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High performance liquid chromatography–tandem mass spectrometry (HPLC/MS/MS) assay for chiral separation of lactic acid enantiomers in urine using a teicoplanin based stationary phase

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Abstract

A novel method for the separation and simultaneous determination of urinary D- and L-lactic acid enantiomers by high performance liquid chromatography–tandem mass spectrometry (HPLC/MS/MS) is presented. The chiral separation was optimized on a Chirobiotic teicoplanin aglycone (TAG) column. Most interestingly, the addition of water in small volume fraction to the polar organic mobile phase was found to significantly improve the chromatography. Calibration curves were linear ($r^2 > 0.9950$) over the range 3–1000 mg/L for L-lactic acid and 0.5–160.8 mg/L for D-lactic acid. The limit of detection (LOD) (S/N = 3) and limit of quantification (LOQ) (S/N = 10) were determined experimentally ($n = 3$) to be 0.2 and 0.5 mg/L for L-lactic acid and 0.4 and 1.3 mg/L for D-lactic acid, respectively. The normal patient range of L-lactic acid was 1–20 μg/mg creatinine with an elevated value of 85 μg/mg creatinine. For D-lactic acid, the range of normal values were between 0 and 5 μg/mg creatinine with an elevated value of 40 μg/mg creatinine. Finally, the validated method allows for rapid analysis with a total run time of 7.5 min.

Keywords: Chiral separation; D-Lactic acid; L-Lactic acid; Enantiomers; HPLC/MS/MS; Urine; Chirobiotic TAG

1. Introduction

Lactic acid (2-hydroxypropanoic acid, pKa 3.86) is a small organic acid of biological importance that was first isolated from sour milk by Scheele in 1780 [1]. Biochemical processes of lactic acid range from anaerobic by-product of exercise to fermentation useful for the production of yogurt, cheese and even wines. There are two optically active chiral enantiomers of lactic acid (Fig. 1) designated L-(+)-lactic acid or (*S*)-lactic acid, and the mirror image D-(−)-lactic acid or (*R*)-lactic acid. These two optical isomers are very different in terms of biological origin and metabolic significance. Whereas L-lactic acid is naturally occurring in mammals produced by anaerobic reduction of pyruvate, only about one percent of D-lactic acid is generated via the methylgloxylase pathway [2]. Instead, D-lactic acid detectable in human physiological fluids often originates from bacteria present in the intestinal tract or gut. Bacterial overproduction of D-lactate in the gut is inherent to patients with short bowel syndrome (SBS) or after jejunoileal surgery. In the human gut, malabsorption of excess carbohydrates due to SBS or surgical procedure can lead to elevated amounts of both D- and L-lactate produced by intestinal flora [3]. Eventually, this build up of excess organic acid can ultimately result in a condition known as metabolic acidosis and if left untreated, significant neurological symptoms can manifest [1,4]. Therefore, the analytical determination of lactic acid enantiomers especially D-lactate is warranted.

Several analytical techniques have been applied for the enantiomeric separation and detection of L- and D-lactic acid including ligand exchange high performance liquid chromatography (HPLC) [5–8], gas chromatography (GC) [9], and capillary electrophoresis (CE) [2,10,11]. More traditional methods including enzymatic assays (EA) [12,13] using ultraviolet (UV) spectrophotometric and immunoturbidimetric detection [14] have also been employed. Although these techniques provide adequate analysis, in many cases there is room for improved methodology in terms of compatibility for the clinical laboratory setting. These requirements include rapid sample preparation,

Abbreviations: TAG, teicoplanin aglycone; HPLC/MS/MS, high performance liquid chromatography–tandem mass spectrometry

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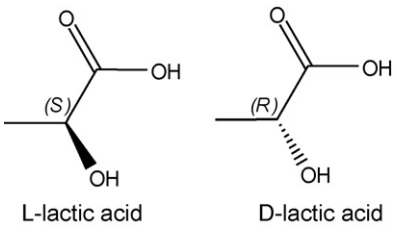

Fig. 1. Chemical structure of lactic acid enantiomers.

the ability for high throughput as well as a detector capable of achieving low limits of detection. Consequently, the current methods that require derivatization (e.g., GC), require rigorous sample clean up (e.g., CE), such as solid phase extraction (SPE), or undergo cross reaction during the analysis (e.g., EA) can be time consuming and problematic. The use of HPLC coupled with tandem mass spectrometry (MS/MS) detection is a well suited alternative that can overcome many of the shortcomings inherent to current methods. Some of the advantages of HPLC/MS/MS include the combination of highly selective separation of HPLC along with the sensitivity and specificity of detection from tandem MS. In many cases, this allows for a simple sample preparation procedure which is advantageous as compared to other techniques. For these reasons, we explored the use of a macrocyclic glycopeptide chiral column coupled to tandem MS.

The capability of macrocyclic glycopeptide chiral columns including teicoplanin and vancomycin for complex chiral separations using HPLC has received growing interest [15–17]. These phases often avoid the use of inorganic buffers and rarely require the use of classical normal phase solvents, such as hexanes. Instead the mobile phase is comprised of short chain alcohols (e.g., methanol, ethanol, isopropanol) containing volatile salts and acids (e.g., ammonium salts, acetic acid). Therefore, these mobile phases are well suited for use with MS detection. Moreover, although our literature search suggests that a lot of fundamental study has been conducted, the application of teicoplanin based chiral separations in the clinical laboratory setting has not been thoroughly explored. In this work, a novel method for simultaneous and sensitive determination of D- and L-lactic acid enantiomers in urine by HPLC/MS/MS is presented. To our knowledge, this is the first application of teicoplanin based column coupled to tandem MS for fast biological analysis of lactic acid enantiomers in human urine.

