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# Evaluation of diabetes-related short-chain organic acids in rat plasma by capillary electrophoresis

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**Abstract**

A capillary zone electrophoresis method was optimised to analyse low-molecular-mass organic acids for the purpose of monitoring diabetes in rat plasma. The method included acetoacetic, 2-hydroxybutyric, lactic and uric acids. A variation in the background electrolyte allowed us to measure pyruvic acid in the same sample. Conditions have been optimised for measuring a large number of plasma samples corresponding to control and diabetic rats. Samples were mixed with acetonitrile (1:1, v/v) to precipitate proteins, centrifuged, diluted and injected. Tropic acid was chosen as an adequate internal standard. Separation was developed with reversed voltage by using a column cartridge pre-treated with polyacrylamide. Two electrophoretic buffers were employed: 0.150 M $H_3PO_4$ made up pH 6.20 with NaOH and 0.3 mM $CaCl_2$ for acetoacetic, hydroxybutyric, lactic and uric acids, and 200 mM phosphate–10 mM acetate pH 4.0 for pyruvic acid, both with direct detection at 200 nm. The method was validated for linearity, accuracy and precision and the limits of quantification were calculated. The method was successfully applied to analyse these organic acids in control and diabetic animals. Acetoacetic and hydroxybutyric acids were clearly increased in diabetic rats, meanwhile no statistically significant difference has been found with the other acids.


*Keywords:* Organic acids; Acetoacetic acid; Hydroxybutyric acid; Lactic acid; Uric acid; Pyruvic acid

## 1. Introduction

The analysis of carboxylic acids in body fluids plays an important role in the screening, diagnosis and monitoring of a variety of pathologies.

Parameters related to diabetes monitoring are interesting not only because it is a disease affecting over 140 million people in the world [1], but also because rats made diabetic by sptreptozotocin injection are an established model to study oxidative stress [2].

Among the metabolites of interest are ketoacids (acetoacetate and β-hydroxybutyrate) that result from the incomplete oxidation of fatty acids in the liver [3]. The alteration of carbohydrate and fat metabolism also leads to an increase in lactate and pyruvate [1], and, finally uric acid is one of the most abundant water-soluble antioxidants in the organism [4].

The usual methods for short-chain organic acid analysis include capillary gas chromatography (GC) with or without mass spectrometry after solvent extraction and derivatization [5,6]. Another routine method for analyzing carboxylic acids is high-performance liquid chromatography (HPLC). Organic acids have been separated underivatized or after fluorescent derivatization in reversed-phase HPLC [7]. Ion exclusion chromatography with UV detection at 210 nm has also been applied for short-chain organic acid analysis [8].

In spite of its unquestionable sensitivity, selectivity and identification ability, two significant drawbacks of GC-MS which is the technique more generally accepted, are the time it takes to prepare samples and make analysis and the need for highly trained personnel. That has hindered its use for quality control. Moreover, some acids are lost during the treatment.

The clinical methods for the determination of these compounds are usually enzymatic methods, which require the individual measurement and, therefore, are time consuming and expensive. Moreover, the amount of sample is too large when working with small experimental animals.

Capillary electrophoresis has been proved to be an extraordinary tool for the measurement of short-chain organic acids. The main features of the technique related to the

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Table 1
Short-chain organic acids in body fluids analysed by CE

