Chapter 8

Diagnosis of L- and D-2-hydroxyglutarias using enantioselective, potentiometric membrane electrodes

8.1 Introduction

Organic acidurias is a group of inherited metabolic disorders characterized by increased excretion of organic acids. 2-Hydroxyglutaric acid (2-HGA) occurs in human urine of healthy individuals in minimal concentrations, and exists in the L- and D- configurations (Figure 8.1). Excess excretion of L- or D-2-hydroxyglutaric acid in urine is a clinical marker for L- or D-2-hydroxyglutaric acidurias. D-2-hydroxyglutaric aciduria is a rare neurometabolic disorder. The severe cases are characterized by encephalopathy of early infantile onset, seizures, hypotonia, cortical blindness and marked developmental delay [1-2]. L-2-hydroxyglutaric aciduria is a rare inborn error of metabolism [3]. It is an autosomal recessively inherited neurodegenerative disorder characterized by psychomotor retardation and progressive ataxia combined with sub-cortical leukoencephalopathy and cerebellar atrophy [4]. D-2-hydroxyglutaric aciduria is a severe neurological syndrome with neonatal onset, while L-2-hydroxyglutaric aciduria is usually associated with slowly progressive encephalopathy presenting in childhood [2]. The differentiation between these diseases requires the enantioanalysis of 2-hydroxyglutaric acid.

L-2-Hydroxyglutaric acid (L-2-HGA) may be found in abnormally higher concentrations in urine as a result of genetic errors or metabolic disorders [5,6] and it is a marker for L-

2-hydroxyglutaric aciduria (a rare neurometabolic disorder) [7-9]. L-2-hydroxyglutaric aciduria was first described by Duran [6]. Its effects are: mental retardation, progressive ataxia combined with subcortical leukoencephalopathy, cerebral atrophy, seizures, pyramidal and extra pyramidal symptoms and severe cerebral dysfunction [8, 10-12].

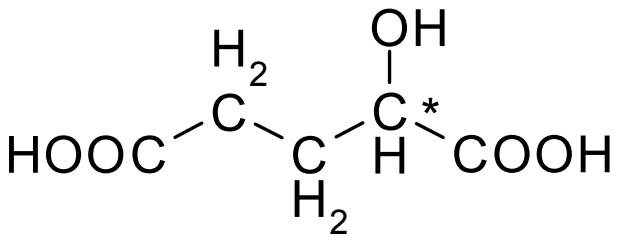

Figure 8.1 2-hydroxyglutaric acid

D-2-hydroxyglytaric aciduria is a rare genetic neurometabolic disorder biochemically characterized by high urinary excretion of D-2-hydroxygluaric acid. It was first described in 1980 by Chalmers et.al. [13]. This disorder is clinically characterized by two wide spectrums of phenotypes. The first one is a severe form with early-infantile-onset encephalopathy, seizures, hypotonia, crotical blindness and marked developmental delay. The mildest case usually has psychomotor retardation, macrocephaly and hypotonia [14]. D-2-hydroxyglutaric acid is a metabolite intermediate in different pathways in mankind [1,15]. It could be produced in mammalian species form 2-keoglutarate and 5-aminolevulinic acid, as shown in Figure 8.2 [2]. The conversion of D-2-hydroxyglutarate to 2-ketoglutarate is catalyzed by the enzyme D-2-hydroxyglutarate dehydrogenase and transhydrogenase that convert succinic semialdehyde into 4-hydroxybutyrate [16,17]. The production of D-2-hydroxyglutarate from 5-aminolevulinic acid is a minor pathway, as 5-aminolevulinic acid is predominately involved in the synthesis of porphyrins [15]. High

excretion of D-2-hydroxyglutaric acid also occurs in multiple acyl-CoA dehydrogenase deficiency that is due to a defect of the electron transfer flavoprotein (EP) or of the mitochondrial enzyme ETF-ubiquinone oxidoreductase [18]. D-2-hydroxyglutaric acid (D-2-HGA) dehydrogenase deficiency was considered as potential cause of this disease, even though the activity of the enzyme was normal or increased in the liver of patients. This suggests that their metabolism may result from a secondary pathway [19,20]. The existence of high metabolism of D-2-HGA in D-2-hydroxyglutaric aciduria patients suggests the vital need of an accurate method for quantitative analysis of D-2-HGA in human fluids.

