Chapter 5

Enantioanalysis of L- and D-pipecolic acid in biological samples

5.1 Introduction

Piperidino-2-carboxylic acid, more commonly known as pipecolic acid, was first identified in the ruminant animals by Onodera and Kandatsu [1]. Pipecolic acid, a cyclic imino acid, is a minor metabolite of lysine, and includes D- and L-enantiomers [2]. Lysine metabolism in mammal is known to occur via sacharopine in the liver and via pipecolic acid in the brain [3-4]. The former is considered to be the major pathway and the latter the minor one for the entire body, although the latter may also be a major pathway in the brain. L-pipecolic acid may be derived from L- and D-lysine by mammalian and bacterial enzyme [5]. However, D-pipecolic acid is considered to originate from D-lysine only by bacterial D-amino acid oxidase [5]. Pipecolic acid is taken up into synaptosomes [6-7], is released form brain slices in a Ca^{2+}-dependaent manner [8], and depress excitatory synaptic transmission [9-10], suggesting that pipecolic acid serves as a modulator of synaptic transmission. In addition, pipecolic acid may facilitate GABAergic transmission by stimulating GABA release [11], inhibiting GABA uptake [12], or enhancing $GABA_A$ receptor responses [13].

L-pipecolic acid (L-2-piperidine carboxylic acid, L-PA, Figure 1.1) is an important biomedical marker for peroxisomal disorder diagnosis, as its concentration in plasma is

increased in neonatal adrenoleukodystrofy (Zellweger syndrome) and infantile Refsum disease [14-16]. Hyperpipecolic acidemia in patients with Zelleweger syndrome is thought to be caused by a disturbance in peroxisomal pipecolic acid oxidase activity [17]. L-PA is a minor intermediate in the L-lysine catabolic pathway in the peroxisomes, where it is oxidized to α-aminoadipic acid. The assay of L-PA is considered as a supplementary test for peroxisomal disease following the analysis of very long chain fatty acids (VLCFAs), bile acids, phytanic acid, and pristanic acid in plasma. D-pipecolic acid (D-PA, Figure 1.1) is a marker of liver cirrhosis and chronic hepatic encephalopathy and it originates from the metabolism of intestinal bacteria and from dietary sources [18-21].

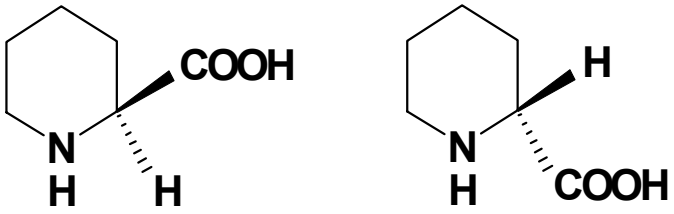

Figure 1.1 Pipecolic acid, (a) L- pipecolic acid and (b) D-pipecolic acid

Different methods were proposed for the determination of pipecolic acid [22-34]. The enantioselective analysis of pipecolic acid was performed using liquid chromatography [22-24], gas chromatography [25], capillary zone electrophoresis [26] and thin-layer chromatography [27]. Chromatographic methods, e.g., HPLC [28-30], and gas chromatography [31-34] were also used for the assay of the total concentration of L- and D-pipecolic acid.

In this chapter, different electrochemical sensors are proposed for the enantioanalysis of L- and D-pipecolic acids: amperometric electrodes and biosensors and enantioselective, potentiometric membrane electrodes.

Amperometric electrodes and amperometric biosensors and enantioselective, potentiometric membrane electrodes represents a good alternative for the chromatographic methods for the enantioanalysis because of the combination of their sensitivity and selectivity. These electrochemical sensors are capable of direct enantioanalysis of pipecolic acid without prior or minor pretreatments of the sample. A feature of the proposed sensors is *in vivo* enantioanalysis of pipecolic acids.

Monocrystalline diamond paste based electrodes proved to be a good alternative for the classical glassy carbon and carbon paste electrodes used in differential pulse voltammetry method [35-38]. Furthermore, the utilization of these electrodes in clinical analysis is increasing the reliability of the analytical information. A monocrystalline natural diamond paste electrode is proposed for the assay of L- and D-pipecolic acids.

Three enantioselective, potentiometric membrane electrodes were proposed for the enantioanalysis of L-pipecolic acid. These EPMEs are based on carbon paste impregnated with maltodextrins as chiral selectors [39].

Enantioselective amperometric biosensors based on physical or chemical immobilization of L-amino acid oxidase (L-AAOD) and/or D-amino acid oxidase (D-AAOD) in

monocrystalline natural diamond paste or in carbon paste are proposed for the direct enantioselective analysis of L- and D-pipecolic acid in serum samples.

5.2 Reagents and chemicals

Natural diamond powder with particle size ca. 1 μm was purchased from Aldrich. Graphite powder, 1-2 μm was supplied by Aldrich (Milwaukee, WI, USA). Paraffin oil was obtained from Fluka (Buchus, Switzerland). De-ionized water from a Modulab system (continental water systems, San Antonio, TX, USA) was used for all solution preparations: L-amino acid oxidase (L-AAOD) (E.C.1.4.3.2. Type I crude Dried Venom from Crotalus adamanteus, 0.53 units/mg solid (Sigma)) solution, D-amino acid oxidase (D-AAOD)(E.C.1.4.3.3.Type I: from porcine kidney, 1.3 units/mg solid (Sigma)) solution, horseradish peroxidase (HRP)(EC 1.11.1.7 Type I from Horseradish, 87 purpurogallin units/mg solid (Sigma)) solution and solutions of L-and D-pipecolic acid (10^{-4} mol/L), respectively. L- and D-pipecolic acids were purchased from Aldrich (Milwaukee, WI, USA). Maltodextrins (DE 4.0-7.0, 13.0-17.0, 16.5-19.5) were purchased from Aldrich (Milwaukee, WI, USA). Phosphate buffer (pH=7.00) and citrate buffer (0.1mol/L, pH = 2.5) were supplied by Merck (Darmstadt, Germany).

De-ionized water from a Modulab system (Continental water systems, San Antonio, TX, USA) was used for all solution preparations. The L- and D-PA solutions were prepared from stock L- and D-PA solutions ($1x10^{-3}$ mol l^{-1}), by serial dilutions.

5.3 Amperometric electrode for enantioselective analysis of pipecolic acid

5.3.1Apparatus

A 663 VA Stand (Metrohm, Herisau, Switzerland) in combination with an Autolab 20 and software (Ecochemie version 4.9) were used for all differential pulse voltammetry measurements. A platinum electrode and an Ag/AgCl (0.1 mol/L KCl) electrode served as the counter and reference electrodes in the cell.

5.3.2 Electrode Design

Diamond paste electrode was prepared by mixing 0.1 g of diamond powder with 20 μL paraffin oil. A portion of the paste was then filled into a plastic pipette tip. The diameter of the sensing part was 3mm. Electric contact was made by inserting a silver wire in the paste. Before each use the electrode surface was smoothed out by polishing with alumina paper (polishing strips 30144-001, Orion). When not in use, the diamond paste was stored at room temperature.

5.3.3 Recommended procedure: Direct DPV assay

The technique used for the direct voltammetric assay was differential pulse voltammetry with the applied potential pulse amplitude of 25mV vs. Ag/AgCl. The diamond paste electrode together with the reference and auxiliary electrodes were dipped into a cell containing the buffer and supporting electrolyte (sodium pyrophosphate or NaCl) in a ratio of 3.5:1, as well as the synthetic mixture between L- and D-pipecolic acids. All

solutions were deoxygenated for 5 min before the measurements with N_2. The peak height measured at 900 and 300 mV vs Ag/AgCl for L- and D-pipecolic acid, respectively, was plotted versus the concentration of L- and D-pipecolic acid, respectively. The unknown concentrations of L- and D-pipecolic acid were determined from the corresponding calibration graphs.

