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Optimization of the separation lactic acid enantiomers in body fluids by capillary electrophoresis

L. Saavedra, C. Barbas*

Department of Analytical Chemistry, Universidad San Pablo-CEU, 28668 Boadilla del Monte, Madrid, Spain

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Abstract

The optimization of the separation conditions of the two optical isomers of lactic acid by a factorial design is reported. Initially, different chiral selectors were systematically investigated and then a experimental design with three quantitative factors (cyclodextrin concentration and background buffer pH and concentration) were evaluated. Optimal conditions for obtaining a resolution higher than 1.5 were: phosphate buffer 200 m*M* at pH=6.0 with 413 m*M* 2-hydroxypropyl-β-cyclodextrin added (HP-β-CD), 20°C, −20 kV of applied potential and polyacrylamide-coated capillary. The method was validated for the measurement in plasma and it was applied to the identification of both isomers in body fluids such as urine, amniotic fluid and cerebrospinal fluid. Samples were centrifuged and diluted (1:4) prior to the analysis.

Keywords: Enantiomer separation; Optimization; Lactic acid

1. Introduction

Most biochemical reactions have selectivity to one enantiomeric form. Different enantiomers of the same compound can activate different metabolic pathways [1]. In humans, L-lactate is formed directly from pyruvic acid as a product of anaerobic carbohydrate metabolism. Some intestinal bacteria metabolize carbohydrate into D-lactate by the action of isomer-specific D-lactate dehydrogenase. Even though mammals may generate small amounts using the methylglyoxal pathway [2], plasma D-lactate is derived mostly from intestinal absorption and requires special techniques for its detection. From this point of view it is possible to determine the origin of several pathologies by an enantiomeric analysis of selected metabolites.

Capillary electrophoresis (CE) has shown to be a good choice for enantiomeric resolutions using chiral selectors in the separation buffer, which can provide very simple and automated method development. Other advantages of CE in body fluids analysis are the small sample volume needed for injection and the reduced sample pretreatment. That point minimizes costs, errors and artefacts. Comprehensive information on the subject can be found in the literature [3–18]. Nevertheless, most compounds described in these reviews have aromatic rings, or at least are hydrophobic, because in the chiral recognition mechanism, the hydrophobic interactions between the cavity of the selector, frequently native or deriva-

*Corresponding author. Fax: +34-91-351-0475.
E-mail address: cbarbas@ceu.es (C. Barbas).

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tized cyclodextrins (CDs) and the hydrophobic part of the compounds plays an important role in the stereoselective interactions.

Several human diseases, in particular metabolic disorders, often lead to accumulation of short chain organic acids in different body fluids.

Lactic acid is the organic acid with a shorter chain and two enantiomeric forms. It can be present in various organisms as a metabolite of carbohydrate metabolism. L-lactic acid originates by anaerobic glycolysis from its precursor pyruvic acid. This reaction is catalyzed by the enzyme lactate dehydrogenase having $NADH+H^+$ as a co-factor. Lactic acid may be elevated in the urine of patients with primary lactic acidosis, hypoperfusion, shock, hypoxia and other conditions. D-lactic acid concentration produced by human metabolism is minimal. It can be produced by the bacterial flora of the gut in pathological conditions, absorbed into the blood and transported to different fluids as urine. D-lactic acid cannot be metabolized by mammals. In very high concentrations, it can cause encephalopathy [19].

For this experiment lactic acid was chosen as a model because its short chain permits to think in a poor stereoselectivity and, therefore, the optimized method could probably be successfully applied for the enantiomeric resolution of other short chain organic acids of metabolic interest.

