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Liquid chromatographic separation of phenolic drugs using catalytic detection: comparison of an enzyme reactor and enzyme electrode*

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Abstract: A catalytic detection system based on the use of immobilized tyrosinase and two different transducers (photometric and electrochemical) is described. Comparison between tyrosinase immobilized in a packed-bed reactor and at the surface of a graphite electrode is discussed in terms of sensitivity in a flow injection system. The enzyme electrode configuration gives the highest sensitivity for the quantitation of dopamine. For the imobilized tyrosinase reactor with photometric detection the range for dopamine is linear up to 0.75 mM (136 μg ml^{-1}) and the immobilized tyrosinase reactor with electrochemical detection and the tyrosinase electrode extends this dynamic range to 1 mM (181 μg ml^{-1}). Liquid chromatographic separation and post-column detection using the tyrosinase electrode is shown for spiked samples of serum.

Keywords: *Dopamine; tyrosinase; immobilized enzyme reactor; enzyme electrode; amperometric detection; liquid chromatography; serum.*

Introduction

Phenolic compounds include a large variety of pharmaceutically and biomedically important compounds. Examples are: L-3,4-dihydroxyphenylalanine (levodopa) which is routinely used in the treatment of Parkinson's disease; 3,4-dihydroxyphenethylamine (dopamine) which is one of the most commonly analysed catecholamines; and *p*-acetylaminophenol (paracetamol) which is used extensively as an analgesic drug.

The selectivity of enzymes makes them extremely useful reagents for chemical analysis. Immobilization of the enzymes results in solid phase reagents which can be easily recovered and used repetitively [1]. Immobilized enzymes have been used as selective detection devices in different configurations, i.e. immobilized enzyme reactors (IMER) and enzyme electrodes [1]. The choice of configuration depends on the equilibrium of the catalysed reaction, the stability of the enzyme, and the molecular complexity of the analyte. It could be stated that large molecules and unfavourable equilibria call for IMER owing to the large amount of enzyme that can be loaded in comparison with the limited amount that can be used with electrodes, resulting in potentially higher sensitivity [1]. An empirical comparison between immobilized enzyme reactors and enzyme electrodes has been made for L-glutamate oxidase [2]. Despite the use of a similar enzymatic load in both configurations, the authors report that the enzyme reactor surpasses the enzyme electrode in respect of sensitivity and analytical speed.

The high sensitivity required for the quantitation of phenolic drugs in biological fluids is commonly achieved by the use of electrochemical detection. Large voltages of +650 mV–+900 mV vs Ag/AgCl are currently applied for the direct oxidation of phenolic compounds at naked glassy carbon electrodes [3–5]. For analytical purposes these potentials give a high risk of interference owing to the oxidation of many organic compounds present in biological fluids. Furthermore, the high potential can lead to electrode fouling. By the use of suitable electrode modification the analysis of these analytes can be done at lower potentials overcoming the problems mentioned above [6]. Enzymatic modification with tyrosinase has been recently described for the detection of phenolic compounds at −50 mV versus a saturated calomel electrode (SCE) [7].

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In this system, the immobilized enzyme catalyses the formation of quinone derivatives which can be electrochemically reduced at low potentials. Enzymatic modification therefore confers the selectivity of enzyme-catalysed reactions and allows the use of lower applied potentials resulting in further selectivity to the assay.

The present work reports a comparative study of three different catalytic detection devices for the characterization in a complex matrix of phenolic drugs using flow systems.

Experimental

Reagents

Tyrosinase (polyphenol oxidase, PhOD EC 1.14.18.1) from mushrooms was purchased as a lyophilized powder (3430 U mg^{-1} protein, Sigma cat. no. T-7755), and was used as received. Water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, and dopamine were obtained from Sigma (cat. no. E-6383 and H-8502). Glutaraldehyde was purchased as a 25% aqueous solution (Sigma cat. no. G-5882) and prior to use the polymerized aldehyde was removed by addition of activated carbon. The mixture was centrifuged at 4°C and the supernatant was stored at −18°C. Paracetamol, chlorpheniramine maleate, phenylephrine hydrochloride and acetylsalicylic acid were purchased from Barcia. L-Dopa was obtained from Madopar® tablets. Standard serum was purchased as lyophilized powder from Tecnicon-Miles Martin. Acetonitrile was HPLC grade from Scharlau. Ultrapure reagent-grade water was obtained by means of a Milli-Q system (Millipore).