5.3.4 Results and Discussion

5.3.4.1 Electrode Response

The electrode response was determined using DPV technique. The response characteristics and the equations of calibration are shown in Table 5.1. Different buffers (citrate buffer, pH=5.0 and phosphate buffer, pH=7.0) and two electrolytes (sodium pyrophosphate (I) or NaCl (II)) were used in order to establish the optimum working conditions for the enantioselective determination of each enantiomer. Accordingly, the highest sensitivity and the lowest limit of detection were recorded when citrate buffer (pH=5.0) was used with sodium pyrophosphate as supporting electrolyte. But, taking into account that the concentration of L-PA in the serum is in the μmol/L magnitude order, the best working conditions will be assured by the utilization of phosphate buffer (pH=7.0) with NaCl as supporting electrolyte.

For the assay of D-PA, the optimum working conditions will be given by utilization of citrate buffer with sodium pyrophosphate as supporting electrolyte, because the electrode has very good sensitivity, a wide concentration range covering the limits within which D-PA is found in the serum samples, and it has the lowest limit of detection. The signal to

background ratio is very high when compared to classical glassy carbon (GC) and carbon paste electrodes. The reproducibility of peak current was excellent (RSD% values recorded were less than 0.1%), when the measurements were done everyday for a period of 6 months.

Table 5.1 Response characteristics of the amperometric electrode for L- and D-pipecolic acid assay.

Analyte	Electrolyte	Buffer	Linear conc. range	Detection limit	Equation of calibration*	r
L-PA	I	Citrate, pH=5.0	0.01-10 pmol/L	1 fmol/L	$^{1,a}H = 5.21 + 0.230C$	0.9980
		Phosphate, pH=7.0	1-100 pmol/L	0.1 pmol/L	$^{1,a}H = 1.63 + 0.003C$	0.9999
	II	Citrate, pH=5.0	1-100 nmol/L	1 pmol/L	$^{1,b}H = 2.22 + 0.014C$	0.9997
		Phosphate, pH=7.0	0.01-10000 nmol/L	1 pmol/L	$^{2,b}H = 204.5 + 1.27C$	0.9966
D-PA	I	Citrate, pH=5.0	0.0001 - 100 nmol/L	10 fmol/L	$^{1,b}H = 1.39 + 0.03C$	0.9997
		Phosphate, pH=7.0	0.1-100 nmol/L	1 pmol/L	$^{1,b}H = 1.39 + 0.01C$	0.9995
	II	Citrate, pH=5.0	0.01 – 1 nmol/L	0.1 pmol/L	$^{1,b}H = 4.03 + 0.11C$	0.9997
		Phosphate, pH=7.0	1-1000 nmol/L	10 pmol/L	$^{2,b}H = -2.17 + 0.49\ C$	0.9970

*H is the peak height in $^{1}\mu A$, ^{2}nA and C is the concentration of L- and D-pipecolic acid, respectively, in ^{a}pmol/L; ^{b}nmol/L.

5.3.4.2 Selectivity of the diamond paste based electrode

The selectivity of the electrode was checked using the mixed solution method. The amperometric selectivity coefficients were determined using the equation proposed by Wang [40].

Table 5.2 Enantioselectivity of the amperometric electrode designed for L- and D-pipecolic acid

Analyte	Interfering enantiomer	Electrolyte	Buffer	pK_{amp}
L-PA	D	I	Citrate, pH=5.0	2.97
			Phosphate, pH=7.0	2.24
	D	II	Citrate, pH=5.0	3.00
			Phosphate, pH=7.0	3.00
D-PA	L	I	Citrate, pH=5.0	2.18
			Phosphate, pH=7.0	2.96
	L	II	Citrate, pH=5.0	2.99
			Phosphate, pH=7.0	2.17

All values are the average of ten determinations.

The ratio between the concentrations (mol/L) of the main analyte and interferent was 1:10. The pK_{amp} values (Table 5.2) show a good enantioselectivity of the electrode in the proposed working conditions. Accordingly, the electrode can be used for the enantioselective analysis of pipecolic acid. Inorganic ions such as Na^+, K^+ and Ca^{2+} did not interfere with the analysis of pipecolic acid.

5.3.4.3 Analytical Applications

The differential pulse voltammetry proved useful for enantioanalysis of pipecolic acid in serum samples.

In order to prove the suitability of the proposed electrodes and method (DPV) for the enantioanalysis of pipecolic acids, recovery tests were performed for the solutions containing different ratios between the two enantiomers. The results (Tables 5.3 and 5.4) show that L- and D-pipecolic acid can be successfully recovered one in the presence of the other.

Table 5.3 Determination of L-pipecolic acid in the presence of D-pipecolic acid

Electrolyte	Recovery L-pipecolic acid, (%)					
	L: D					
	Buffer	2:1	1:1	1:2	1:4	1:9
I	Citrate pH=5.0	99.87 ± 0.03	99.88 ± 0.03	99.85± 0.04	99.89 ± 0.03	99.90 ± 0.04
	Phosphate pH=7.0	99.93 ± 0.04	99.90 ± 0.02	99.92 ± 0.03	99.95 ± 0.03	99.93 ± 0.03
II	Citrate pH=5.0	99.93 ± 0.02	99.90 ± 0.03	99.91 ± 0.03	99.90 ± 0.02	99.89 ± 0.02
	Phosphate PH=7.0	99.93 ± 0.02	99.92 ± 0.03	99.91 ± 0.02	99.92 ± 0.02	99.92 ± 0.02

All values are the average of ten determinations.

No significant differences in the recovery values were recorded for the ratios between L:D or D:L enantiomers varying from 1:9 to 1:99.99.

Table 5.4 Determination of D-pipecolic acid in the presence of L-pipecolic acid

Electrolyte	Recovery D-pipecolic acid, (%)					
	D: L					
	Buffer	2:1	1:1	1:2	1:4	1:9
I	Citrate pH=5.0	99.82 ± 0.04	99.78 ± 0.03	99.76± 0.03	99.80 ± 0.04	99.79 ± 0.03
	Phosphate pH=7.0	99.94 ± 0.03	99.48 ± 0.03	99.54 ± 0.02	99.51 ± 0.03	99.55 ± 0.03
II	Citrate pH=5.0	99.42 ± 0.04	99.69 ± 0.03	99.65 ± 0.03	99.67 ± 0.04	99.66 ± 0.03
	Phosphate pH=7.0	99.56 ± 0.03	99.50 ± 0.02	99.58 ± 0.02	99.56 ± 0.03	99.59 ± 0.02

All values are the average of ten determinations.

Samples were collected from patients suspected of peroxisomal disorders (1-3) and hepatitis B (4-6). L- and D-pipecolic acids were assayed from serum samples using a standard method (41) as well as the proposed diamond paste based electrode (Table 5.5).

Table 5.5 Determination of L-and D-pipecolic acid in serum samples

PA	Sample No.	Standard Method	Proposed Method			
			Sodium pyrophosphate		NaCl	
			Citrate buffer pH=5.0	Phosphate buffer pH=7.0	Citrate buffer pH=5.0	Phosphate buffer pH=7.0
		(: mol/L)	(: mol/L)			
L	1	1.00	0.99 ± 0.02	0.98 ± 0.03	1.01 ± 0.02	0.99 ± 0.04
	2	5.00	4.99 ± 0.04	4.97 ± 0.02	4.98 ± 0.02	4.97 ± 0.03
	3	6.50	6.49 ± 0.02	6.48 ± 0.02	6.49 ± 0.03	6.49 ± 0.02
		(nmol/L)	(nmol/L)			
D	4	14.20	14.18 ± 0.03	14.15 ± 0.03	14.17 ± 0.04	14.19 ± 0.03
	5	11.00	10.94 ± 0.04	10.99 ± 0.02	10.95 ± 0.04	10.98 ± 0.03
	6	28.00	27.93 ± 0.04	27.92 ± 0.02	27.96 ± 0.03	27.98 ± 0.02

All values are the average of ten determinations

Good correlations between the results were obtained using both standard and new developed method.

5.4 Enantioselective, potentiometric membrane electrodes for the determination of L-pipecolic acid in serum

5.4.1 Apparatus

A 663 VA stand (Metrohm, Herisau, Switzerland) connected to a PGSTAT 100 and software (Eco Chemie version 4.9) was used for all potentiometric measurements. An Ag/AgCl (0.1 mol/L KCl) electrode was used as reference electrodes in the cell.

