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A simple, sensitive and efficient assay for the determination of D- and L-lactic acid enantiomers in human plasma by high-performance liquid chromatography

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ABSTRACT

A new method for the simultaneous determination of D- and L-lactic acid in human plasma has been developed using high-performance liquid chromatography (HPLC) with fluorescence detection. This method is based on the reaction of lactic acid with (2*S*)-2-amino-3-methyl-1-[4-(7-nitro-benzo-2,1,3-oxadiazol-4-yl)-piperazin-1-yl]-butan-1-one (NBD-PZ-Val) in the presence of *O*-(7-azobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and *N*-ethyldiisopropylamine (DIEA) to produce fluorescent diastereomeric derivatives that were easily monitored fluorimetrically at λ_{ex} = 490 nm and λ_{em} = 532 nm. The separation was achieved by use of a C18 analytical column (Synergy Hydro 150 mm × 3 mm i.d., 4 μm). The calibration curve was linear over the on-column concentration range of 10–200 μmol/L for D-lactic acid and 0.5–4.0 mmol/L for L-lactic acid. The sensitivity was good with a limit of detection of 5.24 μmol/L for D-lactic acid and 0.15 mmol/L for L-lactic acid. The analytical method was successfully applied to human plasma samples from normal healthy subjects.

1. Introduction

Most biochemical reactions show selectivity to one enantiomeric form and it is well known that the enantiomers of the same compound can activate different metabolic pathways [1]. In healthy humans, lactic acid is almost exclusively present as L-lactic, which is readily metabolized in the liver to pyruvate by L-*lactate dehydrogenase*. Quite interestingly, D-lactic is also produced endogenously in humans, albeit in quite low concentrations, *via* the methylglyoxal pathway [2,3]. Contrary to wide belief, though, also D-lactic is converted quite efficiently to pyruvate by a putative mitochondrial D-*lactate dehydrogenase* and D-*α-hydroxy acid dehydrogenase* [2].

Elevated levels of D-lactic in blood and urine are typically caused by bacterial overproduction in the gut, but may also be a result of infection, ischemia, or traumatic shock [2,4–7]. Also, increase of D-lactic acid concentration has been observed in the blood of patients suffering from short bowel syndrome, which has led to D-lactic acidosis associated with encephalopathy [8,5,9]. As the enantiomeric composition of lactic in bodily fluids is a valuable diagnostic indicator of such conditions, the development of analytical methods suitable for enantiomer analysis of this organic acid is of great importance.

The determination of lactic acid is usually based on the enzymatic reaction between lactic acid and co-factor NAD+. Although the enzymatic assay provides a rapid determination and has been widely used in many laboratories, it has also some disadvantages due to cross-reaction with endogenous compounds [10] and large sample consumption.

For these reasons, a variety of analytical methods for the enantiomeric separation and determination of lactic acid in biological fluids have been reported including: gas chromatography (GC) [11–13], capillary electrophoresis (CE) [14–16], liquid chromatography with mass spectrometric detection (LC–MS) [17], high-performance liquid chromatography (HPLC) with chiral stationary phases [18–20] or chiral mobile phase [21]. However, these methods often suffer from basic limitations in terms of performance, equipment cost, complexity, sample processing and run times, which create challenges or render them impractical for high-throughput routine clinical laboratory. For instance, methods based on mass spectrometric detection provide highly specific and sensitive quantification of compounds of interest, often with less sample manipulation compared to other chromatographic methods. Unfortunately, mass spectrometric detection requires expensive instrumentation that may be out of reach for many laboratories.

