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Review

Metabolic acidosis: separation methods and biological relevance of organic acids and lactic acid enantiomers

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Abstract

Metabolic acidosis can result from accumulation of organic acids in the blood due to anaerobic metabolism or intestinal bacterial fermentation of undigested substrate under certain conditions. These conditions include short-bowel syndrome, grain overfeeding of ruminants and, as recently reported, severe gastroenteritis. Measuring fermentation products such as short-chain fatty acids (SCFAs) and lactic acid in various biological samples is integral to the diagnosis of bacterial overgrowth. Stereospecific measurement of D- and L-lactic acid is necessary for confirmation of the origin and nature of metabolic acidosis. In this paper, methods for the separation of SCFAs and lactic acid are reviewed. Analysis of the organic acids involved in carbohydrate metabolism has been achieved by enzymatic methods, gas chromatography, high-performance liquid chromatography and capillary electrophoresis. Sample preparation techniques developed for these analytes are also discussed.

Keywords: Reviews; Enantiomer separation; Metabolic acidosis; Organic acids; Lactic acid

Contents

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1. Introduction

Organic acids are the main products of bacterial fermentation in the colon. The major organic acids found in the colon are the saturated, volatile short-chain fatty acids (SCFAs), acetic acid (60%), propionic acid (20%) and butyric acid (10%) [1]. They are produced from the fermentation of carbohydrate, specifically di- and oligosaccharides, starch, sugar alcohols and non-starch polysaccharides [1]. The major function of organic acids in the gastrointestinal tract is to provide a fuel for oxidative metabolism and ion pumping for mucosal cells of the colon [2].

DL-Lactic acid, an unsaturated, non-volatile, three-carbon carboxylic acid, is also produced in the anaerobic environment of the gastrointestinal tract by bacterial fermentation. In the healthy state, DL-lactic acid does not enter in systemic circulation, but is saturated to SCFA. However, when a large supply of fermentable carbohydrates is available to the fermentative bacteria of the lower intestine, DL-lactate can accumulate and be absorbed into the systemic circulation. Since L-lactic acid is readily metabolized to pyruvate in the liver and kidney, it tends not to be present in the blood in high levels, however, D-lactic acid is metabolized relatively slowly and can accumulate in the blood to neurotoxic levels [1,2]. L-Lactic acid is also a product of the glycolytic pathway in mammalian cells, and can accumulate in the blood in hypoxic conditions [3]. Propionate and butyrate do not typically pose an acid–base threat in the circulation since they are rapidly metabolized. Propionate is cleared by the liver and is converted to glucose, triglycerides or carbon dioxide and butyrate is oxidized by colonic mucosal cells for ATP [1,2]. Acetate is normally converted to acetyl-CoA in the liver and peripheral tissues, but can accumulate in the circulation in conditions of overfermentation [4,5].

Increases in anaerobic bacterial fermentation may be potentiated by anoxia, malabsorption resulting in increased delivery of nutrients to the bacterial flora of the lower intestines, or as a result of decreased intestinal transit. Under these circumstances, overfermentation may occur, resulting in increased production levels of organic acids. If these are absorbed, a systemic metabolic acidosis can result.

Metabolic acidosis is an increase in the concentration of hydrogen ions in the blood from either the loss of bicarbonate or gain of acid. Loss of bicarbonate may occur via either renal or gastrointestinal mechanisms [3]. Gain of acid may result from increased endogenous production, as in ketoacidosis and lactic acidosis, or from the metabolism of ingested toxins such as ethylene glycol [6]. Organic acids may also be elevated in the systemic circulation due to overproduction by bacterial fermentation in the gastrointestinal tract under certain circumstances, including short bowel syndrome, grain overfeeding in ruminants and severe gastroenteritis [7]. This bacterial origin of organic acids forms the basis for the following methodological review and other causes of metabolic acidosis are beyond the scope of this paper.

Severe metabolic acidosis is of concern because it may cause death due to altered structure and function of proteins performing essential functions in the body [6] or by altering the intracellular to extracellular myocyte potassium ratio and causing heart failure [8].

The anion gap $[(Na^+ + K^+) - (Cl^- + HCO_3^-)]$ is a diagnostic calculation that helps characterize the origins and nature of metabolic acidosis [9]. An increase in anion gap is commonly observed due to an accumulation of strong anions, particularly organic acids such as lactic acid [10]. The anion gap is reduced in hypoalbuminemia [6]. It remains unchanged if the metabolic acidosis is due to loss of bicarbonate since chloride rises reciprocally.

The analysis of fermentation products in serum, faeces and urine is integral to the diagnosis of intestinal bacterial overgrowth, and various analytical techniques have been developed and employed for the measurement of organic acids. Since quantitation of SCFAs generally relies on their volatility, methods are fundamentally different than those for lactic acid. The difficulties encountered in analysing components of faeces has lead to various approaches to sample preparation. Determining the origin of acidosis in various conditions requires the stereospecific separation of lactic acid and numerous methods have been validated for this purpose. This article reviews the sample preparation methods and assays for analysing organic acids in biological samples which are relevant to metabolic acidosis of bacterial origin, with emphasis on the separation of D- and L-lactic acid.

2. Sample purification methods

The methods described here are generally pre-preparative steps for the quantitative analysis of acids in biological matrices. Some techniques allow direct injection of the resultant fluids into chromatographic systems, however, additional preparative steps such as derivatization are often required. Derivatization is used to increase the volatility of components for gas chromatography (GC), to allow detection of acids in high-performance liquid chromatography (HPLC) analyses and to increase sensitivity, particularly for chiral separations. These additional steps are discussed in the sections describing the measurement of various acids that follow.

2.1. Faeces

Since faeces are multiphasic solutions containing solid matter, measurement of solutes within these phases is difficult, especially because of the chemical changes that may occur after stools are passed. Faeces should be collected into a small volume of bacteriostatic agent (such as thimerosal) to minimize bacterial overgrowth and alterations of fermentation products.

Electrolytes and organic acids in stool may be insoluble, dissolved in extracellular fluid, or dissolved in the fluid contained in bacteria, protozoa or other cells [11]. Difficulties in measuring faecal solutes has lead to the development of various sample preparation methods to isolate the organic acid containing water fraction of stool, including dialysis, centrifuging and ultrafiltration. Distillation procedures for the analysis of SCFAs in stool are also discussed.

2.1.1. Fecal distillation and extraction

Distillation procedures have been used to separate SCFAs from stool prior to quantitative or qualitative GC analyses. Although it is important to measure SCFAs, this method is of somewhat limited value, because it leaves behind some of the non-volatile acids that are of interest in the acidotic state, such as lactic acid.

Direct distillation of SCFAs was initially attempted by Ludwig Brieger in 1878 [12] by distilling the acids from an acid mixture and purifying and identifying of their metallic salts. Many modifications have been made to this method, including steam distillation, the Duclaux method of distillation-rate measurement [13] and distillation under vaccuum [14]. Zijlstra et al. [14] determined that vacuum distillation of faeces followed by alkaline freeze–drying resulted in a superior recovery, improved reproducibility and lower relative standard deviations (RSDs) than steam distillation. There are, nonetheless, many objections to the distillation of stool samples. Large fecal samples are required for steam distillation, and products of protein and carbohydrate digestion may contaminate the distillate if strong acids are used to acidify the sample. In addition, decomposition of acetyl groups from other components within the sample can lead to overestimations of acetate values. Thus, no distillation method yields a simple and effective sample preparation.

