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Short communication

High-performance liquid chromatographic assay of lactic, pyruvic and acetic acids and lactic acid stereoisomers in calf feces, rumen fluid and urine

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Abstract

To facilitate clinical investigation of metabolic acidosis, a high-performance liquid chromatographic method was adapted and validated for the chiral separation of D-(−) and L-(+)-lactic acid in calf feces, rumen fluid and urine. A non-chiral method was also adapted and validated for the separation of pyruvic, acetic and DL-(±)-lactic acids in calf feces and DL-(±)-lactic and pyruvic acids in rumen fluid. Separation and quantification were achieved using a reversed phase sulphonated polystyrenedivinylbenzene analytical column for pyruvic, acetic and racemic lactic acids and by a 3 μm octadecylsilane (ODS) packed analytical column coated with *N*,*N*-dioctyl-L-alanine as the chiral selector for the separation of lactic acid enantiomers with Cu(II)-containing eluents by stereoselective ligand exchange chromatography. Endogenous analytes were present in validation samples over a range of concentrations (0.2–14.8 mmol/l). For the stereoselective assay, mean intra-day accuracy ranged from 90.6 to 108.4% and intra-day precision from 0.3 to 13.8%. For the non-stereoselective assay, mean intra-day accuracy ranged from 90.4 to 108.8% and intra-day precision from 1.5 to 11.1%. The limit of quantitation was 1.0 mmol/l for D- and L-lactic acid, 0.06125 mmol/l for pyruvic acid, 1.0 mmol/l for DL-lactic acid and 1 mmol/l for acetic acid. These assays can be used to study the role of the gastrointestinal tract and kidney in metabolic acidosis.

Keywords: Enantiomer separation; Lactic acid; Pyruvic acid; Acetic acid

1. Introduction

The majority of HPLC methods available for the analysis of organic acids and the separation of D-(−)- and L-(+)-lactic acid have been validated for aqueous media, and in a few cases, for use in serum [1–4]. There is a need to better understand the etiology of metabolic acidosis associated with diarrhea, as D-(−)-lactic acid has been recently reported to contribute significantly to the drop in blood pH observed in calves with severe diarrhea [5]. Organic and lactic acid measurements in less common biological fluids would improve the understanding of metabolic disturbances associated with diarrhea. To analyze lactic acid enantiomers in more complex biological fluids such as feces or urine, the only available method is enzymatic, using D-(−)- or L-(+)-lactic acid dehydrogenase. The enzymatic method, however, is subject to various sources of error [6]. Therefore, the development and validation of a stereoselective HPLC method is required for the accurate measurement of lactic acid enantiomers in these biological matrices.

To facilitate further investigation of metabolic acidosis in diarrhea, the objective of this study was to adapt and validate our previously reported HPLC assays [7] for the analysis of lactic acid enantiomers and other organic acids to feces, rumen fluid and urine. Modifications to the original method were required, specifically to sample collection, sample preparation methods and selection of a different internal standard for the non-stereoselective assay.

2. Experimental

2.1. Chemicals and equipment

L-(+)-Lactic, lithium D-(−)-lactic, acetic, pyruvic, malonic and adipic acids were purchased from Sigma (St. Louis,

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MO, USA). Phosphoric acid was purchased from Aldrich (Milwaukee, WI, USA), HPLC-grade acetonitrile from BDH (Toronto, Ont., Canada), copper sulphate from J.T. Baker (Phillipsburg, NJ, USA) and Thimerosal from ICN Biomedicals (Aurora, OH, USA). Ultrafree-MC microcentrifugal filtration units were purchased from Millipore Corporation (Bedford, MA, USA), 0.45 μm membrane mobile phase filters from Scheicher and Schuell (Keene, NH, USA) and Acrodisc PF (0.8/0.2 μm) filter from Pall Corporation (Ann Arbor, MI, USA).

2.2. Apparatus and chromatographic conditions

Both the stereoselective and non-stereoselective assay utilized the same HPLC system, under different conditions. A Waters 600 pump, 486 UV detector and 786 Ultra WISP autoinjector were used (Waters, Mississauga, Ont., Canada). Data collection, calibration and integration was performed using Waters' Millenium chromatography manager v.2 and Millenium^{32} v.4.