5.4.2 Electrode design

Paraffin oil and graphite powder were mixed in a ratio of 1:4 (w/w) followed by the addition of the aqueous solution of maltodextrin (DE 4.0 -7.0 (I), 13.0 - 17.0 (II), or 16.5

- 19.5 (III)) (10^{-3} mol/L) (100 µL chiral selector solution to 100 mg carbon paste). A certain quantity of carbon paste free of maltodextrin was prepared and it was placed into a plastic pipette peak leaving 3 to 4 mm empty in the top to be filled with the carbon paste that contains the chiral selector. The diameter of the EPME was 3 mm. Electric contact was obtained by inserting a Ag/AgCl wire in the carbon paste. A solution of 0.1 mol/L KCl was utilized as internal solution.

The surface of the electrodes was wetted with deionised water and polished with alumina paper (polishing strips 30144-001, Orion) before using them for each of the experiments. When not in use, the electrode was immersed in a 10^{-3} mol/L L-pipecolic acid solution.

5.4.3 Recommended procedure. Direct potentiometry

Direct potentiometry was used for potential measurement of each standard solution (10^{-10} -10^{-3} mol/L). The electrodes were placed in stirred standard solutions. Calibration graphs were obtained by plotting E(mV) versus p(L-PA). Unknown concentrations were determined from the corresponding calibration graph.

5.4.4 Results and discussion

5.4.4.1 Response characteristics of the EPMEs

The responses of the electrodes were determined for both enantiomers L-PA and D-PA, at pH=2.5 (citrate buffer) using a potentiometric method. The responses obtained for D-PA were not linear and non-Nernstian. That proved that the electrodes couldn't be used for the assay of D-PA. The equations of calibration obtained for L-PA are as follows:

(I) E =503.0 - 53.42 p(L-PA) r = 0.9934

(II) E = 432.1 - 52.60 p(L-PA) r = 0.9906

(III) E = 614.8 - 52.19 p(L-PA) r = 0.9918

where E(mV) is the cell potential, p(L-PA) = -log[L-PA], and (I), (II) and (III) correspond to the EPMEs based on the maltodextrins (I), (II), and (III), respectively. The response characteristics for the EPMEs when used for the assay of L-PA are shown in Table 5.6. All electrodes showed very low detection limits, maltodextrin (I) and (II) based electrodes in the magnitude order of 10^{-9} mol/L and the maltodextrin (III) based electrode in the magnitude order of 10^{-12} mol/L. The electrodes responses showed good stability and reproducibility for all the performed tests for 6 months, when used daily for measurements (RSD<1.0%).

Table 5.6 Response characteristics of enantioselective, potentiometric membrane electrodes for L-pipecolic acid

Chiral selector	Slope (mV/pL-PA)	Intercept, E^o (mV)	Linear concentration range (mol/L)	Detection limit (mol/L)
Maltodextrin (I)	58.70	503.0	10^{-8}-10^{-3}	$1.00x10^{-9}$
Maltodextrin (II)	52.60	432.1	10^{-8}-10^{-5}	$6.00x10^{-9}$
Maltodexrin (III)	52.19	614.8	10^{-10}-10^{-6}	$1.66x10^{-12}$

All values are the average of ten determinations.

The response time was less than 1 minute for the concentration range between 10^{-8} and 10^{-5} mol/L, when maltodextrin (II) based electrode was used; for maltodextrin (I) based electrode the response time was higher than 1 min for concentrations between 10^{-8} and 10^{-6} mol/L, while for concentrations between 10^{-5} and 10^{-3} mol/L the response time was lower than 1min; for maltodextrin (III) based electrode, the response times was less than

1min for concentrations between 10^{-10} and 10^{-8} mol/L and higher than 1min for a concentration range between 10^{-7} and 10^{-6} mol/L.

5.4.4.2 The influence of pH on the responses of the electrodes

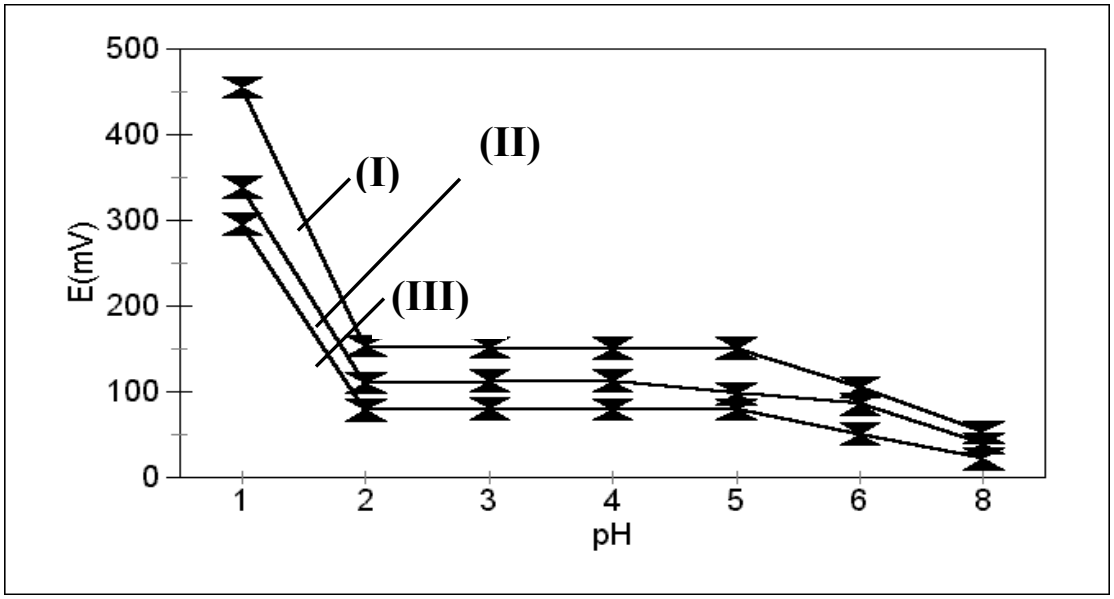

Figure 5.2 Effect of pH on the response of the enantioselective, potentiometric membrane electrodes for L-pipecolic acid (10^{-7} mol/L L-pipecolic acid solution). (I) – maltodextrin (I) based EPME; (II) – maltodextrin (II) based EPME; (III) – maltodextrin (III) based EPME.

The effect of pH on the responses of the electrodes was determined by recording the emf of the cell for a 10^{-7} mol/L solution of L-PA, at different pHs.

The E(mV) vs. pH plots presented in Figure 5.2 show the emf independency of pH in the following pH ranges of 2.0-6.0, 2.0-5.0, and 2.0-4.0 for maltodextrin (I), (II) and (III) based EPMEs, respectively.

5.4.4.3 Selectivity of the EPMEs

The selectivity of all EPMEs was checked using the mixed solutions method. The concentrations of the supposed interfering ion and L-PA were 10^{-6} and 10^{-7} mol/L,

respectively. The enantioselectivity was investigated against D-PA. The potentiometric selectivity coefficients vs D-PA for the maltodextrin (I), (II), and (III) based EPMEs: 3.2 x 10^{-3}, 3.0 x 10^{-3}, and 4.9 x 10^{-3}, respectively, proved that D-PA does not interfere in the determination of L-PA, demonstrating the enantioselectivity property of the electrodes.

Inorganic ions such as Na^{+}, K^{+} and Ca^{2+} do not interfere with the analysis of L-PA, because the potentiometric selectivity coefficients determined using mixed solutios method were less than 10^{-4}. The interference of creatine and creatinine was also checked. Potentiometric selectivity coefficients of $1x10^{-4}$, $1.2x10^{-4}$, $1.1x10^{-4}$ for creatine when the maltodextrin (I), (II), and (III) based EPMEs were used and $1.2x10^{-3}$, $1.5x10^{-3}$, $1.1x10^{-3}$ for creatinine when the maltodextrin (I), (II), and (III) based EPMEs were used, proved that creatine and creatinine do not interfere in the determination of L-PA.

5.4.4.4 Analytical applications

Table 5.7 Determination of L-pipecolic acid in the presence of D-pipecolic acid

Chiral Selector	Average recovery*, (%)				
	L:D (mol:mol)				
	2:1	1:1	1:2	1:4	1:9
Maltodextrin (I)	99.75±0.08	99.25±0.05	99.88±0.05	99.80±0.06	99.81±0.05
Maltodextrin (II)	99.68±0.04	99.20±0.05	99.75±0.05	99.75±0.04	99.73±0.05
Maltodextrin (III)	99.75±0.04	99.80±0.03	100.71±0.05	100.80±0.04	100.78±0.05

* All values are the average of ten determinations.