Various extraction methods have been utilized to enhance the concentration of analytes or to separate

analytes from interfering substances. Acceptable extraction has been achieved with diethyl ether, ethyl acetate, chloroform and dichloromethane. Ethanol extraction has been attempted but does not recover all SCFAs [15]. Extraction is usually followed by evaporation, which allows interferences by even small impurities in the sample. Although liquid–liquid extraction is relatively easy, in general, extraction using organic solvents tends to yield lower recovery rates than other methods [16].

2.1.2. In vivo and in vitro dialysis of faeces

In 1961, a technique was developed for the in vivo dialysis of stool, whereby subjects swallowed bags of cellulose acetate dialysis tubing containing an inert colloidal solution which, when recovered, contained the extracellular component of fecal water, including SCFAs and lactic acid [11]. The in vivo method poses obvious practical difficulties for subjects, is not suitable for severely ill patients or infants, and has not been widely used. In addition, it has been suggested that the stool dialysate in vivo is not in equilibrium with the surrounding fluid [17].

In vitro dialysis of faeces has been shown as a relatively effective method for obtaining fecal water, however, very large samples of stool are required, and the method is time consuming as equilibrium is not reached for 24 h [18].

2.1.3. Centrifugation of faeces

Fecal water can also be separated by high speed centrifugation, although relatively small amounts of fluid are obtained. In a comparison between the in vivo dialysis method with the fluid obtained by high speed centrifugation (40 000 *g* for 30 min), the centrifugation method yielded fecal water that contained more acid, organic anions, sugar and ammonium, and less bicarbonate than the in vivo dialysis method [17].

2.1.4. Ultrafiltration of faeces

Ultrafiltration is a valuable technique for separating the liquid fraction in stool. Membranes between molecular mass (M_r) 5000 and 50 000 are generally used, and require a pressure of 3–4.5 bar or a centrifugal force of 1000–2000 *g* [19]. It is extremely simple and rapid, and has been demonstrated to be as effective as steam distillation in sample clean-up [20]. Ultrafiltration has also been compared with in vitro dialysis of faeces [18]. Dialysis tubing was placed in freshly passed stools for 24 h and the pH, and concentrations of SCFAs and electrolytes contained in the fluid were compared to those obtained by ultrafiltration through an M_r 50 000 membrane. No significant difference was found between the two methods in normal patients, although patients with diarrhoeal diseases had slightly lower concentrations of sodium and chloride in the dialysis bags compared to the ultrafiltrate. This prompted the authors to question in vitro dialysis for clinical use.

The authors use Millipore Ultrafree MC microcentrifugal ultrafiltration units with an M_r 5000 cutoff for sample preparation prior to HPLC analysis. Spun at 5000 *g*, 200-ml samples take 30 min to filter. This method is rapid, simple and effective, with minimal alterations of the sample [21].

2.2. Serum

As with faeces, distillation, extraction and ultrafiltration have been used to purify samples for the analysis of SCFAs and lactic acid.

2.2.1. Deproteinization

Particularly for the analysis of lactic acid enantiomers, but also for measurement of SCFAs, the deproteinization of serum is critical. Deproteinization stops activity of metabolic enzymes, reduces interferences in enzymatic methods, and reduces contamination and deterioration of chromatographic columns.

In some cases, deproteinization yields a sample that can be directly injected into analytical systems, and in others, further preparative steps such as distillation or extraction are required. Derivatization techniques may also be required and are described in the sections that follow.

A common deproteinization method involves precipitation with perchloric acid, however, the resultant low pH may interfere with analyses. If the sample is neutralized, the dilution factor arising from the addition of both perchloric acid and neutralizing solution often results in analyte concentration below the level of detection [22]. Other acids used for deproteinization include tricholoroacetic acid and sulfosaliclyic acid [19].

Cationic protein precipitants can also be used, particularly zinc sulfate solution in combination with barium hydroxide solution. This procedure also

results in significant dilution of samples, and some acids adsorb to the precipitate and are co-precipitated [22].

Organic solvents such as acetonitrile, propan-2-ol and methanol are also used to denature proteins. These require the addition of two volumes of solvent per volume of plasma to precipitate 99% of protein, resulting in significant dilution, but can be evaporated in vacuo to concentrate the sample [19].

2.2.2. Serum distillation and extraction

Distillation has also been applied to serum for GC analysis of SCFAs. Distillation of serum has many of the same shortcomings as distillation of faeces, mainly that it does not isolate other acids of interest, particularly lactic acid. In addition, pyrolysis of acetyl groups from other plasma constituents can lead to overestimations of acetate values.

Liquid extraction of organic acids has been achieved using various solvents and solvent mixtures, typically including chloroform and an alcohol, but as in faeces, tend to yield lower recovery rates [16,19].

Solid-phase extraction using anion-exchange chromatography can be used to isolate organic acids from serum, usually on a Rexyn 201, DEAE-Sephadex or Amberlyst A-26 column [19,23]. Cation exchange has been used to remove interfering plasma cations such as magnesium and calcium on a Dowex 50W-X8 column [23–26].

2.2.3. Ultrafiltration of serum

Ultrafiltration at M_r 10 000–50 000 can be used to deproteinize and prepare samples for analysis. A centrifugal force of approximately 2000 *g* is required, or a pressure of 2–4 bar [19]. It is a rapid, simple method for removing protein, is applicable to small samples, and produces little alteration of the sample. It has the advantage of isolating all the acids of interest. This method is considered by the authors to be a suitable and convenient method of sample purification, providing high accuracy and reliability, and is routinely used in our analyses as described for faeces, except with an M_r 10 000 cutoff.

2.3. Urine and other biological matrices

Urine is different from fecal water in that it contains much less SCFAs and has a much higher ionic content. The Van Slyke and Palmer method [27] for removal of carbonate and phosphate from urine prior to titration measurement of SCFAs is subject to a major source of error due to the presence of creatinine. Collin and McCormick [23] overcame this error by removing non-volatile components such as calcium, magnesium and titrable amine containing materials such as creatinine using a cation-exchange column, then passing through an analytical anion-exchange column. SCFAs tend not to be excreted in urine at significant levels, but D- and L-lactic acid urinary excretion has been investigated in the past [28].

Organic acids have also been measured in other fluids, including cerebrospinal fluid [29], sinus secretions [30] and rumen fluid [31].

3. Measurement of short-chain fatty acids

The analysis of SCFAs in intestinal contents or serum can be used to implicate bacterial colonization in the small or large intestine as the origin of metabolic acidosis. This analysis can also indicate the effects of antibiotics upon the gastrointestinal flora.

