On-line simultaneous determination of *S*- and *R*-perindopril using amperometric biosensors as detectors in flow systems ☆

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Abstract

Two types of flow systems were selected for the simultaneous assay of *S*- and *R*-perindopril (pdp): flow injection analysis (FIA) and sequential injection analysis (SIA). The SIA system was more efficient, because of the highest precision and accuracy, and the lower consumption of sample and buffer. The amperometric biosensors used as detectors in the flow systems were based on L- and D-amino acid oxidase (AAOD). The linear concentration ranges are in the $nmol\,l^{-1}$ range, from $120\,pmol\,l^{-1}$ to $40\,nmol\,l^{-1}$ ($3 \times$ S.D.), with very low detection limits. The biosensors/flow system can be used reliably on-line in synthesis process control, for the simultaneous assay of *S*- and *R*-pdp with a frequency of more than 30 samples per hour. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Flow techniques (e.g. flow injection analysis, FIA; sequential injection analysis, SIA) represent a good alternative for chromatographic techniques used in simultaneous discrimination of enantiomers [1,2]. The main advantages over chromatographic techniques are high precision, sensitivity and accuracy, no need for a complicated sampling process (usually only a dissolution in de-ionized water is required), high rate of sample analysis, and low cost of analysis [1–4].

The role of chirality has become firmly established in the drug industry. Worldwide sales of chiral drugs in single-enantiomer dosage forms continue growing at a rate of >13% per annum. The application of FIA/sensors or SIA/sensors systems for the simultaneous assay of enantiomers is specially needed to be developed for the pharmaceutical industry, due to the necessity to accurately and precisely discriminate between the enantiomers of drugs with a chiral center. The reason for such an analysis is due to the difference in the pharmacokinetics and pharmacodynamics of chiral drugs [5]. Accordingly, for raw materials of the chiral drugs, an enantiopurity test must be performed.

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FIA and SIA are well-known for their high accuracy and precision. The advantages of utilization of SIA over FIA for multi-component analysis are: low consumption of sample and buffer, possibility of using various reagents or/and buffer solutions necessary for the assay of each component separately, the carrier solution when electrochemical sensors are used as detectors is not expensive ($0.1 \, mol \, l^{-1}$ NaCl is used). By introducing electrochemical sensors in their conduits, the reliability of the analytical process increased [6–8]. The following types of electrochemical sensors may be used for the discrimination of the enantiomers: potentiometric, enantioselective membrane electrodes, amperometric biosensors, and amperometric immunosensors [1,4].
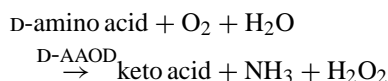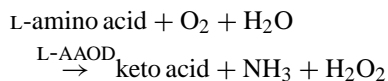
S-Perindopril (*S*-pdp), (2*S*, 3a*S*,7a*S*)-1-[(*S*)-*N*-[(*S*)-1-carbethoxybutyl]alanyl]hexahydro-2-indole carboxylic acid is a long-acting angiotensin-converting enzyme inhibitor. It is a pro-drug that is hydrolyzed to the active metabolite, perindoprilat. Its enantiomer, *R*-pdp, (2*S*,3a*S*,7a*S*)-1-[(*R*)-*N*-[(*S*)-1-carbethoxybutyl]alanyl]hexahydro-2-indole carboxylic acid is a by-product in the synthesis of *S*-pdp [9], that does not have the same pharmacokinetics and pharmacodynamics as the *S*-enantiomer. Therefore, enantiopurity tests for *S*-pdp are required.

The following techniques were proposed for the assay of pdp: gas chromatography [10–12], liquid chromatography [13], radioimmunoassay (RIA) [14], and spectrometric techniques [15,16]. All of these cannot discriminate between *S*- and *R*-enantiomers. For the assay of each enantiomer, biosensors based on L- and D-amino acid oxidase (AAOD) [17,18], a potentiometric, enantioselective membrane electrode [19], as well as FIA/sensor systems [18,20] have been proposed. The proposed biosensors were used for the assay of low concentrations of *S*- and *R*-pdp [17,18] while the potentiometric, enantioselective membrane electrode was used for the assay of higher concentrations of *S*-pdp [19]. The utilization of biosensors in FIA systems for the assay of a single-enantiomer were successful [18,20], but only the simultaneous assay of enantiomers using flow systems will be able to compete with the chromatographic techniques.