In order to determine the suitability of the EPMEs for the enantioanalysis of L-pipecolic acid, solutions containing both enantiomers in different ratios were prepared, and

recovery test for L-PA ($C_{L\text{-}PA}=10^{-7}$mol/L) were performed. The results obtained (Table 5.7) demonstrated the suitability of the electrodes for the enantioanalysis of L-pipecolic acid due to the good recovery and RSD (%) values. No significant differences in the recovery values were recorded for the ratios between L:D enantiomers varying from 1:9 to 1:99.99.

The results obtained for the assay of L–pipecolic acid in serum samples are shown in Table 5.8. Samples (1-3) were collected from different patients suspected of peroxisomal disorders and buffered with citrate buffer (0.1mol/L, pH=2.5, buffer:sample=1:1(v/v)).

Table 5.8 Recovery of L-pipecolic acid in serum samples (nmol/L)

Sample no.	Standard method[14]	Maltodextrin (I)	Maltodextrin (II)	Maltodextrin (III)
1	13.00	12.91±0.02	12.96±0.03	12.92±0.04
2	10.00	9.94±0.01	9.97±0.03	9.91±0.03
3	27.00	26.95±0.02	26.91±0.02	26.98±0.04

All values are the average of 10 determinations.

The results obtained using the proposed EPMEs are in good concordance with those obtained using the standard method (spectrometric method) [41].

5.5 Amperometric biosensors for the enantioselective analysis of L- and D-pipecolic acids in biological fluids

5.5.1 New amperometric biosensors based immobilization of L- and D-amino acid oxidases on diamond paste for the determination of L- and D-pipecolic acids in serum samples

5.5.1.1 Apparatus

A 663 VA Stand (Metrohm, Herisau, Switzerland) in combination with the Autolab 20 and software (Ecochemie version 4.9) were used for all chronoamperometric measurements. A Pt electrode and an Ag/AgCl electrode served as the counter and reference electrodes in the cell.

5.5.1.2 Amperometric biosensors design

The enzyme solutions used for the design of the biosensors were prepared in a 0.1 mol/L phosphate buffer pH=7.0. Two plastic tips were filled with plane diamond paste leaving an empty space of 3-4 mm in the top part filled with the modified diamond paste containing the mixture of L- and D-AAOD as shown below. The diameters of the biosensors were 3 mm. Electric contacts were obtained by inserting silver wires into the diamond paste. The biosensors tips were gently rubbed on fine abrasive paper to produce a flat surface. The surface of the biosensors were wetted with de-ionized water and then polished with an alumina paper (polished strips 30144-001, Orion) before use. The biosensors were stored dry at 4^0C, when not in use.

5.5.1.3 Recommended procedure: Direct DPV assay

The technique used for the direct voltammetric assay was differential pulse voltammetry (DPV) with the applied potential pulse amplitude of 25 mV vs. Ag/AgCl. The diamond paste electrode together with the reference and auxiliary electrodes were dipped into a cell containing phosphate buffer (pH=7.0) and the selected electrolyte (sodium pyrophosphate or NaCl) solution 0.1 mol/L, in a ratio of 3.5:1, as well as the synthetic mixture between L- and D-pipecolic acids. All solutions were deoxygenated for 5 min before measurements with N_2. The peak height measured at 700 mV and 200 mV vs Ag/AgCl for L- and D-pipecolic acid, respectively, was plotted versus their concentrations. The unknown concentrations of L- and D-pipecolic acids were determined from the corresponding calibration graphs.

5.5.1.4 Preparation of the modified diamond paste

Equal volumes of solutions of L-AAOD and D-AAOD (1mg enzyme/mL of phosphate buffer pH=7.0) were mixed. 30 µL paraffin oil and 400 mg natural monocrystalline diamond powder were mixed to form a diamond paste. 50 µL from the solution containing both enzymes was added to the diamond paste to form the modified diamond paste.

5.5.1.5 Results and discussion

5.5.1.5.1 Amperometric biosensors response

Two supporting electrolytes (sodium pyrophosphate (I) and NaCl (II)) that proved previously to give good results when diamond electrodes were used in the DPV technique

[35, 36], were tested for the determination of L- and D-pipecolic acids in order to determine the best working conditions. The biosensor response was determined using DPV technique. The response characteristics are shown in Table 5.9. The lowest limits of detection were found when sodium pyrophosphate was utilized as supporting electrolyte for both L- and D-pipecolic acid. The working concentration ranges are wide, with the exception of the determination of D-PA using NaCl as supporting electrolyte. Accordingly, the electrolyte of choice for the assay of L- and D-PA is sodium pyrophosphate.

Table 5.9 Response characteristics of the amperometric biosensor for L- and D-pipecolic acid assay.

Analyte	Electrolyte	Linear conc. range	Detection limit	Equation of calibration*	r
L-PA	I	0.0001-10 µmol/L	10 pmol/L	$^{1}I = 9.94 + 0.84C$	0.9984
	II	0.1 - 10000 nmol/L	0.01 nmol/L	$^{2}I = 0.19 + 0.031C$	0.9995
D-PA	I	0.0001 - 10 µmol/L	10 pmol/L	$^{1}I = 21.69 + 2.82C$	0.9956
	II	0.01 – 10 µmol/L	0.1 nmol/L	$^{1}I = 11.36 + 0.19C$	0.9995

*I is the intensity of the current in µA, and C is the concentration of L- and D-pipecolic acid, respectively, in [1] µmol/L and [2]nmol/L

5.5.1.5.2 Selectivity of the amperometric biosensors

The selectivity of the biosensor was checked by both the separate and mixed solution methods. Amperometric selectivity coefficients were determined following the method proposed by Wang [40]. In the evaluation, the concentration of the possible interferent, was ten times higher than that of the main analyte. The order of magnitude of all of the amperometric selectivity coefficients was 10^{-3} proving no interference in the

determination of L- and D-PA (Table 5.10), and the proposed electrode can be used for the enantioanalysis of pipecolic acid. Inorganic ions such as Na^+, K^+ and Ca^{2+} did not interfere with the analysis of pipecolic acid.

Table 5.10 Enantioselectivity of the amperometric biosensor designed for L- and D-pipecolic acid

Analyte	Interfering enantiomer	Electrolyte	pK_{amp}
L-PA	D	I	2.11
	D	II	2.98
D-PA	L	I	3.00
	L	II	2.15

All values are the average of ten determinations.

5.5.1.5.3 Analytical Applications

The response characteristics and selectivity of the modified diamond paste based biosensor as well as the potential where the peaks are formed for the assay of L- and D-PA made the amperomeric biosensor suitable for the enantioanalysis of pipecolic acid.

Table 5.11 Determination of L-pipecolic acid in the presence of D-pipecolic acid

L: D	Recovery L-pipecolic acid, (%)	
	Electrolyte	
	I	II
2:1	99.76 ± 0.03	99.75 ±0.03
1:1	99.78 ± 0.02	99.76 ± 0.02
1:2	99.79 ± 0.02	99.73 ± 0.02
1:4	99.76± 0.03	99.77 ± 0.03
1:9	99.77 ± 0.02	99.78 ± 0.03

All values are the average of ten determinations

Table 5.12 Determination of D-pipecolic acid in the presence of L-pipecolic acid

D: L	Recovery D-pipecolic acid, (%)	
	Electrolyte	
	I	II
2:1	99.76 ± 0.03	99.74 ±0.04
1:1	99.89 ± 0.02	99.83 ± 0.06
1:2	99.85 ± 0.02	99.82 ± 0.05
1:4	99.89± 0.03	99.86 ± 0.05
1:9	99.87 ± 0.03	99.85 ± 0.04

All values are the average of ten determinations.

To prove that the amperometric biosensor can be used reliably in enantioanalysis, tests of recovery of L- in the presence of D-PA and of D- in the presence f L-PA, different ratios between the enantiomers were performed. The results shown in Tables 5.11 and 5.12 indicate that the new amperometric biosensor can be used reliably for such tests.

Table 5.13 Determination of L-and D-pipecolic acid in serum samples.