A few papers reporting simultaneous determination of D- and L-lactic acids by HPLC with precolumn fluorescence derivatization and chiral column have been also published [22–24]. However,

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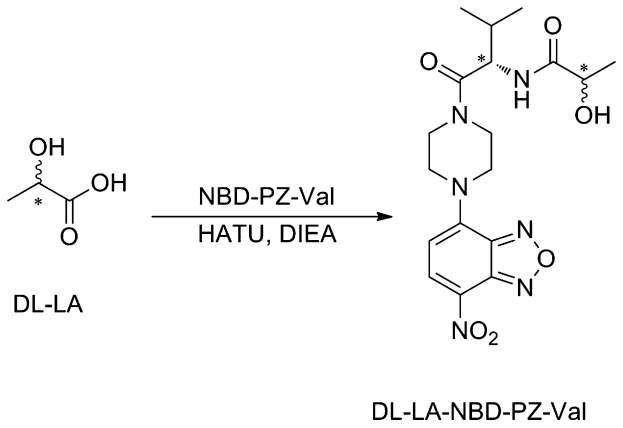

Fig. 1. Derivatization of D- and L-lactic acid by means of NBD-PZ-Val to DL-LA-NBD-PZ-Val diastereomers.

chiral columns suffer from drawbacks (high cost, short lifetime and intrinsic difficulty in selecting the most adequate one) and, therefore, it is of interest to develop alternative, simple, and low cost methods for the simultaneous determination of D- and L-lactic acids.

In this work, we describe a novel RP-HPLC method for enantioseparation of D- and L-lactic acid based on the derivatization of enantiomers with the chiral reagent NBD-PZ-Val (Fig. 1) in the presence of a coupling agent (HATU/DIEA) to yield the corresponding diastereomeric amides (henceforth named DL-LA-NBD-PZ-Val).

2. Experimental

2.1. Chemicals

4-Nitro-7-piperazinobenzofurazan (NBD-PZ) and *N*-ethyldiisopropylamine (DIEA) were purchased from Fluka; *O*-(7-azobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) was purchased from Merck (Germany); sodium D,L-lactate, (*S*)-2-(Boc-amino)-3-methylbutyric acid (Boc-L-valine; enantiomeric purity ≥99.0%), *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyl-uronium hexafluorophosphate (HBTU), trifluoroacetic acid (TFA), *N*,*N*-dimethylformamide (DMF) and hydrochloric acid were obtained from Sigma–Aldrich. NBD-PZ-Val was synthesized in our laboratory. All other reagents were of the highest purity available. The Strata-X-C cartridges (30 mg, 33 μm polymeric strong cation mixed-mode solid phase adsorbent) were from Phenomenex (Torrance, CA, USA).

2.2. Synthesis of (2S)-2-amino-3-methyl-1-[4-(7-nitro-benzo-2,1,3-oxadiazol-4-yl)-piperazin-1-yl]-butan-1-one (NBD-PZ-Val, III)

NBD-PZ-Val was synthesized as depicted in Fig. 2: to a solution of Boc-L-valine (0.1 g; 0.48 mmol) in DMF (1 mL) cooled to 0 °C and kept under N_2, HBTU (0.24 g; 0.64 mmol) dissolved in DMF (2 mL) and DIEA (110 μL; 0.64 mmol) were added. The mixture was stirred for 30 min, then a solution of NBD-PZ (0.08 g; 0.32 mmol) in DMF (2 mL) was added and the resulting solution was stirred at room temperature for extra 1 h. After this time, dichloromethane (10 mL) and water (50 mL) were added and the reaction mixture was extracted with 3× 10 mL portions of 10% HCl. The combined organic extracts were washed with 10 mL of water, then with 2× 10 mL of 5% $NaHCO_3$, dried over anhydrous sodium sulfate and evaporated *in vacuo*. The residue was purified by silica gel chromatography with ethyl acetate as eluent to give 0.13 g; 94% yield, of compound II (Fig. 2) as dark red solid.

This compound (0.13 g; 0.33 mmol) was dissolved in dichloromethane (2 mL) and the resulting solution was added to TFA (2 mL) under stirring at 0 °C. Stirring at 0 °C was continued for 1 h more, and then extra 1 h at room temperature. The reaction mixture was evaporated under reduced pressure, the residue washed 4 times with hexane–dichloromethane (1:1; v/v) to remove TFA completely. The crude product was brought to pH = 10 by adding 10% Na_2CO_3 and then extracted with 2× 10 mL of dichloromethane. The organic layer was washed with water, dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo* to give the desired product III (105 mg; yield 96%) as a red solid. M.P.: 71.3–72.6 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.43(d, *J* = 8.8 Hz, 1H), 6.32 (d, *J* = 8.9 Hz, 1H), 4.36–3.79 (m, 8H), 3.53 (d, *J* = 5.4 Hz, 1H), 1.90 (m, 1H), 0.99 (dd, *J* = 20.5, 6.8 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 174.2, 145.0, 144.9, 144.7, 135.1, 124.5, 102.9, 56.8, 49.2, 48.8, 44.7, 41.2, 32.4, 20.3, 17.0; HRMS (ESI+) calcd for $C_{15}H_{21}N_6O_4$ $[M+H]^+$: 349.1618, found: 349.1630.