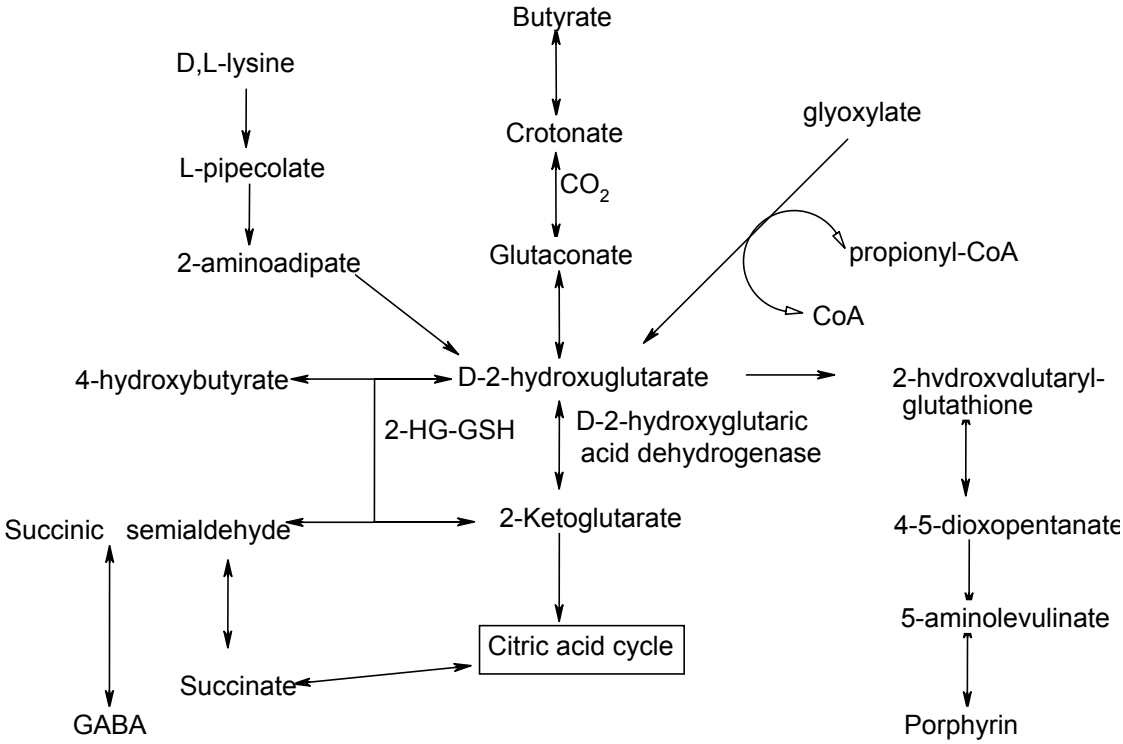

Figure 8.2 Metabolic pathways involving D-2-hydroxyglutaric acid in mammalian and bacterial species (2-HG-GSH = 2-hydroxyglutaryl-gluathione) [2].

Enantioanalysis of 2-hydroxyglutaric acid (HGA) is very important to differentiate between the two inherited metabolic diseases L- and D-2-hydroxyglutaric acidurias.

Configurational analyses of L- and D-2-hydroxyglutaric acids were reported using ^{1}H and ^{13}C NMR and MRI [12, 21-25]. Capillary gas chromatography [20, 26-29] and liquid chromatography [30] have been used for the determination of 2-hydroxyglutaric acid in urine.

In this chapter, five EPMEs based on maltodextrins I, II and III with different dextrose equivalent values (DE: 4.0-7.0 (I), 13.0-17.0 (II), 16.5-19.5 (III)) and cyclodextins (β- and 2-hydroxy-3-trimethylammoniopropyl-β-derivative-CD) are proposed for the enantioanalysis of L-2-hydroxyglutaric acid in urine samples. Also, four EPMEs based on γ-cyclodextrin and macrocyclic antibiotics (vancomycin, teicoplanin and teicoplanin modified with acetonitrile) were proposed for the assay of L-2-hydroxyglutaric acid. Modified carbon paste was proved to be reliable for the construction of EPMEs [31].

8.2 Reagents and materials

Graphite powder (1-2μm) and maltodextrins (DE 4.0-7.0 (I), 13.0-17.0 (II), 16.5-19.5 (III)) were purchased from Aldrich (Milwaukee, WI, USA), paraffin oil was purchased from Fluka (Buchs, Switzerland). L- and D-2-hydroxyglutaric acids creatine and creatinine were purchased from Sigma-Aldrich (Milwaukee, WI, USA). α-, β-, γ-, and 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrins were supplied by Wacher-Chemie GmbH (Germany). Phosphate buffer (pH = 3) was purchased from Merck (Darmstadt, Germany).

10^{-3} mol/L solutions of each maltodextrin (I, II and III), and each cyclodextrin (α-, β-, γ-, and 2-hydroxy-3-trimethylammoniopropyl-β-CD) were prepared. The solution of vancomycin ($2x10^{-3}$ mol/L) was prepared in phosphate buffer (pH 4.00). The solution of teicoplanin ($2x10^{-3}$ mol/L) was prepared using pH 6.00 phosphate buffer. The solution of teicoplanin ($2x10^{-3}$ mol/L) containing acetonitrile was prepared using pH 6.00 phosphate buffer containing 40% (v/v) of acetonitrile.

De-ionized water from a Modulab System (Continental Water Systems, San Antonio, TX, USA) was used for all solution preparations. The L- and D-2-hydroxyglutaric acid solutions were prepared by serial dilutions from standard L- and D-2-HGA solution (10^{-1} mol/L). All diluted and standard solutions of L- and D-2-HGA were buffered at pH=3.00 using phosphate buffer.

Urine samples were donated from healthy persons. Different aliquots of urine samples were spiked with L- and D-2-HGA. All spiked urine samples were buffered at pH=3 using phosphate buffer.

8.3 Enantioselective, potentiometric membrane electrodes based on maltodextrins

8.3.1 Equipments and apparatus

A 663 VA stand (Metrohm, Herisau, Switzerland) connected to a PGSTAT 100 computer-controlled potentiostat (Eco Chemie, Ultrech, The Netherlands) and software

version 4.9 was used for all potentiometric measurements. An Ag/AgCl (0.1mol/L KCl) electrode was used as reference electrode in the cell.

8.3.2 Electrodes design

Paraffin oil and graphite powder were mixed in a ratio 1:4 (w/w) to form the plain carbon paste. The modified carbon pastes were prepared by impregnating 100µL of 10^{-3} mol/L of each maltodextrin in 100mg of the plain carbon paste. A quantity of carbon paste, free of maltodextrins, was filled in a plastic pipette tip, leaving 3-4mm in the upper part to be filled with the modified carbon paste containing the maltodextrin. The diameter of all EPMEs was 3 mm. Electric contact was obtained by inserting silver wires into the carbon paste. The internal solution was 0.1mol/L KCl. The entire electrode surface was gently rubbed on fine abrasive paper to produce a flat surface. The surface of the electrode was wetted with de-ionized water, refreshed with modified carbon paste and then polished with an alumina paper (polished strips 30144-011, Orion) before use for the analysis. When not in use, each sensor was immersed in 10^{-3}mol/L of L-2-HGA solution.