SCFAs can be titrated in water, and this was, historically, the method used to quantify total SCFAs in fecal water until chromatographic methods were developed. Titration was not very specific, since other inorganic titrable compounds in fecal water such as carbonate and phosphate hampered accurate measurement, and difficulties separating SCFAs from one another impeded measurement of individual acids.

3.1. Gas chromatography of short-chain fatty acids

GC has been the most extensively used method to separate SCFAs. Some methods are listed in Table 1. Packed columns are commonly used in many laboratories, however, capillary columns have also become widely utilized since their use was first reported in 1987 [16]. Glass columns are considered unsatisfactory because after around 30 injections, deposits of non-volatile material accumulate and reduce the quality of the chromatograms. In addition, RSDs and chromatograms obtained by the use of a

Table 1
Gas chromatographic methods for the separation of short chain fatty acids, DL-lactic acid and lactic acid enantiomers

Analyte	LLQ	Stationary phase	Carrier gas	Matrix	Sample preparation	Detection	Ref.
C2–C5, LA	60 mg/l	Polyester (SP-1200) on SDB copolymer (Chromosorb A-AW) 183 cm×3 mm I.D., 115 °C	Helium, 40 ml/min	Faeces, urine	Homogenized, centrifuged, ultrafiltered, cation-exchange on a Dowex 50W-X8 column, anion-exchange on Rexyn 201	FID 200 °C	[23] [25]
C2–C5	0.15 nmol	SDB copolymer (Chromosorb 101) 1.4 m×2 mm I.D. 150 °C	Helium, 25 ml/min	Blood	Deproteinized with barium hydroxide, steam distilled, alkalinized with KOH	FID 250 °C	[94]
C2–C5	C2=70 C3=66 C4=38 μmol/g dry mass	Polyethylene glycol (DB-WAX) 15 m×052 mm I.D. 125 °C	Helium, 3.0 ml/min	Faeces	Centrifuged, acidified with 0.36 *M* perchloric acid, vacuum transfer, alkalinized, freeze dried, dissolved in formic acid	FID 200 °C	[16]
C2–C5	0.04 mg/ml	Porous polymer (Porapak N) 150 cm×0.17 mm I.D. 185 °C	Nitrogen, 50 ml/min Hydrogen, 35 ml/min Air, 300 ml/min	Aqueous media	None	FID 240 °C	[95]
C2–C5	50 nmol/ml	Polyethylene glycol (5% Carbowax 20M) 25 mm×0.32 mm I.D.	Hydrogen, 2.5 ml/min Air, 300 ml/min	Plasma, saliva, cecal contents, cecal tissue	Extracted with ethanol, alkalinized with KOH, evaporated	FID 300 °C	[34]
C2–C5	0.3 m*M*	SDB copolymer (Chromosorb 101) 2 m×2 mm I.D. 150–195 °C	Nitrogen, 40 ml/min	Blood	Deproteinized with sulfosalicylic acid, centrifuged, vacuum distilled, alkalinized with NaOH, dried, dissolved in formic acid	FID 135 °C	[96]
C2–C5		FFAP (reaction product between Carbowax 20M and 2-nitroteraphtalic acid) 90–140 °C	Helium	Intestinal fluids, faeces, blood	Acidified with sulfuric acid, extracted with diethyl ether, dried	FID 240 °C	[97]
C2–C5	100 ng/μl	Fluorocarbon (Fluorad FC430) on SDB copolymer (Chromosorb G) 63 cm×3 mm I.D. 100 °C	Nitrogen, 35 ml/min Hydrogen 35 ml/min Air, 600 ml/min	Urine	Cation exchange with Dowex 50-W ($12H^+$) resin	FID 135 °C	[24]
C2–C5	0.05 m*M*	Polyethylene glycol (DB-WAX) 15 m×0.53 mm I.D.	Helium, 30 ml/min	Faeces, amniotic fluid	Acidified with sulfuric acid, homogenized, vacuum distilled, dried, acidified with formic acid	FID 210 °C	[98]
C2–C8	2.5 μmol/l	Polyester (SP-1200) 2 m×22 mm I.D. 145 °C	Nitrogen, 16 ml/min Hydrogen, 230 ml/min Air, 300 ml/min	Serum	Acidified, vacuum distilled, evaporated to sodium salts of SCFA	FID 180 °C	[99]

C2–C7	0.1 m*M*	SDB copolymer (Chromosorb 101) 1.5 m×4 mm I.D. 50–200 °C	Nitrogen, 20 ml/min Hydrogen, 40 ml/min Air, 400 ml/min	Rumen fluid, urine, plasma	Rumen—preserved in mercuric chloride, filtered Urine—preserved with mercuric chloride Plasma—extracted with ethanol, centrifuged, supernatant dried, dissolved in formic acid	FID 330 °C	[100]
C2–C22	20 nmol/l	SP-Sil 5CB 100–300 °C	Helium, 25 cm/s	Serum, urine, rat ventricle	Deproteinized and extracted with ethanol, lipids removed by hexane extraction, cation exchange on Dowex AG 50W-X8 resin, benzyl ester formed via crown ether catalysis	FID 330 °C	[26]
C2–C5	NL	SDB copolymer [Chromosorb P (AW)] coated with Carbowax 2 m×2.2 mm I.D. 90 °C	Nitrogen, 25 ml/min	Plasma	Extracted with ethanol, alkalinized with NaOH, evaporated, rehydrated	FID	[15]
C2–C5	10 ng/μl	2% orthophosphoric acid-modified Phasepak Q 2 m×3 mm I.D.	Nitrogen, 60 ml/min	Carious enamel, saliva	Acidified with 1 *M* hydrochloric acid, vacuum distilled	FID	[101]
C2–C5	1.67 m*M*	SDB copolymer (Chromosorb 101) 2 m×2 mm I.D. 140–215 °C	Hydrogen, 25 ml/min Nitrogen, 15 ml/min	Faeces	Acidified with sulfuric acid, homogenized, vacuum distilled, dried, acidification with formic acid	FID 200 °C	[14]
C2–C5	C2=12.5 C3=5 C4–6=2.5 m*M*	Polyester (SP-1200) on SDB copolymer (100 Chromosorb W AW) 145 °C	Nitrogen, 20 ml/min Hydrogen, 230 ml/min Air, 300 ml/min	Faeces	Homogenized, centrifuged, direct injection of supernatant	FID 180 °C	[32]
C2–C5	NL	Porous polymer (Porapak QS) 2 m 200 °C	Helium, 40–50 ml/min	Serum, plasma	Acidified, steam distilled, alkalinized with NaOH, evaporated, dissolved in 25% metaphosphoric acid	FID	[102]
C2–C5	0.1 m*M*	Polyethylene glycol (Carbopak B-DA/4% Carbowax 20M) 182 cm×2 mm I.D. 175 °C	Nitrogen, 30 ml/min Hydrogen, 30 ml/min Air, 300 ml/min	Faeces	Homogenized, centrifuged, ultrafiltered through a microconcentrator	FID 200 °C	[20]
D-LA L-LA	NL	*N*-Acyl-valine-*tert*.-butyl amide groups	Helium	Aqueous media	Ethyl esterified	FID	[54]
D-LA L-LA	NL	Chirasil-Val 20×0.25 mm I.D.	Hydrogen 0.6 bar	Aqueous media	Alkyl esterified	FID	[103]
D-LA L-LA	NL	SE-30 25 m×0.3 mm I.D. 80 °C	Hydrogen 0.7 bar	Aqueous media	(+)-3-Methyl-2-butyl esters of *O*-trifluoroacetylated or *O*-trimethylsyilylated carboxylic acids	FID	[53]
C2–C5	C2=20 ng	5% polyethylene glycol (Carbowax 20M) on SDB copolymer (Chromosorb P) 2 m×2 mm I.D.	Nitrogen, 40 ml/min	Plasma, urine	Direct injection	FID 135 °C	[104]