2.3. Plasma samples

The plasma samples were obtained by a standardized procedure. Human whole bloods were collected into tubes containing EDTA as an anticoagulant. Plasma was obtained immediately by the centrifugation of the blood at 2000 × *g* for 15 min and samples were stored at −80 °C until analysis.

2.4. Sample preparation

Forty microliters of samples (human plasma or 10–200 μmol/L sodium D-lactate and 0.5–4.0 mmol/L sodium L-lactate in water,

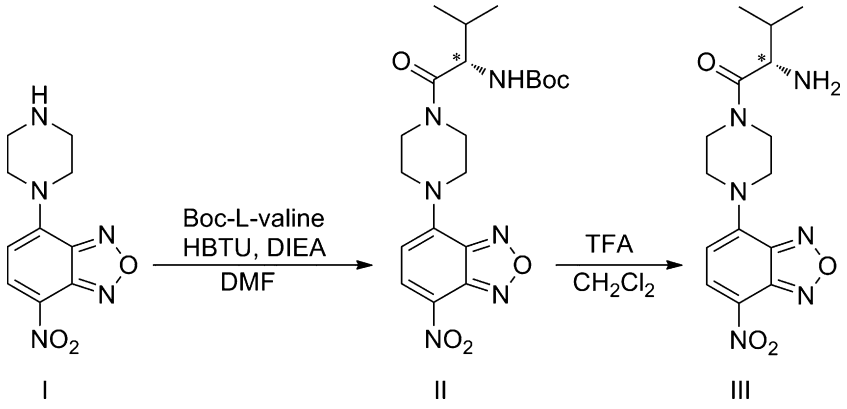

Fig. 2. Synthetic route to NBD-PZ-Val.

respectively) were mixed with 40 μL of water and with 120 μL of the deproteinizing solvent CH_3CN, and then the sample was centrifuged for 6 min at 14,500 × *g*. An aliquot of the resulting solution (40 μL) was mixed with 40 μL of 6 mmol/L NBD-PZ-Val in CH_3CN in the presence of 40 μL of HATU and DIEA, both 8 mmol/L in CH_3CN, incubated at 30 °C for 30 min, and then acidified with 80 μL of 1 mol/L HCl. Subsequently, in order to remove the excess of NBD-PZ-Val, 200 μL of the resulting solution were subjected to a solid-phase extractions with Strata-X-C cartridges. The cartridges, activated and conditioned with 0.6 mL of MeOH and 0.6 mL of water, were loaded with the aforementioned solution (200 μL): after washing with 0.6 mL of 0.1 mol/L HCl, 0.6 mL of MeOH were used to elute the analytes from the cartridge. The eluate was brought to dryness with a rotary evaporator, the residue was redissolved in 200 μL of MeCN–H_2O–HCl (1 mol/L) (60:30:10, v/v/v), and finally a 5 μL aliquot was injected into the HPLC.

2.5. Apparatus and chromatographic conditions

HPLC analyses were performed on a Agilent 1100 system (Agilent Technologies, Inc.) equipped with an online vacuum degasser, a high-pressure gradient quaternary pump, a manual sample injector (loop 5 μL), a column oven and a fluorescence detector. Data analysis was done using Agilent ChemStation software (Agilent Technologies).