The emphasis of this paper is on FIA and SIA systems designed for the simultaneous assay of the *S*- and *R*-pdp. The electrochemical sensors used as detectors are both amperometric biosensors. The L- and

D-AAOD were used for the construction of carbon paste based amperometric biosensors. The selection of this type of sensor was due to their high sensitivity.

The principle of chiral recognition for this type of biosensors is well known [1]. The L- and D-AAOD will catalyze only the reaction of L- or D-amino acid, respectively. Perindopril has an amino acid moiety, so the reactions involved are:

$$\text{L-amino acid} + O_2 + H_2O$$
$$\overset{\text{L-AAOD}}{\rightarrow} \text{keto acid} + NH_3 + H_2O_2$$

$$\text{D-amino acid} + O_2 + H_2O$$
$$\overset{\text{D-AAOD}}{\rightarrow} \text{keto acid} + NH_3 + H_2O_2$$

Hydrogen peroxide is the product of both biochemical reactions, that is measured by the amperometric transducer.

2. Experimental

2.1. Electrodes design

Two electrodes, based on graphite paste, were designed as follows: Paraffin oil and graphite powder were mixed in a ratio 1:4 (w/w) to form a graphite paste. $100 \, \mu l$ from each enzymatic solution (1 mg enzyme/ml in $0.1 \, mol \, l^{-1}$ phosphate buffer, pH = 7.0 (Merck)): L-AAOD (E.C. 1.4.3.2. Type I: Crude Dried Venom from *Crotalus adamanteus*, Sigma) solution and D-AAOD (E.C. 1.4.3.3. Type I: from Porcine Kidney, Sigma) solutions, were respectively added to two separate portions of graphite paste. Two plastic tips were filled with the corresponding graphite–paraffin oil paste leaving an empty space of 3–4 mm in the top part filled with carbon paste containing the enzyme. The diameter of each sensor was 3 mm. Electrical contact was obtained by inserting a silver wire into the carbon paste. Both electrodes tips were gently rubbed on fine paper to produce a flat surface. The surface of the electrode was wetted with de-ionized water and then polished with alumina paper (polished strips 30144-001, Orion) before use. The biosensors were stored dry at $4 \, ^\circ C$.

2.2. Apparatus

A 663 VA Stand (Metrohm, Herisau, Switzerland) in connection with a PGSTAT 20 and software (Eco
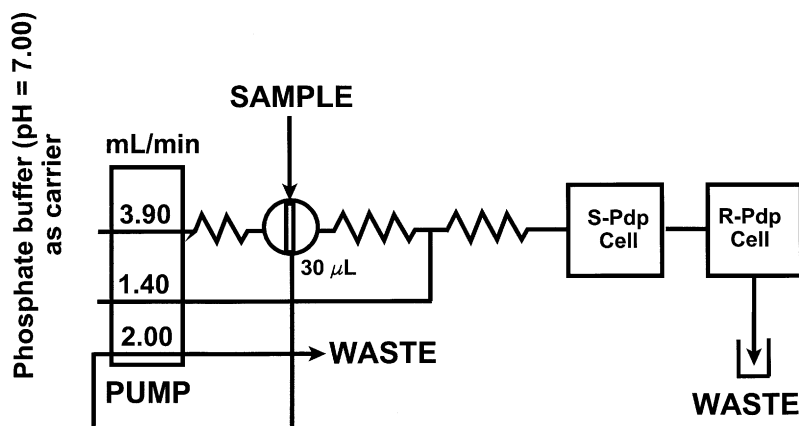
Fig. 1. Schematic flow diagram of FIA system used for the simultaneous determination of *S*- and *R*-pdp.

Chemie version 4.4) was used for all chronoamperometric ($E = 650\,\text{mV}$) measurements. A glassy carbon electrode and a calomel electrode served as the counter and reference electrodes in the cell.