LA, Lactic acid; D-LA, D-lactic acid; L-LA, L-lactic acid; C1, formic acid; C2 acetic acid; C3, propionic acid C4, butyric acid; C5, valeric acid; C6, caproic acid; LLQ, lower limit of quantitation; NL, not listed; SDB, styrene–divinylbenzene; I.D., internal diameter; FID, flame ionization detection.

Table 2
High-performance liquid chromatographic methods for the separation of short-chain fatty acids, DL-lactic acid and lactic acid enantiomers

Analyte	LLQ	Stationary phase	Mobile phase	Matrix	Sample preparation	Detection	Ref.
C1–C8, LA	NL	Al-silica (IEC) 250 mm×4.6 mm I.D. 35 °C	0.5 m*M* sulfuric acid+0.15% heptanol 1 ml/min	Aqueous media	None	UV 220 nm	[39]
C2–C5	1 nmol	ORH-80 organic acid column 30 cm×0.65 cm I.D. 37 °C	0.15 m*M* sulfuric acid 0.5 ml/min	Faeces	Homogenized, centrifuged, direct injection of supernatant	CD	[20]
C2–C5	10–40 nmol	Polyspher OA HY 300 mm×9.5 mm I.D. 60 °C	0.2 *M* sulfuric acid 0.8 ml/min	Faeces, saliva, plasma, duodenal secretions, faeces, tissue	Deproteinized with perchloric acid, alkalinized with KOH. Vacuum transfer of volatile components	UV 214 nm	[35]
D-LA L-LA	0.5 m*M*	*N*,*N*-Dioctyl-L-alanine on ODS 50×4.6 mm I.D.	2 m*M* copper sulfate in 0.1% acetonitrile 0.4 ml/min RT	Serum	Ultrafiltered at M_r 10 000	UV 236 nm	[21]
C2–C4, LA	0.0005 *M*	Brownlee Polypor H 25 cm×4.6 mm I.D.	0.05 m*M* sulfuric acid 0.5 ml/min	Caecal liquor	Solid-phase extraction, Sep-Pak C_{18}, added to phosphate buffer, filtered	UV 210 nm	[36]
LA	NL	LiChrosorb RP 8 250 mm×4.6 mm I.D.	Acetonitrile–water (3:7) 3 ml/min	Single muscle fibres	Homogenized, deproteinized with perchloric acid, neutralized with $KHCO_3$, centrifuged, converted to potassium salt with KOH, dried, dissolved in acetonitrile, ester derivatized with α-*p*-dibromoacetophenone with crown ether as a catalyst	UV 254 nm	[105]
LA	1 μg	μBondapak ODS 250 mm×4.6 mm I.D.	0.01 *M* phosphate buffer 2.0 ml/min	Plasma	Deproteinized with trichloroacetic acid, solid-phase extraction on Sep-Pak C_{18}, extracted with diethyl ether, freeze–dried, reconstituted in phosphate buffer	UV–Vis 190, 220, 245 nm	[60]
D-LA L-LA	NL	CSP II CSP III 50 °C	10^{-4} copper (II) sulfate 1 ml/min	Aqueous media	None	UV 223 nm	[61]
D-LA L-LA	NL	(*R*,*R*)-Tartaric acid monoamide derivatives 150×4.6 mm I.D.	1 m*M* copper (II) sulfate 1.0 ml/min RT	Aqueous media	None	Variable UV	[59]

C2, C4, LA, PA	0.5 m*M*	RSPAK KC-811 300×8 mm I.D. 50 °C	0.1% phosphoric acid 0.7 ml/min	Serum	Ultrafiltration at M_r 10 000	UV 236 nm	[21]
D-LA L-LA	NL	*N*,*N*-Dioctyl-L-alanine 50×4.6 mm I.D.	2 m*M* copper sulfate in 10% acetonitrile 1.0 ml/min RT	Aqueous	None	UV 254 nm	[106]
D-LA L-LA	NL	*N*-(2-Naphthalenesulfonyl)-phenylalanine on porous graphite, 50×4.6 mm I.D.	2. 0 m*M* copper acetate RT	Aqueous	None	UV 254 nm	[107]
D-LA L-LA	NL	Copper(II) complexes of Schiff bases	2 m*M* copper(II) sulfate in water–acetonitrile (85:15), 1.0 ml/min RT	Aqueous	None	Variable UV	[62]
D-LA L-LA	NL	Spherisorb C_8 150×4.6 mm	0.05 *M* acetate–THF (76:24) 2 ml/min	Aqueous	None	UV 265 nm	[52]
D-LA L-LA	62.5 μ*M*	ODS following separation on Phenyl carbamoylated β-cyclodextrin 25 °C 150×4.6 mm I.D.	Methanol–water (80:20) 1.0 ml/min	Aqueous	Derivatized with 4-(*N*,*N*-dimethylaminosulfonyl)-7-piperazino-2,1,3-benzoxadiazole	Fluorimetric 560 nm 450 nm (excitation)	[67]
D-LA	0.5 m*M*	ODS following separation on Phenyl carbamoylated β-cyclodextrin 25 °C 150×4.6 mm I.D.	Acetonitrile–methanol–0.1% TFA (10:20:70) 0.8 ml/min RT	Plasma	Derivatized with 4-nitro-7-piperazino-2,1,3-benzoxadiazole	547 nm 491 nm (excitation)	[66]
LA	36 pmol	RP-ODS bonded 250 mm×4.6 mm I.D.	Acetonitrile–water (30:70, v/v)	Plasma	Acidified with hydrochloric acid, extracted with diethyl ether, dried, derivatized with α-dibromoacetophenone, dried, reconstituted with acetonitrile–water	SPEC 320 nm	[38]

C1, Formic acid; C2, acetic acid; C3, propionic acid; C4, butyric acid; C5, valeric acid; C6, caproic acid; UV, ultraviolet; IEC, ion-exchange chromatography; PA, pyruvic acid; D-LA, D-lactic acid; L-LA, L-lactic acid; I.D., internal diameter; RT, room temperature; ODS, octadecylsilane.

capillary column are superior to those obtained by conventional glass columns [16].

Generally, distillation has been applied to samples prior to injection. However, direct injection of samples into the GC system without pretreatment can be facilitated by the use of a glass liner, stoppered with a glass wool plug to prevent contamination with non-volatile fecal material [32].

