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# High-performance liquid chromatographic assay of (±)-lactic acid and its enantiomers in calf serum

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**Abstract**

Two high-performance liquid chromatographic methods are described for the determination of lactic acid and its enantiomers in calf serum. A 300×8.0 mm I.D. column packed with sulfonated styrene–divinylbenzene copolymer and a 50×4.6 mm ODS column with *N*,*N*-dioctyl-L-alanine were used. UV detection was at 205 and 236 nm for the non-chiral and chiral assays, respectively. Both assays demonstrated excellent linear relationships between peak area ratios and serum concentrations over a range of 0.5 to 20 m*M*, based on 100 μl bovine serum. Recovery was complete. Inter- and within-batch bias and relative standard deviation were <15%. 

*Keywords:* Enantiomer separation; Lactic acid

## 1. Introduction

Neonatal calf diarrhea is a common and costly problem in beef and dairy herds [1]. Dehydration and metabolic acidosis are the primary changes in the pathophysiology of calf scours [2]. Metabolic acidosis is important because of its depressant effects on neurological and cardiovascular function [3,4]. Acidosis contributes to death resulting from heart failure by decreasing myocardial potassium and elevating extracellular potassium [3,4]. It is usually found as a sequela to diarrhea induced dehydration, and it is thought to be a major cause of calf death [5].

L-(+)-Lactic acid [L-(+)-LA, Fig. 1] is an organic acid normally formed from pyruvate in the cells of higher organisms when the amount of oxygen is limiting, as in muscle during intense activity [6]. In the dehydrated state, L-lactic acidosis may be potentiated by limited supply of oxygen, as a result of hemoconcentration and low tissue perfusion [7]. Gut bacterial fermentation of undigested nutrients may also lead to the production of LA, in which case both the D-(−) and L-(+) isomers are produced [8].

A recent pilot study found serum LA levels to be higher in neonatal diarrheic calves versus their healthy counterparts (unpublished data). However, the non-chiral high-performance liquid chromatographic assay used in the pilot study would not discriminate between the different enantiomers of LA, which would be useful in determining their

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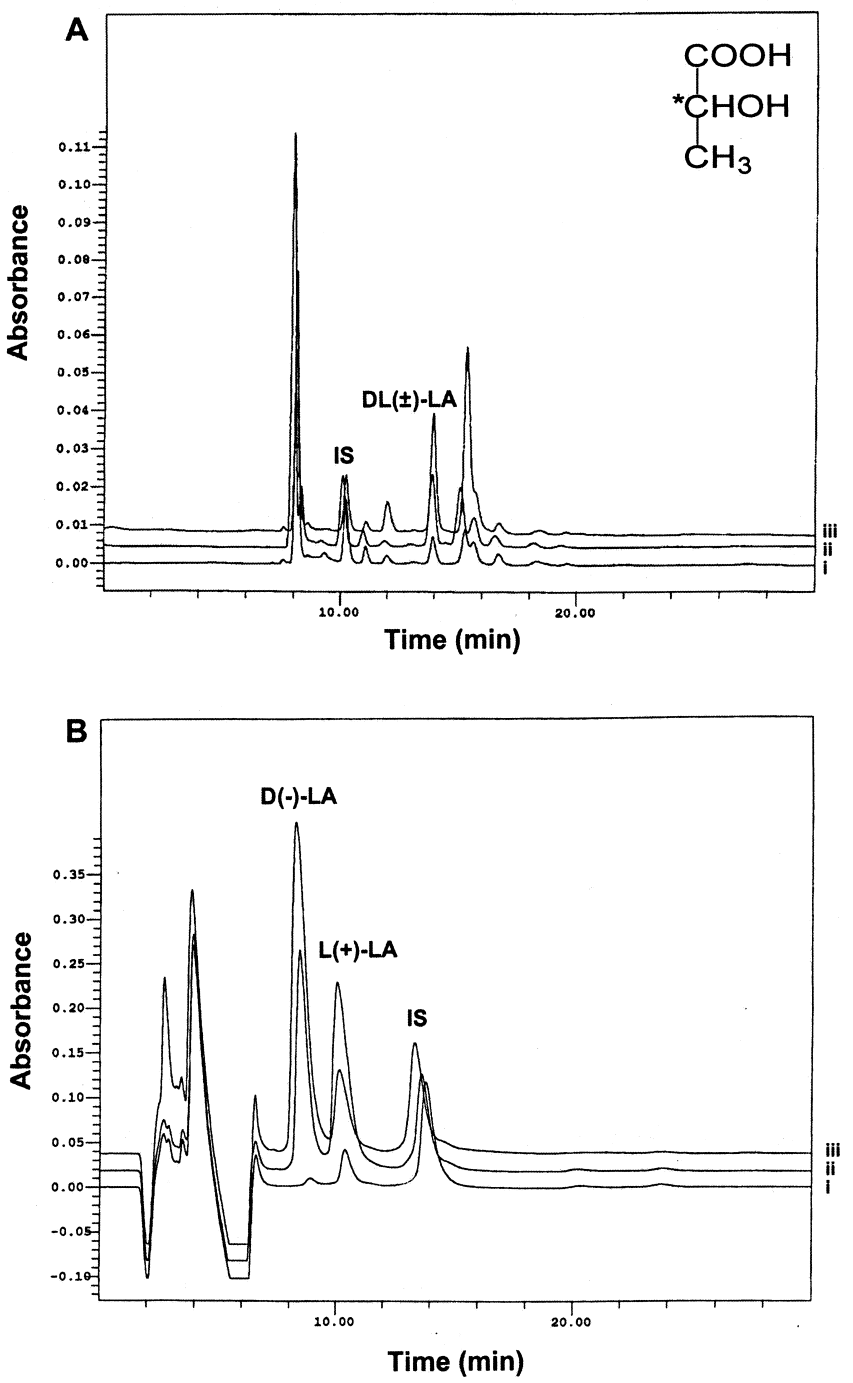