The HPLC separation of the diastereomers of derivatized D,L-lactic acid was performed on a reversed phase C18 column Synergy Hydro (Phenomenex, Torrance, CA, USA) 150 mm × 3 mm (i.d.) with 4 μm particle size, protected by a Phenomenex C18 security guard column (4.0 mm × 3.0 mm). Mobile phases A and B were 0.1% aqueous trifluoroacetic acid and MeOH, respectively. The chromatographic method held the initial mobile phase composition (40% B) constant for 10 min, then the gradient increased linearly up to 55% B in 5 min followed by a isocratic elution for 5 min. Finally the gradient increased until 100% B in 1 min and it was maintained for 3 min for washing the column. Re-equilibration was carried out over 11 min; total analysis time was 35 min. The separation was carried out at a flow rate of 600 μL/min, at room temperature. Typically, 5 μL of sample were injected onto the column. Derivatized lactic acids were detected by fluorescence with excitation at 490 nm and emission at 532 nm.

2.6. Mass spectrometry

LC–MS analyses were performed using an Agilent 1100 MSD Ion Trap SL (Agilent Technologies, Santa Clara, CA, USA) equipped with an orthogonal electrospray ionisation source (ESI) operated in positive ion mode. The MS parameters were optimised for HPLC–MS analysis. The conditions of the ESI source were capillary voltage 2400 V, the end plate offset was fixed at 500 V, nebulizer pressure 50 psi and drying gas flow 12 L/min at a temperature of 350 °C. Nitrogen was used as both drying and nebulizing gas. The experiments were performed in standard scan at normal resolution at *m*/*z* mass range from 110 to 700 Da. MS/MS experiment was performed using auto MS/MS mode and fragment amplitude of 1 V. Agilent LC/MSD Trap Software 5.3 was used for data acquisition and processing.

The accurate mass measurements were carried out using an Applied Biosystems QStar XL quadrupole time-of-flight tandem mass spectrometer (Foster City, CA, USA) equipped with an ionspray source operating in positive ion mode. Instrument calibration was performed by infusion of ESI Tuning Mixture (Agilent Technologies, Palo Alto, CA, USA) and the exact mass analysis was obtained by using internal standard calibration (before analysis each sample was added to the mix of Agilent ESI mix) to improve the accuracy of measurement.

Instrument parameters were set as follows: spray voltage 3200 V, ion source gas 18 (arbitrary units), curtain gas 20 (arbitrary units) and declustering potential 50 V; data acquisition and analysis were performed using the Analyst QS software.

2.7. Calibration and linearity

Five standard solutions of lactic acid covering concentration ranges of 0.1–200 μmol/L for D-lactic acid and 0.5–4.0 mmol/L for L-lactic acid were employed as calibration samples. These were analyzed in triplicate every day for four days. Two calibration curves for both lactic acids were obtained by linear regression analysis of the lactic acid peak area plotted against the nominal lactic acid concentration.

2.8. Recovery and precision

Recovery of D- and L-lactic acid from plasma was assessed by analyzing human plasma obtained from normal healthy subjects after addition of samples of D,L-lactic acid at various concentrations. Concentrations in spiked biological samples were determined in triplicate.

The intraassay (within-day) reproducibility of the methods was established by replicate analyses ($n = 3$) of samples at two different concentrations. The interassay (between-day) was established by replicate analyses of the same samples over four days.

3. Results and discussion

3.1. Synthesis of chiral derivatization reagent

The synthetic route to the chiral derivatization reagent is shown in Fig. 2. The chiral reagent is obtained easily by reacting the commercial reagents NBD-PZ and Boc-L-valine in the presence of HBTU/DIEA at room temperature for 60 min. No racemization appears to occur in the synthetic steps because of the mild reaction conditions. In addition, the reaction of enantiomerically pure L-lactic acid with the chiral reagent produced a single chromatographic peak (data not shown), thus indicating that the chiral derivatization reagent was optically pure and that racemization does not occur during the synthesis of this reagent or during the derivatization reaction.

The reagent, protected from moisture and light, was stable at room temperature for at least 3 months, and acetonitrile solutions were stable at room temperature for at least one week.

3.2. Fluorescence properties

Excitation and emission spectra of the chiral reagent and its derivatives with D- and L-lactic acid in acetonitrile are depicted in Fig. 3.