| Organic acids | References |
|---|---|
| Formic, succinic, acetic, lactic and propionic acids | [25] |
| Acetic, lactic, citric, tartaric, malic and succinic acids | [47] |
| It does not work with fumaric and orotic | |
| Lactate and pyruvate | [14] |
| Oxalic, formic, methylmalonic, fumaric, succinic, 2-ketoglutaric... ($n = 12$) | [15] |
| Oxalic, formic, propionic, fumaric and others acids ($n = 14$) | [23] |
| It does not work with oxalic acid | |
| Methylmalonic and short-chain dicarboxylic acids ($n = 6$) | [31] |
| Uric, hippuric and others acids | [19] |
| Oxalate and citrate | [29] |
| Pyruvic, citric, malic, acetoacetic and lactic acids | [24] |
| Organic and inorganic acids ($n = 13$) | [48] |
| Methylmalonic, citric, 2-ketoglutaric, succinic acids | [26] |
| Methylmalonic acid | [32] |
| Short-chain organic acids ($n = 14$) | [11] |
| Aliphatic (formic and tartaric) and aromatic acids | [16] |
| Methylmalonic, glutaric, *N*-acetylaspartic, aminoadipic, propionic acids ($n = 10$) | [30] |
| Oxalic, malonic, maleic, succinic, pyruvic, lactic, 3-hydroxybutyric and hippuric acids | [13] |
| Orotic acid | [20] |
| Orotic acid | [22] |
| Mevalonic, glutaric, glyceric and methylmalonic acids | [49] |
| Short-chain organic acids | [27] |
| Short-and medium-chain organic acids ($n = 9$) | [17] |
| Short-and medium-chain organic acids ($n = 27$) | [18] |
| Oxalic, ascorbic and uric acids | [50] |
| Orotic acid | [28] |
| Succinic, maleic, malonic and glutaric acids | [51] |
| Homogentisic, piroglutamic acids and others compounds | [52] |
| Oxalic, citric, glyoxylic and glyceric acids | [53] |
| Propionic, benzoic, homogentisic, homovanillic, vanillyl mandelic, glyceric, orotic and more organic acids | [54] |
| D- and L-Lactic acid | [46] |

problem are: (1) the ability to separate small molecules from complex matrices without sample pre-treatment. This is because these molecules run faster and so the capillary is completely emptied and washed after each run. (2) The possibility of measuring the absorbance at 200 nm or below, where the carboxylic group absorbs, because it works in aqueous media. (3) The low consumption of reagents: a few millilitres of an aqueous buffer are enough for 1 day.

A comprehensive survey of capillary electrophoresis methods developed for the measurement of short-chain organic acids and inorganic anions in a wide variety of matrices has been recently published [9,10]. Table 1 includes the groups of short-chain organic acids analysed by CE in body fluids with the corresponding references.

To summarize some aspects, inverted polarity is needed to analyse short-chain organic acids because their electrophoretic mobility towards the anode is usually higher than the electroosmotic flow towards the cathode. This CE mode needs to enclose the use of coated capillaries or a surfactant in the electrolyte acting as dynamic coating to suppress or even reverse the electroosmotic flow. Fourteen short-chain organic acids were studied by capillary electrophoresis with indirect UV detection in three different capillary conditions: polyacrylamide coated, myristyltrimethylammonium bromide dynamically coated and uncoated capillary. The best performance in terms of precision in migration time, highest column efficiency, and better limits of detection were obtained by using the polyacrylamide coated capillary. Nevertheless, when the method was applied to clinical urine samples, several interferences appeared and the authors recognize that this method needs further study on real samples [11]. Our experience, as much with standards as with many different biological samples, is also that polyacrylamide coated capillaries performances related to reproducibility are the best.

UV absorbance is the most common mode of detection in commercial capillary electrophoresis equipment. As previously described, organic acids can be separated in aqueous buffers and if non absorbing electrolytes are employed, direct measurement at 200 nm or below is a good option for the carboxylic group [12–21].Obviously, when organic acids with a characteristic spectra are measured, different wavelengths can be employed, for example orotic acid was measured at 280 nm [22].

Indirect detection can be employed adding at the back ground electrolyte (BGE) an absorbing substance and detecting the less absorbing carboxylic group as a negative peak. Generally it is considered a more sensible mode than direct detection, and it can be so for standards, but when dirty or complex samples have to be measured, such as biological fluids, high dilution rates have to be employed to avoid very noisy baselines and overlapping peaks and, then, the result is not so good. Several compounds have been employed as background absorbing additives: 4-hydroxybenzoate and detection at 254 nm [23]; ε-aminocaproic and phenylhydroxyacetic or mandelic acids at 220 nm [24]; phtalate at 254 nm [25], at 210 nm [26] and at 230 nm [11]; benzoic acid and tris at 220 nm [27]; glutamic acid plus spermine at 254 nm and 280 nm [28]; cromate at 254 nm [29] and some authors employed a commercial BGE of undescribed composition [30].