Preparation of the electrodes

Rods of spectrographic graphite (RW001, Ringsdorff-Werke GmbH) of 3.1-mm diameter were cut, polished on wet, fine emery paper, thoroughly washed with de-ionized water and allowed to dry at ambient temperature. They were then heated to 700°C for 90 s in a muffle furnace. They were cooled and stored in a desiccator until use. When in use, the unmodified electrodes were press fitted into a Teflon holder so that only the flat circular end (0.0731 cm^2) was exposed to the flow.

Preparation of the tyrosinase electrodes

The enzyme was covalently immobilized on the surface of a carbodiimide activated graphite electrode and prepared as described elsewhere [7]. Each electrode was charged with 1372 U of PhOD.

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 silicone-treatment of the glass with 3-aminopropyltriethoxysilane and activation with glutaraldehyde [6]. 34300 U of tyrosinase were charged per gram of activated support. The coupling reaction was allowed to proceed at reduced pressure for 15 min and then at 4°C overnight. The coupling yield was 100%, estimated from the tyrosinase activity measurement of the clear enzyme solution before and after immobilization. The enzyme-charged CPG was packed into Plexiglas reactors with polypropylene nets at the ends. Reactors (25-μl) were used with an inner diameter of 2.5 mm. When not in use, the charged CPG was stored in 0.1 M phosphate buffer (pH 6.0) at 4°C.

Equipment

The different flow manifolds used for this work are presented in Fig. 1. The immobilized tyrosinase reactor was studied in a flow injection system containing a switch valve after the IMER to alternate the flow to a photometric or electrochemical transducer (see Results and Discussion) (Fig. 1 — I). The flow-through cell for photometric detection had a volume of 75 μl and a path length of 10 mm. It was adapted from that in a spectrophotometer (Schimadzu 120-02). The electrochemical transducer comprised a wall-jet flow-through amperometric cell connected to a three-electrode potentiostat (Zäta Electronic) with a SCE reference electrode, and a Pt wire counter electrode. Samples of 25 μl were injected with a pneumatically operated valve (Cheminert type SVA) into the carrier stream which was delivered by a Gilson Minipuls 2 peristaltic pump. The carrier stream contained a 0.1 M phosphate buffer (pH 6.0). The tyrosinase electrode was studied in a single-channel flow injection manifold inserted in the same amperometric cell as the working electrode (Fig. 1 — II).

