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Quantification of lipoic acid in plasma by high-performance liquid chromatography–electrospray ionization mass spectrometry

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Abstract

A sensitive and specific liquid chromatography electrospray ionization mass spectrometry (LC–ESI-MS) method has been developed and validated for the identification and quantification of lipoic acid (LA) in human plasma. LA and the internal standard, naproxen, were extracted from a 500 μl plasma sample by one-step deproteination using acetonitrile. Chromatographic separation was performed on a Zorbax SB-C_{18} Column (100 mm × 3.0 mm i.d. with 3.5 μm particle size) with the mobile phase consisting of acetonitrile and 0.1% acetic acid (pH 4, adjusted with ammonia solution) (65:35, v/v), and the flow rate was set at 0.3 ml/min. Detection was performed on a single quadrupole mass spectrometer by selected ion monitoring (SIM) mode via electrospray ionization (ESI) source. The method was linear over the concentration range of 5–10,000 ng/ml for LA. The intra- and inter-day precisions were less than 7% and accuracy ranged from −7.87 to 9.74% at the LA concentrations tested. The present method provides a relatively simple and sensitive assay with short turn-around time. The method has been successfully applied to a clinical pharmacokinetic study of LA in 10 healthy subjects.

Keywords: Lipoic acid; HPLC–ESI-MS

1. Introduction

α-Lipoic acid (LA), which plays an essential role in mitochondrial dehydrogenase reactions, has recently gained considerable attention as an antioxidant. LA, or its reduced form, dihydrolipoate, reacts with reactive oxygen species such as superoxide radicals, hydroxyl radicals, hypocholorous acid, peroxyl radicals, and singlet oxygen. It also protects membranes by interacting with vitamin C and glutathione, which may in turn recycle vitamin E. LA administration has been shown to be beneficial in a number of oxidative stress models such as ischemia-reperfusion injury, diabetes, cataract formation, HIV activation, neurodegeneration, and radiation injury. Furthermore, LA can function as a redox regulator of proteins such as myoglobin, prolactin, thioredoxin and NF-B transcription factor [1]. The drug is rapidly absorbed and extensively metabolized in the liver and great individual variability in LA concentration occurs among patients. A sensitive and simple LA determination method for pharmacokinetics studies and for therapeutic drug monitoring is then desired. Various methods have been developed for the determination of LA. GC methods based on prior derivatization were the most useful techniques for the determination of LA in biological samples [2]. However, all these methods needed a derivatization procedure and the sample preparation was very laborious. Determination of LA by HPLC with electrochemical detection [3,4] or, more selectively with a dual gold–mercury electrode [5] was highly sensitive and the sample preparation was relative simple, but the concentration-response curve was only linear in a narrow concentration range, and the reconstitution of the Hg-electrode was necessary. Although the sensitivity of the HPLC-ultraviolet detection method for LA was low, due to

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the lack of a strong chromophore, LA can be sensitively detected by HPLC-fluorescence detection based on fluorogenic labeling. Witt et al. [6] and Haj-Yehia et al. [7] reported HPLC methods with fluorescence detection after pre-column derivatization with monobromobimane or strong fluorophore 2-(4-aminophenyl)-6-methylbenzothiazole to analysis LA in human plasma, respectively. However, all these methods included a very tedious sample preparation procedure. In recent years, Teichert et al. developed an isocratic reversed-phase HPLC method for the quantitation of LA in human plasma employing simple solid-phase extraction and pulsed amperometric detection (PAD) and the limit of quantification was 10 ng/ml [8]. In this paper, no internal standard was used and in order to separate LA from endogenous components and its metabolites, the total run time was 22 min per sample. More important, a disadvantage of PAD was the large anodic background current and the fact that the kinetics of surface oxide formation could be influenced by the adsorbed analyte. The calibration curve was strongly influenced by the absorption isotherm of the analyte and therefore, it deviated from linearity at high concentrations. Due to the high concentrations appearing after administration of therapeutic doses and short elimination half-lives of LA, the method must cover a broad concentration range. So in order to minimize the influence of the adsorbed analyte, the author performed all measurements in the same detector in range of 0.1 μA and most of the samples had to be diluted differentially and analyzed repeatedly. In this paper, we describe a more simple, selective and highly sensitive method by using high performance liquid chromatography coupled with electrospray ionization (ESI) single quadrupole mass spectrometry (MS) in the negative selected ion monitoring (SIM) mode for the determination of LA in human plasma and this method has been successfully used for clinical LA pharmacokinetic studies.