Fluorescence has also been employed after derivatizating the carboxylic acids, but, derivatization of short-chain organic acids in aqueous solution is the most challenging because of the low reactivity of the carboxylic group in water [27,31,32].

In our experience direct UV detection provides higher sensitivity and precision in real samples [33]. Other authors have similar results [34].

The objectives of the present work were the identification of small carboxylic acids in rat plasma, optimisation of the sample preparation and separation conditions by reversed polarity CE with direct UV detection, validation of the method and application to control and diabetic animals.

## 2. Materials and methods

### 2.1. Instrumentation

The separation was performed on a capillary electrophoresis P/ACE 5000 (Beckman, Madrid, Spain) with UV detection at 200 ± 10 nm. The injection was by pressure (0.5 p.s.i.; p.s.i. = 6894.76 Pa) for 10 s. The separation was carried out with a polyacrylamide coated capillary (Beckman, Madrid, Spain) (37 cm × 50 μm i.d.) and was operated at −15 kV potential. Temperature was maintained at 25 °C.

The BGE for acetoacetic, hydroxybutyric, lactic and uric acids was prepared with 0.150 M $H_3PO_4$ made up pH 6.20 with NaOH and 0.3 mM $CaCl_2$. The current generated in such conditions was 115 μA.

The capillary was initially conditioned following the manufacturing instructions and it was kept overnight with buffer in the freezer. It was flushed between runs with water for 3 min and the background electrolyte for 3 min. Buffer vials (5 mL) used for separation were replaced each six injections.

The BGE for pyruvic acid with direct detection was prepared with 0.200 M $H_3PO_4$ and 10 mM acetic acid made up pH 4.00 with NaOH. The buffer vials were replaced after each analysis.

The BGE employed for pyruvic acid with indirect detection was from Agilent (Madrid, Spain) designed for organic acids analysis and its pH was 5.6.

### 2.2. Chemicals

Standards were obtained from Sigma (St. Louis, MO, USA). Phosphoric acid 85% was from Merck (Darmstadt, Germany), sodium hydroxide from Panreac (Madrid, Spain) and organic solvents from Scharlau (Barcelona, Spain).

The colorimetric enzymatic test was Trinder–lactate oxidase–PAP from Spin React (Barcelona, Spain).

### 2.3. Animals and samples

Plasma for method development and validation was obtained from Sprague–Dawley male rats each weighing 450 g and bred in our animal quarters. Streptozotocin (50 mg/kg) was used to promote diabetes in one group of animals and the effect was monitored by glucose measurement.

Animals were anaesthetised with 75–100 mg/kg ketamine–2.5 mg/kg azepromacine and blood was obtained by cardiac puncture in EDTA. Blood was rapidly centrifuged to separate plasma and immediately kept at −20 °C until the day of the assay.

One hundred microlitres of plasma were mixed with 100 μL acetonitrile to precipitate proteins and, after centrifugation at 11180 × *g* (Hettich zentrifugen, Mikro 22RTuttlingen, Germany), 150 μL of the supernatant were mixed with the same volume of water and measured.

The standards used for quantification contained 0.5 mM lactic acid, 0.05 mM acetoacetic acid, 0.25 mM hydroxybutyric acid, 0.02 mM uric acid and 0.05 mM pyruvic acid.

### 2.4. Validation

Individual stock solutions of each organic acid 20 mM, except for uric acid which was 4 mM, in purified water were prepared and stored at −20 °C. On the day of the analysis they were adequately diluted.