Although GC has been extensively used to measure SCFAs, it is subject to several sources of error. The major difficulty involves the interaction of acids with the column packing, resulting in tailing of peaks, and retention of the acids on the column. Subsequent injections may cause the adsorbed acids to be released, and ''ghosting'' may occur [33]. Several steps have been taken to reduce these effects, including adding phosphoric acid to the packing, incorporating formic acid in to the carrier gas and by using capillary chromatography [34]. In addition, sample preparation techniques required prior to GC analysis often involve lengthy procedures.

3.2. High-performance liquid chromatography of short-chain fatty acids

SCFAs can be separated by HPLC on a sulfonated polystyrene–divinylbenzene stationary phase following distillation under vacuum and concentration by freeze drying samples under alkaline conditions [35]. Separation has also been achieved on an ORH-801 organic acid column with conductivity detection [20]. Ion-exchange HPLC on a Brownlee Polypor H column with UV detection after solid-phase extraction has been attempted, although with relatively low recovery rates [36]. HPLC methods for the qualification and quantification of SCFAs are often less time consuming than GC methods, with sample preparation being less painstaking. Some HPLC methods are summarized in Table 2.

4. Measurement of DL-lactate and other fatty acids

There are many methods available to measure racemic lactic acid. The first attempts to measure lactate occurred nearly 100 years ago. Initial colorimetric assays involved the conversion of lactate and sulfuric acid to acetic aldehyde, carbon monoxide and water. The acetic aldehyde reacted with *p*-hydroxyphenol in the presence of copper ions, and the resultant colour change was determined spectrophotometrically [37]. More recently, lactate has been derivatized with α-bromoacetophenone, and the resultant ester separated on an octadecylsilane (ODS)-bonded column and detected in the UV range of 242 to 320 nm [38]. Dibromoacetophenone have also been used to derivatize lactic acid as listed in Table 2.

Although measuring DL-lactic acid by itself can provide some understanding of metabolic acidosis, it provides no potentially useful diagnostic information about the source of lactic acid production. Some methods allow for the simultaneous measurement of SCFAs and lactic acid and are more useful in diagnosing the origin of metabolic acidosis.

Simultaneous determination of C_2–C_{22} fatty acids and other carboxylic acids including lactic acid has been achieved [26] by GC of their benzyl esters formed by crown ether catalysis of potassium carboxylates with benzyl bromide. Prior to analysis, non-polar components are removed by hexane extraction, and other cations that create conflicting peaks in the chromatographs are removed by cation-exchange chromatography. While this method is effective in analysing many components simultaneously, sample preparation is time consuming.

An ion-exclusion chromatographic method developed by Ohta and Tanaka [39] employed an aluminum-absorbing silica gel stationary phase with an acidic eluent to separate various acids including SCFAs and lactic acid in aqueous media. A HPLC method was developed for the determination of racemic lactic acid, in conjunction with other carboxylic acids, including pyruvic acid, hydroxybutyric acid and acetic acid using a Shodex RSPAK KC-11 (Shawa Denko, Japan), 0.1% phosphoric acid as the mobile phase and UV detection at 236 nm [21]. Sample preparation involves a rapid ultrafiltration procedure, with no further preparative steps required (Fig. 1).

5. Measurement of lactic acid enantiomers

Lactic acid, or 2-hydroxypropanoic acid was discovered in 1780 by a Swedish chemist, Scheele, who isolated it from sour milk [108]. Lactic acid is the

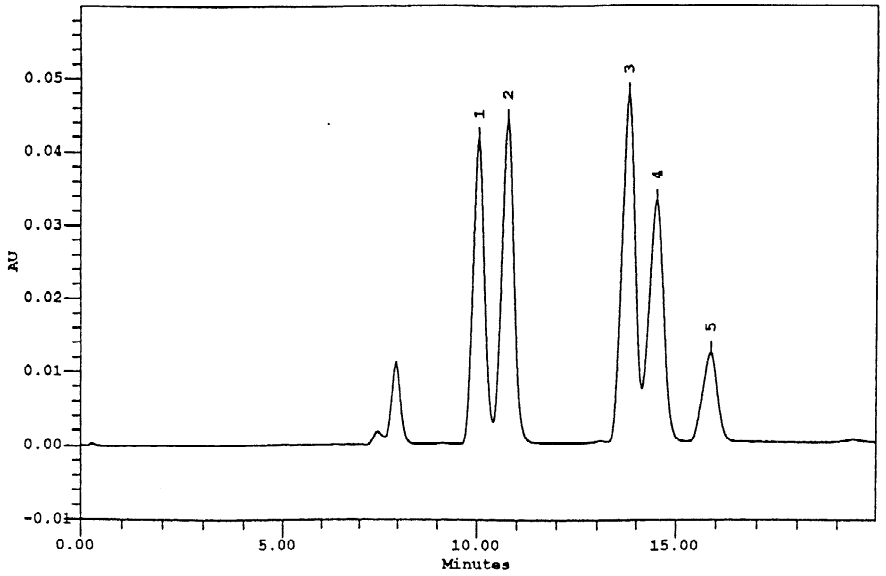

Fig. 1. Separation of organic acids. Conditions: column, RSPAK KC-811 (300×8 mm I.D.), 50 °C; mobile phase, 0.1% phosphoric acid, 0.7 ml/min. UV detection at 205 nm. Peak identification: (1) internal standard—citric acid; (2) pyruvic acid; (3) lactic acid; (4) hydroxybutyric acid; (5) acetic acid. [Laboratory data, Zello et al.].

simplest hydroxycarboxylic acid and exists as two stereoisomers, or enantiomers, due to its asymmetric C2 atom (Fig. 2). These two forms are optical isomers, and rotate light in different directions. Typically, an enantiomer that rotates light in the clockwise direction is called d, for dextrorotary or (+), and the enantiomer that rotates light in the counter-clockwise direction is called l, for levorotary, or (−). In addition, compounds are also classified as D- or L- based on the absolute configuration of D- and L-glyceraldehyde. However, lactic acid is an exception to these rules, existing as a levorotary D-isomer and a dextrorotary L-isomer [40]. Both enantiomers have similar physical and chemical properties [41]. Each isomer can contribute to metabolic acidosis, but since the origins of L-lactic acid and D-lactic acid are different, distinguishing between the two isomers is important in understanding their relative contribution.

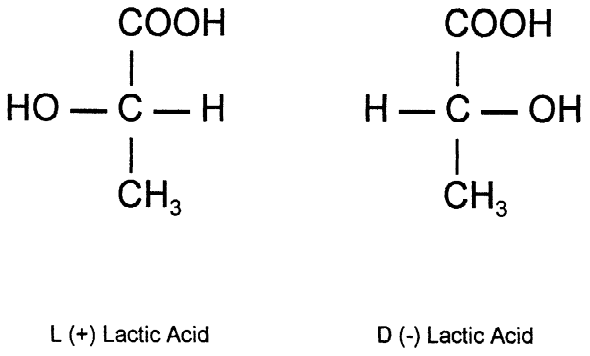

Fig. 2. Lactic acid enantiomers.