2. Experimental

2.1. Chemicals and reagents

Triethylamine (TEA) 99%, L(+)-lactic acid 98%, and thymol 99.5% were obtained from Sigma–Aldrich (St. Louis, MO, USA). D-lactic acid sodium salt 99% was purchased from Fluka, a division of Sigma–Aldrich. HPLC grade ethanol (EtOH) and methanol (MeOH) were obtained from Burdett and Jackson (Muskegon, MI, USA). Glacial acetic acid (HOAc) was purchased from J.T. Baker (Phillipsburg, NJ, USA). Saline solution 0.85–0.90% (w/v) was obtained from VWR (West Chester, PA, USA). The internal standard, sodium L-lactate-3,3,3-D_3 (99.8% atom %D) was purchased from CDN isotopes (Quebec, Canada).

2.2. Instrumentation

Samples were analyzed on a Waters Quattro-LC tandem mass spectrometer equipped with an electrospray ionization source. All analytes were detected in electrospray negative (ESI−) ionization mode. Chromatographic separations were performed on a Waters (Milford MA, USA) 2695 high performance liquid chromatograph (HPLC). The collected data was processed using MassLynx (version 4.0).

2.3. Mass spectrometry conditions

Initially, the direct in-line infusion of standard L-lactic acid (100.0 mg/L) and D-lactic acid (16.1 mg/L) into the MS was performed in order to establish the most sensitive operating conditions. Table 1 shows the optimized MS parameters for electrospray negative mode. For multiple reaction monitoring (MRM), the parent molecule $[M\text{-}H]^-$ of lactic acid was equal to 89.2 *m/z* and the daughter ion was found to be 43.4 *m/z* which were both collected at unit mass resolution with a dwell time of 0.33 s. The MRM of the deuterated internal standard (Deuterated (D_3)–L-lactate) was 92.0 and 45 *m/z* for parent and daughter ions, respectively. For increased sensitivity, the cone gas was set to zero. These settings were utilized for all further study.

2.4. Chromatographic conditions

Chromatographic separation was conducted using a Chirobiotic teicoplanin aglycone (TAG) chiral column, 150 mm × 2.1 mm (Advanced Separation Technologies, Whippany, NJ, USA). The chromatographic chiral separation of the

Table 1
Optimized mass spectrometry settings

Source (ES-)	
Capillary (kV)	1.50
Cone (V)	25
Extractor (V)	1
RF lens (V)	0.1
Source temperature (°C)	145
Desolvation temperature (°C)	350
Cone gas flow (L/Hr)	OFF
Desolvation gas flow (L/Hr)	700
Nebuliser gas flow (L/Hr)	80
Analyzer:	
LM 1 Resolution	15
HM 1 Resolution	15
Ion energy 1	1
Entrance	−5
Collision	11
Exit	1
LM 2 Resolution	15
HM 2 Resolution	15
Ion energy 2	1.0
Multiplier (V)	650

D- and L-lactic acid enantiomers was optimized by systematic tuning of the liquid chromatography (LC) mobile phase composition. For the initial screening and investigation, a suitable starting mobile phase composition consisting of MeOH along with small volume addition of HOAc and TEA were selected. Using three mobile phase reservoirs, the ratio of acid/base was manipulated to achieve the optimum conditions. The first mobile phase was pure MeOH, the second mobile phase was MeOH containing 0.5% HOAc, the third mobile phase was MeOH containing 0.5% TEA. This setup allowed the varying percentage of acid or base to be delivered into the pure MeOH. Following this experiment, different solvents were investigated, such as EtOH and also addition of H_2O. For these, the acid and base were contained in two respective EtOH reservoirs, while the H_2O was delivered using the third reservoir. Overall, the final optimized isocratic mobile phase conditions consisted of 17% H_2O and 83% EtOH/0.12% HOAc/0.30% TEA. The sample injection volume was 25 μL and the total run time was 7.5 min. The column temperature and flow rate were optimized to 25 °C and 0.2 mL/min, respectively.

2.5. Calibration standards and sample preparation

Nine standard calibrator solutions (Table 2) covering high and low range were prepared and used to establish the accuracy and linearity of the method. The standards contained L-lactic acid at nine concentrations of 3.1 to 1000 mg/mL, and D-lactic acid at nine concentrations of 0.5 to 160.8 mg/mL. In order to more closely match the urine sample matrix of the patient sample, calibrators were constituted in saline solution. Calibrators were sequentially prepared from a stock solution (Cal. 9) using saline for equal volume dilution of each calibrant. For the internal standard (I.S.), 40 μg/mL of deuterated D_3-L-lactic acid was prepared by weighing 2 mg into 50 mL volumetric flask using saline to fill to the mark. Next, 9.1 mL of this solution was transferred to a 100 mL volumetric flask using a glass volumetric pipette. This was followed by addition of EtOH to fill to the mark.

The final working solution for calibration was prepared by transferring 300 μL of calibrator to a sample vial followed by addition of 900 μL of the I.S. solution. This solution was briefly mixed prior to injection. Patient urine samples were collected in tubes containing 20 μL of thymol (0.05 mg/mL) as a preservative, and stored at −20 °C. For patient sample preparation, 300 μL of urine was transferred to a 1.7 mL micro centrifuge tube followed by addition of 900 μL I.S. solution. The tube was then briefly mixed. Finally, the tube was centrifuged at 7000 rpm for 5 min and the resulting supernatant was transferred using glass pipette to the sample vial avoiding collection of any solid precipitate.

Table 2
Concentrations of the nine standard calibrant solutions used for validation

	L-Lactic acid	D-Lactic acid
Cal #9	1000.0	160.8
Cal #8	500.0	80.4
Cal #7	200.0	32.2
Cal #6	100.0	16.1
Cal #5	50.0	8.0
Cal #4	25.0	4.0
Cal #3	12.5	2.0
Cal #2	6.3	1.0
Cal #1	3.1	0.5

Concentration in mg/L.

