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Short communication

Determination of lactic acid enantiomers in human urine by high-performance immunoaffinity LC–MS

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ABSTRACT

In this study, a monoclonal anti-D-hydroxy acid antibody was used as chiral selector for chromatographic enantiomer separation and quantification of lactic acid contained in human urine samples. The immunoaffinity column was directly coupled to an electrospray ionization mass spectrometer for detection. Separations were performed at room temperature and under isocratic conditions using ammonium bicarbonate buffer (pH 7.8; 10 mM) as mobile phase. No elaborate sample preparation or analyte derivatization was required and individual runs were completed in less than 10 min.

1. Introduction

The α-hydroxy acid lactic acid is a key metabolite in both prokaryotic and eukaryotic organisms [1,2]. Its quantitative determination is of great relevance for monitoring metabolic states and possible disorders [3–5], as well as fermentation processes [6–8]. Lactic acid is furthermore an important product of microbial metabolic engineering [9] and a chiral building block for the production of, e.g., biodegradable polymers [10].

In healthy humans, lactic acid is almost exclusively L-lactate, which is readily metabolized to pyruvate in the liver by L-lactate dehydrogenase. Contrary to wide belief, though, also D-lactate is converted quite efficiently to pyruvate by a putative mitochondrial D-lactate dehydrogenase and D-α-hydroxy acid dehydrogenase, respectively [11]. Interestingly, D-lactate is even produced endogenously in humans, albeit at relatively low concentrations, *via* the methylglyoxal pathway [11,12].

Elevated levels of D-lactate in blood and urine are typically caused by bacterial overproduction in the gut, but may also be a result of infection, ischemia, or traumatic shock [3–5,11,13]. As the enantiomeric composition of lactate in bodily fluids is a valuable diagnostic indicator of such conditions, the development of analytical methods suitable for enantiomer analysis of this organic acid is of great importance.

A number of techniques have been utilized for chiral analysis of α-hydroxy acids; these range from enzymatic assays [14] to chromatographic and electrophoretic techniques such as HPLC, GC, CEC, and CE [15–24]. Direct enantiomer separation of lactate contained in human samples was previously achieved using, e.g., macrocyclic antibiotics and cyclodextrins as chiral selectors [20,24].

We have recently reported direct enantiomer resolution of α-hydroxy acids on an antibody-based chiral stationary phase (CSP) [25]. A monoclonal anti-D-hydroxy acid antibody was covalently linked to a synthetic high-flow-through type support material and used in an HPLC system coupled to a UV detector. With this setup, several aromatic and aliphatic α-hydroxy acids, including lactic acid, could be separated under mild isocratic conditions; however, we were unable to analyze weakly retained compounds contained in complex biological matrices due to overlapping peaks. The objective of the present study was to test the feasibility of interfacing the antibody column with a mass spectrometer in order to establish a high-performance immunoaffinity LC–MS setup suitable for the determination of lactate enantiomers contained in human urine samples.

2. Experimental

2.1. Chemicals

HPLC grade methanol was purchased from Fluka (Allentown, PA). Inorganic salts were from ACROS/Fisher (Fair Lawn, NJ). Water was purified using a MilliQ water purification system (Millipore, Bedford, MA). L-Lactic acid was obtained from Fluka (Allentown,

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PA) and D-lactic acid from ACROS/Fisher (Fair Lawn, NJ). Sodium L-lactate-3,3,3-D_3 (98% atom %D) was obtained from CDN isotopes (Quebec, Canada). Creatinine standard solutions were obtained from Quidel Corporation (San Diego, CA). Saturated picric acid aqueous solution (1.2%, w/v) was purchased from Ricca Chemical Company (Arlington, TX).

2.2. Urine samples

Human urine samples were graciously provided by Delnor Hospital (Geneva, IL). The samples were collected anonymously and stored at −20 °C prior to analysis. This project was approved by the Institutional Review Board of Northern Illinois University (ORC # 2963).