5.1. Enzymatic methods

The enzymatic method of analysis is commonly employed for the determination of lactate isomers in biological fluids [30]. The assay is based on the formation of lactate by the reduction of pyruvate by NADH, which is catalyzed by lactate dehydrogenase (LDH) (EC 1.1.1.27). The concentration of NADH using spectrophotometry at an absorbance of 340 nm is directly proportional to the concentration of L-lactate in the sample [42]. Fluorometric detection is also possible, and yields a twofold decrease in the limit of detection for D-lactate measurement [43]. D-lactate is measured enzymatically using the same reactions as those for L-lactate, with D-lactate as substrate in reaction (1), and D-lactate dehydrogenase (EC 1.1.1.28) instead of LDH [44]:

Reaction 1: L-lactate + $NAD^+ \xrightarrow{LDH}$ pyruvate + NAD + H^+ (1)

Reaction 2: pyruvate + L-glutamate $\xrightarrow{LDH}$ L-alanine + 2-oxoglutarate (2)

The equilibrium of reaction (1) is in favour of lactate. If pyruvate is removed, as in reaction (2), lactate is completely hydrogenated by LDH [45]. In reaction (2), a pH of 8.9 favours the formation of pyruvate. In the past, alkaline medium, which causes a rapid inactivation of the enzyme, and carbamyl reagents, which trap generated pyruvate, were used to promote equilibrium. However, the carbamyl reagents also react with NAD to form compounds that absorb UV light in the same range as NADH [45]. Pyruvate can be trapped more efficiently through conversion to alanine by reacting with glutamate in a reaction catalyzed by L-alanine 2-oxoglutarate aminotransferase enzyme [45].

Galban et al. [46] proposed a fluorimetric method based on the enzymatic reaction of lactate with lactate oxidase, which has intrinsic fluorescent optical properties due to tyrosine and tryptophan residues.

Girotti et al. [47] determined D-lactate in biological samples using a bioluminescent flow sensor. D-Lactate dehydrogenase catalyses the oxidation of lactate by NAD^+ to yield NADH:

D-lactate + $NAD^+ \xrightarrow{D\text{-}LDH}$ pyruvate + NADH + H^+

NADH is then quantified by bacterial bioluminescent enzymes NADH/flavin mononucleotide oxidoreductase and luciferase:

NADH + FMN + $H^+ \xrightarrow{NADH/FMN\ oxidoreductase}$ NAD^+ + $FMNH_2$

$FMNH_2$ + RCHO + $O_2 \xrightarrow{luciferase}$ FMN + RCOOH + H_2O + light

The simultaneous determination of D- and L-lactic acid has been achieved using a flow system with two enzyme reactors and an octadecylsilica column [48]. LDH and glutamic-pyruvic transaminase (GPT) were used to measure L-lactate acid, and D-lactate dehydrogenase and GPT were used to measure D-lactate, in two separate enzyme reactors placed in tandem. The ODS column was positioned between the two enzyme reactors to separate the NADH produced by the D-lactate dehydrogenase (Fig. 3).

Langton et al. [49] used a Clark oxygen sensing electrode with a specialized, three-layer membrane. Ultimately, L-lactic acid contacts gluteraldehyde cross-linked L-lactate oxidase which converts L-lactate to hydrogen peroxide and pyruvate. The hydrogen peroxide diffuses through to the anode of the electrode where it is reduced, yielding a current that is proportional to lactate concentration. Results obtained by this method correlate very well with traditional enzymatic methods, with a coefficient of correlation of 0.998.

The major disadvantage of enzymatic methods is the length of time required for the reaction to reach completion (15–30 min) [45]. However, a more rapid, automated analysis was established with the use of a centrifugal analyzer [50]. An automated micromethod has also been developed to simultaneously measure D- and L-lactate, using an autosampler pump system [51].

Sources of error for enzymatic methods include contamination or impurities of reagents, especially contamination of D-lactate dehydrogenase with L-lactate dehydrogenase, and disturbances in measurement techniques at pH less than 8.9 [45].

5.2. Gas chromatography of lactic acid enantiomers

Separation of lactic acid enantiomers was first achieved by GC of diastereomeric derivatives on an achiral stationary phase. These derivatives include mainly esters, such as (−)-menthyloxycarbonylmethyl esters, acetyl/(−)-menthyl esters and tri-

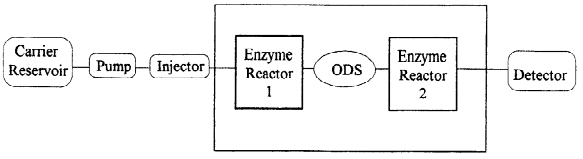

Fig. 3. Flow system for the simultaneous enzymatic analysis of D- and L-lactic acid. Enzyme reactor 1 contains L-lactate dehydrogenase, enzyme reactor 2 contains D-lactate dehydrogenase. ODS, octadecylsilica; detector is spectrophotometric. Adapted from [48].

fluoroacetyl or trimethylsilyl/(+)-3-methyl-2 butyl ester [52,53]. Diamide chiral stationary phases for GC enantiomeric separations have been throughly investigated, in particular, Chirasil-Val. The chiral selecting mechanism of this stationary phase is unknown. Betschinger et al. [54] separated the ethyl esters of lactic acid enantiomers on a tripodal C3 symmetric structural skeleton, with *N*-acyl-valine-*tert*.-butyl amide groups as the chiral selector. For diagnosing inborn errors of metabolism, Heil et al. [55] developed a multidimensional GC–mass spectrometry (MS) method that provided an adequately sophisticated analysis to measure various compounds, including D- and L-lactic acid. After separating samples on a 30 m×0.25 mm I.D. high-temperature fused capillary precolumn coated with 0.38 μm PS 268, pre-column flame ionization detection was used and selected compounds were transferred to a main column with different selectivity and polarity. Mass spectrometric detection in the ion impact mode was then employed.

5.3. High-performance liquid chromatography of lactic acid enantiomers

Methods of chiral resolution of lactic acid using HPLC have been developed in the last 20 years. There are three general mechanisms for the separation of enantiomers, namely, direct separation on a chiral stationary phase, direct separation on an achiral stationary phase with a chiral mobile phase, and indirect separation by derivatization of stereoisomers with a chiral agent on an achiral stationary phase [56]. Lactic acid contains a carbonyl group, making its detection with the variable-wavelength UV–visible detector susceptible to interference from other species containing double bonds absorbing UV light in the same region [57]. Therefore, many HPLC techniques for lactic acid employ derivatization to produce stronger absorbing chromophores to improve sensitivity [58,59]. However, lactate has been determined in the low UV range without derivatization [60,21].