2.6. Matrix effects study

For determination of ion suppression effects due to variation in sample matrix, a comparison of the area under the curve (AUC) versus the response factor (ratio of analyte area divided by internal standard area) was conducted for calibrators in saline versus two urine matrices. Following the experimental protocol described by Annesley [18], a similar study was designed for this work. First, six tubes containing 500 μL of pooled urine were evaporated to dryness using N_2 at 50 °C for 40 min. Next, five calibrators (Cals. #9, 7, 5, 3, 1 of Table 2) in saline plus a blank were used to reconstitute the dried urine. For comparison, the second urine matrix was comprised of standards D-and L-lactic acid weighed directly into pooled urine. All matrix study samples were then prepared in the same fashion as normal calibrators. A comparison of the instrument response to three sets of calibrators was then evaluated.

2.7. Method validation

Method validation was based upon linearity, patient ranges, quality control, accuracy, precision, and sample stability. Linearity was evaluated by using a nine point calibration curve. The accuracy was determined by conducting a spiked recovery experiment. The precision was assessed for both intraday (within run) and interday (between run) using normal and elevated controls. Finally, the short term stability of sample preparation was determined from quantitative results of three samples stored at 5 °C on the HPLC instrument over a 3 day period. The long term stability was evaluated using normal and elevated controls under various storage conditions.

2.7.1. Linearity and sensitivity

The linear calibration curve was comprised of nine calibration levels as shown in Table 2. The linearity was determined by linear regression, including the intercept ($y = mx + b$) and weighted by $1/x$ in order to correct for larger standard deviation at the upper end of the calibration curve. All calculations were performed using EP Evaluator 6 software, (RHOADS, Kennett Square, PA, USA) and MassLynx (version 4.0).

Sensitivity was evaluated by the determination of limit of detection (LOD) and limit of quantification (LOQ) using the linear regression approach [19]. The LOD and LOQ were calculated at a signal to noise ratio of 3 and 10, respectively.

2.7.2. Patient ranges and quality control samples

Patient ranges were established using intra-laboratory urine samples. All concentration measurements were normalized to

creatinine in order to correct for urinary dilution [20]. Data were collected from approximately 130 samples to calculate a working within laboratory range for both normal and elevated results. The ranges established are relevant to adult patients 13 years of age and older. Urinary creatinine concentration was measured on a Cobas Mira Plus using a creatinine assay kit obtained from Roche (Quebec, Canada) following modification of Jaffe's picric acid method [20].

For quality control (QC) sample, normal and elevated controls were prepared from pooled urine. Elevated controls were prepared by weighing 60.0 mg/L of L-lactic acid, and 25.1 mg/L of D-lactic acid into the normal control urine. Both the normal and elevated controls were aliquoted then stored in the freezer at −20 °C.

2.7.3. Accuracy and precision

The accuracy of the method was evaluated by performing a spiked recovery experiment. For this, a mixture of pooled patient samples was spiked with a standard solution of no more than 10% initial patient volume. A baseline was obtained by running pooled urine spiked with 10% (urine volume) of saline. The results were calculated based on the average of four successive measurements for each level.

Within run precision was calculated using normal and elevated ($n = 10$) controls over 1 day. Between run precision was determined using normal and elevated controls ($n = 20$) over a 10 day period.

2.7.4. Short term stability of sample preparation

The short term stability of the patient sample following preparation was determined over a period of 3 days (72 h). Three patient samples were prepared and stored on the HPLC instrument compartment at 5 °C. Following the initial measurement on day 1, the samples were analyzed at 24 h intervals for 3 consecutive days. The stability was evaluated by a comparison of the mean value of each sample with the initial value and reported as a percentage of the initial value.

2.7.5. Long term stability of sample storage

In order to assess the stability of shipped and received samples, the long term stability of the patient sample was evaluated over a period of 3 weeks. For this, a pooled patient urine was collected and spiked with three levels of D- and L-lactic acid as follows. Sample A was prepared by adding 100 mg/L L-lactic acid and 40 mg/L D-lactic acid to the pooled urine, sample B was 50 mg/L L-, 20 mg/L D-, sample C was 25 mg/L L-, 10 mg/L D-, and sample D was unspiked. Aliquots of 14 mL pooled urine were then placed into specimen collection tubes containing 20 μL of thymol preservative. The unspiked samples were then run experimentally in order to measure the baseline (day 1 = baseline). Next, all specimens were frozen overnight, the next day removed and put into shipping boxes containing ice packs in order to simulate being shipped to the laboratory. After being stored an additional day at room temperature in the shipping boxes (day 2), the samples were then run experimentally