Chiral resolution of lactic acid has been conducted by enzymic methods [20,21], thin-layer chromatography (TLC) [22], gas chromatography (GC) [23,24], high-performance liquid chromatography (HPLC) with chiral ligand-exchange phases [25–30], or chiral fluorescent derivatization [31–35]. Multidimensional GC–mass spectrometry (MS) has also been employed for several urinary organic acids. It requires different derivatization conditions depending on the organic acid analyzed, because some of them undergo racemization, cyclication or other effects during the sample treatment [24,36,37]. It is a common problem in most of the chromatographic method proposed. Moreover, most of these methods are not applied in biological samples. Recently it has been reported that direct chiral resolution of lactic acid, 2-hydroxybutyric acid, 2-hydroxy-3-methylbutyric acid and 2-hydroxyisocaproic acid were possible by CE using 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) [38] and the method was optimized for the analysis of D- and L-lactic acids in food products [39,40].

Commonly, the optimization process is one of the most complex stages during method development. The success of this preliminary study depends on a laborious routine that investigates the influence of selected variables on the chosen analytical response. The classical approach is represented by the separate study of each experimental variable, using the so-called ''one-variable-at-a-time'' strategy. Although frequently applied, this classical methodology may be inefficient in determining the true optimal conditions, because it neglects interaction between variables.

The basic idea of the so-called multivariate study is to devise a small set of experiments, in which all pertinent factors are changed systematically, to simultaneously study the individual variables and their combined effects.

Various chemometric experimental designs have been employed for the optimization of CE methods [41]. For example: fractional factorial designs [42,43], factorial design [44], central composite design [45,46], Plackett–Burman design [47], overlapping resolution mapping [48–51], and a simplex design [52].

In this work, the optimization of the separation conditions of the two optical isomers of lactic acid by a factorial design are reported. Initially, we systematically investigated different chiral selectors and then an experimental design with three quantitative factors (cyclodextrin concentration and background buffer pH and concentration) was evaluated. The method was applied to determine both isomers in body fluids such as urine, plasma, amniotic fluid and cerebrospinal fluid (CSF).

2. Materials and methods

2.1. Instrumentation

The separation was performed on a capillary electrophoresis P/ACE 5500 (Beckman, Palo Alto, CA, USA) with UV detection at 200 nm. The injection was by pressure (0.035 bar) for 50 s. The separation was carried out with a polyacrylamide-

coated fused-silica capillary [57 cm×50 μm I.D. (Beckman)] and was operated at −20 kV potential. Temperature was maintained at 20°C.

2.2. Chemicals

α-, β- and γ-cyclodextrins were from Sigma (St Louis, MO, USA). 2-Hydroxypropyl-β-cyclodextrin, degree of substitution 0.6, was purchased from Fluka (Buchs, Switzerland). 6-Monodeoxy-6-monoamino-β-CD was from Cyclolab (Budapest, Hungary). 2-Hydroxyethyl-β-cyclodextrin was from Fluka. Dextrin was from Sigma. Phosphoric acid 85% was from Merck (Darmstadt, Germany). Sodium hydroxide was from Sharlau (Barcelona, Spain). D- and L-lactic acids were from Fluka.

2.3. Buffer and sample preparation

For optimizing the pH of the separation, different buffers were prepared with H_3PO_4 ranging from 50 to 500 m*M*, and pH adjusted by adding NaOH in the range from 5 to 7. The best final conditions were 200 m*M* phosphate buffer at pH 6.0. The buffer preparation was achieved by adding the required volume of buffer to the previously weighted amount of HP-β-CD in order to minimize the consumption of the product.

Stock solutions of 80 m*M* DL-, D- and L-lactic acid were individually prepared and stocked at −20°C. The day of the analysis a standard solution was prepared by diluting (1:10) the stock solutions with Milli-Q water quality (Millipore Ibérica, Madrid, Spain).

Samples were only centrifuged for 5 min at 4000 *g*, diluted 1:4 (v/v) with Milli-Q water and injected.

2.4. Experimental design

Factorial designs are experimental designs involving simultaneous alteration of all parameters according to a predefined matrix of experiments. They are well adapted to the determination of the relative importance of each variable in comparison to the estimated responses. In this study, three quantitative factors (cyclodextrin concentration and background buffer pH and concentration) were evaluated. The factors, their levels and the complete 3^3 factorial design are presented in Table 1. The chosen levels for the first assay were 50, 100 and 200 m*M* for the buffer concentration; 100, 200 and 300 m*M* for the HP-β-CD concentration; and 4, 5 and 6 for the pH.