3.3. Derivatization reaction with the chiral reagent

The derivatization of lactic acid requires the preliminary activation of the carboxylic group. Various activation agents have been developed for this purpose, for example: diethyl phosphorocyanidate (DEPC) [25], dicyclohexylcarbodiimide–1-hydroxybenzotriazole (DCC-HOBt) [26], diphenylphosphoryl azide (DPPA) [27], and Mukaiyama A (DPDS-TPP) [22–24,28,29]. Synthetic progress over the last four decades has resulted in a variety of new coupling reagents, which allow for the synthesis of products in high yield and high enantiomeric purity. Among them HATU and HBTU have become two of the most popular coupling reagents in solid phase as well as in solution phase peptide synthesis. We have investigated the use of HBTU and HATU in this particular reaction,

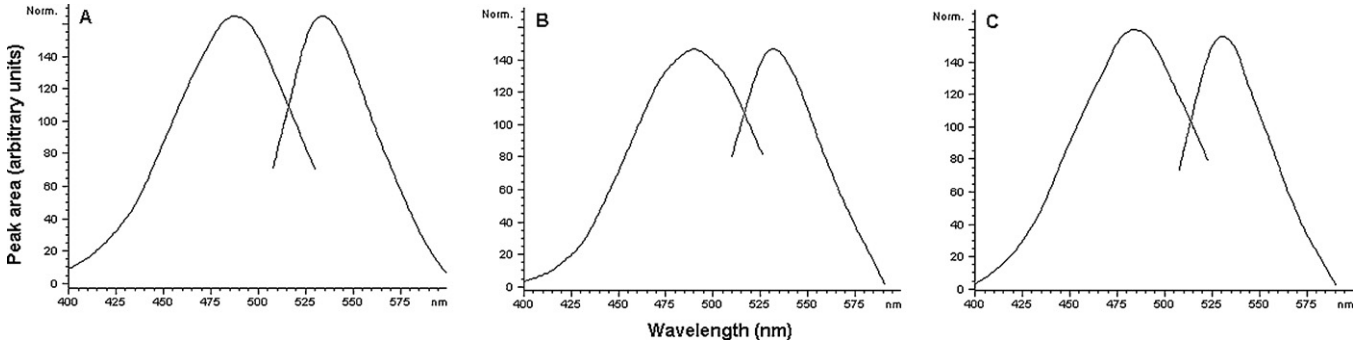

Fig. 3. Excitation and emission spectra of (A) NBD-PZ-Val; (B) D-LA-NBD-PZ-Val and (C) L-LA-NBD-PZ-Val.

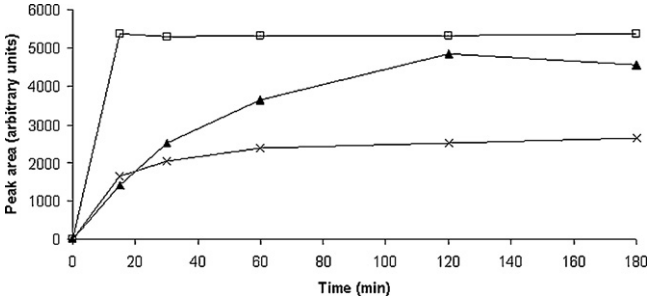

Fig. 4. Derivatization reaction of D-lactic acid with NBD-PZ-Val in acetonitrile in the presence of: (×) Mukaiyama A (70 mmol/L), (▲) HBTU (70 mmol/L), and (□) HATU (70 mmol/L).

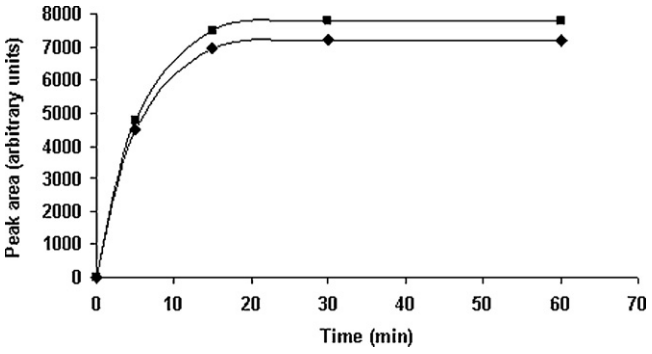

Fig. 6. Time courses of the derivatization reactions of D-lactic acid (♦) and L-lactic acid (■) with NBD-PZ-Val.

and compared them to Mukaiyama A coupling reagent (Fig. 4). The results showed that HATU was superior to HBTU and Mukayiama A in terms of yield (highest final fluorescence intensity) and reduced reaction time.