8.3.3 Recommended procedure

Direct potentiometric method was employed for potential measurements, E (mV), of each standard L-2-HGA (10^{-10}-10^{-2} mol/L) solution and urine sample. The electrodes were placed in the standard solutions. Calibration graphs were obtained by plotting E (mV) versus pL-2-HGA. Unknown concentrations of L-2-HGA in urine samples were determined from the calibration graphs.

8.3.4 Results and discussion

8.3.4.1 EPMEs response

The response characteristics exhibited by the EPMEs based on different types of maltodextrins for the analysis of L-2-hydroxyglutaic acid are summarized in Table 8.1. All the proposed membrane electrodes exhibited linear and near-Nernestian responses (57-60 mV per decade of concentration) for EPMEs based on maltodextrins I, II and III, respectively, for the determination of L-2-HGA. The same EPMEs were shown non-Nernestian responses when used for the assay of D-2-hydroxyglutaric acid. The detection limits recorded for L-2-HGA are low, as shown in Table 8.1. Enantioselectivity using maltodextrins is based on the formation of inclusion complexes [33-35]. The stability of the complexes formed between the chiral selector and analytes increases with the value of DE, because increasing the DE value will result in an increase of the diameter of the helix. The response obtained for all three electrodes show good stability and reproducibility for tests performed for more than 2 months (RSD<1%).

Table 8.1 Response characteristics of enantioselective, potentiometric membrane electrodes for L-2-HGA[a]

EPME based on	Parameters			
	Slope (mV/decade of conc.)	Intercept E^o (mV)	Linear conc. range (mol/L)	Detection limit (mol/L)
Maltodextrin I	57.3	546.86	10^{-9}-10^{-5}	2.86×10^{-10}
Maltodextrin II	59.7	392.4	10^{-6}-10^{-3}	2.67×10^{-7}
Maltodextrin III	59.3	536.7	10^{-8}-10^{-5}	8.90×10^{-10}

[a]All measurements were made at room temperature; all values are the average of ten determinations.

The response times of EPMEs based on maltodextrins I and II are higher than 1 min for concentration of L-2-HGA between 10^{-9} and 10^{-5}, and 10^{-6} and 10^{-3} mol/L, respectively.

For EPME based on maltodextrins III, the response time is lower than 1 min for the concentration of L-2-HGA between 10^{-8} and 10^{-5} mol/L.

8.3.4.2 The pH influence on the response of the EPMEs

The effect of the pH variation on the response of the EPMEs based on maltodextrins I, II, and III has been tested by recording the emf of the cell, using direct potentiometric method.

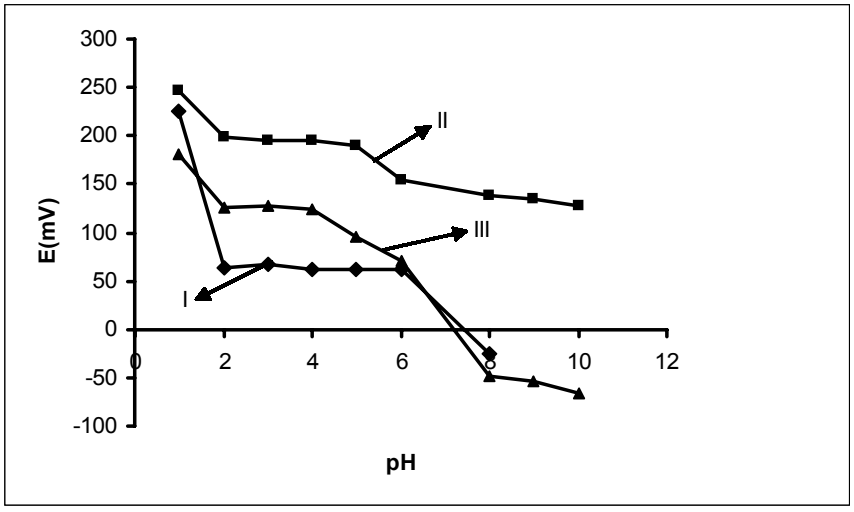

Figure 8.3 Effect of pH on the response of the enantioselective, potentiometric membrane electrodes based on maltodextrin I (I), II (II) and III (III), respectively, for the determination of L-2-HGA ($C_{L\text{-}2\text{-}HGA} = 10^{-6}$ mol/L).

All measurements were performed for a concentration of L-2-HGA of 10^{-6} mol/L, at different pH values selected between 1 and 10. These solutions were prepared by adding small volumes of HCl (0.1 mol/L) and/or NaOH solution (0.1 mol/L) to a solution of L-2-HGA. The E (mV) vs. pH plots presented in Figure 8.3 shows that the response of the EPMEs are pH-independent in the pH ranges of 2.0-6.0 (maltodextrins I based EPME), 2.0-5.0 (maltodextrin II based EPME) and 2.0-4.0 (maltodextrin III based EPME).

8.3.4.3 Selectivity of the electrodes

The selectivity of all EPMEs was checked using mixed solutions method proposed. The ratio between the concentrations of interfering ion and L-2-HGA was 10:1. The selectivity was investigated against D-2-hydroxglutaric acid (D-2-HGA), creatine, creatinine, Na^+, K^+ and Ca^{2+}. The selectivity coefficients for the enantioselective, potentiometric membrane electrodes, K_{sel}^{pot}, obtained are summarized in Table 8.2. The values obtained for D-2-HGA, creatine, creatinine, and the inorganic cations (Na^+, K^+ and Ca^{2+}, $K_{sel}^{pot} < 10^{-4}$) demonstrated the enantioselectivity and selectivity properties of the proposed EPMEs for the assay of L-2-HGA.

