 mM chlorpheni-

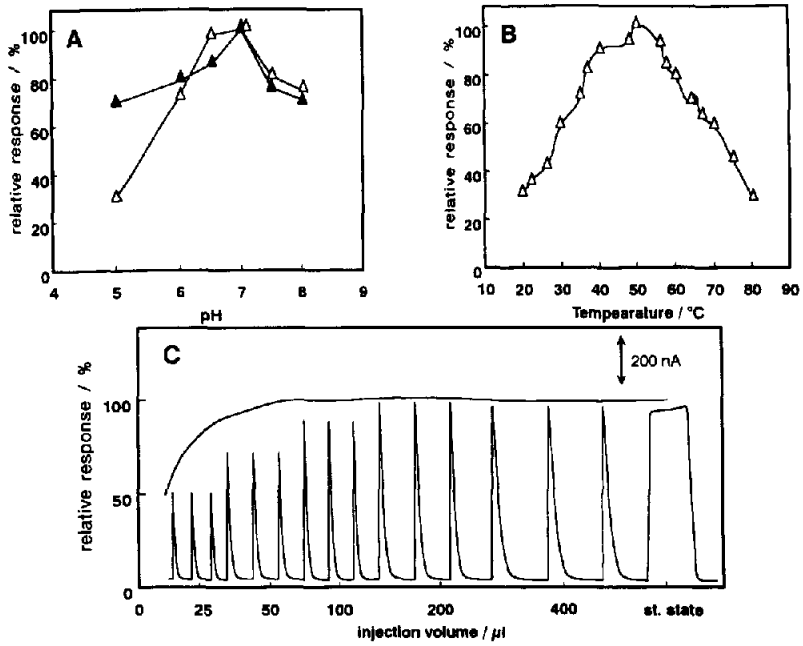

Fig. 5. Effect of pH (A), temperature (B), and injection volume (C) on the catalytic detection with photometric transduction: (△) immobilized tyrosinase; (▲) free tyrosinase. Same flow conditions as in Fig. 4.

ramine, 0.4 mM dopamine, 0.8 mM paracetamol, 1.2 mM acetyl salicylic acid, 0.4 mM caffeine and 0.4 mM dimenhydrinate was injected. Fig. 6 shows the chromatogram obtained with catalytic detection and electrochemical tansduction (A), and with UV detection at 270 nm (B). Catalytic detection results in a selective peak for dopamine despite the simultaneous elution with chlorpheniramine and the presence of other drugs.

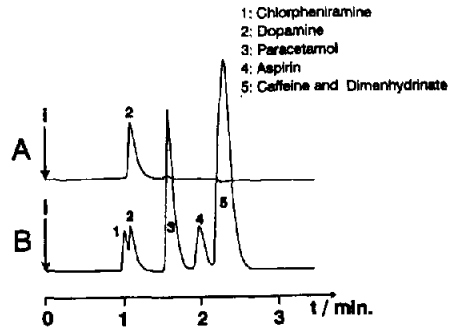

Fig. 6. Chromatographic separation of 20 µl injections of a complex mixture containing 2 mM chlorpheniramine (peak 1), 0.4 mM dopamine (peak 2), 0.8 mM paracetamol (peak 3), 1.2 mM acetyl salicylic acid (peak 4), 0.4 mM caffeine (peak 5) and 0.4 mM dimenhydrinate (peak 5). (A) Catalytic detection with electrochemical transduction; (B) direct photometric detection at 270 nm. For more details, see text.

Acknowledgements

Financial support from the University of Alcalá de Henares (Project 021/95) is gratefully acknowledged.

References

[1] M.W. Duncan, P. Compton, L. Lazarus and G.A. Smythe, N. Engl. J. Med., 319 (1988) 136–142.
[2] B.-M Eriksson and B.-A. Persson, J. Chromatogr. 612 (1993) 1–5.
[3] M.D. Hawley, S.V. Tatawawadi, S. Piekarski and R.N. Adams, J. Am. Chem. Soc., 89 (1967) 447–450.
[4] V. M. Abate, Curr. Sep., 13 (1994) 53–55.
[5] L. Gorton, E. Csöregi, E. Dominguez, J. Emnéus, G. Jönsson-Pettersson, G. Marko-Varga and B. Persson, Anal. Chim. Acta, 250 (1991) 203–248.
[6] F. Ortega, E. Dominguez, G. Jönsson-Pettersson and L. Gorton, J. Biotechnol., 31 (1993) 289–300.

[7] F. Ortega, J.L. Cuevas, J.I. Centenera and E. Dominguez, J. Pharm. Biomed. Anal., 10 (1992) 789–796.
[8] F. Ortega, E. Dominguez, E. Burestedt, J. Emnéus, L. Gorton and G. Marko-Varga, J. Chromatogr. *A*, 675 (1994) 64–78.
[9] E. Burestedt, J. Emnéus, L. Gorton, G. Marko-Varga, E. Dominguez, F. Ortega, A. Narváez, H. Irth, M. Lutz, D. Puig and D. Barceló, Chromatographia, 41 (1995) 207–215.
[10] L. Gorton, G. Marko-Varga, E. Dominguez and J. Emnéus, in S. Lam and G. Malikin (Eds.), Analytical Applications of Immobilized Enzyme Reactors, Blackie, London, 1994, pp. 1–21.
[11] H. Huck, Bioelectrochem. Bioenerg., 21 (1989) 99–105.