2.3. Chromatography and instrumentation

The antibody-based CSP was prepared as described previously [25]. In brief, the monoclonal anti-D-hydroxy acid antibody 8E10.9 was immobilized onto POROS-OH support material (20 μm particles; PerSeptive Biosystems, Cambridge, MA) following activation with disuccinimidyl carbonate [26]. The amount of immobilized antibody was determined to be 29 mg per gram of support. A stainless steel column (4.6 mm × 150 mm) was slurry packed with the antibody-derivatized support in phosphate buffered saline (PBS), pH 7.4, at a maximum pressure of 160 bar using an Alltech Slurry Packer Model 1666 (Alltech, Deerfield, IL). The column was stored under azide-containing PBS at 4 °C when not used for an extended period of time.

The chromatographic system utilized a Jasco PU-1586 preparative scale HPLC pump (Jasco Inc., Easton, MD) and a Rheodyne 7725i injection valve with a 20 μl loop (Hitachi, Naperville, IL); post-column addition of methanol was achieved using an Agilent 1100 series pump (Agilent, Wilmington, DE). The combined flow was split and directed into an Esquire 3000 quadrupole ion trap mass spectrometer with an electrospray ion source (Bruker Daltonics, Billerica, MA), which was operated in negative ion mode with a spray voltage of 4 kV. Drying gas was nitrogen (250 °C, 18 psi, 7 l/min). The maximum accumulation time was 200 ms and the ICC target was set to 30,000; five spectra were averaged. Chromatograms were analyzed using DataAnalysis™ software version 2.0 (Bruker Daltonics, Billerica, MA).

All chromatographic separations were performed at room temperature and at a flow rate of 0.5 ml/min using ammonium bicarbonate buffer (pH 7.8; 10 mM) as mobile phase. In order to improve ionization, methanol was added post-column in an equal volume ratio. The total combined flow into the mass spectrometer was reduced to 0.25 ml/min by diverting the appropriate fraction to waste. Ionization conditions of D- and L-lactic acid and deuterated L-lactic acid-3,3,3-D_3 used as internal standard were optimized off-column. Both lactic acid enantiomers produced anions with m/z of 89. The internal standard yielded anions with m/z 92.

2.4. Sample preparation and analysis

Calibration standard solutions of D- and L-lactic acid were prepared in ammonium bicarbonate buffer at concentrations ranging from 0.25 to 4 mM. A 1 mM solution of L-lactate-3,3,3-D_3 to be used as internal standard was prepared in the same solvent. Frozen human urine samples were allowed to thaw, and 500 μl aliquots were filtered using Microcon® Centrifugal Filter Devices with a nominal molecular weight cut-off of 10,000 (Millipore, Bedford, MA). Three hundred microliters of filtrate were mixed with 300 μl of internal standard solution prior to injection. Spiked samples were prepared by directly adding D-lactic acid to urine samples. Remaining aliquots were frozen at −20 °C until needed.

In order to correct for matrix-induced ion suppression, the concentrations of lactate enantiomers in urine were first determined as the ratio (expressed as response factor) of analyte peak area to internal standard peak area as suggested in Ref. [20], and then correlated with the response factors of calibration standards. Calibration standards were injected in triplicate; urine samples were analyzed in duplicate. Lactate concentrations were normalized against creatinine in standard clinical format, which corrects for urinary dilution. Creatinine concentrations of each urine sample were determined spectrophotometrically by a modified version of Jaffé's alkaline picric acid method [27,28] following the procedure outlined in Ref. [29].

3. Results and discussion

The antibody-based CSP used in this study was previously shown to allow chiral separation of various α-hydroxy acids in an HPLC system coupled to a UV detector [25]. These studies, which were performed using pure, commercially available analytes, demonstrated that the monoclonal anti-D-hydroxy acid antibody used as chiral selector binds to the D-enantiomers of both aromatic and aliphatic members of this class of compounds, but not to the corresponding L-enantiomers. However, since the antibody had been raised against an aromatic hapten, chromatographic retention times of the D-enantiomers of aliphatic analytes, such as D-lactate, were significantly shorter than those of analytes with more bulky side chains. The relatively short retention times of more weakly bound analytes made the analysis of complex biological samples impractical as the large amount of unretained matrix components resulted in overlapping peaks (Fig. 1).