Acetic, pyruvic and racemic lactic acids were separated by a reverse-phase, Shodex RSPAK KC-811 (300 mm × 8 mm) analytical column, with a KC-811 pre-column (Showa Denko K.K., Tokyo, Japan). D-(−)- and L-(+)-lactic acid were separated by a 3 μm ODS packed (50 mm × 4.6 mm) analytical column, coated with *N*,*N*-dioctyl-L-alanine (ChiralPak MA+, Chiral Technologies, Exton, PA, USA) using a Waters Guard-Pak precolumn (Waters, Mississauga, Ont., Canada).

For the non-stereospecific assay, 7 mmol/l adipic acid was used as the internal standard and 0.1% phosphoric acid as the mobile phase. Mobile phase was pumped at 0.7 ml/min, and the column maintained at 50 °C. UV detection was at 205 nm. For the stereospecific assay, 2 mmol/l malonic acid was used as the internal standard and 2 mmol/l copper sulphate in 1% acetonitrile as the mobile phase. Mobile phase was pumped at 1 ml/min at room temperature, and UV detection was at 236 nm.

2.3. Sample preparation

2.3.1. Fecal samples

Approximately 50 g of calf feces were collected into 15 ml of 250 μmol/l thimerosal, a bacteriostatic agent, and immediately frozen at −20 °C until analysis. Samples were thawed at 4 °C and then shaken for 20 min on an automatic shaker. One gram of feces was added to 9 ml of double distilled water (DDW) (or increasing concentrations of the acid to be validated as described in Section 2.4) and homogenized for

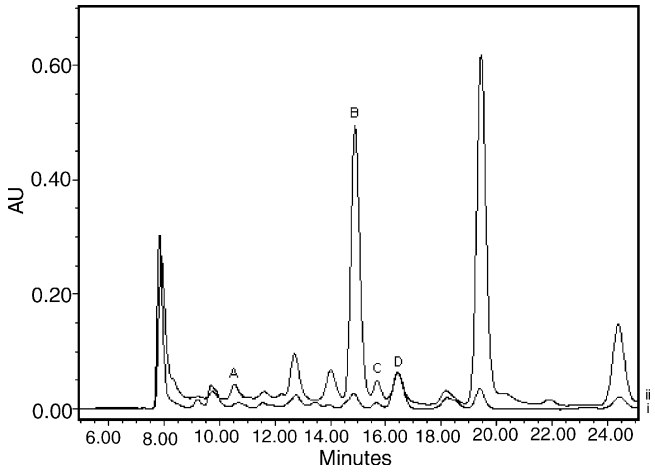

Fig. 1. Non-stereoselective assay; chromatograms of (i) healthy calf feces; (ii) diarrheic calf feces: (A) pyruvic acid; (B) DL-(±)-lactic acid; (C) acetic acid; (D) adipic acid (internal standard). Concentrations represented by peaks: (A) (i) nq; (A) (ii) 0.28 mmol/l; (B) (i) nq; (B) (ii) 18.9 mmol/l; (C) (i) 0.3 mmol/l; (C) (ii) 3.4 mmol/l; (D) (i) and (D) (ii) 2.0 mmol/l; nq, not quantifiable; AU, absorbance units.