Chromatographic analyses were performed using a HPLC system HP1050 (Hewlett–

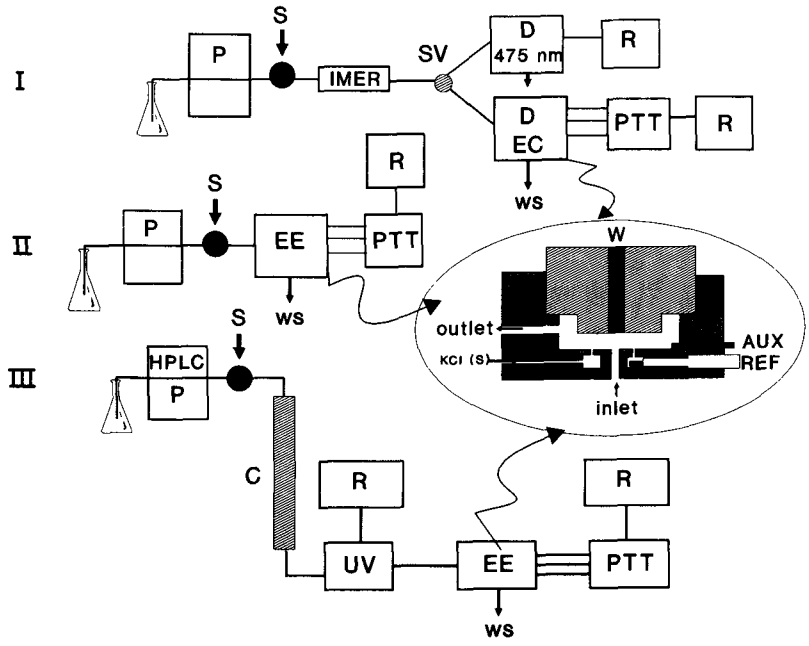


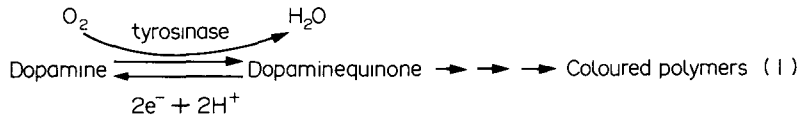
Figure 1
Flow injection manifolds used in this work. P, peristaltic pump; S, sample; IMER, immobilized enzyme reactor, SV, switch valve; D, detector; R, recorder; EC, electrochemical cell; PTT, potentiostat; ws, waste; EE, enzyme electrode; W, working electrode; AUX, auxiliary electrode; REF, reference electrode; HPLC P, high-pressure liquid chromatography pump; C, analytical column; UV, ultraviolet. Manifolds I and II were used to study the catalytic detection systems in the FIA mode. Manifold III was used to validate the enzyme electrode for selective detection in the analysis of phenolic drugs. For details see text.

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 a HP 9153 C disk drive. A stainless-steel column (250 × 4 mm i.d.) packed with 5-μm Nucleosil® 120 C18 material (Scharlau) was used. The mobile phase was pumped in the isocratic mode at 1 ml min^{-1} and comprised acetonitrile–phosphate buffer (0.1 M), (5:95, v/v) (pH* 6.2). Detection was effected at 270 nm. The efluent of the photodiode array detector was connected to the inlet of the amperometric cell containing the tyrosinase electrode (Fig. 1 — III).

Results and Discussion

Reaction detection scheme

Tyrosinase catalyses the oxidation of phenolic compounds via hydroxylation with molecular oxygen to catechols and subsequent dehydrogenation to *o*-quinone. Quinones are highly unstable in water and readily polymerize to coloured polyaromatic compounds which allows the catalysed reaction to be monitored colorimetrically at 475 nm. Quinones are also electroactive species which can be electrochemically reduced at low potentials [7]. Dopamine is a catechol derivative which can be oxidized by tyrosinase [7]. The reaction detection scheme proposed for dopamine can be summarized in the following coupled reactions:



Taking into consideration this overall detection system three strategies were considered: IMER with photometric detection at 475 nm; IMER with electrochemical detection at −50 mV vs SCE; and an enzyme electrode working at −50 mV vs SCE.

The effect of flow rate

The influence of flow rate in the above

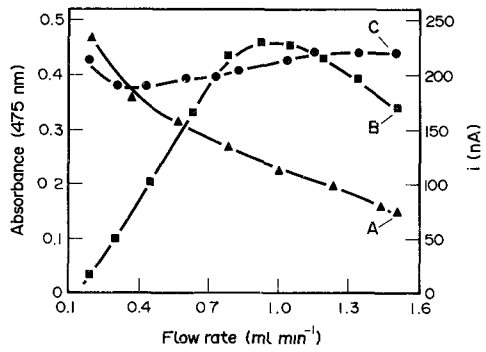


Figure 2
Effect of the flow rate on the different catalytic detection systems studied. (A) IMER and photometric detection at 475 nm. (B) IMER and electrochemical detection with an unmodified graphite electrode working at −50 mV vs SCE. For both (A) and (B), 25-μl injections of 1 mM of dopamine were made into the carrier stream. For (C) enzyme electrode (tyrosinase graphite electrode) working at −50 mV vs SCE, 25-μl injections of 50 μM of dopamine were made into the carrier stream.