To address this problem, we investigated the feasibility of coupling the analytical size immunoaffinity column directly to a mass spectrometer for detection. Using an anti-D-amino acid antibody immobilized onto silica as CSP, we had previously demonstrated that microbore immunoaffinity columns are suitable for enantiomer separation of amino acids under isocratic conditions in LC–MS [30]. Similar to this previous study we first investigated

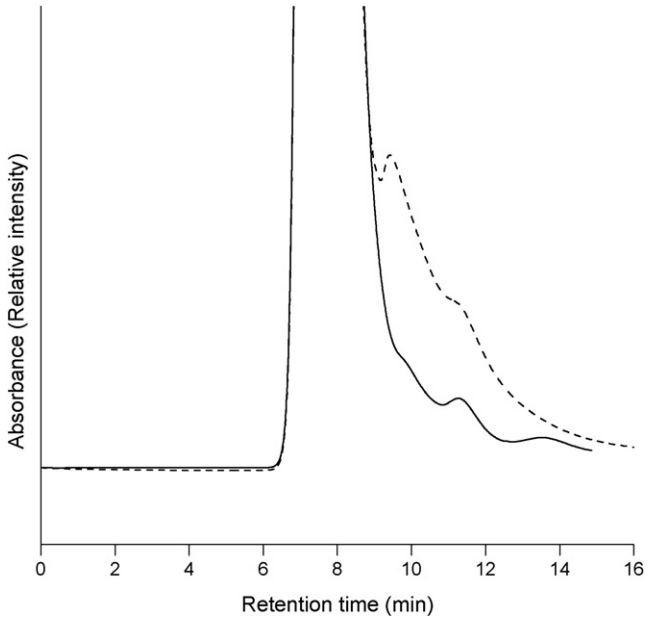

Fig. 1. Chromatograms of a urine sample before (solid line) and after spiking with lactic acid (dotted line) obtained using an anti-hydroxy acid immunoaffinity column and UV detection.

the utility of a low ionic strength ammonium bicarbonate buffer as sole mobile phase; this volatile buffer is compatible with both MS detection and the antibody CSP. It was found not only that the enantiomers of lactate can be separated in ammonium bicarbonate buffer (pH 7.8; 10 mM) but also that the resolution actually increases in comparison to separations performed in PBS (R_s = 1.51 *vs.* R_s = 1.28). This can be explained with increased electrostatic interactions between antibody and analyte at low ionic strengths [25]. Direct infusion of the aqueous mobile phase into the mass spectrometer, though, resulted in relatively poor ionization efficiencies. Addition of methanol greatly improved ionization and led to stable and reproducible responses for the characteristic ions *m/z* 89 for lactate and *m/z* 92 for the deuterated internal standard sodium L-lactate-3,3,3-D_3. Since it was known from previous studies [31] that organic modifiers have a potentially harmful effect on antibody CSPs, methanol was added post-column. Calibration curves obtained with standard solutions of D-lactate ($y = 1.05x - 0.05$; $R^2 = 0.9979$; $n = 5$) and L-lactate ($y = 1.07x + 0.02$; $R^2 = 0.9994$; $n = 5$), respectively, in the presence of internal standard yielded linear regression lines over the range of concentrations investigated.

Fifty human urine samples were then analyzed and the characteristic ion peaks for D-lactate, L-lactate, and the deuterated internal standard were quantified. The urinary levels, expressed in millimoles per mole of creatinine, for both D- and L-lactate are shown in Fig. 2. L-Lactate levels ranged between 19.6 and 4737 mmol/mol creatinine, with two samples exceeding 1000 mmol/mol creatinine. D-Lactate levels were between 0 and 119 mmol/mol creatinine. One urine sample that only contained L-lactate was spiked with the D-enantiomer to confirm the absence of this analyte and to validate those samples having no detectable concentrations of D-lactate (Fig. 3a).

The limit of detection (LOD) and limit of quantification (LOQ) for D-lactate were determined to be 63 and 250 μM, respectively. For L-lactate, the LOD was 31 μM while the LOQ was found to be 81 μM. Interassay deviations did not exceed 6% over a period of 2 weeks.