Fig. 1. (A) Non-stereospecific assay; chromatograms of (i) healthy calf serum, (ii) bovine serum spiked with 5 m*M* DL-(±)-LA, (iii) diarrheic calf serum. (B) Stereospecific assay; chromatograms of (i) healthy calf serum, (ii) bovine serum spiked with 5 m*M* of DL-(±)-LA (i.e., 2.5 m*M* of each enantiomer), (iii) diarrheic calf serum. Structure of DL-LA in top-left corner of A; chiral center denoted by an asterisk.

likely source, whether anaerobic respiration or gut bacterial fermentation. In a related study [9], measured concentrations of D-(−)-LA in diarrheic sucker calves ($n=12$), using the enzymatic method of determination was higher ($p<0.05$) than in healthy controls ($n=10$).

A rapid, sensitive and stereospecific high-performance liquid chromatographic assay, suitable for the measurement of LA enantiomers in calf serum was adapted and validated to determine the source of the lactic acidosis present in neonatal diarrheic calves. A non-stereospecific assay for the determination of DL-(±)-LA was also validated. The methods described in this report may be applied to determining LA and its enantiomers in physiological fluids, and may be useful in investigations of acidosis in animals and perhaps humans.

## 2. Experimental

### 2.1. Materials and chemicals

DL-(±)-LA, citric acid and malonic acid were obtained from Sigma (St. Louis, MO, USA). Phosphoric acid was purchased from Aldrich (Milwaukee, WI, USA), acetonitrile (HPLC-grade) from BDH (Toronto, Canada), copper sulphate from J.T. Baker (Phillipsburg, NJ, USA) and 0.45-μm membrane filters from Scheicher and Schuell (Keene, NH, USA).

### 2.2. Chromatography

Both assays made use of the same high-performance liquid chromatography (HPLC) system, under different conditions. It consisted of a Waters Model 600 pump, a Waters 486 tunable-wavelength UV absorbance detector and a Waters 710 Ultra WISP autoinjector (Waters, Mississauga, Canada). Data collection, integration and calibration were performed using a Waters Millenium chromatography manager version 2.1 (Waters). Ultrafree molecular-mass cut-offs were purchased from Millipore (Waters, Millipore, Milford, MA, USA).

The non-stereospecific analysis employed a reversed-phase, 300×8 mm analytical column (Shodex RSPAK KC-811, Showa Denko K.K., Tokyo, Japan) for the chromatographic separation of racemic LA and citric acid (internal standard). The mobile phase, consisting of 0.1% phosphoric acid and double distilled water (DDW) was pumped at 0.7 ml/min in the isocratic mode, with the column temperature maintained at 50°C. UV detection was at 205 nm, representing the UV maxima of a solution of LA in the mobile phase.