catalytic detection systems is shown in Fig. 2. The study was made in the flow injection manifolds shown in Fig. 1 (I and II). The response obtained for injections of 1 mM of dopamine with the immobilized tyrosinase reactor and photometric detection decreases with the flow rate in the range of 0.2–1.5 ml min^{-1} [Fig. 2(A)]. This flow rate profile is characteristic for small reactor volumes where conversion efficiency is mainly controlled by the kinetics of the enzyme [6, 8]. The faster the sample plug passes the immobilized tyrosinase reactor the smaller is the fraction of dopamine oxidized and consequently the fraction of coloured compounds formed (reaction 1). Conversion efficiency values of 65 and 20% were achieved at 0.2 and 1.5 ml min^{-1}, respectively. These percentages were calculated in relation to the absorbance obtained when a solution of 1 mM of dopamine was treated with an excess of free tyrosinase and the reaction allowed to proceed until steady absorbance value was attained; this was considered to represent 100% conversion. When the enzymatic reaction in the IMER was amperometrically monitored at −50 mV vs SCE by measurement of the electrochemical reduction of dopaminequinone (reaction 1), the flow rate profile was completely different to that observed with photometric detection [Fig. 2(B)]. The current intensity for injections of 1 mM of dopamine increases with flow rate to a maximum value at 1 ml min^{-1}. The response obtained at 0.2 ml min^{-1} represents 7% of the response at 1 ml min^{-1}. At flow rates higher than 1 ml min^{-1}, a decreased signal is observed. According to the overall reaction 1, polymerization of dopaminequinone competes with the electrochemical reduction of dopaminequinone at the surface of the unmodified graphite electrode. This competition is favoured at low flow rates when polymerization of dopaminequinone takes place until the quinone plug reaches the electrode. The faster the dopaminequinone reaches the electrode the higher is the signal obtained as a consequence of a higher quinone concentration and an efficient electrocatalytical reduction at −50 mV vs SCE. When the flow rate is higher than 1 ml min^{-1} the enzymatic oxidation of the analyte becomes the limiting step owing to the short residence time of dopamine in the IMER. The flow rate profile for the enzyme electrode was studied for injections of 50 μM of dopamine in order to obtain similar current intensities as those registered with IMER and electrochemical detection. The influence of the flow rate on the behaviour of the tyrosinase electrode is shown in Fig. 2(C). Under these conditions, insignificant signal variations with the flow rate were observed. The low concentration of dopamine injected, the high conversion efficiency of tyrosinase and the efficient coupling between the catalytic oxidation and the electrochemical reduction in the tyrosinase graphite electrode may be responsible for total dopamine conversion between 0.2 and 1.5 ml min^{-1}.

Sensitivity of the catalytic detection systems

The sensitivity of the three catalytic detection systems was studied in the flow injection manifolds of Fig. 1 (I and II) by comparison of the response obtained for different dopamine concentrations. The IMER with photometric detection and the enzyme electrode were tested at 0.7 ml min^{-1} and the flow rate in the IMER with electrochemical detection was 1 ml min^{-1}, as optimum conditions of flow. The results are presented in Fig. 3. Despite the fact that the immobilized tyrosinase reactor contains higher amount of enzyme than the tyrosinase electrode, the highest sensitivity was obtained for the enzyme electrode. This can be explained by considering the close contact between the catalytic and the electrochemical reactions that take place at the enzyme electrode (Fig. 4). In this configur-