Linearity of response for standards was tested assaying by triplicate using five levels of concentrations, ranging from 0.0250 to 0.5 mM for acetoacetic; from 0.25 to 1 mM for lactic; from 0.12 to 0.5 mM for hydroxybutyric; from 0.01 to 0.04 mM for uric; from 0.025 to 0.1 mM for pyruvic acids.

Recovery was estimated comparing the values obtained in the linearity test for spiked samples with the corresponding standards linearity, taking into account the plasma concentrations, which had been previously quantified.

Within-day precision was tested both to check the constancy of instrumental response to a given analyte and the repetitiveness of concentrations. For this purpose, the assay was performed with six solutions of standards and six of samples, in the medium concentration of the calibration curve for all the compounds.

Limits of detection (LODs) were calculated with 3 × signal/noise ratio.

### 2.5. Lactic acid enzymatic assay

L-Lactic acid was also measured with the classical enzymatic assay, employing lactate oxidase. In brief, for calibration, the reactive solution containing L-lactate oxidase, peroxidase, 4-aminophenazone and 4-chlorophenol was prepared following the manufacturer's instructions. One millilitre of this solution was added to 10 μL of L-lactate standard and incubated for 5 min at 37 °C. The absorbance was read against a blank solution at 505 nm. The same procedure was followed for samples but using 10 μL of plasma.

## 3. Results and discussion

### 3.1. Optimization of BGE

The optimization strategy started with our experience of short-chain organic acid analysis in different matrices quoted previously and it was addressed towards small variations to obtain maximum resolution between the acids present in this type of samples and the possible interferences.

With this objective pH was varied from 5.8 to 6.4 with 0.20 increments and pH 6.20 gave the best results. Phosphate concentration was tested from 150 to 300 mM. These values are high for usual practice in capillary electrophoresis, but currents are maintained under acceptable limits and it is necessary to give sufficient resolution of analytes while still maintaining acceptable dispersive properties.

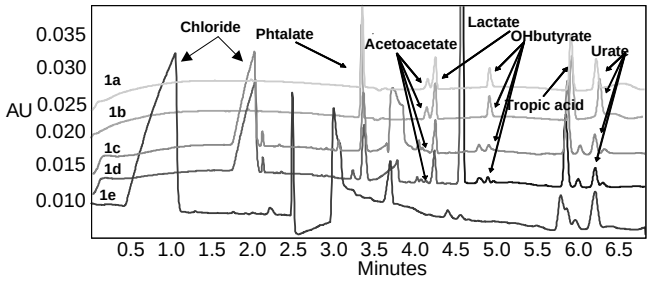


Fig. 1. (a) Standards with 150 mM phosphate buffer at pH 6.2 (0.5 mM lactic acid, 0.05 mM acetoacetic acid, 0.25 mM hydroxy-butyric acid, 0.02 mM uric acid). (b) Standards at the same concentration with $CaCl_2$ 0.3 mM and 150 mM phosphate buffer pH 6.2. (c) Sample with 150 mM phosphate buffer at pH 6.2. (d) Sample with $CaCl_2$ 0.3 mM, 150 mM phosphate buffer pH 6.2. (e) Sample with 0.3 mM $CaCl_2$ added into the sample and 150 mM phosphate buffer at pH 6.2.

Nevertheless, although the separation for standards was very good including phtalic and tropic acids as possible internal standards (Fig. 1a), when samples were analysed, a broad peak appeared that interfered with acetoacetic acid measurement (Fig. 1c). It was identified as EDTA, employed to avoid coagulation during sample collection. Different anticoagulants were not adequate, because citrate interfered to a higher extent, and heparine is described as showing pro-oxidant properties [35] and the blood sample will be used as part of a project for oxidative stress monitoring (Fig. 2).

Therefore, different approaches were checked to eliminate EDTA peak. At first instance $CaCl_2$ was added to the sample to react with the chelant agent, but the profile clearly worsened (Fig. 1e), probably due to an increase in the ionic strength of the sample. Thus, $CaCl_2$ was added to the buffer. In such conditions EDTA peak disappeared (Fig. 1d) meanwhile, the carboxylic acids peak areas did not decrease due to complex formation or precipitation (Fig. 1b).