PA	Sample No.	Standard Method	Proposed Method	
			Sodium pyrophosphate	NaCl
		(: mol/L)	(: mol/L)	
L	1	1.10	1.09 ± 0.02	1.07 ± 0.04
	2	4.40	4.38 ± 0.02	4.37 ± 0.03
	3	6.00	5.97 ± 0.03	5.96 ± 0.03
		(nmol/L)	(nmol/L)	
D	4	13.90	13.87 ± 0.04	13.85 ± 0.04
	5	11.30	11.26 ± 0.03	11.24 ± 0.03
	6	30.00	29.96 ± 0.03	29.93 ± 0.03

All values are the average of ten determinations

Samples were collected from patients suspected of cerebrohepatorenal syndrome (1-3) and hepatitis B (4-6). These samples were analysed using the new amperometric biosensors and the results were compared with those obtained using a standard method

[41] (Table 5.13). A good agreement was obtained between the results that show that the electrodes can be successfully used in the diagnosis of these diseases.

5.5.2 Carbon paste based amperometric biosensors for the enantioselective analysis of pipecolic acid

5.5.2.1 Apparatus

A663 VA Stand (Metrohm, Herisau, Switzerland) in combination with a μAutolab and software Ecochemie (version 4.9) were used for all chronoamperometric measurements. A Pt electrode and an Ag/AgCl electrode served as the counter and reference electrodes in the cell.

5.5.2.2 Amperometric biosensors design

Four plastic tips were filled with plane carbon paste leaving an empty space of 3-4 mm (which is the sensing part of the biosensors) to be filled with carbon paste containing the different enzymes. The diameters of all biosensors were 3 mm. Electric contacts were obtained by inserting silver wires into the carbon paste. The biosensors tips were gently rubbed on fine abrasive paper to produce a flat surface. The surface of the biosensors was wetted with de-ionized water and then polished with an alumina paper (polished strips 30144-011, Orion) before use. The biosensors were stored dry at 4^0C, when not in use. All the enzyme solutions used for the biosensors design were prepared in 0.1 mol/L phosphate buffer of pH=7.

5.5.2.2.1 Monoenzyme amperometric biosensors

Two biosensors, 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 carbon paste. 100 μL from the solution (1mg/enzyme/mL) of L-amino acid oxidase (L-AAOD) or D-amino acid oxidase (D-AAOD), respectively, were added to two separate portions of carbon paste.

5.5.2.2.2 Bienzyme amperometric biosensors

Two mixtures of enzymes were used for the design of amperometric biosensors: (1) 1mg of horseradish peroxidase (HRP) was dissolved in 50 μL of L-AAOD solution (0.25 mg/mL); (2) 1mg of HRP was dissolved in 50 μL of D-AAOD solution (0.25 mg/mL). Each mixture was incorporated in the carbon paste (100 mg graphite powder and 40 μL paraffin oil), to obtain two bienzyme electrodes.

5.5.2.3 Recommended procedures: Direct amperometry

The chronoamperometric technique was used for all the measurements. The electrodes were dipped into a cell containing 10 mL of phosphate buffer, pH =7.00 and different aliquots of L- or D-pipecolic acid solution. The intensity of current measured was plotted versus the concentration of L- or D-pipecolic acid. The unknown concentrations of L- and D-pipecolic acid were determined from the calibration graphs.

5.5.2.4 Determination of L- and D-pipecolic acid in serum samples

Six serum samples were collected from different patients suspected of peroxisomal disorders (samples 1-3) and liver cirrhosis (samples 4-6). Different aliquots of serum

samples were diluted with buffer solution (pH=7.00). Direct amperometry was involved to determine the content of L- and D-pipecolic acid in serum samples.

5.5.2.5 Results and discussion

5.5.2.5.1 Amperometric biosensors response

The response characteristics of the biosensors were measured at different potentials in order to determine the best working potential (higher sensitivity, lower limit of detection, shorter response time, etc.) for the assay of L- and D-pipecolic acid (Table 5.14).

Table 5.14 Response characteristics for the amperometric biosensors designed for L- and D-pipecolic Acid

Enzyme(s) used for the biosensor design	E (mV)	Linear conc. range	Detection limit	Response time, $t_{R90\%}$ (s)	Equations of calibration*	r
L-AAOD	650	0.02-0.1µmol/L	20pmol/L	60	^{1,a}I=-0.73+33.19C	0.9866
	50	2-600nmol/L	400pmol/L	30	^{2,b}I =168.9+18.23C	0.9993
	400	0.8-10pmol/L	1pmol/L	120	^{1,c}I =3.75+0.482C	0.9956
L-AAOD + HRP	400	4-200pmol/L	0.2pmol/L	30	^{2,c}I =-71.99+21.78C	0.9993
	650	20-200pmol/L	0.4pmol/L	120	^{1,c}I =0.24+9C	0.9980
D-AAOD	650	0.002-0.06 fmol/L	$1x10^{-4}$ fmol/L	30	^{1,d}I =0.02+67.01C	0.9999
	130	1-8nmol/L	20pmol/L	120	^{2,b}I =5.03+5.08C	0.9976
	550	20-800fmol/L	10fmol/L	120	^{1,d}I =9.75+0.035C	0.9988
D-AAOD+ HRP	650	2-10fmol/L	0.02fmol/L	120	^{2,d}I =404.3+46.45C	0.9935

* I is the intensity of the current in ^{1}µA, ^{2}nA, and C is the concentration of L-and D-pipecolic acid, respectively, in ^{a}µmol/L, ^{b}nmol/L, ^{c}pmol/L and ^{d}fmol/L.

The biosensor response was highly stable and reproducible over 35 days, when the biosensors were intensively used everyday for measurements and were kept into the fridge at 4^{0}C when not in use. The lower limits of detection for the analysis of L- and D-pipecolic acid were obtained at 400mV and at 650 mV when L-AAOD+HRP and D-AAOD, respectively, were used for the design of the biosensors. The higher sensitivity

(defined by Otto as the slope of the equation of calibration [22]) was recorded at 650mV when L-AAOD+HRP and D-AAOD where used for the assay of L-PA and D-PA, respectively.

5.5.2.5.2 Enantioselectivity of the Amperometric Biosensors

The enantioselectivity of all biosensors was checked using both separate and mixed solution methods with respect to L- and D-pipecolic acid. Amperometric enantioselectivity coefficients (K_{amp}) were determined following the method proposed by Wang [40] for the same potentials used for the determination of the response characteristics of the amperometric biosensors. The ratios between the concentrations of the main enantiomer and the other enantiomer were 1:10. The values of the amperometric selectivity coefficients (pK_{amp}), obtained using the mixed solution method, for all the biosensors designed for L- and D-PA are higher than 2.00 (Table 5.15), showing that the proposed biosensors are enantioselective.

Table 5.15 Enantioselectivity of the amperometric biosensors designed for L- and D-pipecolic acid

Enzyme(s) used for the design of the biosensor	Interfering enantiomer	E(mV)	pK_{amp}
L-AAOD	D	650	3.00
	D	400	2.55
	D	50	2.82
L-AAOD + HRP	D	650	2.17
	D	400	3.82
D-AAOD	L	650	2.43
	L	550	2.48
	L	130	2.96
D-AAOD + HRP	L	650	2.47

All values are the average of ten determinations.

The biosensors based on L-AAOD+HRP has got the best enantioselectivity for L-pipecolic acid analysis, when measurements are performed at 400mV while the biosensors based on D-AAOD enzyme has got the best enantioselectivity for D-pipecolic acid analysis, when measurements are performed at 130mV.

5.5.2.5.3 Analytical Applications

In order to determine the suitability of the proposed biosensors for the enantioanalysis of pipecolic acid, solutions containing both enantiomers in different ratios were prepared, and a recovery test for each enantiomer was performed.

Table 5.16 Determination of L-pipecolic acid in the presence of D-pipecolic acid

Enzyme(s) used for the design of the biosensors	E(mV)	Average recovery, L-PA (%)				
		L:D				
		2:1	1:1	1:2	1:4	1:9
L-AAOD	650	99.21±0.01	99.80±0.02	99.97±0.02	99.58±0.03	99.60±0.02
	400	99.37±0.02	99.89±0.03	99.86±0.02	99.40±0.01	99.70±0.02
	50	99.88±0.03	99.97±0.02	99.21±0.02	99.47±0.03	99.50±0.03
L-AAOD + HRP	650	99.42±0.03	99.52±0.03	99.52±0.01	99.53±0.02	99.53±0.02
	400	99.42±0.02	99.49±0.02	99.60±0.02	99.55±0.01	99.35±0.02

All values are the average of ten determinations.