The effect of varying amounts of HATU on the derivatization of D-lactic acid with NBD-PZ-Val was also investigated (data not shown). From these results, derivatization conditions were chosen as described in Section 2.

A derivatized sample, after SPE treatment, was analyzed by LC–MS to confirm that the expected diastereomers were actually formed. Full scan mass spectra were acquired using electrospray ionisation (ESI) in positive ion mode (Fig. 5). The extracted ion chromatogram (EIC, Fig. 5A) shows the signal of the separated diastereoisomers at 13.6 min (D-LA-NBD-PZ-Val) and 15.5 min (L-LA-NBD-PZ-Val), respectively. The mass spectrum of each diastereomer (Fig. 5B and 5C) showed the expected protonated molecular ion at 421 *m*/*z*, the potassium adduct at 459 *m*/*z* and an intense sodium adduct at 443 *m*/*z*.

3.4. Time course study

Time course studies on the derivatization reaction of racemic mixture of lactic acid with NBD-PZ-Val were carried out in the time range of 15–60 min and at 30 °C; sample preparation was the same as described in Section 2.4. The highest increase of the peak signal was observed close to 20 min. The peak area did not increase significantly even extending the reaction time up to 60 min. A graph exhibiting the results of this study is shown in Fig. 6. Therefore the time of 30 min was chosen for the rest of this study.

3.5. Chromatographic separation

Chromatograms of standard solutions of the derivatized enantiomers of lactic acid in water and human plasma spiked with standard D- and L-lactic acid derivatives are shown in Fig. 7. The eluted peaks related to the D- and L-lactic acid were well separated with retention times of 13.8 and 15.4 min, respectively.

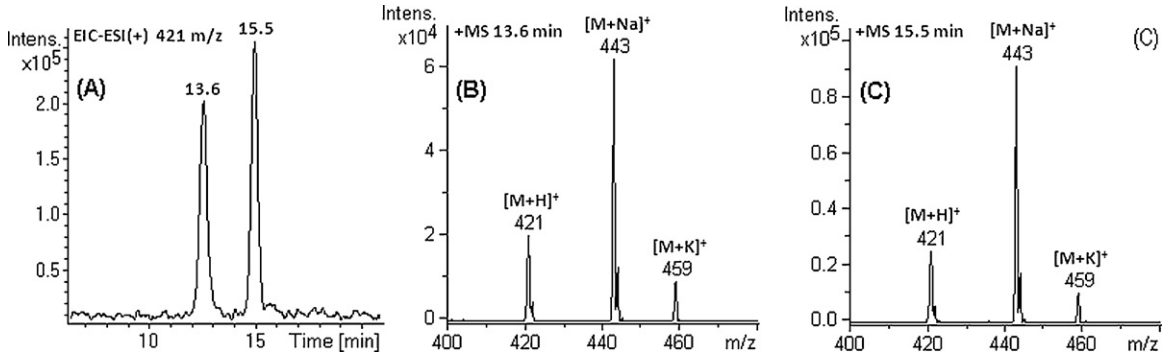

Fig. 5. (A) Extracted ion chromatogram (EIC, 421 *m*/*z*): D-LA-NBD-PZ-Val (13.6′), L-LA-NBD-PZ-Val (15.5′), (B) full scan spectra of D-LA-NBD-PZ-Val and (C) full scan spectra of L-LA-NBD-PZ-Val.