The development of two chiral ligand exchange phases, CSP II [L-hydroxyproline bound to silica gel reacted with 2-(3,4-epoxycyclohexyl)ethyltrimethoxysilane] and CSP III (L-hydroxyproline on silica gel reacted with 3-glycidoxypropyltrimethoxysilane) facilitated separation ($\alpha = 0.82$) of lactic acid enantiomers by HPLC using 10^{-4} (II) sulfate as the mobile phase and UV detection [61]. Oi et al. [62] successfully separated lactic acid enantiomers using copper(II) complexes of Schiff bases of chiral amino alcohols as chiral ligand-exchange stationary phases on Sumipax ODS columns. They also used (*R*,*R*)-tartaric acid monoamide derivatives as chiral selectors [59].

A rapid, simple method was developed using a reversed-phase ligand exchange stainless steel 3 μm ODS packed analytical column coated with *N*,*N*-dioctyl-L-alanine as the chiral selector, 2 m*M* copper sulfate in 1% acetonitrile as the mobile phase and UV detection at 236 nm. Sample preparation involved only a rapid ultrafiltration procedure [21] (Fig. 4).

The separation of carboxylic acid enantiomers, including lactic acid has been achieved using various derivatization techniques. A fluorescence compound, (*S*)-(+)-methyl-2-(6,7-dimethoxy-2,3-naphthalimido) - ethyl trifluoromethanesulfonate, has been used as a derivatizing agent which demonstrates intense fluorescence at 394 nm [63]. The agent is highly reactive with carboxylic acids and no racemization occurs during the reaction. Resultant compounds are separated on a reversed-phase Wakosil-II RS column [63]. Ohmori and Iwamoto [64], measured D-lactic acid by converting it to quinoxanol and quantifying using a Unisil ODS QT-5K column with fluorescence detection. Lactic acid enantiomers have also been separated on a β-cyclodextrin stationary phase with a non-aqueous polar mobile phase following the formation of UV and fluorescent derivatives with 2-quinoxaloyl chloride with UV detection at 315 nm [58]. Ichihara et al. [65] measured D- and L-lactate in rat serum by fluorescence derivatization with 4-(*N*,*N*-dimethylaminosulfonyl)-7-piperazion-2,1,3-benzoxadiazole, followed by *O*-acetylation. The derivatives are separated on an octadecylsilica and a cellulose-type column. Determination of D- and L-lactic acid has also been achieved by derivatization with 4-(*N*,*N*-dimethylaminosulfonyl)-7-piperazino-2,1,3-benzoxadiazole or 4-nitro-7-piperazino-2,1,3-benzoxadiazole on an ODS column following separation on a phenyl carbamoylated β-cyclodextrin chiral column [66,67].

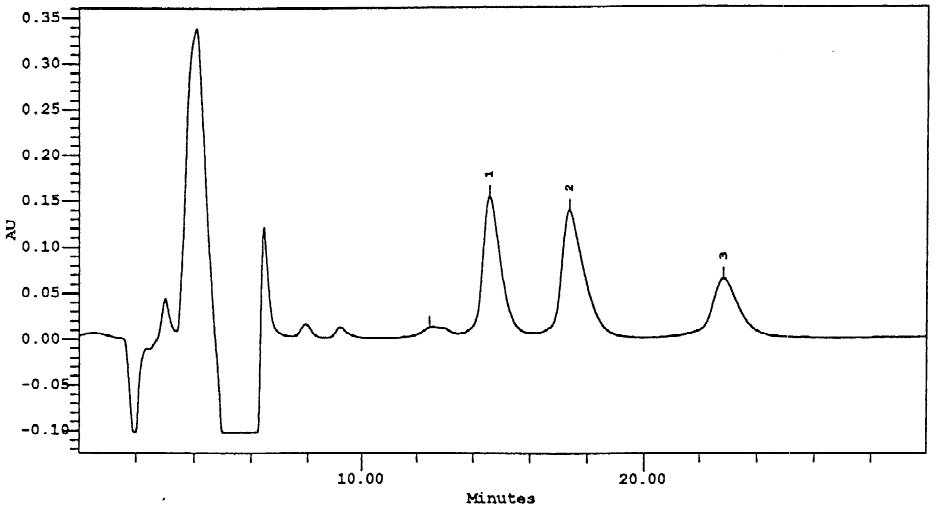

Fig. 4. Separation of lactic acid enantiomers. Conditions: Column, *N,N*-dioctyl-L-alanine on octadecylsilica (50×4.6 mm I.D.), ambient temperature; mobile phase, 2 m*M* copper sulphate in 0.1% acetonitrile, 0.4 ml/min. UV detection at 236 nm. Peak identification: (1) D-lactic acid; (2) L-lactic acid; (3) internal standard—malonic acid. [Laboratory data, Zello et al.].

5.4. Capillary electrophoresis

Capillary electrophoresis (CE) can provide a simple and automated method of separation for enantiomeric analytes using chiral selectors in the separation buffer. CE requires little sample pretreatment, and a small volume of sample. The separation of many compounds, including D- and L-lactic acid has been achieved by using cyclodextrins derivatives and modified crown ethers as chiral selectors [68].

Kodama and co-workers [69,70] developed a method for the separation of lactic acid enantiomers in foods, using capillary electrophoresis with 2-hydroxypropyl-β-cyclodextrin as the chiral selector, with UV detection at 200 nm. This methodology has been [71] adapted and optimized for use in body fluids. Various antibiotics, including ristocetin A have been shown to be excellent chiral selectors, and have been applied to the CE separation of lactic acid enantiomers with UV detection [72].

6. Biological and clinical relevance

The presence of SCFAs in faeces at abnormally high levels can be diagnostic of bacterial overgrowth associated with metabolic acidosis. The origin and nature of metabolic acidosis in short-bowel syndrome and gastroenteritis also requires rapid and reliable methods for the separation and quantification of lactic acid enantiomers.

6.1. Lactic acid production and metabolism

Serum lactate in healthy humans is approximately 2 m*M* and is considered entirely L-lactate, as lactate produced in the body is almost exclusively this isomer [73]. L-Lactic acidosis is most commonly caused by increased peripheral production in tissues due to hypoxia from poor tissue perfusion or by decreased hepatic metabolism of lactic acid [6,74,75]. Under these conditions, glucose is glycolytically converted to pyruvate and L-lactate is used as substrate to produce ATP by the enzyme L-lactate dehydrogenase, with NADH as a cofactor, in the muscle and red blood cells [76] (Fig. 5). Once produced, it can either be slowly oxidized in cells or rapidly converted back to glucose in the liver [6] (Fig. 6). In addition, L-lactic acid may be produced by bacterial fermentation in the gastrointestinal tract [7].

In mammals, D-lactate is formed via the glyoxalase system [77–79] (Fig. 7). It is a ubiquitous reaction in biological life, but produces only physio-

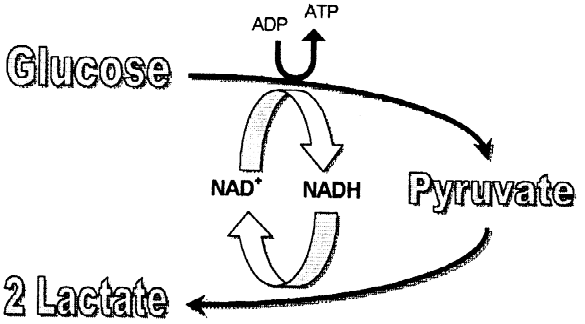

Fig. 5. Glucose is converted to pyruvate and L-lactate to produce ATP by the enzyme L-lactate dehydrogenase, with NADH as a cofactor.