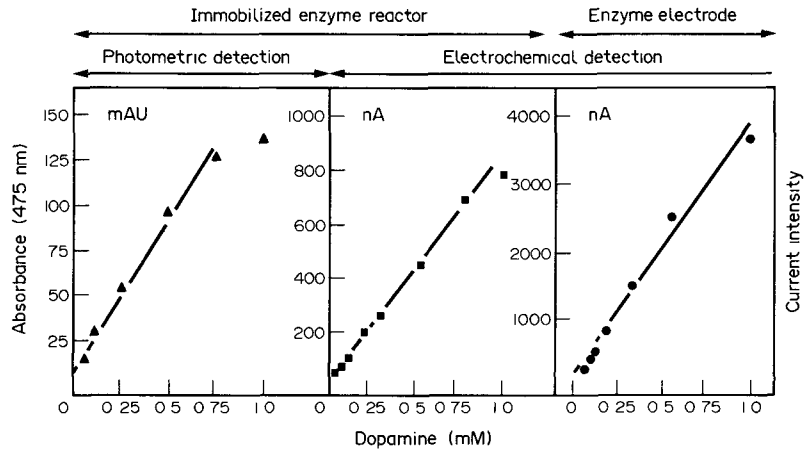


Figure 3
Sensitivities and dynamic range achieved with the different catalytic detection systems. The flow rate for the IMER with photometric detection and the enzyme electrode was 0.7 ml min^{-1}. The flow rate for the IMER with electrochemical detection was 1 ml min^{-1}.

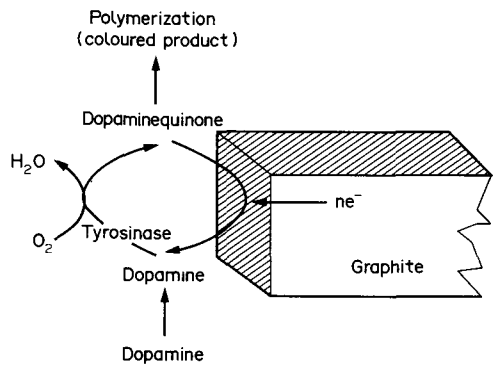


Figure 4
Catalytic and electrochemical coupled reactions for the detection of dopamine with a tyrosinase graphite electrode.

ation dopamine is oxidized to the quinone form which is readily electrochemically reduced to dopamine giving the possibility of signal amplification. The fact that dopamine enters a catalytic oxidation and electrochemical reduction cycle could be responsible for the high sensitivity achieved in this configuration. When the enzyme is not in intimate contact with the electrode (IMER and electrochemical detection) no cyclic sequence is established and consequently the sensitivity decreases [9]. Additionally, the efficient coupling between the catalytic and the electrochemical steps may decrease the polymerization reaction rate. The formation of these polymeric compounds leads to a risk of electrode fouling and moreover these polymers have proved to inactivate the enzyme [10]. The sensitivities of the IMER with photometric and electrochemical detection calculated as the slope of the regression calibration graphs gave values of 137 mAU per mM (30 mAU μg^{-1}) dopamine and 790 nA per mM (174 nA μg^{-1}) dopamine, respectively. The sensitivity for the enzyme electrode was found to be 4769 nA per mM (1054 nA μg^{-1}) dopamine. A detection limit of 4.5 ng dopamine (25-µl injection volume) was determined with the tyrosinase electrode in the flow injection system using a signal-to-noise ratio of 3.

FIA peaks and stability

The peaks obtained after 50 consecutive injections of 0.5 mM of dopamine in the flow system containing the IMER and electrochemical detection are presented in Fig. 5. A decrease of 15% in the response is attributed to fouling of the electrode by the polymeric compounds. This conclusion is drawn from evaluation of the stability of the IMER with photometric detection. After 50 consecutive injections, the peak height remained constant (data not shown) indicating that the immobilized tyrosinase was stable in the reactor configuration. After 30 consecutive injections of 0.04 mM of dopamine a decrease of 10% in the response was observed.

Liquid chromatographic separation of phenolic drugs and post-column tyrosinase electrode detection

The catalytic detection configuration which showed highest sensitivity in the flow injection system was tested as a selective detection system in LC (post-column mode). For this