Table 8.2 Selectivity coefficients for the enantioselective, potentiometric membrane electrodes used for the assay of L-2-HGA[a]

Interference species (J)	pK_{sel}^{pot}		
	EPMEs based on		
	Maltodextrin I	Maltodextrin II	Maltodextrin III
D-2-HGA	2.40	2.42	2.42
Creatine	2.40	2.41	2.40
Creatinine	2.39	2.42	2.40

[a]All measurements were made at room temperature; all values are the average of ten determinations.

8.3.4.4 Analytical applications

The high selectivity and enantioselectvity of proposed EPMEs based on maltodextrins made them suitable for the enantioanalysis of L-2-HGA in urine in order to diagnose 2-hydroxyglutaric aciduria. The analysis of L-2-hydroxyglutaric acid was investigated in the presence of D-2-hydroxyglutaric acid by using different ratios between L- and D-2-HGAs. The results obtained (Table 8.3) proved again the suitability of the proposed potentiometric membrane electrodes for the enantioanalysis of L-2-HGA. No significant

differences in the recovery values were recorded for the different ratios between the enantiomers.

Table 8.3 The results obtained for the analysis of L-2-HGA in the presence of D-2-HGA[a]

L:D (mol/mol)	% L-2-HGA, Recovery		
	EPMEs based on		
	Maltodextrin I	Maltodextrin II	Maltodextrin III
2:1	99.60±0.03	99.60±0.01	99.78±0.01
1:1	99.91±0.01	99.46±0.02	99.75±0.01
1:2	99.35±0.01	99.54±0.01	99.04±0.01
1:4	99.82±0.01	99.92±0.03	99.80±0.02
1:9	99.76±0.03	99.71±0.02	99.43±0.01

[a] All measurements were made at room temperature; all values are the average of ten determinations.

Table 8.4 Recovery of L-2-HGA in urine samples[a]

Sample no.	LC/MS method [30]	% L-2-HGA, Recovery		
		EPMEs based on		
		Maltodextrin I	Maltodextrin II	Maltodextrin III
1	99.84	99.16±0.05	99.92±0.03	99.93±0.02
2	99.67	99.37±0.01	99.93±0.01	98.52±0.01
3	99.43	99.08±0.05	99.27±0.02	99.95±0.01
4	99.65	99.30±0.02	99.66±0.01	99.76±0.04
5	99.76	99.72±0.01	99.80±0.01	99.67±0.02
6	99.80	99.71±0.02	99.68±0.03	99.82±0.03

[a]All measurements were made at room temperature; all values are the average of ten determinations.

Urine samples (1-6) were donated from healthy persons and spiked with different amounts of L-2-HGA. All urine samples were buffered at pH=3 using phosphate buffer. The results recorded for the assay of L-2-HGA in urine samples are shown in Table 8.4 and they are in good agreement with those obtained using the method proposed by Rashed et al [30].

8.4 Enantioselective, potentiometric membrane electrodes based on cyclodextrins for the determination of L and D-2-hydroxyglutaric acid in urine samples

8.4.1 Equipments and apparatus

All potentiometric measurements were performed using a 663 VA Stand (Metrohm, Herisau, Switzerland) connected to an Autolab PGSTAT 100 (Eco Chemie, Netherlands) and software version 4.9. An Ag/AgCl (0.1 mol/l KCl) electrode and was used as reference electrode in the cell.

8.4.2 Electrodes design

The paraffin oil and graphite powder were thoroughly mixed in a ratio of 1:4 (w/w) followed by the addition of the aqueous solutions of β- cyclodextrin, β- cyclodextrin-derivative (2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrin) and γ-cyclodextrin (10^{-3} mol/L 100 µL chiral solution to 100mg carbon paste was added). Plain carbon paste was prepared by mixing 100mg of graphite powder with 40 µl paraffin oil. The plain carbon paste was filled into a plastic pipette peak leaving a space of 3-4 mm into the top to be filled with the modified carbon paste. The diameter of enantioselective, potentiometric membrane electrodes was 3 mm. Electric contact was obtained by inserting Ag/AgCl wire into the carbon paste. The internal solution was 0.1 mol/L KCl. All the EPMEs tips were gently rubbed on fine abrasive sand paper to produce a flat surface. The surface of the EPMEs was wetted with de-ionized water and then polished with an alumina paper (polished strips 30144-001, Orion) before use in each experiment.

When not in use, the electrodes were immersed in 10^{-3} mol/l L- or D-2-hydroxyglutaric acid solution.

8.4.3 Recommended procedure

The direct potentiometric method was used for the measurement of the potentials (E) of each standard solution (10^{-10} – 10^{-2} mol/L). Calibration graphs were obtained by plotting E(mV) versus p(L-HGA) or p(D-HGA). The unknown concentrations were determined from calibration graphs.

Ten urine samples were collected from patients suffering of L- or/and D-2-hydroxyglutaric acidurias. Different aliquots of the urine samples were buffered using phosphate buffer solution (pH=3). All samples were kept in refrigerator during experimental period, at 4^{o}C. Direct potentiometry was used for the enantioanalysis of L- and D-2-hydroxyglutaric acid in the urine samples.