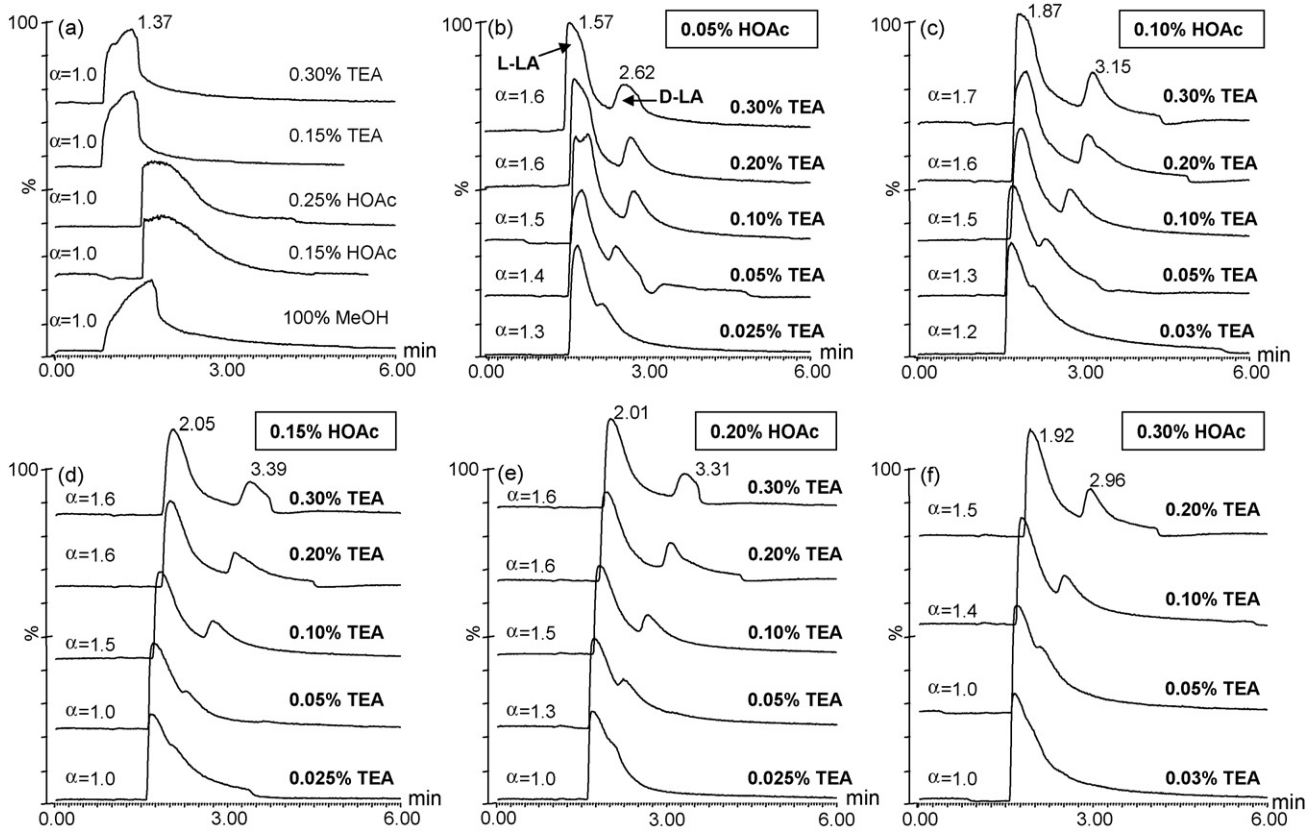

Fig. 2. Chromatograms showing the effects of varying mobile phase MeOH/HOAc/TEA ratio (v/v) on the separation of D- and L-lactic acid enantiomers. Conditions: flow rate 0.35 mL/min, temperature 25 °C, injection volume 25 μL. For ESI-MS conditions please see Section 2. Analytes: Cal. 6 of Table 2.

(day 3 = received). Then all samples were aliquoted in order to test the effects of storage conditions. All four levels of sample were measured after storing at room temperature (RT) (25 °C), frozen (F) (−20 °C), refrigerated (R) (5 °C), and freeze thaw (FT). Initially, all samples were run for 3 consecutive days (day 3, 4, 5) to evaluate stability. Determination of 3 week stability (W1–W3) was then conducted for RT, F, and R storage. The effects of FT were evaluated on day 3–5 to establish the effects of freezing then thawing the patient sample. Frozen samples were allowed to equilibrate to room temperature, without heating, prior to analysis. The % difference of each measurement from the initial value was reported.

For evaluation of interference due to addition of thymol sample preservative, internal standard in EtOH (900 μL) was added to appropriate aliquot of thymol (300 μL) then analyzed as a patient sample.

3. Results and discussion

3.1. Optimization of the HPLC chiral separation

The chiral separation was optimized utilizing isocratic conditions as these provide faster analysis due to the absence of column re-equilibration steps. A Chirobiotic TAG column was chosen because the polar ionic stationary phase is well suited for the separation of small polar molecules, such as lactic acid. Since the chiral stationary phase contains ionizable groups, such as amines, amides, carboxylic acids, and phenols, the addition of acid and base (i.e., HOAc versus TEA) allows for the possibility of retention based on an ion exchange mechanism.

The optimization of the ion exchange mechanism was evaluated by experimentally varying the percentage of MeOH/0.5%HOAc and MeOH/0.5%TEA on column. The results of the experiment can be seen in Fig. 2. No separation was observed when using 100% MeOH or when varying percentages of HOAc and TEA alone (Fig. 2a). However, it is clear that addition of HOAc promotes greater retention while the addition of TEA reduces peak tailing. Next, the effects of maintaining a fixed HOAc percentage while varying the TEA concentration were examined (Fig. 2b–f). In general, increasing the mobile phase concentration of TEA resulted in greater chiral separation when the concentration of HOAc was held constant. A decrease in tailing for D-lactic acid and improved peak shape from 0.10 to 0.20% HOAc was observed. Overall, we established that 0.15% HOAc and 0.30% TEA was an adequate starting point for further optimization of the chromatography.

As seen in Fig. 2, the retention time of both enantiomers is near the void, suggesting that the small polar lactic acid has a high affinity for the polar MeOH mobile phase. Therefore, the effects of adding or combining a more non-polar organic solvent to the mobile phase was investigated. First, the addition of EtOH to the MeOH containing HOAc/TEA mobile phase was conducted and dramatic improvements in the separation were observed due to increased retention. From this observation, while the ratio of HOAc/TEA was maintained, MeOH was replaced with EtOH in all mobile phases. Along with an increase in retention of each enantiomer, higher resolution at the cost of peak broadening and total anaylsis time was observed. Therefore, in order to increase the overall efficiency, the addition of water to the mobile phase was investigated.

The effects of adding H_2O to EtOH can be seen in Fig. 3. Pure EtOH (Fig. 3a) results in longer retention and poor sensitivity for D-lactic acid shown by the inset expanded view. Impressively, upon addition of H_2O over the range of 0–25% (Fig. 3a–g), a reduction in retention was achieved along with significant improvement in both the resolution and sensitivity of D-lactic acid. It was observed that when going from 17 to 25% H_2O (Fig. 3e and f), the retention continued to decrease, however the extent of baseline resolution was slightly diminished as shown inset. From this study, the final optimized conditions of 17% H_2O, 83% EtOH/0.12% HOAc/0.30% TEA (Fig. 3e) were established as the final mobile phase conditions.