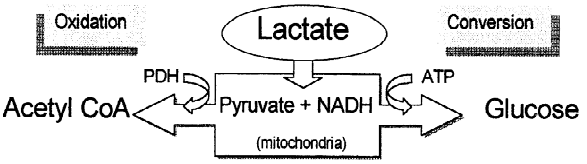

Fig. 6. Lactate utilization. Lactate can be slowly oxidized in the cells to acetyl CoA for the production of ATP or rapidly converted to glucose in the liver via pyruvate with NADH as a cofactor. PDH, pyruvate dehydrogenase; NADH, nicotinamide dinucleotide; ATP, adenosine triphosphate. Adapted from [6].

logically insignificant quantities of D-lactic acid [79]. D-lactate is also produced in the gut by *Lactobacilli* spp. and *Escherichia coli* [80]. D-Lactate is metabolized to pyruvate by the enzyme D-α-hydroxyacid dehydrogenase, which metabolizes D-lactate about one-fifth as fast as L-lactate dehydrogenase metabolizes L-lactate [28,81] (Fig. 8).

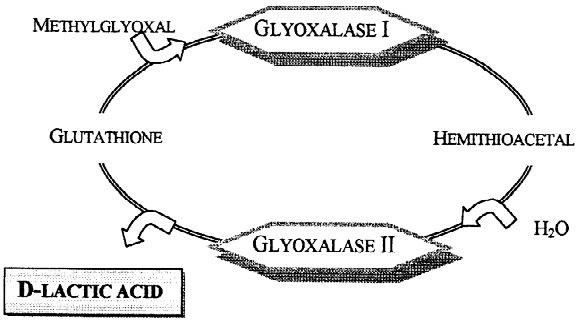

Fig. 7. The glyoxalase pathway. Methylglyoxal is converted to D-lactic acid via the intermediate S-D-lactoylglutathione by two enzymes, glyoxalase I and glyoxalase II, in the cytosol of cells and organelles, expecially the mitochondria. Adapted from [79].

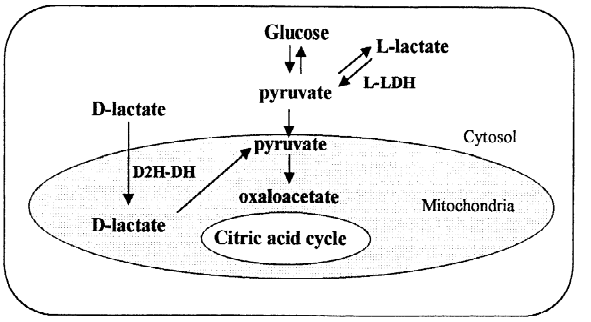

Fig. 8. D- and L-lactic acid metabolism. In contrast to the L-isomer, D-lactate must pass the mitochondrial cell membrane for oxidation. D2H-DH: D-2-hydroxy acid dehydrogenase; L-LDH: L-lactate dehydrogenase.

D-Lactic acidosis as a consequence of short bowel syndrome (SBS) was first described in 1979 [82]. Patients who have had extensive resectioning of the small bowel, leaving behind less than 150 cm are classified as having SBS. Diarrhoea, dehydration, acid–base disturbances and nutrient deficiencies are common, and occasionally, a profound D-lactic acidosis occurs [83]. D-Lactic acidosis has been defined metabolic acidosis accompanied by an increase in serum D-lactate of 3 m*M* or more [73]. Patients with D-lactic acidosis have neurological dysfunction characterized by ataxia, slurred speech and confusion, in association with a high anion gap metabolic acidosis [73,84].

The pathogenesis of D-lactic acidosis in short bowel syndrome is well elucidated. A short or bypassed small intestine causes poor digestion of carbohydrate, which leads to the delivery of sugars to the colon. Bacterial fermentation occurs, leading to increased SCFA production and a decrease in pH. The degree of SCFA production determines the ability of substrate to cause lactate accumulation [85]. The acidic environment permits acid-resistant species like *Lactobacilli* to grow preferentially, yielding both D- and L-lactate, which are then absorbed into the circulation [1,86].

A similar D-lactic acidosis occurs in overfed ruminants, where engorgement on grain causes abnormal fermentation in the rumen. The pathogenesis is nearly identical to that of D-lactic acidosis in SBS, with the exception that the site of fermentation is the rumen, rather than the colon. High levels of SCFAs

have been observed in ruminal acidosis, although how serum concentrations change in acidosis has not yet been investigated [87].

6.2. Acidosis in acute gastroenteritis

Metabolic acidosis is a common sequela of acute gastroenteritis. Recently, knowledge of the pathogenesis of this problem has improved [88,7]. High anion gap acidosis is frequently observed in diarrhoea with moderate or severe dehydration [89]. However, in calves there is no established relationship between acidosis and dehydration and acidosis can occur in the absence of dehydration. Historically, the genesis of metabolic acidosis in diarrhoea has been attributed either to L-lactic acid accumulation in the blood or from loss of bicarbonate in the faeces. Recent studies indicate that L-lactic acidosis is only part of the total acidosis, and that D-lactic acid is a major contributor to the increase in anion gap in the diarrhoeic calf model [7,88]. Increased serum levels of acetic acid and pyruvic acid were also observed. Further investigations into the levels of SCFAs in serum and faeces are required.

Since many of the causative agents implicated in human diarrhea cause villous atrophy, nutrients likely escape small intestinal absorption. It can be theorized that the mechanism is likely similar to that elucidated for D-lactic acidosis in short bowel syndrome except that malabsorption is the result of the infectious agent, not surgical removal of small intestine. In support of our hypothesis, Sack et al. [90] showed that carbohydrate malabsorption in rotavirus diarrhea exacerbates acidosis; however, the pathophysiological mechanism was not identified.

6.3. Other relevant biological conditions

The diagnosis of bacterial fermentation with SCFAs and lactate production is important in other conditions involving bacterial fermentation such as stagnant loop syndrome [91], inflammatory bowel disease [92], sinusitis [30] and vaginal conditions [93].

7. Conclusion

A variety of techniques have been developed over the years for measurement of SCFAs and other acids relevant to bacterial fermentation. GC techniques are most widely used, but newer, more simple HPLC methods are gaining popularity. Methods that determine concentrations of SCFAs and lactic acid simultaneously are of great value in the study of metabolic acidosis of bacterial origin. Many sample preparation methods are now outdated, as more rapid and effective approaches have been developed and validated.

The separation of lactic acid enantiomers has been possible for about the last 30 years. Enzymatic techniques are still used in many laboratories, however, rapid and simple HPLC techniques have eliminated some of the short-comings of enzymatic analyzes. Capillary electrophoresis is also effective in separation lactic acid enantiomers. The application of these methods to biological conditions has enabled the identification of D-lactic acid as a novel origin of acidosis in diarrhoeic calves.

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