8.4.4 Results and discussion

8.4.4.1 EPMEs response

The three cyclodextrin-carbon paste electrodes were tested for their response characteristics towards L- and D-2-hydroxyglutaric acid at pH=3.0 (phosphate buffer). Enantioanalyses was based on the formation of inclusion complexes between the cyclodextrin (host) and L- or D-2HGA (guest). The response obtained for L-HGA was linear and near-Nernstian only using β-CD and 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrins based EPMEs, while the response obtained for D-HGA was linear and

near-Nernstian only when γ-CD based EPME was used. The equations of calibration and correlation coefficients (r) for L-HGA and D-HGA are as follows:

L-2-HGA: $E = 57.5\ p\ (L\text{-}HGA) - 315.5;\ r = 0.9999$

L-2-HGA: $E = 58.6\ p\ (L\text{-}HGA) + 465.2;\ r = 0.9990$

D-2-HGA: $E = 50.0\ p\ (D\text{-}HGA) + 460;\ r = 0.9946$

where E is the cell potential in mV and p(L-HGA) = -log [L-HGA] and p (D-HGA) = -log [D-HGA]. The response characteristics of the EPMEs are summarized in Table 8.5. The limits of detection are very low 1.0×10^{-9} and 1.47×10^{-8} mol/L for L-2-hydroxyglutaric acid when EPMEs based on β- and 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrins are used and 6.30×10^{-7} mol/L for D-2-hydroxyglutaric acid when γ-CD based is used. The EPMEs responses exhibited a good stability and reproducibility for the tests performed for 4 months, when daily used for measurements (RSD<1.0%).

The response times were: 1 min for L-HGA assay using β-CD based EPME in the concentration range 10^{-8} – 10^{-6} mol/L, 2 min for L-HGA assay using 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrin based EPME in the concentration range 10^{-7}-10^{-5} mol/L and for D-HGA using γ-CD based EPME in the concentration range 10^{-6}-10^{-4} mol/L

Table 8.5 Response characteristics of enantioselective, potentiometric membrane electrodes for L-and D-2-hydroxyglutaric acid [a]

Analyte	Chiral selector	Parameters			
		Slope (mV/decade of conc.)	Intercept, E^o (mV)	Linear conc. range (mol/L)	Detection limit (mol/L)
L-2-HGA	β-Cyclodextrin	57.5	-315.5	10^{-8}-10^{-6}	$1.00x10^{-9}$
L-2-HGA	β-Cyclodextrin derivative	59.4	465.2	10^{-7}-10^{-5}	$1.47x10^{-8}$
D-2-HGA	γ-Cyclodextrin	50.0	460.0	10^{-6}-10^{-4}	$6.30x10^{-7}$

[a] All measurements were made at room temperature; all values are the average of ten determinations.

8.4.4.2 The pH influence on the response of the EPMEs

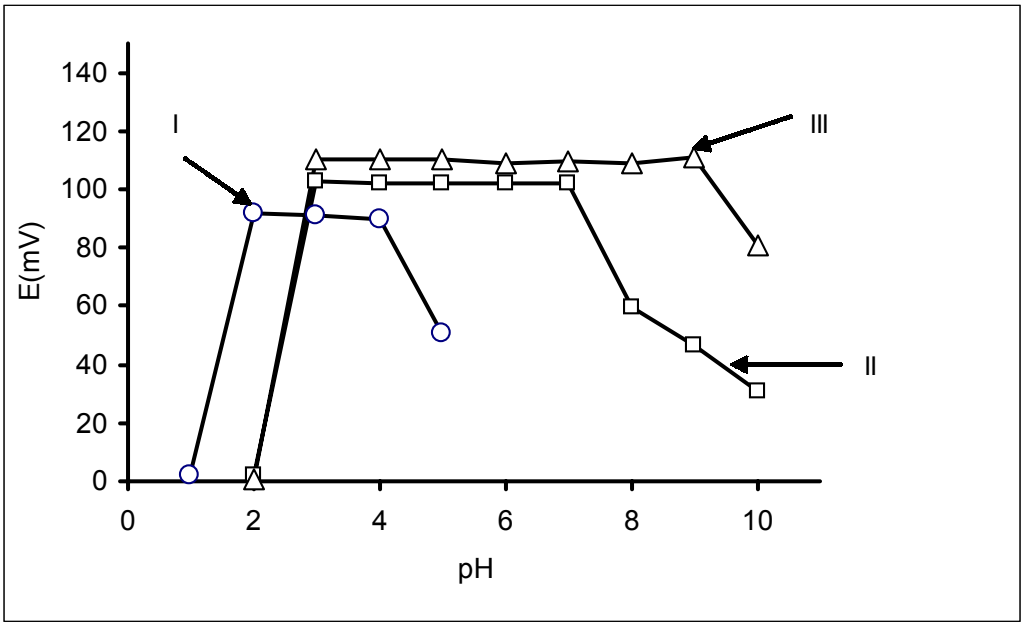

Figure 8.4. Effect of pH on the response of the enantioselective potentiometric membrane electrodes for L- and D-2-hydroxyglutaric acid ($C_{L\text{-}HGA} = 10^{-7}$ and $C_{D\text{-}HGA} = 10^{-5}$ mol/L). I &II for L-2-hydroxyglutaric acid using β and β-derivative cyclodextrin, respectively, and III for L-2-hydroxyglutaric acid using γ cyclodextrin.

Different solutions of L-HGA and D-HGA in the pH from 1 to 10, were prepared by adding small volumes of HCl (0.1 mol/l) or NaOH (0.1 mol/l) solutions to stock solutions

of L-HGA and D-HGA, respectively. The E (mV) vs. pH plots presented in Figure 8.4 show that the emf is not dependant on the pH in the following ranges 2.0-4.0 for β-CD based EPME, 3.0-10.0 for 2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrin based EPME and 3-7 for γ-CD based EPME.