2.3. Flow injection system

The electrodes were incorporated into the conduits of a flow injection system (Fig. 1), similar to that previously described [18,20]. A Carle microvolume two-position sampling valve (Carle No. 2014) containing two identical sample loops was used. Each loop has a volume of 30 µl. A Cenco peristaltic pump operating at 10 rpm supplied the carrier streams to the manifold system. Tygon tubing (0.51 mm i.d.) was used to construct the manifold; coils were wound round suitable lengths of glass tubing (15 mm o.d.). Phosphate buffer was used as carrier solution. The sample was injected in a phosphate buffer (pH = 7.00) stream. A 60 s cycle sampling time was used, giving the system a capacity of about 60 samples per hour.

2.4. Sequential injection system

The biosensors were incorporated into the conduits of a SIA system (Fig. 2) constructed from a Gilson Minipuls peristaltic pump and a 10-port electrically actuated selection valve (Model ECSD10P, Valco Instruments, Houston, TX). Tygon tubing (0.76 mm i.d. for both holding coils and 0.89 mm i.d. for both mixing coils) was used to construct the manifold; coils were

wound round suitable lengths of glass tubing (15 mm o.d.); $0.1\,\text{mol}\,\text{l}^{-1}$ NaCl was used as carrier. The capacity of the system is about 38 samples per hour. The device operating sequence is shown in Table 1.

Table 1
Device sequence for one cycle of the SIA system

| Time (s)	Pump	Valve	Description
0	Off	Buffer	Pump stops, select buffer stream (valve position 1)
5	Reverse	Buffer	Draw up buffer solution
9.5	Off		Pump stops
10.5		Sample	Select sample stream (valve position 2)
11.5	Reverse	Sample	Draw up sample solution
16	Off		Pump stops
17		S-Pdp cell	Select S-pdp cell line (valve position 3)
18	Forward		Pump stack of zones to S-pdp cell
48	Off		Pump stops
49		Buffer	Select buffer stream (valve position 4)
50	Reverse	Buffer	Draw up buffer solution
54.5	Off		Pump stops
55.5		Sample	Select sample stream (valve position 5)
56.5	Reverse	Sample	Draw up sample solution
61	Off		Pump stops
62		R-Pdp cell	Select R-pdp cell line (valve position 6)
63	Forward		Pump stack of zones to R-pdp cell
93	Off	Home	Pump stops, return valve to starting position (valve position 1)
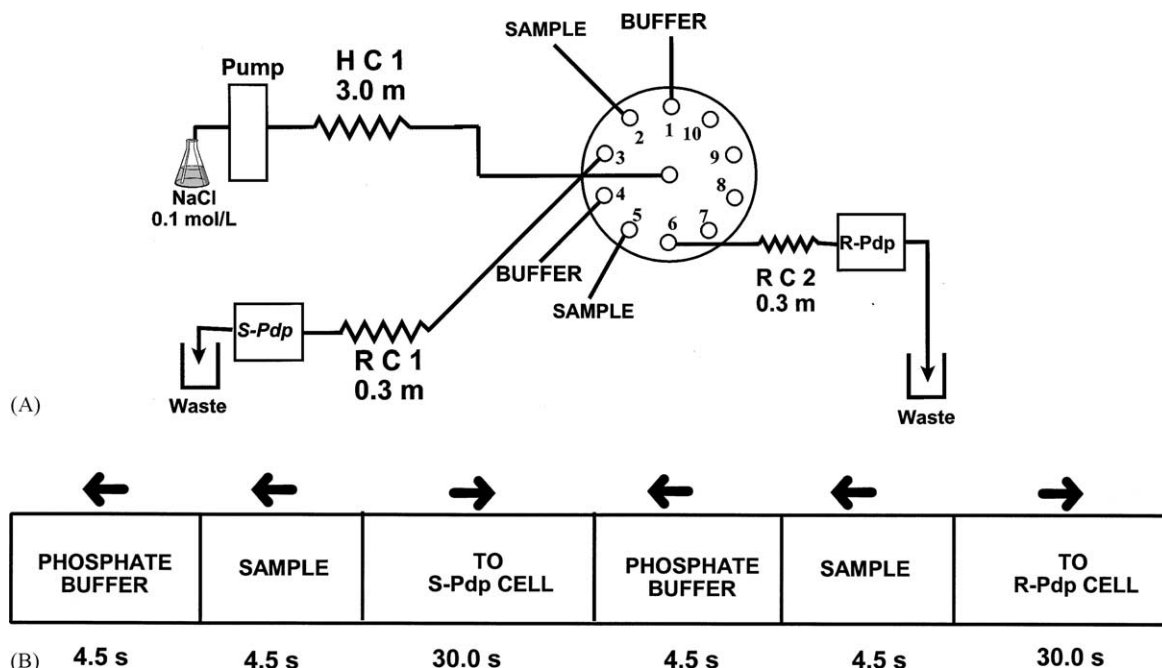
Fig. 2. SIA system used for the simultaneous determination of *S*- and *R*-pdp. (A) Schematic flow diagram; (B) sequence of rinse, sample, phosphate buffer, and electrochemical cells for *S*- and *R*-pdp.