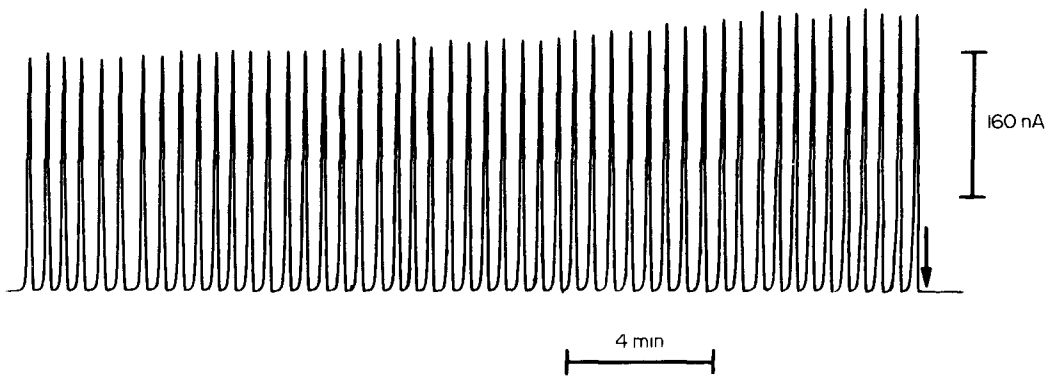


Figure 5
FIA peaks after 50 consecutive injections of 0.5 mM of dopamine obtained with the IMER and electrochemical detection. The flow rate was 0.7 ml min^{-1}.

study, the inlet of the electrochemical cell was connected to the outlet of a diode array detector (Fig. 1 — III). Spiked serum samples (20 μl) containing levodopa, dopamine, paracetamol, chlorpheniramine, phenylephrine and acetylsalicylic acid were injected. The serum was diluted 10-fold and filtered through sterile 0.45-μm Millipore membranes before being injected into the LC system. All the compounds were added at a concentration of 1 mM.

The selectivity of catalytic detection was first evaluated for serum blank injections. The blank chromatograms are presented in Fig. 6.

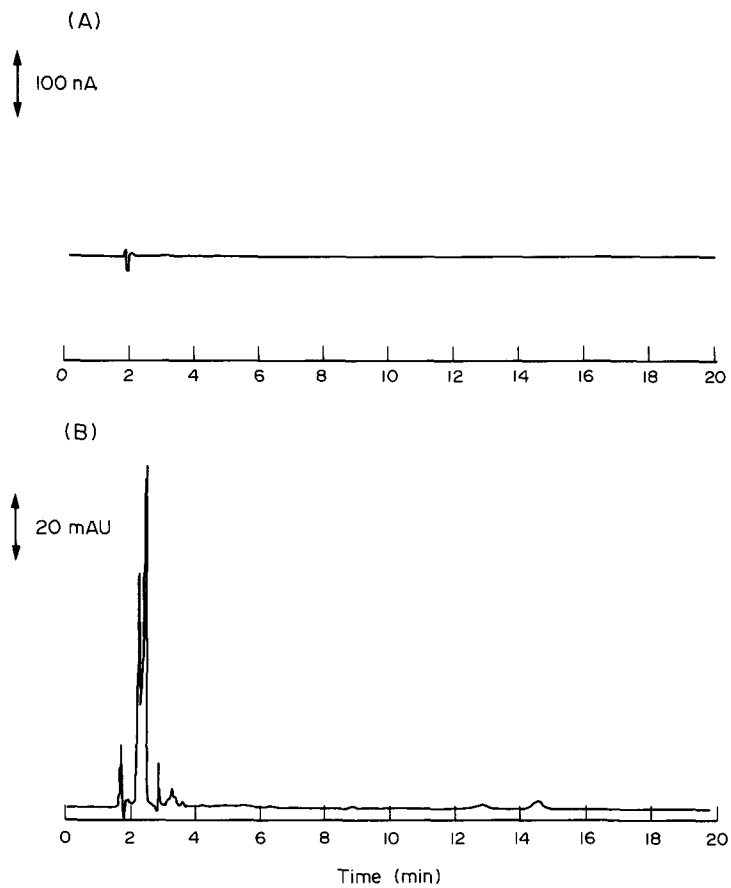


Figure 6
Chromatograms obtained for 20-μl injections of human serum (10-fold diluted and filtered through a 0.45-μm membrane) with post-column (A) catalytic detection (tyrosinase electrode) working at −50 mV vs SCE, and (B) UV detection at 270 nm.