8.4.4.3 Selectivity of the electrodes

The selectivity of the electrodes was checked using the mixed solutions method proposed by Renk [33], over L- or D-HGA, creatine, creatinine and some inorganic cations. The ratio between the concentration of interfering ion and enantiomer was 10:1. The potentiometric selectivity coefficients, K_{sel}^{pot}, (Table 8.6) proved the high enantioselectivity and selectivity of the proposed EPMEs. Inorganic cations such a Na^+, K^+, and Ca^{2+} do not interfere in the analysis of L- and D-HGA.

Table 8.6 Potentiometric selectivity coefficients for the enantioselective, potentiometric membrane electrodes.[a]

Interference species (J)	K^{pot}_{sel}		
	EPME based on		
	β-Cyclodextrin	2-hydroxy-3-trimethylammoniopropyl-β-cyclodextrin)	γ-Cyclodextrin
L-2-HGA	-	-	3.80×10^{-3}
D-2-HGA	4.09×10^{-3}	3.80×10^{-3}	-
Creatine	3.93×10^{-3}	3.95×10^{-3}	9.65×10^{-3}
Creatinine	8.34×10^{-3}	8.06×10^{-3}	4.71×10^{-3}

[a] All measurements were made at room temperature; all values are the average of ten determinations.

8.4.4.4 Analytical applications

The electrodes can be applied for the enantioanlaysis of L- and D-2-hydroxyglyutaric acid in urine matrices using direct potentiometric method. The recovery tests

demonstrated the suitability of these EPMEs for the assay of enantiopurity assay of L- and D-2-hydroxyglyutaric acid (Tables 8.7 and 8.8). The assay of one enantiomer in presence of its antipode was conducted by using different ratios between enantiomers. 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 8.7 The results obtained for the analysis of L-2-Hydroxyglutaric acid in the presence of D-2-Hydroxyglutaric acid [a]

L:D (moL/moL)	Recovery, % L-2-HGA	
	EMPE based on	
	β-Cyclodextrin	β-Cyclodextrin derivative
2:1	99.87±0.01	99.85±0.04
1:1	99.60±0.03	99.84±0.06
1:2	99.73±0.02	99.63±0.06
1:4	99.46±0.01	100.0±0.05
1:9	99.88±0.06	99.88±0.07

[a] All measurements were made at room temperature; all values are the average of ten determinations.

Table 8.8 The results obtained for the analysis of D-2-Hydroxyglutaric acid in the presence of L-2-Hydroxyglutaric acid [a]

D:L (mol/mol)	Recovery, % D-2-HGA
	γ-cyclodextrin based EPME
2:1	99.70±0.02
1:1	99.76±0.04
1:2	99.75±0.05
1:4	99.67±0.03
1:9	99.75±0.04

[a] All measurements were made at room temperature; all values are the average of ten determinations.

Table 8.9 Recovery of L-2-Hydroxyglutaric acid in urine samples, (%) [a]

Sample no.	Standard method [32] (ng/L)	Recovery %, L-2-HGA	
		Chiral selector	
		β-Cyclodextrin	β-Cyclodextrin derivative
1	29.60	98.04±0.09	99.98±0.09
2	74.00	99.97±0.12	98.78±0.07
3	118.40	99.43±0.04	99.69±0.03
4	148.00	99.42±0.02	99.30±0.01
5	370.00	99.54±0.09	99.96±0.05

[a] All measurements were made at room temperature; all values are the average of ten determinations.

Table 8.10 Recovery of D-2-Hydroxyglutaric acid in urine samples, (%) [a]

Sample no.	Standard method [32] (mg/L)	Chiral selector
		γ-cyclodextrin
		Recovery % D-2-HGA
6	59.20	99.18±0.02
7	118.40	99.48±0.01
8	148.00	100.00±0.02
9	592.00	99.40±0.03
10	1036.00	99.12±0.02

[a] All measurements were made at room temperature; all values are the average of ten determinations.

The results obtained for the analysis of L- and D-2-hydroxyglutaric acid in urine samples are shown in Table 8.9 and 8.10, respectively. The results obtained by using the proposed EPMEs are in good concordance with those obtained using the standard method [32]. The advantage of the proposed method is the simplicity and high precision

8.5 Determination of D-2-hydroxyglutaric acid in urine samples using enantioselective, potentiometric membrane electrodes based on antibiotics

8.5.1 Apparatus

A 663 VA stand (Metrohm, Herisau, Switzerland) connected to a PGSTAT 100 computer-controlled potentiostat (Eco Chemie, Ultrech, The Netherlands) and software version 4.9 was used for all potentiometric measurements. An Ag/AgCl (0.1 mol l^{-1} KCl) electrode was used as reference electrode in the cell.

8.5.2 Electrodes design

Paraffin oil and graphite powder were mixed in a ratio 1:4 (w/w) to form the plain carbon paste. The modified carbon pastes were prepared by impregnating 100 µl of 10^{-3} mol/L solution of antibiotic (vancomycin, teicoplanin, or teicoplanin modified with acetonitrile), in 100 mg of the plain carbon paste. A certain quantity of carbon paste, free of antibiotics, was filled in a plastic pipette peak, leaving 3-4 mm in the top to be filled with the modified carbon paste. The diameter of the EPMEs was 3 mm. Electric contact was obtained by inserting silver wires into the carbon paste. The internal solution was 0.1 mol/L KCl.

The entire electrode surface was gently rubbed on fine abrasive paper to produce a flat surface. The surface of the electrode was wetted with de-ionized water, refreshed with modified carbon paste and then polished with an alumina paper (polished strips 30144-

011, Orion) before use for the analysis. When not in use, each sensor was immersed in 10^{-3} mol l^{-1} of D-2-hydroxyglutaric acid solution.