For both flow systems, the device control was achieved using a PC30-B interface board (Eagle Electric, Cape Town, South Africa). The FlowTEK [21] software package (obtainable from MINTEK) for computer-aided flow analysis was used throughout for device control.

2.5. Reagents and materials

The *S*- and *R*-pdp were supplied by Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ). Graphite powder, $1–2\,\mu m$, synthetic, was supplied by Aldrich (Buchs, Switzerland). Paraffin oil was supplied by Fluka. Phosphate buffer (pH $= 7.00$) was supplied by Merck (Darmstadt, Germany).

De-ionized water from a Modulab system (Continental Water Systems, San Antonio, TX) was used for all solutions. The *S*- and *R*-pdp solutions were prepared from standard *S*- and *R*-pdp solutions $(1 \times 10^{-2}\,\text{mol}\,l^{-1})$, respectively, by serial dilution.

3. Results and discussion

An optimum flow rate of $3.61\,\text{ml}\,\text{min}^{-1}$ was used to propel the solutions in both flow systems. The timing and flow direction for the SIA system is shown in Fig. 2B. It follows from this that in the SIA system, the sample and buffer consumption is only $270\,\mu l$ each per measurement of *S*- and *R*-enantiomer, which is very economical.

3.1. The optimum working pH of the amperometric biosensors

The variation of peak current (measured as relative peak height (H) by using the SIA system) with pH is given in Figs. 3 and 4 for the FIA and SIA systems, respectively. As seen from the graph, the response of the system varied with pH. The optimum pH value for the amperometric biosensor was determined for 40 and $600\,\text{nmol}\,l^{-1}$ *S*-pdp, in the FIA and SIA systems, respectively and for 10 and $80\,\text{nmol}\,l^{-1}$ *R*-pdp in the FIA and SIA systems, respectively, using
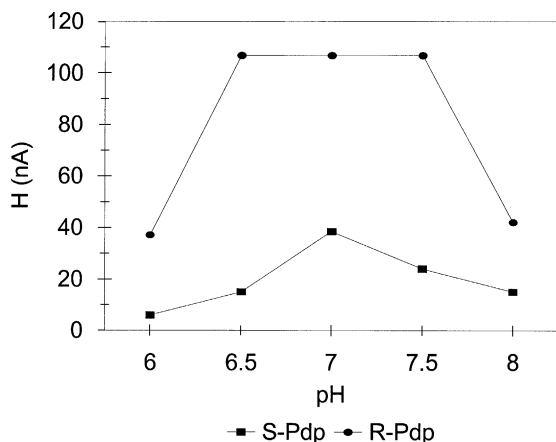
Fig. 3. The variation of peak heights, *H*, with the pH for the FIA/amperometric biosensors system.

various phosphate buffers with pH varying between 6.00 and 8.00.