Analysis of the response factors, determined as the chromatographic resolution, was carried out by Statgraphic™ plus for Windows 4.1 (Rockville, MD, USA) program. Resolution was calculated with the difference in migration time and peak width of the two analytes $[R_S = 2\ ((t_m)_B - (t_m)_A)/(W_A + W_B)]$.

A second factorial design was assayed with only two factors of variation. pH was fixed at the value of 6.0 because response got a maximum at this point. The assayed levels in this second experiment were 300, 400 and 500 m*M* for HP-β-CD; 200, 350 and 500 m*M* for buffer concentration. The experiment organisation is shown in Table 2.

Table 1
Experimental design no. 1

Exp. no.	Buffer concn.	CD concn.	pH	Resolution
1	50	100	4	0
2	100	100	4	0
3	200	100	4	0
4	20	200	4	0
5	100	200	4	0
6	200	200	4	0
7	50	300	4	0
8	100	300	4	0
9	200	300	4	0
10	50	100	5	0
11	100	100	5	0
12	200	100	5	0
13	50	200	5	0.49
14	100	200	5	0.40
15	200	200	5	0.415
16	50	300	5	0.825
17	100	300	5	0.85
18	200	300	5	0.84
19	50	100	6	0
20	100	100	6	0
21	200	100	6	0
22	50	200	6	0.53
23	100	200	6	0.455
24	200	200	6	0.555
25	50	300	6	1.0
26	100	300	6	0.77
27	200	300	6	1.055

Table 2
Experimental design no. 2

Exp. no.	Buffer concn.	CD concn.	Resolution
1	200	300	1.055
2	350	300	0.97
3	500	300	1.033
4	200	400	1.296
5	350	400	1.494
6	500	400	1.356
7	200	500	1.583
8	350	500	0
9	500	500	0

2.5. Validation

D- and L-lactic acid stock solutions (2.5 mg/ml) were prepared independently and kept at −20°C. The day of the assay they were tempered and adequately diluted with water. Linearity of response for standards was tested assaying by triplicate five levels of concentrations, ranging from 0.12 to 1.5 m*M*. This range was established after measuring a pool of plasmas from healthy people. The lower end was placed around the limit of quantitation of the method and the upper level was selected to permit the measurement of the increase foreseen in pathological situations. Linearity of response for samples was tested in the same way but replacing water with plasma.

Recovery was estimated comparing the values obtained in the linearity of the calibration line for plasma with the standards linearity, taking into account the endogenous concentration of D-lactate, which had been previously quantified.

Precision was tested to check the constancy of instrumental response in samples and therefore only D-lactate was measured because plasma samples did not contain the L-isomer. For this purpose, the assay was performed injecting 10 samples of the same pool of plasma and the corresponding standards for quantitation in 1 day for repetitivity and in two different days for intermediate precision.

Limits of detection were calculated with two methods. Method 1 followed IUPAC (International Union of Pure and Applied Chemistry) recommendations $[(a+3.S_B)/b]$ for chromatographic methods [53] by extrapolating to zero concentration the SDs of the last three points of linearity and interpolating this value in the corresponding equation. Method 2 measured the noise of the baseline in a length approx. 20 times the width of the analyte peak [54].

3. Results and discussion

It is well known that at least three-point simultaneous interactions are generally needed between a chiral selector and an enantiomer to cause physical separation. Kodama et al. suggested that they could occur with aliphatic α-hydroxyacids and HP-β-CD [38] but reported resolution was 1.02 for lactic acid. The chiral selectors tested were: α-cyclodextrin in concentrations ranging from 0.001 to 3 m*M*; β-cyclodextrin from 0.005 to 10 m*M*; γ-cyclodextrin from 0.001 to 1 m*M*; 6-monoamino-6-deoxy-β-cyclodextrin from 20 to 250 m*M*; 2-hydroxypropyl-β-CD from 100 to 500 m*M*; dextrin from 1 to 2% (w/w); ciclodex 2 from 5 to 10% (w/w) and 2-hydroxyethyl-β-CD 200 m*M*.