Table 5.17 Determination of D-pipecolic acid in the presence of L-pipecolic acid

Enzyme(s) used for the design of the biosensors	E(mV)	Average recovery, D-PA (%)				
		D:L				
		2:1	1:1	1:2	1:4	1:9
D-AAOD	650	99.85±0.01	99.81±0.01	99.68±0.01	99.99±0.02	99.99±0.01
	400	99.61±0.01	99.46±0.02	99.99±0.01	99.95±0.02	99.98±0.01
	50	99.60±0.02	99.65±0.03	99.89±0.01	99.80±0.03	99.85±0.02
D-AAOD + HRP	650	99.25±0.01	99.89±0.03	99.85±0.02	99.99±0.02	99.92±0.02

All values are the average of ten determinations.

The results obtained (Tables 5.16 and 5.17) demonstrated the suitability of the proposed amperometric biosensors for the enantioanalysis of pipecolic acid due to the good recovery values obtained for the assay of one of the enantiomers in the presence of its antipode. No significant differences in the recovery values were recorded for the ratios between L:D or D:L enantiomers varying from 1:9 to 1:99.99.

The results obtained for the assay of L- and D-pipecolic acid in serum samples are shown in Tables 5.18 and 5.19. Samples 1-3 were used for the assay of L-pipecolic acid while samples 4-6 were used for the assay of D-pipecolic acid. The results obtained using the proposed biosensors at different working potentials is in concordance with those obtained using the standard method [41].

Table 5.18 Recovery of L-pipecolic acid in serum samples

Average recovery, L-PA (: mol/L)						
Sample No.	Standard method	L-AAOD			L-AAOD+HRP	
		650 mV	400mV	50mV	650mV	400mV
1	0.91	0.82±0.01	0.88±0.03	0.89±0.03	0.89±0.02	0.88±0.04
2	2.41	2.41±0.01	2.39±0.03	2.38±0.02	2.37±0.01	2.35±0.04
3	3.30	3.29±0.01	3.26±0.03	3.28±0.03	3.29±0.02	3.27±0.03

All values are the average of ten determinations.

Table 5.19 Recovery of D-pipecolic acid in serum samples

Average recovery, D-PA (nmol/L)					
Sample No.	Standard method	D-AAOD			D-AAOD+HRP
		650 mV	550mV	130mV	650mV
4	13.0	12.90±0.02	12.92±0.01	12.93±0.03	12.95±0.01
5	19.0	18.87±0.02	18.92±0.01	18.89±0.03	18.93±0.03
6	23.0	22.83±0.02	22.80±0.02	22.89±0.02	22.97±0.03

All values are the average of ten determinations.

5.5.3 Diamond paste based amperometric biosensors based on L-AAOD and D-AAOD for the determination of L- and D-pipecolic acid

5.5.3.1 Apparatus

A 663 VA Stand (Metrohm, Herisau, Switzerland) in combination with a PGSTAT 20 and software Ecochemie (version 4.8) were used for all chronoamperometric measurements. A Pt electrode and an Ag/AgCl electrode served as counter and reference electrodes in the cell.

5.5.3.2 Amperometric Biosensors Design

Two plastic tips were filled with plane diamond paste (0.1 g of each diamond powder were mixed with 20 μL paraffin oil) leaving an empty space of 3-4 mm in the top part to be filled with modified diamond paste (containing the different enzymes). The diameters of all biosensors were 3 mm. Electric contacts were obtained by inserting silver wires into the diamond paste. The biosensors tips were gently rubbed on fine abrasive paper to produce a flat surface. The surface of the biosensors were wetted with de-ionized water and then polished with an alumina paper (polished strips 30144-011, Orion) before use. The biosensors were stored dry at 4^0C, when not in use. All the enzyme solutions used for the biosensors design were prepared in 0.1 mol/L phosphate buffer of pH=7.0.

Two modified diamond paste were prepared as follows: 30 μL paraffin oil and 400 mg natural monocrystalline diamond powder were mixed to form a diamond paste. 50 μL

from the solution (1mg/enzyme/mL) of L-AAOD or D-AAOD, respectively, were added to two separate portions of diamond paste.

5.5.3.3 Recommended procedures: Direct amperometry

The chronoamperometric technique was used for the measurement of the intensity of current. The electrodes were dipped into a cell containing 10 mL of phosphate buffer, pH =7.00 and different aliquots of L- or D-pipecolic acid solution. The intensity of current measured was plotted versus the concentration of L- or D-pipecolic acid. The unknown concentrations of L- and D-pipecolic acid were determined from the calibration graphs.

5.5.3.4 Determination of L- and D-pipecolic acid in serum samples

Different aliquots of serum samples were diluted with buffer solution (pH=7.00). Direct amperometry was involved to determine the content of L- and D-pipecolic acid in serum samples.

5.5.3.5 Results and discussion

5.5.3.5.1 Response characteristics of the amperometric biosensors

The response characteristics of the biosensors were measured at different potentials in order to determine the best working potential (higher sensitivity, lower limit of detection, shorter response time, etc.) for the assay of L- and D-pipecolic acid (Table 5.20).

Table 5.20 Response characteristics for the amperometric biosensors designed for L- and D-pipecolic acid

Enzyme used for the design of the biosensor	E (mV)	Linear conc. Range	Detection limit	t_R (s)	Equation of calibration*	r
L-AAOD	650	$2x10^{-4}$- $1x10^{-3}$ fmol/L	$2x10^{-5}$ fmol/L	60	[1,a] $I = 1x10^{-3} + 58.6C$	0.9955
	400	20-100 pmol/L	0.2 pmol/L	120	[2,b] $I = 0.9 + 1.2C$	0.9937
D-AAOD	650	0.2 - 1.0 pmol/L	0.02 pmol/L	180	[2,b] $I = 0.7 + 8.1C$	0.9813
	300	20 – 100 fmol/L	0.6 fmol/L	120	[2,a] $I = 0.01 + 0.1C$	0.9987

*I is the intensity of the current in [1]mA and [2]μA, and C is the concentration of L- and D-pipecolic acid, respectively, in [a]fmol/L, [b]pmol/L

The biosensor response was highly stable and reproducible over one month, when the biosensors were intensively used everyday for measurements and were kept into the fridge at 4^0C when not in use. The best response characteristics (larger concentration range, lower limit of detection and highest sensitivity) for the assay of L-pipecolic acid and D-pipecolic acid were obtained at 650 mV and 300 mV, respectively. The low limits of detections and the high reliability of the biosensors is due to the type of the matrix used for the biosensors design (diamond paste), way adopted for the design of biosensors as well as to the selection of the working potential.

5.5.3.5.2 Enantioselectivity of the Amperometric Biosensors

The enantioselectivity of all biosensors was checked using both separate and mixed solution method with respect to L- and D-pipecolic acid. Amperometric enantioselectivity coefficients were determined following the method proposed by Wang [40], for the same potentials used for the determination of the response characteristics of the proposed amperometric biosensors. The ratio between the concentration of the main

enantiomer and the other enantiomer was 1:10. The values of the amperometric selectivity coefficients (pK_{amp}) (obtained using mixed solution method) for all the biosensors designed for L- and D-pipecolic acid are higher than 2.00, showing that the proposed biosensors are enantioselective. The biosensor based on L-AAOD enzyme has got the best enantioselectivity for L-pipecolic assay, when measurements are performed at 650 mV (pK_{amp}=2.82) while the biosensor based on D-AAOD has got the best enantioselectivity for D-pipecolic acid assay, when measurements are performed at 300 mV (pK_{amp}=2.77).