Pyruvic acid, more acidic than the others, needed a different pH to be separated and detected. pH 4.00 gave the best result (Fig. 3), and although phosphate was included in the buffer to maintain the ionic strength, acetate was also

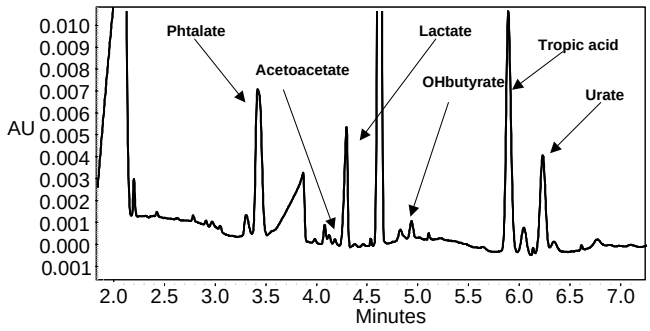


Fig. 2. Zoom for sample at working conditions ($CaCl_2$ 0.3 mM and 150 mM phosphate buffer pH 6.2).

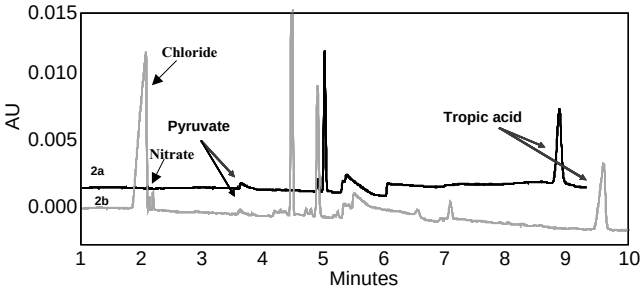


Fig. 3. Pyruvate analysis with direct detection at 200 nm with 200 mM phosphate 10 mM acetate buffer pH 4.0. (a) Standard 0.05 mM; (b) Sample.

added to have some buffer capacity, but its concentration is low because it absorbs at 200 nm, where the analyte will be detected. Due to the low buffer capacity shown by this system the vials have to be replaced at each measurement to obtain a good reproducibility. In this profile chloride, nitrite and nitrate could also be measured. A good resolution was obtained for standards of these compounds, however, sensitivity in this samples was not enough for nitrite, meanwhile, chloride and nitrate can be observed in Fig. 3.

As could be also observed in this figure, the pyruvic acid peak in samples was very small, and a commercial buffer designed for indirect detection was tested, in an attempt to obtain higher sensitivity. The result exceeded expectations, but pyruvic peak area in samples was more than 500 times higher than the standard prepared with the concentration described in literature for this compound [36] and obtained with direct detection. Therefore, direct detection was preferred because an interference seems to be present in samples in these conditions.

### 3.2. Validation

Validation was performed following ICH guidelines [37,38]. Prior to validation several internal standards were tested. Tropic acid could be employed for both conditions pH 6.20 and 4.00, although phtalic acid is even more adequate for pH 6.20 because it is nearer to the analytes. The presence of the internal standard corrects the small bias due to sample injection and sample evaporation, which is easier in the presence of acetonitrile. Calculations described in the manuscript have been performed including phtalic acid, as internal standard at pH 6.20, and tropic acid at pH 4.00, however, small differences were found when they were omitted.

Once the compounds present in the sample were identified by migration time as compared with the standards and by spiking, selectivity was proved by injecting acids very close in migration time (such as oxalic, fumaric, citric and pyroglutamic acids). The high efficiency obtained in capillary electrophoresis permits the clear separation of most of them. All the calculations were performed with peak areas.