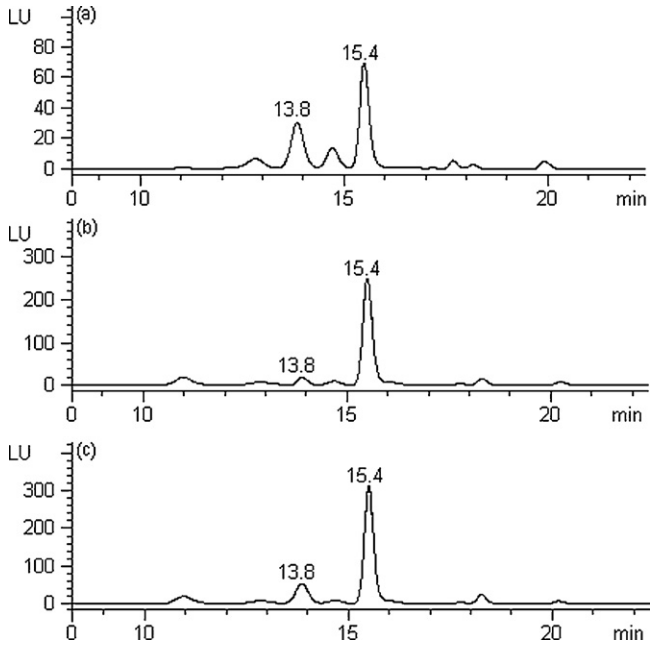

Fig. 7. Chromatograms of derivatized D- and L-lactic acid: (A) In water, 0.5 mmol/L each, (B) in human plasma and (C) the same as B, spiked with 0.5 mmol/L each.

3.6. Linearity, limit of quantification (LOQ) and limit of detection (LOD)

Calibration curves were obtained for both analytes in the concentration ranges of 10–200 μmol/L for D-lactic acid and 0.5–4.0 mmol/L for L-lactic acid. The regression equations were $y = 1.93x + 8.81$ ($r^2 = 0.9996$) and $y = 2986x - 192$ ($r^2 = 0.9990$), respectively.

The LOD and LOQ values were calculated by dividing three or ten times the standard deviation of the blank to the slope of calibration curve, respectively. Standard deviation of the blank can be estimated by the regression residual standard deviation. The calculated LOD were verified by analyzing a sample spiked at the LOD concentration to ensure visual detection of the analytes. The LOD (LOQ) values were 5.24 μmol/L (17.47 μmol/L) for D-lactic acid and 0.15 mmol/L (0.51 mmol/L) for L-lactic acid.

In comparison with the other few methods proposed for the determination of D-lactic acid in plasma, the target of this work, the method here reported is more sensitive than that based on capillary electrophoresis, where LOD value for D-lactic acid was reported as 80 μM [14]. Although our detection limit is slightly higher than those obtained using chiral columns (LOD for D-lactic acid was about 1 μM) [22,23] it is noteworthy to stress that such columns suffer from severe drawbacks such as low efficiency, short lifetime and high cost, compared to achiral columns.

Table 1
Recovery of D-lactic acid and L-lactic acid from human plasma.

Concentration of D-lactic acid (μmol/L)			Concentration of L-lactic acid (mmol/L)		
Spiked	Measured ± SD	% Rec	Spiked	Measured ± SD	% Rec
0	74.23 ± 4.06	–	0	1.92 ± 0.14	–
25	100.73 ± 4.15	101.5	0.5	2.28 ± 0.14	94.3
50	116.33 ± 4.30	93.6	1.0	2.90 ± 0.14	99.4
100	154.87 ± 4.94	88.9	1.5	3.17 ± 0.15	92.8
150	226.94 ± 6.78	101.2	2.0	3.76 ± 0.17	96.1

3.7. Matrix effect

To investigate the matrix effect, standard calibration and addition standard curves were obtained. The standard calibration samples in water were prepared as described in Section 2.4. Standard-added calibration samples were obtained by addition of different amounts of lactic acid standard solution to a constant volume of human plasma. Aliquots of each sample were deproteinized, and derivatized following the previously mentioned procedure.

The absolute matrix effect was assessed by comparing the slope of matrix standard curve with that of the standard calibration curve shown above (Section 3.6). As shown in Fig. 8, curves obtained from standard solutions in water and from human plasma were linear and parallel for either enantiomer of lactic acid, thus meaning that there is no matrix effect.

3.8. Recovery and precision

Analytical recoveries were examined by measuring the concentrations of the two analytes in human plasma spiked with four different concentrations of standard solution of D-lactic and L-lactic acid. Analytical recoveries were 88.9–101.5% for D-lactic and 92.8–99.4% for L-lactic (Table 1).