5.5.3.5.3 Analytical Applications

Table 5.21 Determination of D-pipecolic acid in the presence of L-pipecolic acid

E (mV)	Recovery L-pipecolic acid, (%)				
	L: D				
	2:1	1:1	1:2	1:4	1:9
650	99.59 ± 0.03	99.70 ± 0.03	99.69 ± 0.02	99.82± 0.03	99.80 ± 0.02
400	99.65 ±0.03	99.69 ± 0.02	99.27 ± 0.02	99.97 ± 0.03	99.67 ± 0.02

Table 5.22 Determination of L-pipecolic acid in the presence of D-pipecolic acid

E (mV)	Recovery D-pipecolic acid, (%)				
	D: L				
	2:1	1:1	1:2	1:4	1:9
650	99.90 ± 0.02	99.98 ± 0.03	99.96 ± 0.02	99.89 ± 0.01	99.94 ± 0.02
300	99.89 ± 0.01	99.96 ± 0.02	99.90 ± 0.02	99.88 ± 0.01	99.92 ± 0.03

The assay of L- and D-pipecolic acid was conducted by use of different ratios between L- and D-pipecolic acid. The results obtained (Table 5.21 and 5.22) demonstrated the suitability of the proposed amperometric biosensors for the enantioanalysis of pipecolic acid due to the good recovery values obtained for the assay of one of the enantiomers in

the presence of its antipode. No significant differences in the recovery values were recorded for the ratios between L:D or D:L enantiomers varying from 1:9 to 1:99.9.

The results obtained for the assay of L- and D-pipecolic acid in serum samples are shown in Table 5.23. The serum samples were collected from different patients suspected of peroxisomal disorders (samples 1-3, for the assay of L-PA) and liver cirrhosis (samples 4-6, for the assay of D-PA). The results obtained using the proposed biosensors at different working potentials are in good concordance with those obtained using the standard method [41].

Table 5.23 Determination of L-and D-pipecolic acid in serum samples

PA	Sample No.	Standard Method (: mol/L)	400 mV	Proposed Method (: mol/L) 650 mV	300 mV
L	1	1.00	0.99 ± 0.02	1.01 ± 0.03	-
	2	4.00	3.91 ± 0.03	3.87 ± 0.01	-
	3	5.50	5.49 ± 0.02	5.42 ± 0.01	
		(nmol/L)		(nmol/L)	
D	4	14.00	-	13.92 ± 0.03	13.91 ± 0.01
	5	12.00	-	11.97 ± 0.02	11.98 ± 0.02
	6	30.00		29.98 ± 0.03	29.90 ±0.01

All values are the average of ten determinations.

5.6 Sequential injection analysis utilizing amperometric biosensors as detectors for the simultaneous determination of L- and D-pipecolic acid

5.6.1 Apparatus

A 663 VA stand (Metrohm, Herisau, Switzerland) in combination with a PGSTAT 20 and software (Eco chemie version 4.9) was used for all chronoamperometric

measurements. A platinum electrode and Ag/AgCl electrodes were used as counter and reference electrodes in the cell.

5.6.2 Biosensor's design

Carbon pastes based biosensors described in section (3.5.2) were used as detectors in the SIA system for the simultaneous assay of L- and D-pipecolic acids. Monoenzymatic biosensors are based on the physical immobilization of L-AAOD or D-AAOD in carbon paste and the bi-enzymatic biosensor is based on the physical immobilization of L-AAOD and HRP or D-AAOD and HRP in carbon paste.

5.6.3 Sequential injection system

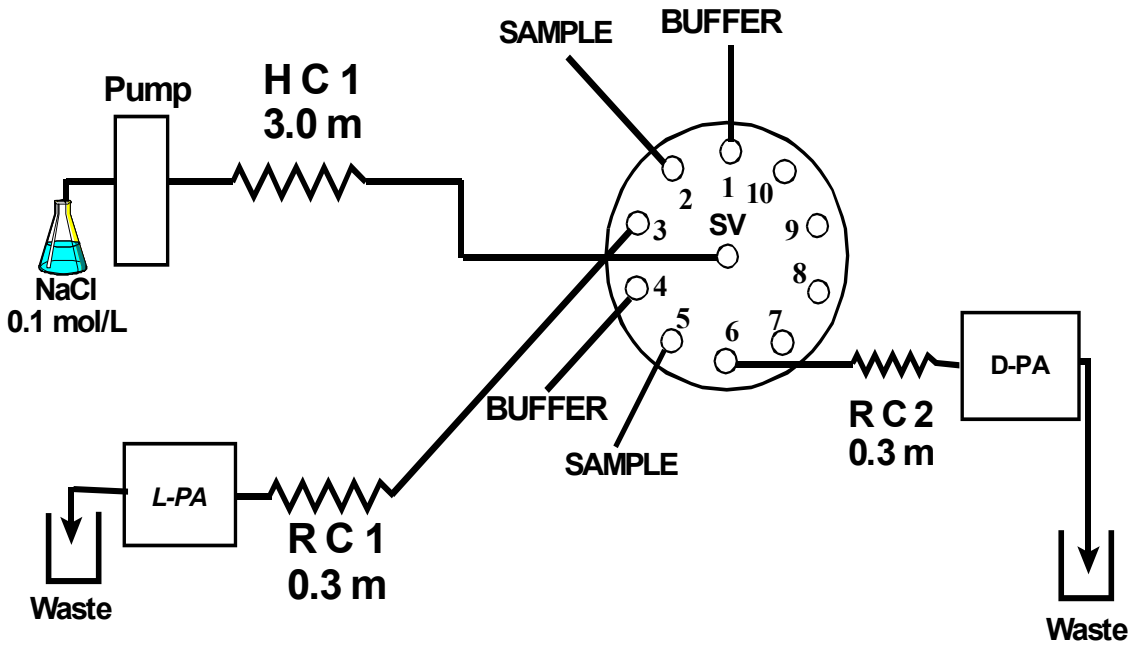

Figure 5.2 Schematic flow diagram of the sequential injection analysis/amperometric biosensors system used for the simultaneous determination of L- and D-pipecolic acid.

Table 5.24 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		L-pipecolic cell	Select L-pipecolic cell line (valve position 3)
18	Forward		Pump stack of zones to L-pipecolic acid 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		D-pipecolic acid cell	Select D-pipecolic acid cell line (valve position 6)
63	Forward		Pump stack of zones to D-pipecolic acid cell
93	Off	Home	Pump stops, return valve to the starting position (valve position 1)

The amperometric biosensors were incorporated into the conduits of a SIA system (Figure 5.2) constructed from a 10-port electrically actuated selection valve (Model ECSD10P, Valco Instruments, Houston, TX) and a Gilson Minipuls peristaltic pump. Tygon tubing (0.76 mm 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 mol/L NaCl was used as carrier. The capacity of the system is about 38 samples per hour.

The device operating sequence is shown in Table 5.24. The device control was achieved using a PC30-B interface board (Eagle electric, Cape Town, South Africa). The FlowTEK [42] software package (obtained from MINTEK) for computer-aided flow analysis was used through out the system. An optimum flow rate of 3.61 ml/min was used to propel the solutions. The sample and buffer consumption is only 270 µl of each per measurement of L- and D-enantiomers.

5.6.4 Results and discussions

5.6.4.1 The response characteristics of the biosensors in the SIA system

The response of all the biosensors was determined at different potentials using a chronoamperometric technique in order to obtain the best response characteristics (e.g., higher sensitivity, lower limit of detection, wide linear concentration range, etc.) for the simultaneous detection of L-and D-pipecolic acid (Table 5.25). The working concentration ranges as well as the limits of detection demonstrated the suitability of the

proposed amperometric biosensors for the simultaneous on-line monitoring of both enantiomers.

The best response characteristics were obtained when the biosensors based on L-AAOD and HRP (at 400mV) and the biosensor based on D-AAOD (at 650mV) were used as detectors for the simultaneous assay of L- and D-PA, respectively in the SIA system. The response obtained for all the biosensors showed good stability and reproducibility for tests performed daily, for 3 weeks.