Linearity has been determined by a series of two replicates of five levels of standards whose concentrations span

Table 2
Main validation parameters of the method

| | Range (mM) | Acetoacetic 0.025–0.1 | Lactic 0.25–1 | OHbutyric 0.125–0.500 | Uric 0.01–0.04 | Pyruvic 0.025–0.1 |
|---|---|---|---|---|---|---|
| Linearity | Standards | | | | | |
| | Intercept ± C.I. | 0.014 ± 0.007 | 0.05 ± 0.04 | 0.01 ± 0.02 | 0.2 ± 0.1 | 0.01 ± 0.003 |
| | Slope ± C.I. | 2.1 ± 0.1 | 0.70 ± 0.06 | 0.93 ± 0.07 | 43.8± 4.2 | 1.17 ± 0.13 |
| | *r* | 0.998 | 0.995 | 0.997 | 0.993 | 0.994 |
| | Samples | | | | | |
| | Intercept ± C.I. | 0.002 ± 0.005 | 0.05 ± 0.04 | 0.03 ± 0.01 | 0.06 ± 0.05 | |
| | Slope ± C.I. | 2.2 ± 0.2 | 0.7 ± 0.1 | 1.0 ± 0.1 | 43.2 ± 4.3 | 1.75 ± 0.25 |
| | *r* | 0.996 | 0.993 | 0.993 | 0.995 | 0.990 |
| Accuracy | Standards (n = 5) | | | | | |
| | Recovery (%) | 99.5 | 99.3 | 99.4 | 99.4 | 99.2 |
| | R.S.D. (%) | 4.8 | 6.8 | 7.2 | 8.6 | 4.3 |
| | Samples (*n* = 5) | | | | | |
| | Recovery (%) | 101.8 | 101.0 | 102.7 | 101.3 | 99.1 |
| | R.S.D. (%) | 7.6 | 4.3 | 9.1 | 5.5 | 6.7 |
| Instrumental precision standards | Intra-assay | | | | | |
| | *n* | 6 | 6 | 6 | 6 | 6 |
| | Mean (mM) | 0.05 | 0.5 | 0.25 | 0.02 | 0.05 |
| | R.S.D. (%) | 1.5 | 0.4 | 2.1 | 1.6 | 6.2 |
| | Intermediate | | | | | |
| | *n* | 12 | 12 | 12 | 12 | 12 |
| | Mean (mM) | 0.05 | 0.5 | 0.25 | 0.02 | 0.05 |
| | R.S.D. (%) | 2.4 | 1.1 | 2.3 | 3.2 | 6.8 |
| Method precision samples | Intra-assay | | | | | |
| | *n* | 6 | 6 | 6 | 6 | 6 |
| | Mean (mM) | 0.007 | 0.585 | 0.083 | 0.007 | 0.036 |
| | R.S.D. (%) | 4.6 | 3.3 | 6.8 | 7.8 | 6.2 |
| | Intermediate | | | | | |
| | *n* | 12 | 12 | 12 | 12 | 12 |
| | Mean (mM) | 0.007 | 0.592 | 0.087 | 0.007 | 0.035 |
| | R.S.D. (%) | 9.7 | 3.1 | 8.5 | 9.1 | 8.0 |
| LOD (mM) | Standards | 0.002 | 0.006 | 0.004 | 0.0001 | 0.002 |

up around 50–200% of the expected concentration range. As can be seen in Table 2, the linear regression equation applied to the results gave an intercept not significantly different from zero in most cases. When a significant non zero intercept was obtained the bias was very small and it showed no effect on the accuracy of the method. The slopes are different from zero in all cases and correlation coefficients are over 0.99 in all cases. Samples linearity is also adequate for the same reason, but this experiment was mainly developed to study the recoveries. All the recoveries include 100% and R.S.D.s presented very adequate values. Intra-assay instrumental precision for standards provided R.S.D. values ranging from 0.4 to 2.1% but it reached 6.8% for pyruvic acid, because it is a very small peak. Similar values were obtained for intermediate instrumental precision. For method precision for samples R.S.D. ranged from 3.3 to 7.8% in 1 day and from 3.1 to 9.7% on two different days. To summarize, all the values can be considered adequate for the levels of analytes and characteristics of the method.