8.5.3 Recommended procedure

Direct potentiometric method was employed for all potential measurements, E (mV), of each solution of D-2-hydroxyglutaric acid (10^{-10} -10^{-2} mol L^{-1}) and of urine samples. Calibration graphs were obtained by plotting E (mV) versus p(D-2-HGA). Unknown concentrations of D-2-HGA in urine samples were determined by interpolating of the potential value in the calibration graph.

8.5.4 Results and discussion

8.5.4.1 EPMEs response characteristics

The calibration equations obtained for the D-enantiomer are:

(I) E = 192.0 + 59.3 p (D-HGA), r = 0.9994 (vancomycin)

(II) E = 167.01 + 57.74 p (D-HGA), r = 0.9766 (teicoplanin)

(III) E = 85.6 + 54.0 p (D-HGA), r = 0.9993 (teicoplanin modified with acetonitrile)

The response characteristics exhibited by the EPMEs based on different types of antibiotics (vancomycin, teicoplanin and teicoplanin modified with acetonitrile) for the determination of D-2-hydroxyglutaric acid are summarized in Table 8.11. All the proposed membrane electrodes exhibited near-Nernstian responses (54-59.30 mV per decade of concentration) for the determination of D-2-hydroxyglutaric acid. Non-

Nernestian responses were recorded for L-2-hydroxyglutaric acid. Detection limits were low.

The response of the EPME based on vancomycin and teicoplanin modified with acetonitrile are lower than 1 min for concentration of D-2-hydroxyglutaric acid between 10^{-7} and 10^{-3} and between 10^{-6}-10^{-2} mol/L, respectively. For EPME based on teicoplanin, the response time is higher than 1 min for the concentration range between 10^{-7} and 10^{-2} mol/L of D-2-hydroxyglutaric acid. The response obtained for all three electrodes show good stability and reproducibility for tests performed for more than 1 months (RSD <0.1%). All electrodes were stored at 4^{o}C when not in use.

Table 8.11 Response characteristics of enantioselective, potentiometric membrane electrodes for D-2-hydroxyglutaric acid [a]

EPME based on	Parameters			
	Slope (mV/decade of concentration)	Intercept, E^{o} (mV)	Linear concentration range (mol/L)	Detection limit (mol/L)
Vancomycin	59.30	192.0	10^{-7}-10^{-3}	$1.00x10^{-8}$
Teicoplanin	57.74	167.0	10^{-7}-10^{-2}	$1.00x10^{-8}$
Teicoplanin & acetonitrile	54.00	85.60	10^{-6}-10^{-2}	$1.00x10^{-7}$

[a] All measurements were made at room temperature; all values are the average of ten determinations.

8.5.4.2 Effect of pH on the EPMEs response

The effect of pH variations on the response of the constructed electrodes, based on vancomycin, teicoplanin and teicoplanin modified with acetonitrile, was investigated for the assay of D-2-hydroxyglutaric acid by recording the emf of the cell, containing its

solutions at different pHs (1-10) the concentration of D-2-hydroxyglutaric acid was $1x10^{-6}$ mol/L for all measurements. These solutions were prepared by adding small volumes of HCl (0.1 mol/L) and/or NaOH solution (0.1 mol/L) to D-2-hydroxyglutaric acid solutions. In Figure 8.5, the plot investigates the relation between the cell potential, E (mV) and the pH variations in the solutions of D-2-hydroxyglutaric acid. EPME based on teicoplanin has the widest pH independency range from 2.0 to 10.0, while the one based on vancomycin is independent in the 2.0 to 5.0 pH range. EPME based on teicoplanin mixed with acetonitrile is pH independent in the range 2.0-6.0.

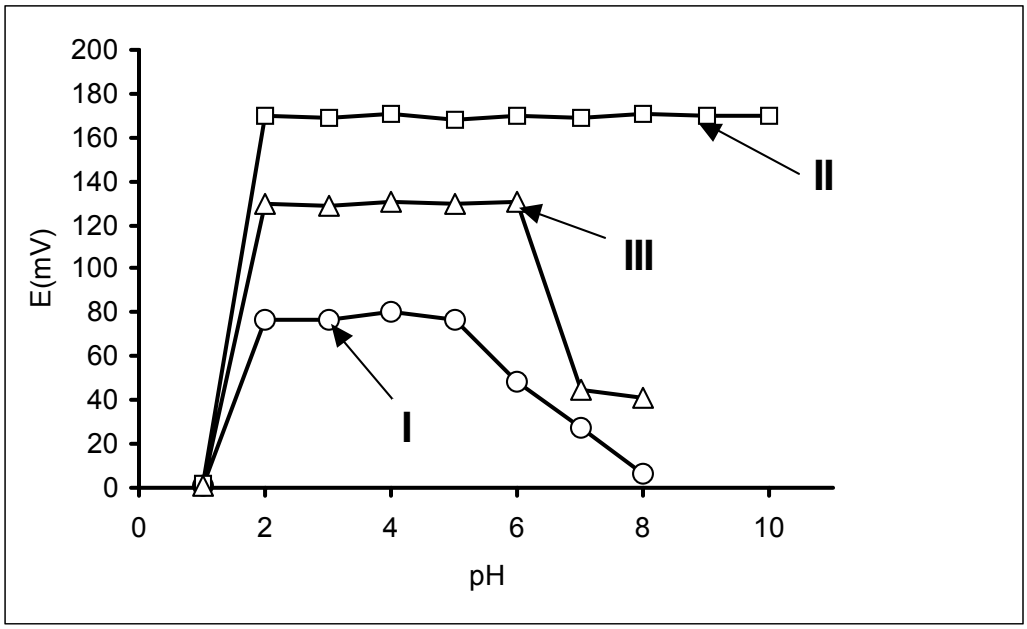

Figure 8.5 Effect of pH on the response of the enantioselective potentiometric membrane electrodes based on vancomycin (I), teicoplanin (II) and teicoplanin modified with acetonitrile (III).