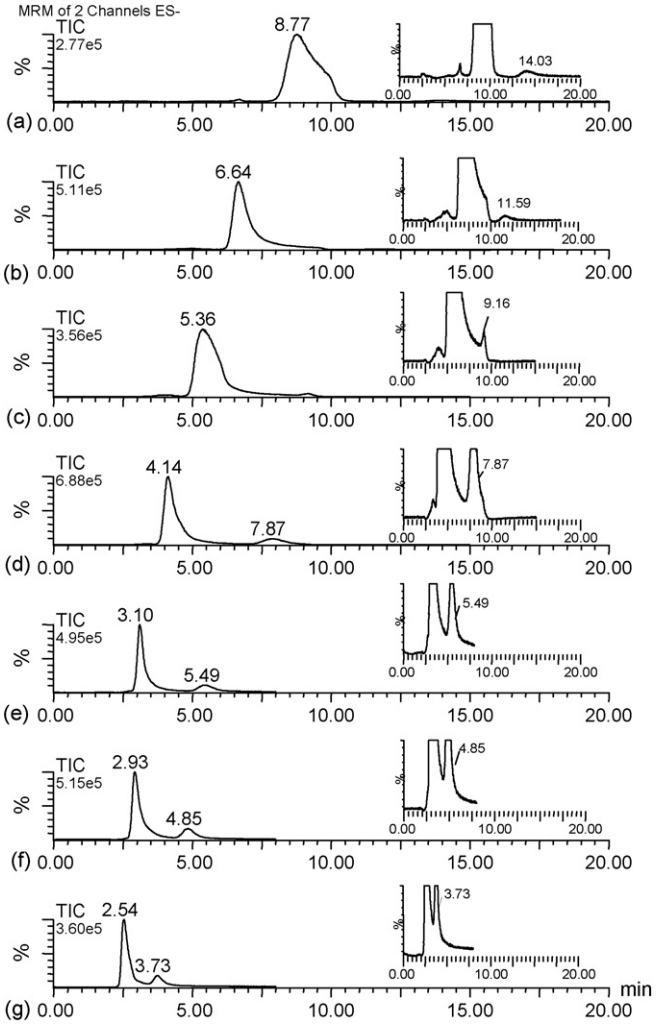

Fig. 3. Chromatograms showing the effects of adding H_2O to the polar ionic mobile phase. Conditions are the same as Fig. 2 except the flow rate was 0.2 mL/min and (a) 0% H_2O, 100% EtOH/0.15% HOAc/0.35% TEA, (b) 3% H_2O, 97% EtOH/0.15% HOAc/0.34% TEA, (c) 6% H_2O, 94% EtOH/0.15% HOAc/0.32% TEA, (d) 10% H_2O, 90% EtOH/0.13% HOAc/0.32% TEA, (e) 17% H_2O, 83% EtOH/0.12% HOAc/0.30% TEA, (f) 20% H_2O, 80% EtOH/0.12% HOAc/0.29% TEA, (g) 25% H_2O, 75% EtOH/0.10% HOAc/0.28% TEA, analytes: Cal. 5 of Table 2.

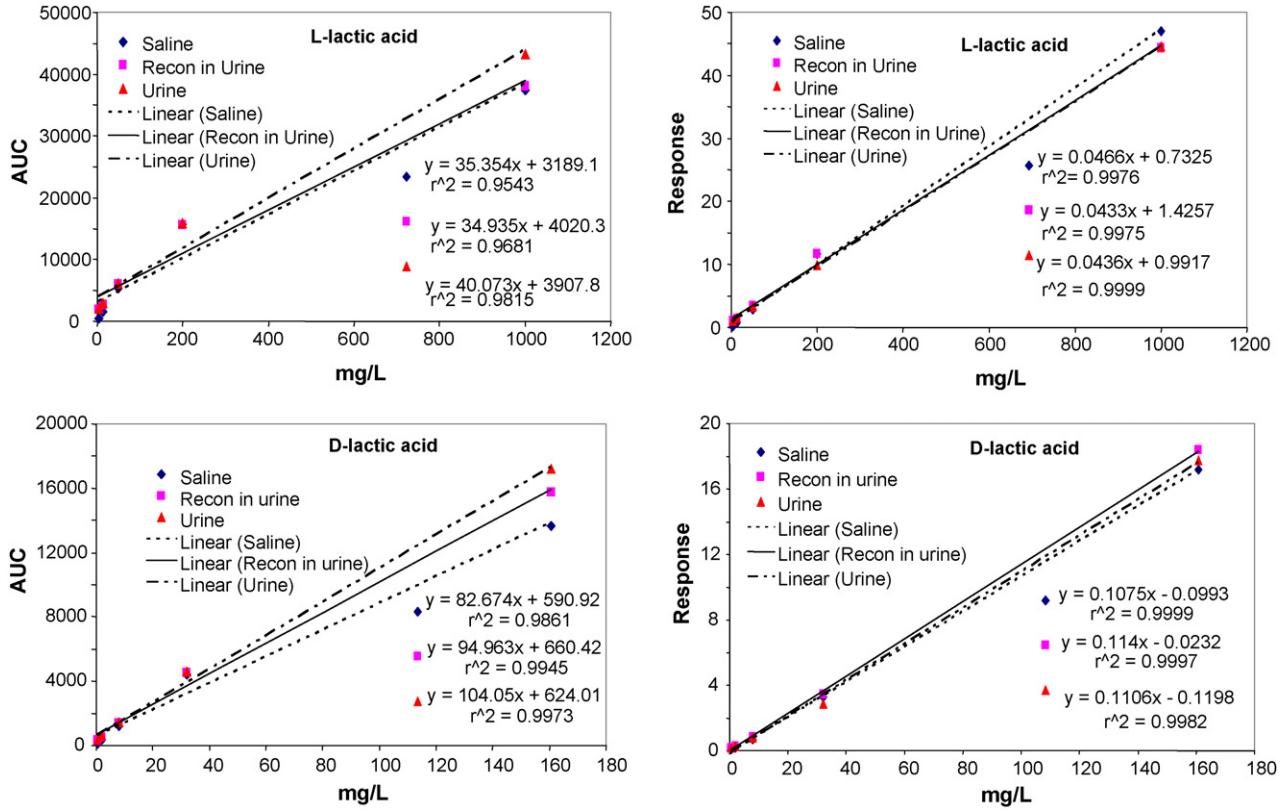

Fig. 4. Effects of sample matrix on the peak area under curve (AUC) and the instrument response factor relative to internal standard. Conditions: same as Fig. 3e. Analytes: Cals. 9, 7, 5, 3, 1 of Table 2.