2. Experimental

2.1. Reagents and chemicals

LA was obtained from Shanghai Institute of Pharmaceutical Industry (Shanghai, China). Naproxen, used as internal standard (I.S.), was obtained from Shanghai Institute for Drug Control (Shanghai, China). Chemical structures were presented in Fig. 1. The purity of LA and Naproxen were all >99.5%. Acetonitrile was chromatographic pure grade and purchased from Merck (Merck Company, Germany), acetic acid and ammonia solution (analytical reagent grade) were purchased from Shanghai Chemical Reagent Company (Shanghai, PR China). Double distilled water was purified by Millipore Simplicity™ (Millipore, Bedford, MA, USA). The drug-free human heparinized plasma was obtained from Shanghai Blood Center (Shanghai, PR China).

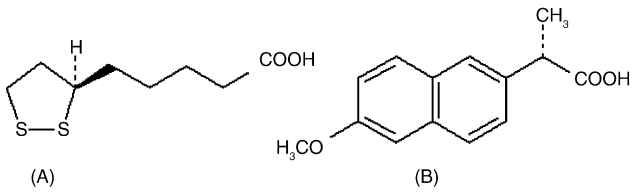

Fig. 1. Chemical structures of LA and naproxen.

2.2. Preparation of standard solution

The primary stock solutions of LA were prepared by dissolving 50.0 mg of LA in 50 ml methanol, producing a concentration of 1.0 mg/ml and was stored at 4 °C. Working solutions of LA were prepared by appropriately diluting the stock solution with water at the concentrations of 0.1, 0.5, 1, 5, 10, 50 and 200 μg/ml. The internal standard stock solution was prepared by dissolving 10.0 mg of naproxen in 10 ml methanol, producing a concentration of 1.0 mg/ml and was stored at 4 °C. This solution was further diluted with the same solvent to prepare the internal standard working solution containing 20 μg/ml of naproxen. All these solutions were stored at 4 °C and no change in stability over a period of 1 month was observed.

2.3. Equipment

The experiments were carried out with a HP1100 system (Agilent Technology, Palo Alto, CA, USA). The system consisted of a G1312A binary pump, a mobile phase vacuum degassing unit, a G1329A autosampler, a temperature-controlled column compartment, and a HP1100 single-quadrupole mass spectrometric (MS) detector equipped with an electrospray source. Data were acquired and integrated by the ChemStation software runn on a HP Vectra 150/PC with a Windows NT operating system. The stationary phase was composed of Zorbax SB-C_{18} material (Agilent Technology) packed in a stainless steel column (100 mm × 3.0 mm i.d. with 3.5 μm particle size).

2.4. Chromatographic and MS conditions

Chromatographic separations were achieved using a mobile phase consisting of acetonitrile and 0.1% acetic acid (pH 4, adjusted with ammonia solution) (65:35, v/v), with a flow rate set at 0.3 ml/min. The analytical column was kept at 40 °C. The column effluent was connected to an electrospray ionization MS interface without splitting. Electrospray ionization was performed using nitrogen as nebulizing gas at 10 l/min flow rate, 40 psi nebulizing pressure, and 350 °C drying gas temperature. Capillary voltage was set at 3000 V. Fragment voltage applied between capillary outlet and the first skimmer produced fragment ions by in-source collision-induced dissociation by nitrogen. Optimum fragment voltage of 70 V was selected after varying between 50 and 150 V. Negative-ion selected ion monitoring (SIM) mode was used to detect *m/z* 205.0 ($[LA-H]^-$), and *m/z* 229.0 ($[Naproxen-H]^-$).

2.5. Sample preparation

To a 0.5 ml plasma in a 2.0 ml plastic Eppendorf tube, 25 μl of I.S. solution (20 μg/ml of Naproxen) was added. The sample was vortexed briefly, and then deproteinated by addition of 1.0 ml acetonitrile. After vortexmixing for 30 s, the mixture was centrifuged at 16,000 × *g* for 15 min. The aqueous supernatant was transferred into an auto-sampler vial, and 5 μl was subsequently injected into the LC–MS system.