Table 5.25 Response characteristics of the amperometric biosensors designed for the simultaneous assay of L- and D-pipecolic acids in a SIA system. All values are the average of ten determinations

Enzyme used for the biosensor design	E (mV)	Linear conc. Range	Detection limit	Equations of calibration*	r
L-AAOD	650	10-100 nmol/L	2 nmol/L	$^{1,c}H$=-0.89+0.056C	0.9788
	50	10-400 nmol/L	8 nmol/L	$^{1,c}H$ =-0.83+0.11C	0.9948
	400	0.04-20 pmol/L	0.02 pmol/L	$^{1,b}H$ =2.13+0.31C	0.9998
L-AAOD + HRP	+400	2-100 pmol/L	0.08 pmol/L	$^{2,b}H$ =-260.78+80.0C	0.9982
	650	8-100 pmol/L	2 pmol/L	$^{3,b}H$ =3.23+0.1C	0.9974
D-AAOD	650	0.001- 0.8 fmol/L	$8x10^{-4}$ fmol/L	$^{1,a}H$ =0.31+0.41C	0.9953
	130	20-100 nmol/L	8 nmol/L	$^{2,c}H$ =24.99+0.14C	0.9980
	550	0.6-40 fmol/L	0.4 fmol/L	$^{1,a}H$ =0.11+0.27C	0.9992
D-AAOD+ HRP	650	0.002-0.4 fmol/L	0.001 fmol/L	$^{2,a}H$ =0.015+2.045C	0.9939

* H is the peak height in $^{1}\mu A$, ^{2}nA, and ^{3}mA and C is the concentration of L-and D-pipecolic acid, respectively, in $^{a}fmol/L$, $^{b}pmol/L$ and $^{c}nmol/L$.

5.6.4.2 Selectivity of the amperometric biosensors

The selectivity of all biosensors was checked using both the mixed solutions and separate solutions methods with respect to L- and D-pipecolic acid. Amperometric selectivity coefficients (K_{amp}) were determined at the same potentials used for the determination of the response characteristics of the amperometric biosensors following the method proposed by Wang [40]. The ratio between the concentration of the enantiomer of interest and the other enantiomer was 1:10.

Table 5.26 Enantioselectivity of the amperometric biosensors designed for the simultaneous assay L- and D-pipecolic acids in a SIA system. All values are the average of ten determinations

Enzyme(s) used for the design of the biosensor	Interferent	E(mV)	pK_{amp}
L-AAOD	D-PA	650 400 50	3.11 2.45 2.63
L-AAOD + HRP		650 400	3.01 3.14
D-AAOD	L-PA	650 550 130	3.33 2.97 2.73
L-AAOD + HRP		650	3.96

The values of the amperometric selectivity coefficients (pK_{amp}) (obtained using the mixed solution method) for all the biosensors designed for L- and D-PA are higher than 2.00, revealing that all the proposed biosensors are enantioselective when used as detectors in SIA system (Table 5.26). The best enantioselectivity for the assay of L-PA was achieved by the bienzymatic sensor is based on L-AAOD and HRP when measurements are performed at 400 mV, while the best enantioselectivity for the assay of D-PA was achieved by using the bienzymatic sensor based on D-AAOD and HRP when

measurements are performed at 650 mV. Also the biosensor based on D-AAOD exhibit a good enantioselectivity for the assay of D-PA at 650 mV.

5.6.4.3 Analytical applications

The SIA system incorporated with the amperometric biosensors proved to be useful for the simultaneous assay of L- and D-pipecolic acid. The results (Tables 5.27 and 5.28) obtained by using different ratios between L- and D-pipecolic acid demonstrated the suitability of the proposed SIA/amperometric biosensors system for the enantioanalysis of pipecolic acid due to the good recovery values obtained for the assay of one of the enantiomers in the presence of its antipode.

Table 5.27 Determination of L-pipecolic acid in the presence of D-pipecolic acid

Enzyme(s) used for the design of the biosensors	E(mV)	Average recovery*, (%)				
		L:D (mol:mol)				
		2:1	1:1	1:2	1:4	1:9
L-AAOD	650	99.56±0.02	98.99±0.04	99.77±0.05	99.22±0.06	99.01±0.05
	400	99.55±0.03	99.17±0.04	99.63±0.03	99.98±0.02	99.20±0.03
	50	99.65±0.07	99.87±0.05	99.77±0.05	99.22±0.06	99.01±0.05
L-AAOD + HRP	650	99.72±0.03	99.53±0.04	99.37±0.04	99.96±0.04	99.90±0.03
	400	99.59±0.04	99.82±0.03	99.79±0.03	99.13±0.03	99.24±0.04

All values are the average of 10 determinations.

No significant differences in the recovery values were recorded for the ratios between L:D or D:L enantiomers varying from 1:9 to 1:99.99.

Table 5.28 Determination of D-pipecolic acid in the presence of L-pipecolic acid

Enzyme(s) used for the design of the biosensors	E (mV)	Average recovery L-PA*, (%) D:L (mol:mol)				
		2:1	1:1	1:2	1:4	1:9
D-AAOD	650	99.46±0.03	99.38±0.03	99.77±0.02	99.45±0.02	99.48±0.04
	550	99.84±0.04	99.80±0.03	99.26±0.03	99.18±0.03	99.47±0.02
	130	99.83±0.06	99.72±0.05	99.61±0.05	99.62±0.04	99.88±0.04
D-AAOD + HRP	650	99.20±0.04	99.15±0.05	99.69±0.03	99.39±0.04	99.42±0.05

All values are the average of 10 determinations.

The results obtained for the assay of L- and D-pipecolic acid in serum samples are shown in Tables 5.29 and 5.30. The samples were collected from different patients suspected of peroxisomal disorders and liver cirrhosis for the simultaneous assay of L- and D-pipecolic acid, respectively. The results using the proposed SIA/amperometric biosensors system at different working potentials are in good concordance with those obtained using the standard method [41].

Table 5.29 Recovery of L-pipecolic acid in serum samples (μmol/L)

Sample No.	Standard method	L-AAOD			L-AAOD+HRP	
		650 mV	400mV	50mV	650mV	400mV
1	1.10	1.09±0.01	1.08±0.03	1.09±0.01	1.07±0.02	1.10±0.01
2	2.30	2.28±0.02	2.29±0.02	2.28±0.03	2.30±0.02	2.27±0.02
3	0.80	0.79±0.02	0.80±0.02	0.79±0.03	0.8±0.01	0.78±0.03

All values are the average of 10 determinations.

Table 5.30 Recovery of D-pipecolic acid in serum samples (nmol/L)

Sample No.	Standard method	D-AAOD			D-AAOD + HRP
		650 mV	550mV	130mV	650mV
1	30.00	29.83±0.03	29.40±0.03	29.81±0.02	29.70±0.03
2	13.00	12.88±0.02	12.87±0.01	12.91±0.02	12.89±0.02
3	27.50	27.41±0.02	27.39±0.02	27.98±0.03	27.47±0.02

All values are the average of 10 determinations.

5.7 Conclusion

Different types of electrochemical techniques have been applied for the enantioanalysis of L- and D-pipecolic acid in biological fluids using carbon or diamond paste based electrodes. The construction of the proposed electrodes is simple, fast and reproducible and it is also assuring reliable response characteristics for the proposed electrodes.

The proposed EPMEs proved to be successful for the enantioanalysis of L-pipecolic acid in serum samples, helping for a fast and reliable diagnosis of peroxisomal disorders. The serum samples did not need any pre-treatment before the analysis.

The proposed amperometric biosensors and the amperometric electrode based diamond paste have excellent features for enantioselective clinical analysis. The selection of the new type of matrix (diamond paste) as well as of the best working potential in the assay of the enantiomers of L- and D-pipecolic acid proved to have a high effect on the performances of the amperometric biosensors, in terms of sensitivity, limit of detection, linear concentration range, response time and enantioselectivity. Due to the high biocompatibility of the diamond, the biosensors will be able to be used, after miniaturization, for *in vivo* assay of L- and D-pipecolic acid.

34 serum samples may be analyzed in 1 hour, without the need of any pre-treatment by using SIA/amperometric biosensor system for the simultaneous assay of L- and D-pipecolic acids in serum samples. The main advantages of the system are high reliability of analytical information, rapidity, simplicity and low cost of analysis. The selection of

the enzyme(s) and working potentials in the assay of the enantiomers of L- and D-pipecolic acid proved to have a high influence on the performance of amperometric biosensors in terms of sensitivity, limit of detection, linear concentration range, and enantioselectivity. The best amperometric biosensors that can be used as detectors in the proposed sequential injection analysis system proved to be the one based on L-AAOD and HRP and the one based on D-AAOD for the assay of L- and D-PA, when the measurements are performed at 400 and 650 mV, respectively.

5.8 References

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