It has previously been shown by Inoue et al. [16] that the so-called D-configuration ratio (see Eq. (1)) in urine is a better indicator for the onset of D-lactic acidosis in clinical follow-up testing of patients with short bowel syndrome, compared with simply determining the concentration or level of D-lactate in blood and urine, respectively, or the D-configuration ratio in blood.

$$\text{D-configuration ratio (\%)} = \frac{\text{D-lactate peak area}}{\text{D-lactate peak area+L-lactate peak area}} \times 100 \quad (1)$$

The results reported by Inoue et al. imply that urinary D-lactate levels in patients experiencing D-lactic acidosis typically contain several thousand millimoles per mole of creatinine [16]. As seen in Fig. 2b, 12 of the 50 samples investigated in this study contained D-lactate levels greater than 20 mmol/mol of creatinine. The D-configuration ratios calculated for these 12 samples are presented in Table 1 along with the corresponding D-lactate levels. The D-configuration ratios of all samples tested were below 35%, which is also significantly lower than those of patients with D-lactic acidosis (>95%). Based on these results, none of the sample donors suffered from D-lactic acidosis; as this is a rare metabolic condition, though, it is not surprising that none of the randomly collected urine samples contained any pathologically elevated concentrations of D-lactate. The observed variations in D-lactate concentrations may be explained by, e.g., different gastrointestinal activities and diets [11,32]. It has furthermore been shown that urinary D-lactate levels may be age-dependant [14]. The results, thus, may simply reflect the diversity of the donor population.

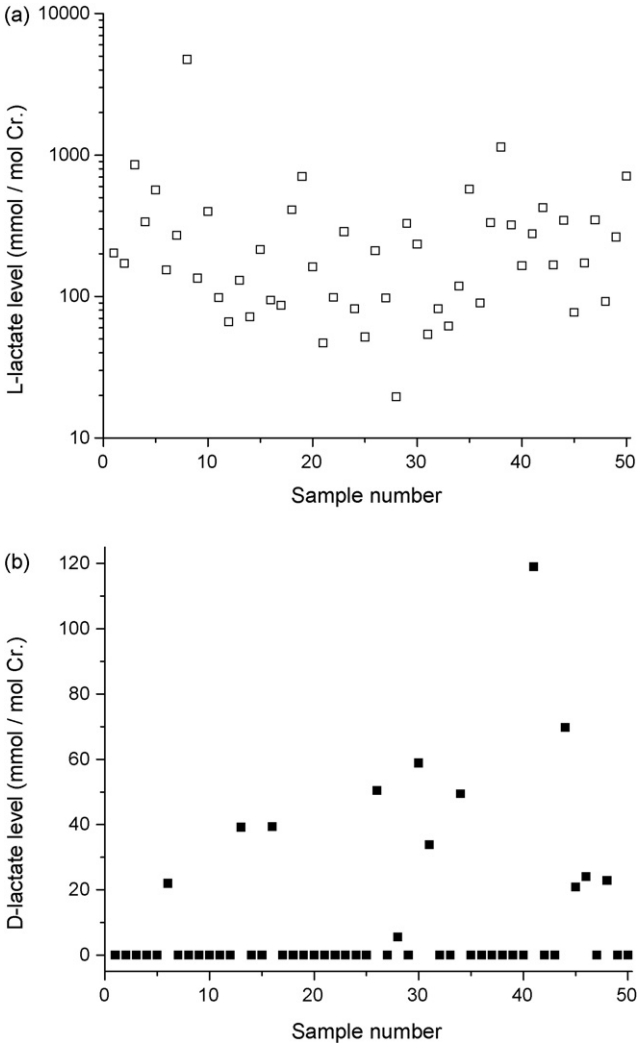

Fig. 2. Urinary levels of (a) L-lactate and (b) D-lactate contained in samples from 50 anonymous donors. Lactate levels are expressed in units of millimoles per mole of creatinine (Cr.).