In the present assay only 6-monoamino-6-deoxy-β-CD and 2HP-β-CD, among the tested selectors, provided some selectivity, probably due to the interaction with the lateral chains in the CD and 2HP-β-CD was chosen because it is more easily available. Results related to the experimental designs are shown in Tables 1 and 2 and they were analysed with the corresponding statistical tests, as described below.

Fig. 1 shows a graphical representation (Pareto plot) of the ''size effect'' of each of the parameters

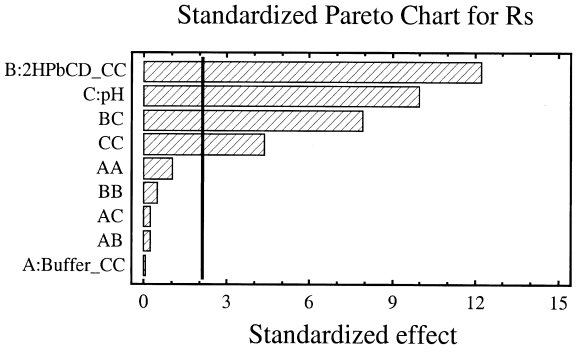

Fig. 1. Standardized Pareto plot for resolution. (A) Buffer concentration; (B) 2HP-β-CD concentration; (C) pH. Two letters represent the interaction between both parameters.

investigated upon the resolution of both peaks. In this treatment a parameter is deemed to have a significant influence if the size of the effect is greater than 2. Therefore, in Fig. 1 cyclodextrin concentration, pH and the interaction between both parameters show a significant effect on resolution, while buffer concentration did not present a significant effect. The main effects plot (Fig. 2) showed that resolution increased with cyclodextrin concentration without getting to a maximum, while the increase of resolution with pH reached the top value at 6.0, when the acids are fully ionized ($pK_a = 3.83$), and again buffer concentration showed slight effect.

Temperature was not included in the optimization process and it was fixed at the lowest value that the equipment was able to maintain constant, which was 20°C. It is known that temperature increases thermal agitation of molecules and debilitates the interaction between the selector and the isomers. This fact was experimentally tested in previous assays and it was found that 15°C of temperature provided a better separation, but the equipment was not able to maintain it constant for a long time.

In relation to the applied voltage, the peak resolution is proportional to the square root of the applied voltage, but Joule heating is a problem with increasing voltage. Thus, voltage must be maintained at the higher value that permits a reasonable current intensity not to exceed the heat removal capacity of the system. In this case it was −20 kV.

Having established the key factors affecting the performance of the method by using a screening design it is then appropriate to optimize the method

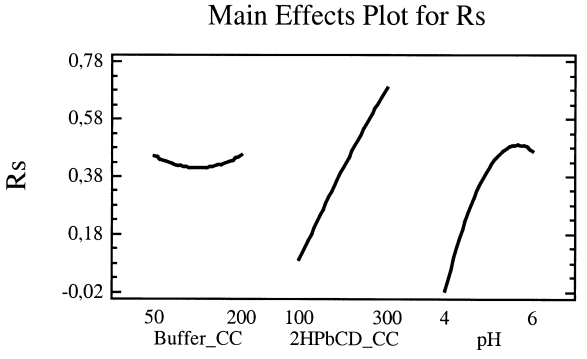

Fig. 2. Main effects plot for resolution. Buffer CC, buffer concentration; 2HPbCd CC, 2HP-β-CD concentration.