We developed an assay for the stereospecific analysis of DL-(±)-LA enantiomers based on the separation previously performed by Daicel [10]. Our technique utilized a stainless steel 3 μm ODS packed analytical column (50×4.6 mm I.D., ChiralPak MA+), coated with *N*,*N*-dioctyl-L-alanine as chiral selector (Chiral Technologies, Exton, PA, USA). The mobile phase consisted of 2 m*M* copper sulphate containing 1% acetonitrile, and was pumped in the isocratic mode at 0.4 ml/min and room temperature. UV detection was at 236 nm. This wavelength represented the UV maxima of a solution of LA in the stereospecific mobile phase. The mobile phases of both assays, the eluents were filtered through 0.45-μm membrane filters and degassed under vacuum to remove oxygen and contaminants prior to use.

### 2.3. Standard and stock solutions

Stock analyte solution for both assays was prepared by making up 1.14 ml of DL-(±)-LA syrup to 200 ml with DDW. This solution was stored at 4°C between use and under this condition, LA remained stable for at least eight months. Internal standard stock solutions were prepared by dissolving 0.306 g of citric acid sodium salt (non-stereospecific assay) and 0.296 g of malonic acid (stereospecific assay) in 100 ml of DDW each. The resulting solutions were also stored at 4°C between use. Working standard solutions were routinely prepared from the stock solutions by sequential dilution with DDW to yield final concentrations ranging from 1 to 20 m*M* for DL-(±)-LA, and 2 m*M* for the internal standards.

### 2.4. Ultrafiltration

Serum samples were deproteinized by ultrafiltration. To an Ultrafree-MC centrifugal filter unit, 100 μl of standard bovine serum was added along with

50 μl internal standard solution and made up to 200 μl with DDW for quantitation purposes. Aliquots (50 μl each) of aqueous solution of DL-(±)-LA were used instead of DDW for calibration purposes. The resulting mixture was then spun down at 5500 *g* for 30 min. Aliquots of 20 μl from the filtrate were then injected into the HPLC system.

### 2.5. Recovery

To examine the efficiency of the ultrafiltration unit, serum was spiked with nominal concentrations of DL-(±)-LA and subjected to ultrafiltration. This first ultrafiltrate was divided into two parts. One part was injected into the HPLC system directly while the other was subjected to ultrafiltration a second time before being injected into the column. Peak areas from both analyses were compared to determine the efficiency of the ultrafiltration unit according to the equation:

$$\%\ \text{recovery} = 100 \times \frac{\text{peak area in second ultrafiltrate}}{\text{peak area in first ultrafiltrate}}$$

Recovery was determined in quadruplicate at 0.5 and 20 m*M* concentrations of DL-(±)-LA.

### 2.6. Calibration, accuracy and precision

Calibration curves were generated using peak area ratios of analyte to internal standard, plotted against standard analyte concentration. Since bovine serum contains some background amount of LA, response ratios from blank serum were subtracted from that of analyte spiked serum before calibration curves were regenerated. Four standard points within the calibration curve range, 0.5, 2.5, 5.0 and 20 m*M* for DL-(±)-LA, and 0.5, 1.25, 2.5, 10 m*M* of each enantiomer were used each day in quadruplicate over a three-day period to examine the inter- and intra-day reproducibility of this method.

For the purposes of quantitation, a separate, independent, standard curve was incorporated into each run. Intra-day accuracy was determined based on the mean percentage error, according to the equation:

$$\text{intra-day accuracy}\ (\%) = 100 \times \frac{\text{mean of measured concentration}}{\text{nominal concentration}}$$

and the inter-day accuracy was calculated as the mean of the intra-day accuracy determinations. The precision around mean values, expressed as a percentage, was estimated by calculating the intra- and inter-day relative standard deviations (RSDs) thus:

$$\text{RSD} = 100 \times \frac{\text{SD}}{\text{mean}}$$

### 2.7. Application

Jugular venous blood samples were collected from one of healthy and one diarrheic neonatal calf less than 28 days of age to examine the utility of both assay methods. Blood pH values for the calves were 7.38 and 7.07, respectively. The resultant serum samples were frozen at −20°C until assayed. Serum concentrations of LA and its enantiomers were determined using a calibration curve incorporated into the run.