In order to verify that our method would also be suitable for diagnosing D-lactic acidosis, a urine sample was spiked with D-lactic acid to simulate this condition. Chromatographic enantiomer resolution on the antibody column (Fig. 3b) yielded both the D-lactate level (20.259 ± 145 mmol/mol Cr.) and D-configuration ratio (95.8 ± 0.5%) with satisfactory precision from triplicate determinations.

Table 1
D-Configuration ratios for samples with D-lactate levels greater than 20 millimoles per mole of creatinine.

Sample #	D-Lactate (mmol/mol Cr.)	D-Configuration ratio (%)
6	22.03 ± 3.02	6.92 ± 0.60
13	39.17 ± 3.58	12.06 ± 3.03
16	39.34 ± 0.40	21.08 ± 2.34
26	50.43 ± 7.80	10.63 ± 1.44
30	58.89 ± 0.83	8.54 ± 1.14
31	33.80 ± 6.07	30.89 ± 2.50
34	49.46 ± 13.02	22.13 ± 2.44
41	118.93 ± 7.71	26.07 ± 6.11
44	69.71 ± 7.65	8.55 ± 1.51
45	20.89 ± 0.71	13.65 ± 4.03
46	24.06 ± 3.95	5.04 ± 2.67
48	22.89 ± 1.10	9.63 ± 1.80

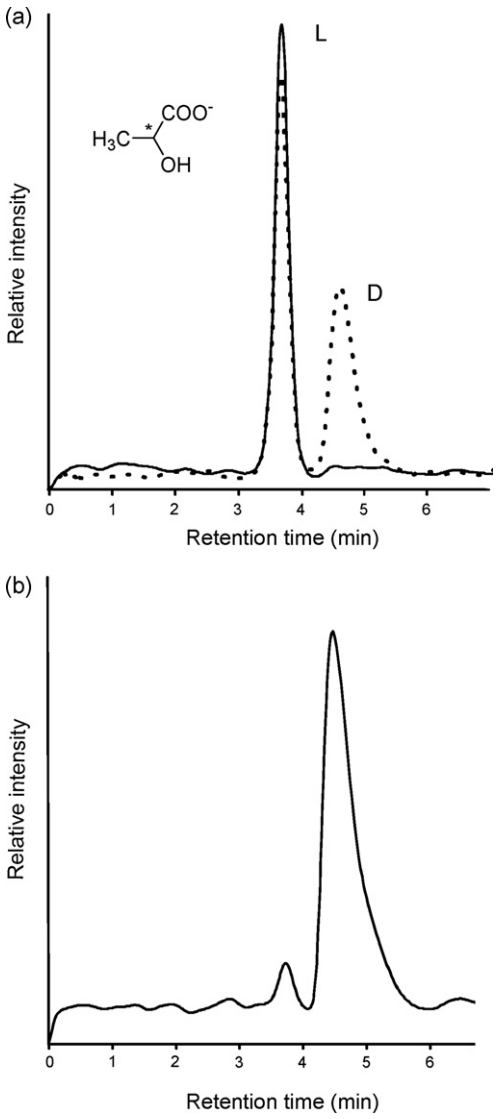

Fig. 3. Extracted ion chromatograms (*m*/*z* 89) of (a) a urine sample containing only L-lactate (solid line), and after spiking with D-lactate (dotted line), and (b) a spiked urine sample containing a pathological concentration of D-lactate.

4. Conclusions

Direct separation and quantification of enantiomeric metabolites contained in biological samples requires the availability of suitable chiral selectors and detection systems. This manuscript describes the first antibody-based approach to directly couple an immunoaffinity column to a mass spectrometer for detection and routine analysis of lactate enantiomers in human urine samples. Post-column addition of methanol to the aqueous mobile phase greatly enhanced ionization and resulted in good response factors. Calibration curves showed satisfactory linearity for D- and L-lactate, respectively, thus allowing quantification of both enantiomers. As isocratic conditions were used for the whole chromatographic process and no column regeneration was needed, individual separations were completed in less than 10 min. Although neither the D-lactate levels nor the D-configuration ratios determined for the 50 samples investigated here would be symptomatic of severe medical disorders, it was shown with spiked samples that also pathological levels of lactate can be quantified.

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