For the FIA system, the optimum pH values for the assay of *S*- and *R*-enantiomers were found to be 7.0 and between 6.5 and 7.5 (maximum and constant value for the peak height recorded), respectively. For the SIA system, the optimum pH values for the assay of *S*- and *R*-enantiomers were found to be 7.0 and between 6.5 and 7.0 (maximum and constant value for the peak height recorded), respectively. Accordingly, a pH of 7.0 was selected to perform the measurements in both flow systems for both enantiomers.
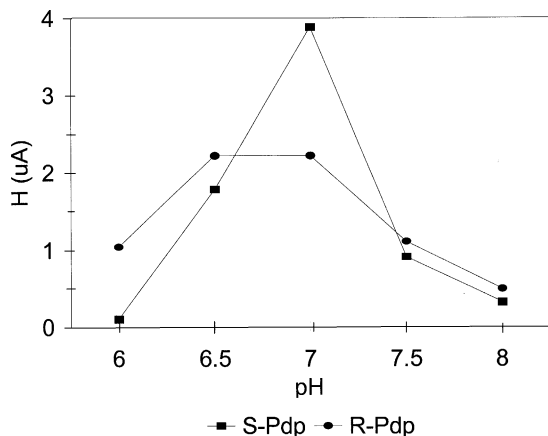

Fig. 4. The variation of peak heights, *H*, with the pH for the SIA/amperometric biosensors system.

The amperometric biosensors/FIA and amperometric biosensors/SIA systems are working at non-equilibrium conditions. Therefore, a major advantage of the flow systems is the repeatable handling of sampling due to the control of the flow pattern.

3.2. Electrodes response

The response of both electrodes was determined using a chronoamperometric technique ($E = 650\,\mathrm{mV}$), using Pt and saturated calomel electrodes as auxiliary and reference electrodes, respectively at a pH = 7.00 (phosphate buffer). The calibration equations obtained for the amperometric biosensors ($n = 10$) are as follows:

FIA/amperometric biosensors system:

$$S\text{-perindopril}: H = 20.0 + 0.08c; \quad r = 0.9994$$

$$R\text{-perindopril}: H = 26.5 + 8.27c; \quad r = 0.9993$$

SIA/amperometric biosensors system:

$$S\text{-perindopril}: H = 0.32 + 0.008c; \quad r = 0.9963$$

$$R\text{-perindopril}: H = 9.00 + 0.10c; \quad r = 0.9938$$

where, *H* is the peak height in nA and *c* is the concentration of *S*- and *R*-captopril in $\mathrm{nmol\,l^{-1}}$.

Linear concentration ranges between $240\,\mathrm{pmol\,l^{-1}}$ and $60\,\mathrm{nmol\,l^{-1}}$, and between 6 and $40\,\mathrm{nmol\,l^{-1}}$ for *S*- and *R*-pdp, respectively, with limits ($3 \times$ S.D.) of detection of $120\,\mathrm{pmol\,l^{-1}}$ and $4\,\mathrm{nmol\,l^{-1}}$, respectively were obtained when the FIA/amperometric biosensors system was used. For the SIA/amperometric biosensors system, the linear concentration ranges for the *S*- and *R*-enantiomers were between 60 and $800\,\mathrm{nmol\,l^{-1}}$ and between 60 and $900\,\mathrm{nmol\,l^{-1}}$, respectively, with a limit of detection of $40\,\mathrm{nmol\,l^{-1}}$ for both enantiomers. The working concentration ranges as well as the limits of detection demonstrated the suitability of the proposed amperometric biosensors for the on-line monitoring of both enantiomers.

The response obtained for both biosensors revealed good stability and reproducibility for tests performed over 2 weeks. (The RSD values obtained during this period were <0.1% for the intercepts and slopes).

Table 2
Selectivity coefficients pK_{amp} for the amperometric biosensors in the FIA and SIA systems

Interfering species (J)	S-Pdp	R-Pdp	L-Proline	D-Proline	PVP
S-Pdp					
FIA	–	4.00	1.12	3.40	3.70
SIA	–	3.62	1.10	2.31	2.40
R-Pdp					
FIA	4.74	–	3.96	1.96	4.04
SIA	3.92	–	4.00	1.89	3.68

All measurements were made at room temperature; all values are the average of 10 determinations.