## 3. Results

In the non-stereospecific chromatographic assay, the internal standard, citric acid and DL-(±)-LA eluted approximately at 10.1 and 13.9 min, respectively, were baseline resolved and without interfering peaks (Fig. 1A). Mean values of recovery from the ultrafiltration unit for DL-(±)-LA from 100 μl of bovine serum were 108% and 109%, at concentrations of 0.5 and 20 m*M*, respectively. Excellent linear relationships ($r^2 \geq 0.999$) were obtained between peak area ratios and their corresponding serum concentrations over a range of 0.5 to 10 m*M* of DL-(±)-LA; mean regression line from the validation runs was described by DL-(±)-LA (m*M*) = (peak area ratio − 0.0123)/0.1992. For this assay, the validated lower limit of quantitation (LLQ) was 0.5 m*M* (Table 1). Intra- and inter-batch RSDs were less than 15% between 0.5 and 20 m*M* of DL-(±)-LA (Table 1).

With the stereospecific assay, retention times for the internal standard malonic acid, D-(−) and L-(+)-LA were 13.8, 10.3 and 8.2 min, respectively (Fig. 1B). Linearity was demonstrated between peak area ratios and the corresponding serum concentrations over a range of 0.5 to 10 m*M* of each enantiomer, with $r^2$ values >0.997 for both D-(−)- and L-(+)-

Table 1
Validation data for the non-chiral HPLC assay

| Nominal spiked concentration (m*M*) | Intra-batch measured concentration (mean±SD) (m*M*) | | | Inter-batch measured concentration (mean±SD) (m*M*) | Mean inter-batch bias (%) | Mean inter-batch RSD (%) |
|---|---|---|---|---|---|---|
| | Batch 1 ($n=4$) | Batch 2 ($n=4$) | Batch 3 ($n=4$) | | | |
| 0.5 | 0.49±0.03 | 0.51±0.04 | 0.47±0.07 | 0.49±0.02 | 98.4 | 3.76 |
| 2.5 | 2.39±0.04 | 2.52±0.09 | 2.50±0.05 | 2.47±0.07 | 98.7 | 2.77 |
| 5 | 4.84±0.02 | 4.90±0.03 | 4.91±0.08 | 4.88±0.04 | 97.6 | 0.77 |
| 20 | 19.9±0.7 | 20.1±0.0 | 20.1±1.9 | 20.0±0.1 | 100.1 | 0.59 |

LA. Mean regression lines from validation runs were described by D-(−)-LA (m*M*)=(peak area ratio−0.0301)/0.2921 and L-(+)-LA (m*M*)=(peak area ratio−0.0077)/0.2942. Validation of the assay was also attempted at 0.25 m*M*, however, data obtained for mean bias and RSD were unacceptably high (>30%). Hence, the LLQ for each enantiomer was 0.5 m*M*. Both intra- and inter-batch RSD and bias values were less than 15% (Table 2).

On application of both assays, all peaks of interest were resolved to the baseline. Serum concentrations of LA and its enantiomers were found to differ in the healthy, versus the diarrheic calf on comparison. Levels of DL-(±), D-(−) and L-(+)-LA were 2.8, 0.36, 3.31 and 15.06, 13.20 and 1.3 m*M* in the healthy and diarrheic calves, respectively.

## 4. Discussion

The above described HPLC assays are rapid, sensitive and suitable for the determination of DL-(±)-LA and its enantiomers in bovine serum. Most other analytical methods have focused on the enzymatic method of determination, based on the reaction of lactate with β-$NAD^+$ in the presence of lactate dehydrogenase to produce pyruvate and NADH [9]. However, the enzymatic assays may be sensitive to slight deviations from the conditions for optimum enzyme activity, and may be hampered by protein denaturation. Moreover, a major shortcoming of the standard clinical enzymatic approach in determining LA is in its inability to assay a large number of samples within a short time period.

Ohmori and Iwamoto [11] employed HPLC with fluorescent detection to determine D-(−)-LA in biological samples; however, the series of reactions resulting in the formation of the quinoxalin derivatives are complex. Although linearity was reported for the calibration curve for up to 60 mmol/ml with a detection limit of 600 fmol, the medium in which calibration was performed was not reported. Sensitivity is usually higher for assays standardized in water, as the matrix of determination compromises sensitivity to an extent.