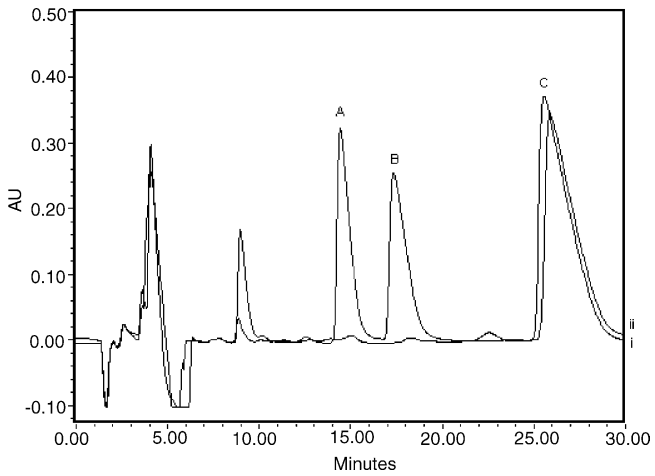

Fig. 2. Stereoselective assay; chromatograms of (i) healthy calf rumen fluid; (ii) diarrheic calf rumen fluid: (A) D-(−)-lactic acid; (B) L-(+)-lactic acid; (C) malonic acid (internal standard). Concentrations represented by peaks: (A) (i) nq; (A) (ii) 9.9 mmol/l; (B) (i) nq; (B) (ii) 8.0 mmol/l; (C) (i) and (ii) 7.0 mmol/l; nq, not quantifiable; AU, absorbance units.

Table 1
Endogenous concentrations of samples used for validation

	D-Lactate (mmol/l) ($n = 3$)	L-Lactate (mmol/l) ($n = 3$)	DL-Lactate (mmol/l) ($n = 3$)	Pyruvate (mmol/l) ($n = 3$)	Acetate (mmol/l) ($n = 3$)
Feces	5.77 ± 0.14	2.98 ± 0.34	14.18 ± 0.46	0.26 ± 0.01	10.20 ± 0.31
Rumen	3.56 ± 0.27	0.36 ± 0.13	1.22 ± 0.11	0.10 ± 0.01	–
Urine	0.20 ± 0.07	1.29 ± 0.36	–	–	–

Mean ± S.D.

1 min, and centrifuged at 20,000 × *g* for 30 min. The supernatant was removed and syringe filtered through an Acrodisc PF (0.8/0.2 μm) filter. One hundred microlitres of filtrate were added to 50 μl of internal standard (7 mmol/l malonic acid for stereoselective assay, 2 mmol/l adipic acid for the non-stereoselective assay) and 50 μl DDW in an Ultrafree MC filter unit and centrifuged at 5500 × *g* for 30 min; 20 μl aliquots of the final filtrate were injected into the HPLC system. Fecal samples were run for 30 min for both the stereoselective and non-stereoselective assay.

2.3.2. Rumen samples

Ruminal fluid was collected and prepared as for feces, with the exception of 1:1 dilution with DDW or spike, instead of 1:9.

2.3.3. Urine samples

Urine samples were diluted 1:5 with mobile phase. One hundred microlitres of diluted urine were added to 50 μl of internal standard and 50 μl DDW in an Ultrafree MC filter unit and centrifuged at 5500 × *g* for 30 min; 20 μl aliquots of the final filtrate were injected into the HPLC system. Urine samples were run for 40 min, due to late eluting peaks. Only stereoselective validation was performed.

2.4. Assay validation

The analytical methods were analyzed for precision, accuracy, quantitation limit (QL) and stability [8,9]. The FDA guidelines for accuracy (80–120%) and precision (<20%) were used [8].

Table 2
Validation data for stereoselective assay

Nominal concentration (mmol/l)	Intra-day accuracy ($n = 5$)		Mean intra-day accuracy ($n = 15$)	
	Percentage	R.S.D.	Percentage	Range (%)
D-Lactate in urine				
1.25	90.4	3.94	93.6	90.4–96.8
2.5	104.4	1.78	108.4	104.4–111.6
5	101.6	0.46	103.4	101.6–105.0
7	101.7	0.84	102.8	101.7–104.1
10	94.7	1.08	95.2	94.7–95.8
D-Lactate in rumen fluid				
1	120.0	1.82	105.6	99.0–120.0
2.5	96.4	7.43	95.6	92.8–96.8
5	91.8	6.60	92.4	91.8–93.8
7	98.7	4.50	104.3	98.7–100.8
10	100.5	7.48	90.6	99.9–101.0
D-Lactate in feces				
1	98.0	13.81	92.5	81.0–99.0
2.5	95.6	4.67	97.1	95.6–98.8
5	101.8	1.47	98.1	95.6–101.8
7	100.8	2.66	98.7	96.1–100.8
10	106.1	2.20	104.2	102.6–106.1
L-Lactate in urine				
1.25	102.4	4.00	101.6	99.2–103.2
2.5	110.8	1.64	110.8	110.4–111.6
5	104.2	0.34	104.2	104.0–104.6
7	103.4	0.85	103.5	103.4–104.0
10	95.8	1.06	95.7	95.3–95.8
L-Lactate in rumen fluid				
1	97.0	3.42	97.4	97.0–97.1
2.5	95.6	6.41	98.0	95.6–101.6
5	100.6	5.57	100.3	99.6–100.8
7	102.1	4.21	102.6	102.1–103.0
10	85.2	9.88	95.5	94.0–97.2
L-Lactate in feces				
1	93.0	6.55	95.1	93.0–99.0
2.5	98.4	5.23	97.1	95.6–98.4
5	98.0	2.17	95.4	94.6–98.0
7	99.3	3.42	97.0	95.7–99.2
10	104.2	2.75	102.6	101.6–104.2