8.5.4.3 Selectivity of the electrodes

The selectivity of all EPMEs was investigated using the mixed solution method proposed by Ren [42]. The concentrations of D-2-hydroxglutaric acid and interfering ion were 10^{-5} and 10^{-4} mol/L, respectively. The selectivity was investigated against L-2-hydroxglutaric

acid, creatine and creatinine. The selectivity coefficients for the enantioselective, potentiometric membrane electrodes, K_{sel}^{pot}, obtained are summarized in Table 8.12. The K_{sel}^{pot} values prove that the constructed potentiometric membrane electrodes are selective over creatine and creatinine. Inorganic cations such a Na^+, K^+, and Ca^{2+} do not interfere in the analysis of D-HGA.

Table 8.12 Selectivity coefficients of the enantioselective, potentiometric membrane electrodes based on macrocyclic antibiotics.[a]

Interference species (J)	pK_{sel}^{pot}		
	EPME based on		
	Vancomycin	Teicoplanin	Teicoplanin modified with acetonitrile
L-2-Hydroxyglutaric	3.81×10^{-3}	4.07×10^{-3}	4.17×10^{-3}
Creatine	3.81×10^{-3}	7.67×10^{-3}	4.17×10^{-3}
Creatinine	3.96×10^{-3}	3.91×10^{-3}	4.36×10^{-3}

[a] All measurements were made at room temperature; all values are the average of ten determinations.

8.5.4.4 Analytical applications

The electrodes proved to be useful for the determination of the enantiopurity of D-HGA raw material by direct potentiometric techniques and for its assay in urine samples. The analysis of D-2-hydroxyglutaric acid was investigated in the presence of L-2-hydroxyglutaric acid by using different ratios between D- and L-enantiomers.

The results obtained (Table 8.13) proved the suitability of the proposed potentiometric membrane electrodes for the enantioanalysis of D-2-hydroxyglutaric acid in the presence of its antipode. No significant differences in the recovery values were recorded for the ratios between 1:9 and 1:99.9 (L:D).

Table 8.13 The results obtained for the analysis of D-2-Hydroxyglutaric acid in the presence of L-2-Hydroxyglutaric acid [a]

Chiral selector	Recovery, % D-2-HGA		
	EPME based on		
D:L (moL:moL)	Vancomycin	Teicoplanin	Teicoplanin modified with acetonitrile
2:1	100.0±0.01	100.00±0.02	99.63±0.01
1:1	99.92±0.02	99.97±0.01	99.99±0.02
1:2	99.76±0.01	100.00±0.01	100.0±0.03
1:4	99.93±0.03	100.01±0.02	99.80±0.03
1:9	99.92±0.02	99.92±0.01	99.11±0.01

[a] All measurements were made at room temperature; all values are the average of ten determinations.

Table 8.14 Recovery of D-2-Hydroxyglutaric acid in urine samples, (%) [a]

Sample no.	Standard method [32] (µg/L)	Recovery, % D-2-HGA		
		EPME based on		
		Vancomycin	Teicoplanin	Teicoplanin with acetonitrile
1	44.40	99.56±0.02	99.30±0.05	99.51±0.02
2	88.80	99.18±0.02	99.27±0.02	98.59±0.03
3	133.20	99.59±0.01	99.29±0.03	99.60±0.01
4	177.60	99.83±0.01	99.52±0.02	99.54±0.02
5	370.0	99.35±0.03	99.87±0.01	99.50±0.03
6	1480.0	99.38±0.02	99.90±0.02	99.62±0.02

[a] All measurements were made at room temperature; all values are the average of ten determinations.

Urine samples (1-6) were donated from healthy persons and spiked with different aliquots of D-2-hydroxyglutaric acid. All spiked urine samples were buffered at pH=3 using phosphate buffer. The results recorded for the assay of D-2-hydroxyglutaric acid in urine samples are shown in Table 8.14. These results are in good concordance with those

obtained using the standard method [41]. The results obtained for samples (1-6) using the proposed EPMEs based on the different types of antibiotics (vancomycin, teicoplanin, and teicoplanin with acetonitrile) show the suitability of EPMEs for enantioanalysis of D-HGA and diagnosis of D-2-hydroxyglutaric acidurias.

8.6 Conclusions

The EPMEs based on vancomycin, teicoplanin, and teicoplanin modified with acetonitrile proved to be suitable for the enantioanalysis of D-2-hydroxyglutaric acid in solutions and urine samples by direct potentiometric technique. These electrodes can be reliable used for the diagnosis of D-2-hydroxyglutaric acidurias patients.

The EPMEs based on maltodextrins proved to be useful for the enantioanalysis of L-2-HGA in urine samples using direct potentiometric method. Therefore, these electrodes can be reliable used for the diagnosis of L-2-hydroxyglutaric aciduria.

β- and 2-Hydroxy-3-trimethylammoniopropyl-β-cyclodextrins based EPMEs can be reliable applied for the determination of L-2-hydroxyglutaric acid in urine samples and γ-cyclodextrin based EPME for the analysis of D-2-hydroxygluaric acid. The construction of the electrodes is simple, fast and reproducible. These electrodes have high precision, rapid response and the cost of construction and analysis is low. They can be successfully used for fast and reliable diagnosis of L- and D-2-hydroxyglutaric acidurias. EPME also have some advantages over the proposed chromatographic technique, such as high

precision, high enantioselectiviy, rapidity, low cost of analysis and no need for special sample pre-treatment before analysis.

8.7 References

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