From the results of our systematic optimization of the mobile phase compositions, several conclusions regarding the mechanism of retention and separation can be reached. The chiral separation of lactic acid enantiomers is believed to be the result of three stationary phase interactions. The first involves the electrostatic interaction of lactic acid with stationary phase amines. This interaction is promoted by the addition of TEA and HOAc to the mobile phase. The basic character of TEA facilitates the ionization of lactic acid to the respective conjugate base, while the addition of HOAc helps to ionize basic stationary phase amines to their respective conjugate acid. The action of these mobile phase additives on both analyte and stationary phase results in the electrostatic interactions between the two. The second interaction relates to the possibility of lactic acid forming hydrogen bonds between the alpha hydroxyl group and the peptide amido groups of the stationary phase. As these two hydroxyl groups are orientated differently on the D- and L-lactic acid molecules, this results in the selective chiral docking with the stationary phase. Ultimately, the enantiomeric separation is observed with a greater molecular recognition for D-lactic acid indicated by increased retention. The third and final point can be summarized as small hydrophobic and weak steric interactions which are considered negligible given that lactic acid is a very hydrophilic small molecule. Overall, the optimized ratio of 0.12% HOAc with 0.30% TEA are consistent with the three point mechanism. For the addition of H_2O to the mobile phase, it can be suggested that the improved solubility of hydrophilic lactic acid in the more polar mobile phase enhances the peak shape and reduces retention. Furthermore, it is possible for the stationary phase to undergo a conformational change in the presence of water that alters the distance for hydrogen bonding as mentioned in step two above. In this manner, the careful and systematic evaluation of the chromatography was assessed.

3.2. Evaluation of matrix effects

Variation of the sample matrix has been shown to contribute to ion suppression using MS detection [18,21]. Therefore, a comparison of sample matrix effects on the MS response was conducted in this work. Normal working solutions of calibrators dissolved in saline were compared to the same calibrators prepared in two urine matrices. The first urine matrix was made by drying down pooled urine followed by reconstituting with calibrator in saline. The second urine matrix was prepared by weighing standard L- and D-lactic acid directly into pooled urine. Matrix effects were evaluated by comparing the area under the curve (AUC) to the response factor ratio of standard area divided by internal standard area. From Fig. 4, a comparison of the linear plots for the peak area under the curve (AUC) versus concentration for both L- and D-lactic acid shows variation between different matrices. These deviations are seen in both slope and correlation (r^2), comparatively. Using a deuterated internal standard, as seen in the linear plot of response versus concentration, the above mentioned variations are greatly

reduced. The observed slopes for the internal standard corrected plots compared well for all matrices (C.V. < 5%) and showed linear correlations of $r^2 > 0.997$.

3.3. Method validation

3.3.1. Linearity and sensitivity

The linearity was evaluated based on the average of nine calibrators run in duplicate including a blank which were all calculated from a standard curve. For both enantiomers, acceptable linearity was achieved. Using EP Evaluator (version 6), the observable error based upon the linearity of calibration was found to be less than 8% for both enantiomers. For linearity of L-lactic acid, the slope was 1.01 and the intercept was −0.06. For D-lactic acid, the slope was 1.02 and the intercept was −0.04. The MassLynx4.0 correlation factors (r^2) were found to be greater than 0.995 for both enantiomers.

For sensitivity, the LOD (S/N = 3) and LOQ (S/N = 10) were determined experimentally ($n = 3$) based upon the linear regression from the established linear range of calibration [19]. For L-lactic acid, the LOD was found to be 0.2 mg/L with LOQ of 0.5 mg/L. For D-lactic acid, the LOD was found to be 0.4 mg/L with LOQ of 1.3 mg/L.

3.3.2. Patient ranges

Adult normal and elevated patient ranges were determined from approximately 130 intra-laboratory samples. The normal range was established using the 95% confidence interval based upon the collected patient data. Any values above this range were considered elevated. The established ranges, corrected for creatinine, are presented in Fig. 5a and b. For L-lactic acid, Fig. 5a shows that the majority of samples were between 0 and 20 μg/mg creatinine, and the highest sample was 85 μg/mg creatinine. For D-lactic acid, Fig. 5b indicates that that the range of normal values were between 0 and 5 μg/mg creatinine, and the highest value was 40 μg/mg creatinine. The elevated control values were chosen to represent elevated values outside of the 95% confidence interval (C.I.) of these patient samples. Fig. 5c shows the correlation between L- and D-lactic acid for the patient samples. Finally, an example chromatogram showing the normal and elevated controls can be seen in Fig. 6 which clearly demonstrates the increase in signal and peak area upon addition of the standard enantiomers into normal control urine.

3.3.3. Accuracy and precision

The accuracy of the assay was evaluated based on the percent recovery for five levels of spiked urine samples compared with a baseline of pooled urine. The average percent recoveries are presented in Table 3 which shows that the percent deviation from the theoretical value for the recovered spike was less than 15% R.S.D. except in one case at level 1 for L-lactic acid. Overall, both analytes showed average recovery greater than 86%.