R.S.D., relative standard deviation.

Calibration curves were produced using linear regression for each assay by plotting peak area ratios of analyte to internal standard against nominal concentrations of DL-(±)-lactic acid, D-(−)-lactic acid, L-(+)-lactic acid, acetic acid or pyruvic acid. Since urine, feces and ruminal fluid contain the analytes of interest, calibration curves were prepared in each biological matrix and response ratios from blank samples of these fluids were subtracted from the peak area ratios of standards prior to the generation of calibration curves. Intra-day accuracy and precision was evaluated using five concentrations of each acid (D-(−)- and L-(+)-lactic acid, 0.5, 1.25, 2.5, 5, 10 mmol/l; pyruvic acid, 0.0625, 0.125, 0.3125, 0.625, 1.25 mmol/l; acetic acid, 0.5, 1, 2.5, 5, 10 mmol/l; racemic lactic acid, 1.25, 2.5, 5, 10, 20 mmol/l) per day in quintuplicate. Blanks of each validation sample were included and endogenous concentrations of analytes subtracted. Endogenous concentrations of the samples used for validation are presented in Table 1. The procedure was repeated on three separate days to determine mean intra-day accuracy. Intra-day accuracy was determined as mean percentage error, and the mean intra-day accuracy was calculated as the mean of the pooled 3 days determinations. The precision, expressed as a percentage, was determined by calculating the intra-day relative standard deviation.

Stability of the analytes was assessed by determining the effect of three freeze–thaw cycles of 24 h each. In addition, analyte stability at room temperature every 12 h over a 72 h period (i.e., in the autosampler) was evaluated. Control samples were spiked with analyte at two concentrations (high and low, $n = 3$ each) within the calibration curve range. Stability was expressed as a percentage of the initial value.

3. Results

All analytes yielded excellent linear relationships in each biological matrix over the various standard curve concentrations ($r^2 \geq 0.99$, 0.06125–20 mmol/l).

For the non-stereoselective assay, pyruvic acid, lactic acid, acetic acid and adipic acid eluted at 10.7, 14.6, 15.8 and 16.4 min, respectively (Fig. 1). For the stereoselective assay, D-(−)-lactic acid, L-(+)-lactic acid and malonic acid eluted at 14.7, 17.2 and 26.0 min, respectively (Fig. 2).