3.3. Selectivity of the biosensors

The selectivity of both biosensors was checked using the mixed solutions method, with respect to $R(S)$-pdp, D-proline, L-proline, and polyvinylpyrolidone (PVP). Amperometric selectivity coefficients were determined following the method proposed by Wang [22]. The L- and D-proline were selected due to their possible status as by-products. PVP is very often used as a compression compound for tablets. In the evaluation, the concentration of the interferent was selected to be four times that for the enantiomer of interest. As is shown in Table 2, the proposed biosensors are enantioselective when used as detectors in a SIA system. The results obtained, revealed that both sensors also have good selectivity over PVP. Inorganic cations such as Na^+, K^+, Ca^{2+} do not interfere in the determination.

3.4. Analytical applications

The flow systems obtained by incorporation of the amperometric biosensors in the FIA and SIA conduits, proved to be useful for the simultaneous assay of S- and R-pdp. The recovery tests for each enantiomer in the FIA systems were $99.92 \pm 0.34\%$ for S-pdp ($n = 10$) and $99.86 \pm 0.10\%$ for R-pdp ($n = 10$) and in the SIA systems were $99.62 \pm 0.05\%$ for S-pdp ($n = 10$) and $99.93 \pm 0.03\%$ for R-pdp ($n = 10$). This demonstrated the suitability of the proposed biosensors/FIA and biosensor/SIA systems for simultaneous assay of the enantiomers. Simultaneous detection of the enantiomers was done using different ratios between R- and S-pdp. The results obtained (Tables 3 and 4) demon-

Table 3
The results obtained for the assay of S-pdp in the presence of R-pdp

S:R (mol:mol)	Recovery S-pdp[a] (%)	
	FIA	SIA
2:1	99.87 ± 0.02	99.90 ± 0.01
1:1	99.90 ± 0.01	99.97 ± 0.02
1:2	99.89 ± 0.01	99.94 ± 0.02
1:3	99.90 ± 0.03	99.96 ± 0.01
1:99.9	99.90 ± 0.02	99.99 ± 0.01

[a] Mean ± S.D. of 10 determinations.

Table 4
The results obtained for the assay of R-pdp in the presence of S-pdp

R:S (mol:mol)	Recovery R-pdp[a] (%)	
	FIA	SIA
2:1	99.74 ± 0.05	99.46 ± 0.01
1:1	99.87 ± 0.03	99.42 ± 0.02
1:2	99.86 ± 0.02	99.91 ± 0.01
1:3	99.80 ± 0.04	99.92 ± 0.01
1:99.9	99.86 ± 0.02	99.93 ± 0.01

[a] Mean ± S.D. of 10 determinations.

strated the suitability of the proposed flow system for the on-line purity tests of S-pdp. No differences were recorded in recovery tests between 1:3 and 1:99.9 (mole ratio, in the favor of each enantiomer).

The recovery test for both S- and R-enantiomers was done only for the raw substance, because the recommendation for the pharmaceutical product is that it must contain only the S-pdp. Therefore, there is no need to perform an uniformity content test with the proposed system for the pharmaceutical formulations.

4. Conclusions

The paper opens a new and very important field in the use of biosensors/FIA and biosensors/SIA systems for simultaneous assay of enantiomers.

The main advantages of the proposed systems are: simplicity of construction and operation—that involved its introduction for on-line monitoring of enantiomers during the synthesis of enantiomers, high reliability of analytical information, rapidity, and low cost of the analysis. Due to its higher reliability and versatility, as well as due to its lower consumption

of reagents, the SIA/biosensors system is preferred to the FIA/biosensors system for the simultaneous assay of enantiomers. The high precision of the flow based systems is due to the fact that all the measurements are done after the same interval of time. The surface of the biosensors are continuously washed by the sodium chloride or phosphate buffer carrier streams.

The main disadvantage of the utilization of biosensors as detectors in flow system is their short lifetime. Due to the low working concentrations levels the raw material must sometime be diluted on-line. The only possible interferences from the real raw material samples are L- and D-proline. The enantioselectivity versus these compounds was checked, and it was proved that it was better in a flow system, compared with a static method of analysis.

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