LODs were below the expected values for the five analytes.

Some of these compounds are usually measured by commercially available kits, such is the case of lactic acid, and

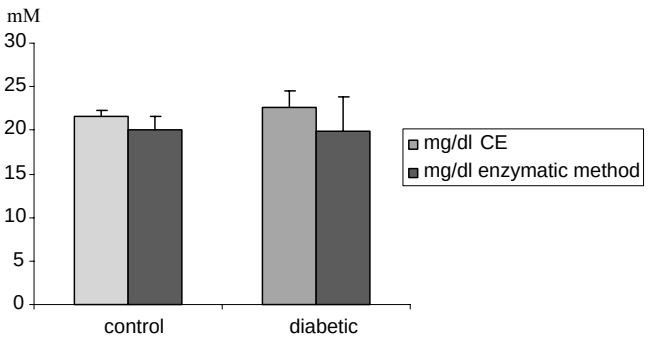


Fig. 4. Lactate values by CE and enzymatic method in control and diabetic rats.

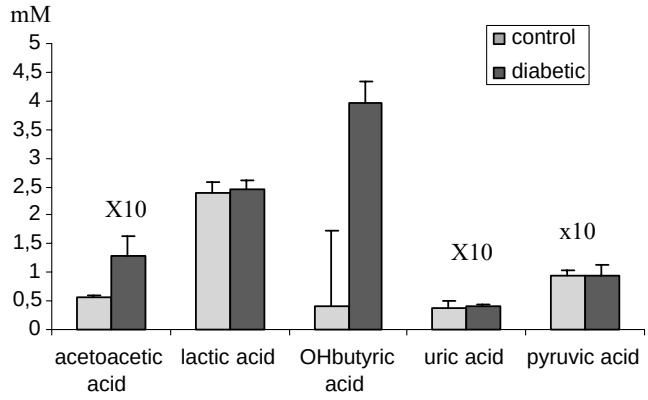


Fig. 5. Results acetoacetate, lactate, hydroxy butyrate, urate and pyruvate in control and diabetic rat plasma.

therefore, capillary electrophoresis results were compared with those obtained with the enzymatic method for five control and six diabetic animals. As can be seen in Fig. 4, no statistically significant difference was found (Student *t*-test, $P > 0.95$) within the same group between the two methods, although dispersions were higher with the enzymatic test. Slightly higher values obtained with the capillary electrophoresis method could be due to the measurement of both D- and L-isomers with this technique.

Aliquots of the same samples were measured through 20 days and all the acids can be considered stable at least during this time because the R.S.D.s of the results were lower than 5% related to the initial value.

Therefore, the capillary electrophoresis method was employed for the measurement of samples, not only because it saves sample manipulation and costs compared to the enzymatic kits that require the individual determination, but also because it permits the measurement of five acids with a single aliquot of plasma consuming several nanoliters, which is very important when several determinations have to be done with small experimental animals.

After validation the method was applied to plasmas of 10 control and 10 diabetic rats. Results can be observed in Fig. 5. All of them are within the ranges described in the literature for these compounds [36,39–41]. Furthermore, acetoacetic and hydroxybutyric acids, ketony bodies, were clearly increased in diabetic rats, whereas no statistically significant difference has been found with the other acids. Nevertheless, although it was not statistically significant, lactic acid tends to be higher in diabetic rats. It has been reported that D-lactic acid was significantly increased in serum of diabetic animals [42,43], produced from methylglyoxal in the metabolic pathway of glucose [44,45]. Probably, as the amount of the D-lactic isomer is approximately 1% relative to L-lactic, the weight of this increase is not enough to be detected in the total concentration. Therefore, in future works the two isomers will be measured separately with the method previously developed in our laboratory by direct CE measurement [46].

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