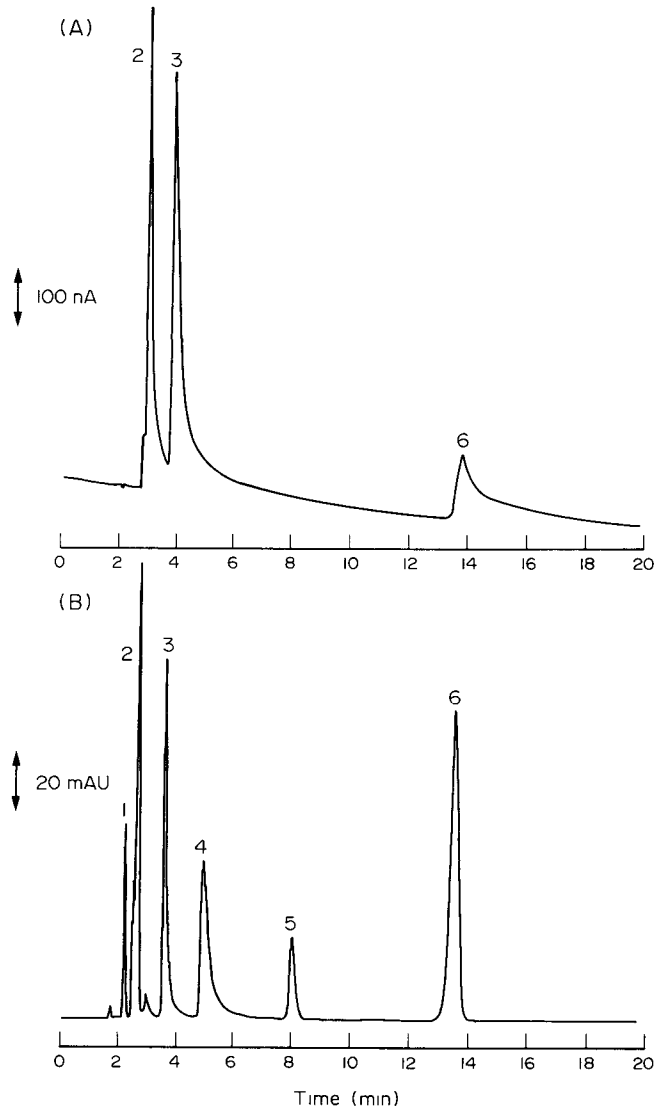


Figure 7
Chromatographic separation of 20-μl injections of spiked serum (10-fold diluted and filtered through a 0.45-μm membrane) samples with (A) post-column catalytic detection (tyrosinase electrode) working at −50 mV vs SCE, and (B) UV detection at 270 nm. Chlorpheniramine (peak 1), levodopa (peak 2), dopamine (peak 3), phenylephrine (peak 4), acetylsalicylic acid (peak 5), and paracetamol (peak 6).

Chromatogram (A) corresponds to tyrosinase electrode detection at −50 mV vs SCE while chromatogram (B) corresponds to UV detection at 270 nm. No interference in the base line can be observed for serum samples when operating with catalytic electrochemical detection. Several unidentified peaks are detected with UV detection. The chromatograms of spiked serum samples are presented in Fig. 7. Chromatograms (A) and (B) correspond to tyrosinase electrode and UV detection, respectively. Phenolic drugs, levodopa (peak 2), dopamine (peak 3) and paracetamol (peak 4), are selectively detected with the enzyme electrode after separation in the analytical column. Figure 7(B) shows the peaks corresponding to clorpheniramine (peak 1), levodopa (peak 2), dopamine (peak 3), phenylephrine (peak 4), acetylsalicylic acid (peak 5), and paracetamol (peak 6). The unknown peaks from the blank [Fig. 6(B)] interfere with levodopa, dopamine and paracetamol proving the necessity of selective detection systems for the characterization of these drugs in complex samples. The tyrosinase graphite electrode shows the required selectivity for the analysis of these samples by LC.

Conclusions

The present work demonstrates the usefulness of post-column catalytic reaction systems for the selective detection of phenolic drugs. Close coupling between biochemical and electrochemical transducers yields a sensitive and selective detection system. The tyrosinase graphite electrode allows the characterization of these drugs in biological fluids.

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