We first tried to separate D-, L-LAs by pre-column derivatization with chiral chloroformates/conventional $C_{18}$ column. We then attempted to use another chiral column (chiral urea naphthylethylamine). Neither approach proved successful. We then looked at

Table 2
Validation data (inter-batch accuracy and precision) for the chiral HPLC assay

| Nominal enantiomer concentration (m*M*) | Measured enantiomer concentration (mean±SD) (m*M*) | | Mean RSD (%) | | Mean accuracy (%) | |
|---|---|---|---|---|---|---|
| | D-LA | L-LA | D-LA | L-LA | D-LA | L-LA |
| 0.5 | 0.50±0.04 | 0.51±0.07 | 8.11 | 13.25 | 100.3 | 102.3 |
| 1.25 | 1.21±0.02 | 1.18±0.07 | 1.79 | 5.56 | 96.4 | 94.7 |
| 2.5 | 2.51±0.02 | 2.47±0.03 | 0.83 | 1.07 | 100.4 | 98.7 |
| 10 | 10.1±0.2 | 10.1±0.2 | 2.06 | 1.79 | 100.5 | 101.2 |

the columns that Daicel had to offer. Although the separation of LA enantiomers using the ChiralPak MA+ column has been previously described [11], applicability of the method to biological samples was not established, and appropriate validation data were not provided. In conjunction with our extraction method, we have shown the separation conditions for LA enantiomers using the ChiralPak MA+ column [11] to be relevant for separation and measurement of LA enantiomers from bovine serum, when used in combination with our extraction procedure. We did not try any other columns. Chiral columns are expensive and this limited our ability to try other chiral columns.

With our method, all calibration curves were determined in calf serum. Sample preparation with the techniques described in this report was fast and simple. Preparation of 24 samples took less than 2 h prior to injection into the HPLC, and all peaks of interest eluted within 15 min for both methods. Recovery of lactate from multiple filtered samples was excellent and both assays demonstrated acceptable reproducibility and accuracy over the investigated range of concentrations. The lower and higher limits of quantitation for both assays are ideal for studying the development of metabolic acidosis in neonatal diarrheic calves and perhaps other species.

Comparison of the results from both assays show that the sum of LA enantiomers in the diarrheic calf (14.6 m*M*) agreed closely with the value of DL-(±)-LA (15.1 m*M*) obtained from the non-chiral assay (Table 3). There was some deviation between the two assays in the serum from the single healthy calf serum, which involved LA concentrations lower than the LLQ for D-(−)-LA (<0.5 m*M*). Levels of serum LA and its enantiomers were found to differ with both assays, being higher in the diarrheic versus the healthy calf. L-(+)-LA is thought to be a cause of acidosis in less than one week old calves [5]. D-(−)-LA was much higher in the diarrheic calf. This suggests that D-lactic acidosis may be a contributory factor in calves with high anion gap acidosis (Table 3). This has not been previously reported in calves with diarrhea.

Schelcher et al. [9] also recently showed hyper D-lactatemia (12.92±3.84 mmol/l; $n=12$) to be responsible for metabolic acidosis with high anion gap in a controlled clinical study in sucker calves with minimal or no signs of diarrhea. Although they employed the enzymatic approach of assay, levels of D-(−)-LA measured in their study compares well with the amount of D-(−)-LA we found in our diarrheic calf (Table 3).

Malabsorption of nutrients in the small intestine of short bowel syndrome (SBS) patients, with subsequent fermentation by colonic bacteria results in D-lactic acidosis in humans [12,13]. Accumulation of undigested nutrients and malabsorption may also cause the same process in diarrheic calves. Unlike L-(+)-LA [14], little or no metabolism of D-(−)-LA takes place in humans and other vertebrates after production and absorption, thus accumulation of D-(−)-LA may be a contributory factor to acidosis in neonatal calves with diarrhea.

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Table 3
Comparison of blood gas and lactic acids in two calves

| Calves | pH | $HCO_3^-$ (mmol/l) | BE (mmol/l) | AG (mmol/l) | DL-LA (m*M*) | D-LA (m*M*) | L-LA (m*M*) |
|---|---|---|---|---|---|---|---|
| Healthy | 7.38 | 32.50 | 6.80 | 4.40 | 2.80 | NQ[a] | 3.31 |
| Diarrheic | 7.07 | 11.30 | −17.90 | 32.40 | 15.06 | 13.20 | 1.30 |

[a] NQ=Not quantifiable; less than the validated lower limit of quantitation (i.e., 0.50 m*M*).

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