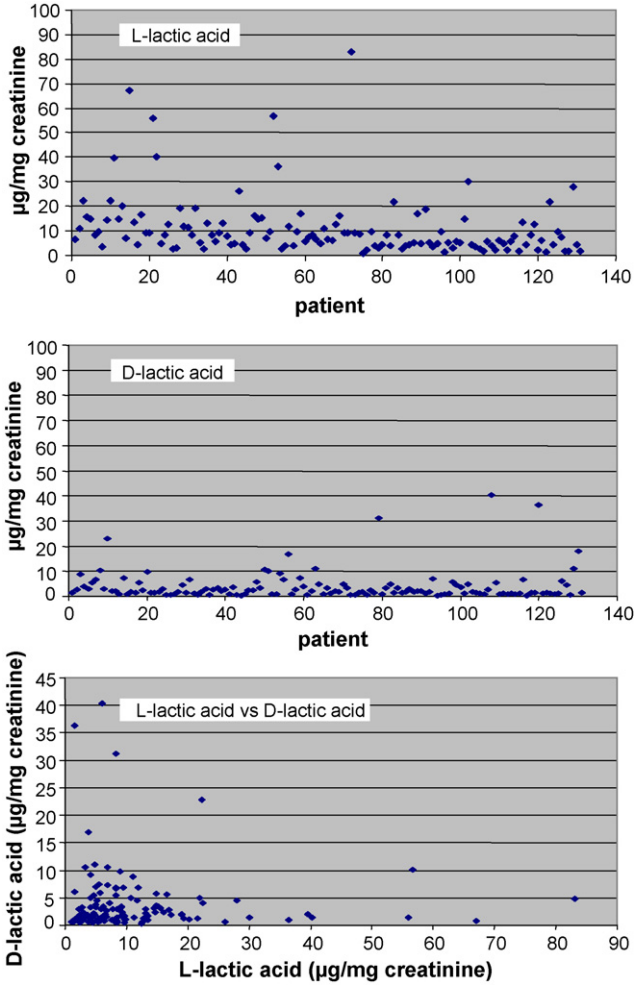

Fig. 5. Scatter plots show the ranges of (a) L-lactic acid and (b) D-lactic acid obtained for approximately 130 patient urine samples. Plot (c) illustrates correlation between D- and L-lactic acid for individual patients. Conditions are the same as Fig. 3e.

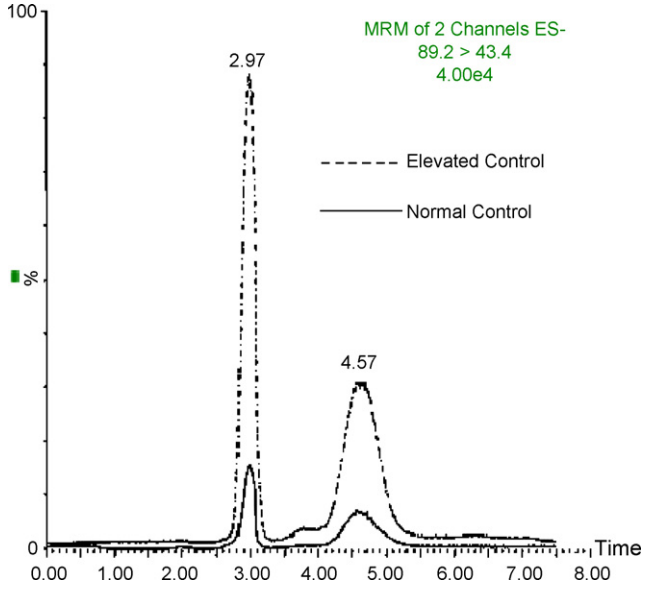

Fig. 6. Chromatograms of the normal and elevated controls. Normal control comprised of patient pooled urine. Elevated control is normal control spiked with 60.0 mg/L L-lactic acid and 25.1 mg/L D-lactic acid. Conditions: same as Fig. 3e.

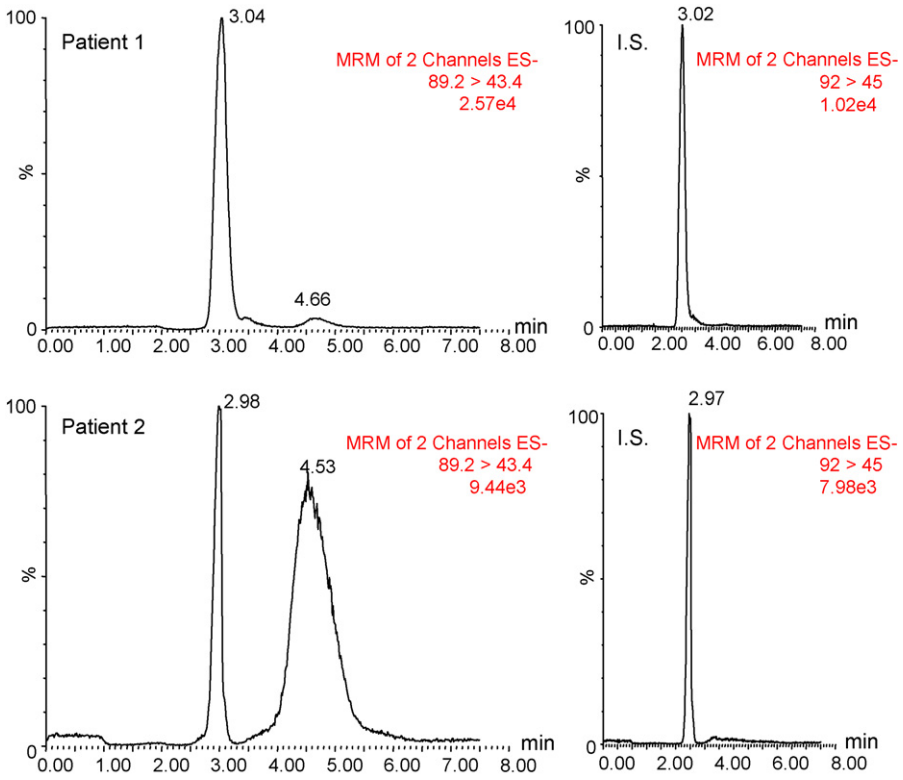

Fig. 7. Chromatograms comparing patient 1 with low D-lactic acid, and patient 2 with elevated D-lactic acid. Conditions: same as Fig. 3e.