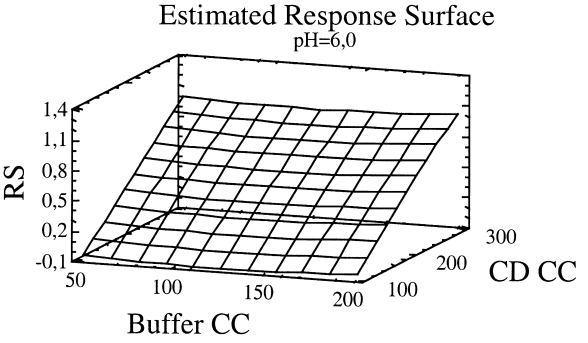

Fig. 3. Estimated response surface for resolution at pH = 6.0 in experiment 1. Buffer CC, buffer concentration; CD CC, cyclodextrin concentration.

by obtaining response surfaces. These plots can provide a graphical representation of the data over the ranges studied and can be used to predict areas of optimal performance [41]. It was obtained with data from the previous factorial design. As estimated, response surface at pH 6.0 achieved the maximum in the higher assayed values; therefore a second assay was performed with concentrations ranging from 200 to 500 m*M* for buffer concentration, and from 300 to 500 m*M* for HP-β-CD as chiral selector was added (Figs. 3 and 4).

A further response surface analysis enabled to set the optimum conditions.

Phosphate buffer 200 m*M* at pH 6.0 with 413 m*M* of 2-hydroxypropyl-β-cyclodextrin added as chiral selector. These conditions were applied to different body fluids. In all cases the presence of an isomer was confirmed by migration times as compared with the standard and by spiking the sample with the pure

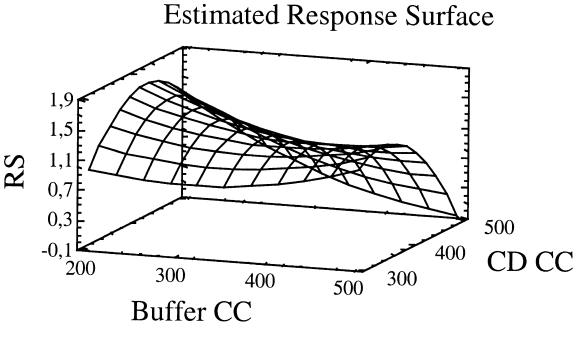

Fig. 4. Estimated response surface for resolution at pH = 6.0 in experiment 2.

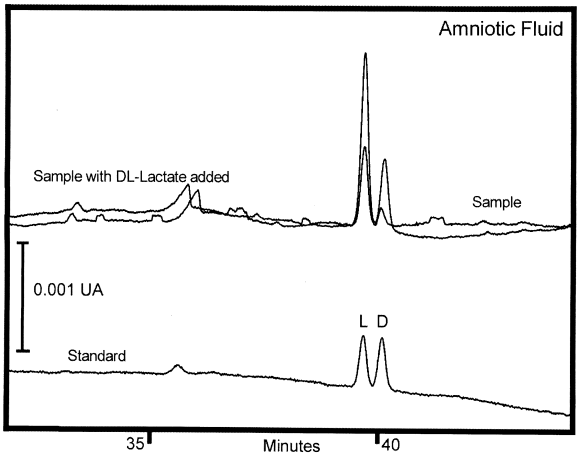

Fig. 5. Electrophoretogram of the amniotic fluid sample; DL-lactate standard; the sample spiked with the standard. Conditions: phosphate buffer 200 m*M* at pH=6.0 with 413 m*M* 2-hydroxypropyl-β-cyclodextrin added (HP-β-CD), −20 kV of applied voltage and polyacrylamide-coated capillary. UV detection at 200 nm.

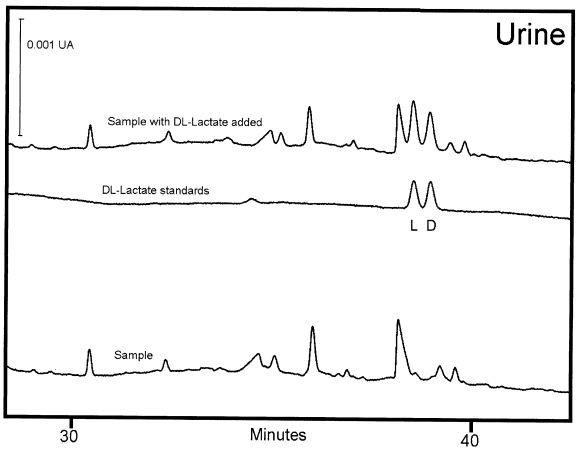

Fig. 7. Electrophoretogram of the urine sample; DL-lactate standard; the sample spiked with the standard. Conditions: phosphate buffer 200 m*M* at pH=6.0 with 413 m*M* 2-hydroxypropyl-β-cyclodextrin added (HP-β-CD), −20 kV of applied voltage and polyacrylamide-coated capillary. UV detection at 200 nm.

standard. Fig. 5 shows the electrophoretogram obtained for amniotic fluid where both isomers were present.