Intra-day and inter-day precisions were assessed by analyzing human plasma obtained from two normal healthy subjects (Table 2): intra-day values are below 4% and inter-day values are lower than 11.41%.

3.9. Analysis of D- and L-lactic acid derivatives in human plasma

The concentrations of D- and L-lactic acid in human plasma from 6 healthy subjects were determined to assess the applicability of the proposed method to such biological fluid: results were reported in Table 3. These values are in good agreement with those from previous work (15.0–25.0 μmol/L [9], 12.7–47.6 μmol/L [30]).

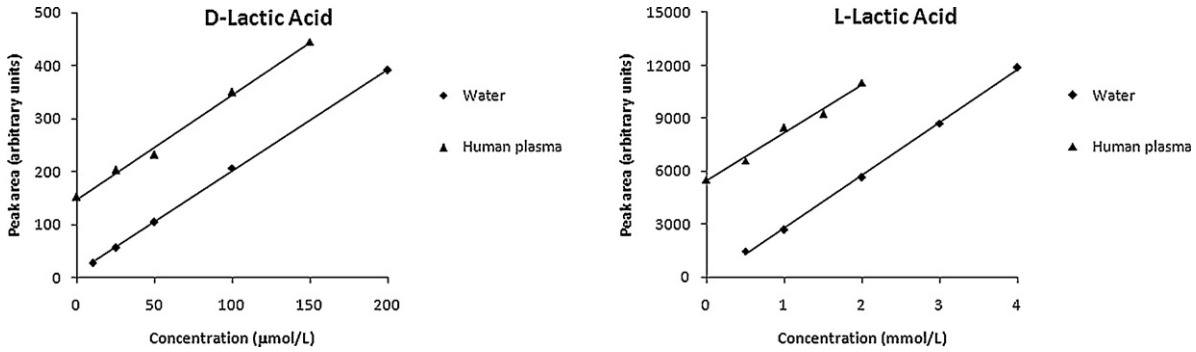

Fig. 8. Assessment of matrix effect in D- and L-lactic acid determination.

Table 2
Intra- and inter-day reproducibility of D-lactic acid and L-lactic acid in human plasma.

	Concentration ± SD	Intra-day RSD(%)	Inter-day RSD(%)
D-Lactic acid (μmol/L)	21.58 ± 4.53	2.77 ± 0.21	11.41 ± 0.91
	61.54 ± 4.10	2.65 ± 0.20	8.15 ± 0.42
L-Lactic acid (mmol/L)	0.76 ± 0.14	3.67 ± 0.61	8.80 ± 1.44
	1.84 ± 0.12	2.63 ± 0.29	7.78 ± 1.16

Table 3
Determination of D- and L-lactic acid in human plasma of healthy subjects.

Subject	Population	Age	Concentration[a] ± SD	
			D-LA (μmol/L)	L-LA (mmol/L)
1	M	61	35.59 ± 4.02	2.04 ± 0.12
2	F	27	61.54 ± 4.10	1.58 ± 0.12
3	F	28	33.62 ± 4.05	2.69 ± 0.12
4	F	32	58.58 ± 3.79	3.64 ± 0.15
5	F	26	68.28 ± 3.74	1.90 ± 0.12
6	M	61	23.54 ± 4.50	0.77 ± 0.14

[a] Calculated from calibration curves in water.

4. Conclusions

In this work we describe a new chiral fluorescent derivatization reagent and an useful indirect RP-HPLC method with fluorescence detection for the simultaneous quantitative determination of D- and L-lactic acid in human plasma, in particular at the trace levels characteristic of D enantiomer. To the best of our knowledge, a similar approach has never been reported. The proposed HPLC method, possessing quite satisfactory linearity and precision, has been successfully applied for the determination of D- and L-lactic acid enantiomers in very small volumes (40 μL) of human plasma samples. This method provides a simple, sensitive and efficient assay for detection of D- and L-lactic acid in plasma that circumvents the need for expensive columns with chiral stationary phases and therefore useful for ordinary, routine clinical laboratory.

Future work has been planned to develop a protocol employing NBD-PZ-Val as chiral derivatization reagent for lactic acid in other biological samples (*e.g.* cerebrospinal fluid, urine).

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