Table 3
Summary of the spiking and recovery

	Added (mg/L)		Added (mg/L)	
	L-Lactic acid	%Recovery	D-Lactic acid	%Recovery
Level 5	400	85.4	64.0	113.0
Level 4	200	85.2	32.0	102.7
Level 3	100	90.8	16.0	101.3
Level 2	50.0	89.2	8.00	89.1
Level 1	10.0	81.7	1.60	95.9
Average		86.5		100.4
%R.S.D.		4.1		8.8

All samples run four times ($n=4$), average is reported, % recovery calculated as follows: ((experimental − base)/calculated) × 100. Conditions: same as Fig. 3(e).

Precision was assessed using normal and elevated controls. For the intraday precision, 10 samples of each control were evaluated in one run. As shown in Table 4, the within-run (intraday) precision was less than 6% R.S.D. for all normal controls and no greater than 4% R.S.D. for elevated controls. For interday (between run) precision, two normal ($n=2$) and elevated controls ($n=2$) were run over a period of 10 days. The precision was less than 7% R.S.D. for L-lactic acid both normal and elevated control. For D-lactic acid the between run variation was higher although less than 15% R.S.D.

3.3.4. Sample stability and analysis of patient urine

First, the short term preparation stability of the sample was evaluated by analysis of three patient samples over a period of 72 h when stored in the refrigerated HPLC sample compartment at 5 °C. Table 5 shows that for each patient sample, an acceptable deviation of concentration was obtained which was less

Table 4
Precision of normal and elevated controls in urine

	Normal control		Elevated control	
	L-Lactic acid	D-Lactic acid	L-Lactic acid	D-Lactic acid
Intraday (within run)				
Mean concentration, mg/L	11.4	3.6	69.0	23.7
%R.S.D.	2.9	5.3	3.9	3.4
Interday (between run)				
Mean Concentration, mg/L	10.6	3.8	67.1	24.3
%R.S.D.	6.9	14.6	4.8	10.7

Normal control no addition. Elevated control added 60.0 mg/L L-lactic acid and 25.1 mg/L D-lactic acid. Intraday $n=10$. Interday $n=20$. Conditions: same as Fig. 3(e).

Table 5
Short term 3 day preparation stability of patient sample

	Initial value		Average concentration (±average percentage of initial value)					
			24 h		48 h		72 h	
	L-Lactic acid	D-Lactic acid	L-Lactic acid	D-Lactic acid	L-Lactic acid	D-Lactic acid	L-Lactic acid	d-Lactic acid
Patient sample 1	16.5	60.5	16.1 (−2.6)	60.1 (−0.7)	15.3 (−7.6)	64.5 (6.5)	16.0 (−3.4)	60.4 (−0.3)
Patient sample 2	16.9	25.2	16.9 (0.2)	25.5 (1.0)	16.3 (−3.8)	25.6 (1.3)	16.3 (−3.4)	25.0 (−0.8)
Patient sample 3	60.5	114.8	63.7 (−5.1)	114.5 (−0.2)	61.7 (−1.9)	117.6 (2.5)	61.4 (−1.4)	113.5 (−1.1)

$n = 3$, mg/L. Conditions: same as Fig. 3(e).

than 8% R.S.D. Therefore, it can be concluded that samples were stable for at least 3 days under the experimental conditions. For longer term stability, the effects of storing the urine sample at RT, F, R, and FT cycles were examined. An acceptable deviation from the initial value was achieved when using either R, F and FT cycles over a 2 week period. After 3 weeks, all samples were found to be stable with the exception of sample B which had ∼25% deviation from the initial value. The long term storage of samples at RT was shown not to be stable. Finally, the effect of thymol sample preservative was found to have no interference. The results showed that only the signal from internal standard was observed, and that no signal was present for MRM of lactic acid (89.2 > 43.4). For analysis of patient sample with elevated D-lactic acid, Fig. 7 provides representative chromatograms showing the patient 1 with normal level of D-lactic acid, and patient 2 with elevated level. The MRM of the internal standard is provided for reference.

4. Conclusions

The developed method allows for rapid and sensitive analysis of both L- and D-lactic acid in urine following a quick and easy sample preparation. Optimized mobile phase conditions were 17% H_2O, 83% EtOH/0.12% HOAc/0.30% TEA delivered isocratically at 0.2 mL/min. A matrix study was performed which showed that minor variation due to sample matrix was corrected for using the deuterated internal standard. The LOD for L-lactic acid was found to be 0.2 mg/L with LOQ of 0.5 mg/L. For D-lactic acid, the LOD was found to be 0.4 mg/L with LOQ of 1.3 mg/L. The linearity of the calibration curves was found to be acceptable with low observable error and $r^2 > 0.9950$ for both enantiomers. Patient ranges varied from 1 to 85 μg/mg creatinine of L-lactic acid and from 0 to 40 μg/mg creatinine of D-lactic acid which are comparable to those established in the literature [9]. A summary of the spiking and recovery experiment showed average recovery to be 86.5 ± 4.1% for L-lactic acid and 100.4 ± 8.8% for D-lactic acid. The precision of the method was evaluated using normal and elevated controls. For the within run precision ($n = 10$ replicate runs), the %R.S.D. was found to be overall lower than 6% R.S.D. The between run precision ($n = 2$) measured over 10 days was slightly higher with %R.S.D. no greater than 14.6%. Finally, the short and long term stability of the patient sample was evaluated and was deemed to be stable for 3 days stored on-instrument and also when refrigerated or frozen for greater than 2 weeks. Overall, this method has been validated for clinical applications and represents a rapid and robust assay for the quantification of both L- and D-lactic acid in urine.

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