Cerebrospinal fluid (CSF), coming from a healthy patient, showed only the L-isomer (Fig. 6). Increased

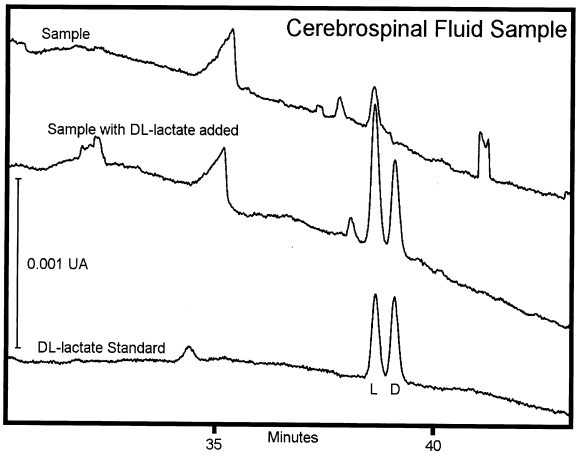

Fig. 6. Electrophoretogram of the cerebrospinal fluid sample; DL-lactate standard; the sample spiked with the standard. Conditions: phosphate buffer 200 m*M* at pH=6.0 with 413 m*M* 2-hydroxypropyl-β-cyclodextrin added (HP-β-CD), −20 kV of applied voltage and polyacrylamide-coated capillary. UV detection at 200 nm.

D-lactate is characteristic of patients with purulent meningitis [21]. The same happened with the urine sample (Fig. 7), where only L-lactate could be observed. Urine was the fluid with lower lactic acid content and efforts on stacking effect will be carried out in order to get better detection limits. Anyway, in pathological samples the analyte concentration increases and it simplifies the problem.

The method was validated for plasma samples, which is the most frequent type of sample to be analysed. As shown in Table 3, standards and samples fit the linear model ($r>0.99$) for both isomers. No bias was found as the intercept (a) with its limits of confidence (lc) includes the zero value and the recoveries were near 100% in the whole range.

Calculated limits of detection were around 0.1 mg/ml for L-lactate with method 1 and for both isomers with method 2, while it was lower (0.04 mg/ml) for D-lactate with method 1. It must be considered that these values come from statistical approaches and in practice 0.02 mg/ml was the lowest concentration clearly detected for both analytes in samples.

When running 10 runs per day of both standards and samples, daily RSDs in concentrations are low enough to consider the method acceptable (7.7%

Table 3
Main validation parameters

	L-lactate	D-lactate
Standards linearity		
Range (mg/ml)	0.13–1.52	0.12–1.50
r	0.993	0.995
Slope	12470±890	12353±810
Intercept	382±758	411±685
Samples linearity		
Range (mg/ml)	0.02–1.40	0.05–1.44
r	0.99	0.99
Slope	13900±1541	13548±876
Intercept	523±1344	355±765
Accuracy		
Recovery in standards (%)	99±7	99±6
Recovery in samples (%)	99±7	96±5
Precision		
Repetitivity		
RSD (%) ($n=10$)	7.7	
Intermediate precision		
RSD (%) ($n=20$)	10.0	
LOD		
Method 1 (mg/ml)	0.09	0.04
Method 2 (mg/ml)	0.13	0.13
LOQ		
Method 1 (mg/ml)	0.40	0.20
Method 2 (mg/ml)	0.43	0.43

RSD). The intermediate precision evaluated on different days with a total of 20 runs provided RSD values slightly superior to intra-assay precision (10% RSD), as could be expected.

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