Table 3
Validation data for non-stereoselective assay

Nominal concentration (mmol/l)	Intra-day accuracy ($n = 5$)		Mean intra-day accuracy ($n = 15$)	
	Percentage	R.S.D.	Percentage	Range (%)
DL-Lactate in rumen fluid				
1.25	117.6	10.31	108.8	104.0–106.4
2.5	108.0	4.99	102.4	99.6–108.0
5	106.6	2.36	99.0	95.2–106.0
10	98.1	1.51	95.3	93.3–98.1
20	108.0	2.90	98.8	107.1–108.0
DL-Lactate in feces				
1	128.0	6.47	108.3	98.0–128.0
2.5	92.8	5.30	96.3	92.8–100.8
5	100.2	5.54	99.8	97.4–102.0
10	96.0	4.56	97.6	95.3–101.9
20	94.1	5.00	98.5	94.1–101.8
Pyruvate in rumen fluid				
0.0625	106.6	4.12	108.2	104.1–113.8
0.156	101.3	3.75	98.6	94.8–101.3
0.313	96.5	2.56	94.5	91.0–96.5
0.625	94.4	2.20	95.0	93.7–96.8
1.25	98.4	2.91	96.0	93.6–98.4
Pyruvate in feces				
0.0625	95.4	7.89	101.6	95.4–109.4
0.156	89.6	1.90	90.4	89.6–101.3
0.313	93.3	3.27	97.7	92.6–106.7
0.625	97.1	2.23	96.4	94.9–97.3
1.25	99.2	2.73	100.7	98.4–99.2
Acetate in feces				
1	94.0	11.06	97.9	94.0–100.0
2.5	94.8	6.38	96.1	94.8–97.2
5	96.0	2.91	96.1	96.0– 97.0
10	97.4	2.73	99.6	97.4–102.9

R.S.D., relative standard deviation.

For the stereoselective assay, mean intra-day accuracy ranged from 90.6 to 108.4% and intra-day precision from 0.3 to 13.8%. For the non-stereoselective assay, mean intra-day accuracy ranged from 90.4 to 108.8% and intra-day precision from 1.5 to 11.1% (Tables 2 and 3).

Validation of the stereoselective lactic acid assay was attempted at 0.5 mmol/l, but yielded unacceptable accuracy (>20% deviation) and precision (>20% R.S.D.), making the QL 1.0 mmol/l for this assay in rumen fluid, feces and urine. Validation of the non-stereoselective assay was attempted at 0.03125 mmol/l for pyruvic acid, 0.5 mmol/l for lactic acid and 0.5 mmol/l in acetic acid, but also yielded poor accuracy and precision. Thus the QL for pyruvic acid, lactic acid and acetic acid in rumen fluid and feces are 0.0625, 1.0 and 1.0 mmol/l, respectively. Acceptable accuracy and precision was not obtained for acetic acid in rumen fluid which was attempted over the same range as in feces (0.25–10 mmol/l) and at a higher range (3.5–40 mmol/l).

Analysis of three freeze–thaw cycles resulted in no significant differences in analyte concentration. Time in the autosampler did not impact the accuracy and precision of the analysis (data not shown).

4. Discussion

Both assays in all biological fluids (except acetic acid in rumen fluid) demonstrated good linearity, accuracy and precision. Concentrations typically found in each biological matrix exceed the determined QL for each assay.

Several modifications of the original methods [7] were made. First, the addition of thimerosal to rumen and fecal samples immediately upon collection was critical to preventing significant increases in fermentation products after freezing and thawing (data not shown). Secondly, the dissolution of urine into the acetonitrile-containing mobile phase was necessary, as this facilitated precipitation of salts prior to filtration and prevented clogging of the column. Finally, since citrate may be present in fecal and rumen samples due to its presence in oral electrolyte solutions used to treat diarrhea, it was not a suitable internal standard for this application. Adipic acid was utilized instead.

The poor accuracy and precision obtained for the analysis of acetic acid in rumen fluid suggests that there may be some conversion of other organic acids present in the sample that were not measured, such as propionic acid and butyric acid, to acetic acid.

Metabolic acidosis and dehydration are typical outcomes of severe diarrhea [10]. Until the last several years, the major organic acid reported as a contributor to metabolic acidosis in diarrhea was L-(+)-lactic acid, which is produced by anaerobic respiration resulting from hypovolemia and low oxygen supply to tissues [11]. However, in diarrheic calves, we have recently shown high serum levels of D-(−)-lactic acid, with less L-(+)-lactic acid [5]. D-(−)-Lactic acid is produced almost exclusively by microbes, particularly *Lactobacillus* spp. and is assumed to originate in the gastrointestinal tract. The ability to quantify organic acids and lactic acid stereoisomers in the unique biological matrices of rumen fluid, feces and urine will facilitate studies investigating the origin and nature of metabolic acidosis and lead to appropriate therapies.

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