2.6. Validation of the assay method

2.6.1. Specificity

Chromatographic interference from endogenous plasma components was investigated using pooled blank plasma samples as well as samples from healthy subjects who participated in a clinical pharmacokinetic study of LA.

2.6.2. Linearity

Calibration curves were prepared by spiking different samples of blank plasma each with proper volume of one of the above-mentioned working solutions to produce the calibration curve points equivalent to 5, 10, 50, 100, 500, 1000, 5000 and 10,000 ng/ml of LA. The samples were assayed using the method described above. The standard calibration curves for LA were constructed using the analyte/I.S. peak area ratios versus the nominal concentrations of the analytes. Linear least-squares regression analysis with weighting factor of $1/x^2$ was performed to assess the linearity as well as to generate the standard calibration equation: $y = ax + b$, where y is the peak area ratio, x the concentration, a the slope and b is the intercept of the regression line.

2.6.3. Precision, accuracy, and absolute recovery

QC samples (5, 50, 500 and 5000 ng/ml) in five replicates were analyzed on the same day to determine the intra-day precision and accuracy, and on each of five separate days to determine inter-day precision and accuracy. The absolute recovery was determined in five replicates by comparing the peak areas of the extracted samples to those of the unextracted standards at equivalent concentration. The unextracted sample was prepared by mixing 0.5 ml of water, 25 μl of I.S. solution, and 1.0 ml acetonitrile.

2.6.4. Sensitivity

The lower limit of quantification (LLOQ) was determined for LA, based on the criteria that: (1) the analyte response at LLOQ is five times of baseline noise; (2) the analyte response at LLOQ can be determined with sufficient precision and accuracy, i.e. precision of 20% and accuracy of 80–120%. The limit of detection (LOD) was determined as the lowest concentration, which gives a signal-to-noise ratio of 3 for LA.

2.6.5. Matrix effect

The "absolute" matrix effect was evaluated by comparing the peak area of LA spiked in pre-extracted plasma samples (prepared in five replicates at each QC concentration using pooled blank plasma) to that of the aqueous standards at equivalent concentrations. Percent ion suppression was calculated as $100 \times (A_s - A_p)/A_s$, where, A_p was the mean peak area of LA from pre-extracted plasma samples (blank plasma extracted and spiked with LA after extraction) and A_s was the mean peak area of LA from the directly injected aqueous standards. To assess the lot-to-lot matrix variation, six different lots of blank plasma were used to prepare the QC samples (triplicates for each lot) at a concentration of 500 ng/ml. The relative standard deviation (R.S.D.) in peak area ratio among the six lots of plasma was calculated as an indicator of the inter-lot matrix variability.

2.6.6. Stability of analytes

The stability of LA was determined in three ways: (1) for storage stability, samples (five replicates at each QC concentration) were prepared and stored at −20 °C for 30 days. On day 30, all samples were thawed and analyzed along with the calibration standard samples, which were freshly prepared. (2) For freeze–thaw stability testing, the samples (five replicates at each QC concentration) were determined after three freeze (−20 °C) and thaw (25 °C) cycles, and the concentrations were compared to their nominal concentrations. (3) To assess the injector stability of the processed samples, the plasma samples (five replicates at each QC concentration) were extracted and placed in the auto-sampler at 25 °C for 24 h, and then injected into the LC–MS system for analysis.

3. Results

3.1. Separation, specificity and matrix effect

Negative ion electrospray mass scan spectra of LA and IS were shown in Fig. 2. The major ions observed were $[M-H]^-$, $m/z = 205.0$ for LA and $[M-H]^-$, $m/z = 229.0$ for Naproxen. The ions of $[M-H]^-$, $m/z = 205.0$ for LA and $[M-H]^-$, $m/z = 229.0$ for Naproxen were selected for the SIM(−) due to their high stability and intensity.

Fig. 3 shows the representative chromatograms of blank plasma, plasma samples spiked with LA at 1000 ng/ml and at LLOQ (5 ng/ml), and plasma sample obtained from a healthy subject following an oral 600 mg dose of LA. Thanks to the high specificity of SIM detection, the analytes were well separated using the present chromatographic conditions. The retention times were 3.3 min for LA and 3.9 min for I.S. No interfering peaks from the endogenous plasma components were observed at the retention time of LA or I.S. Moreover, LA and I.S. also could be well separated under this condition.

Due to negative SIM detection, no matrix components in plasma caused significant changes in the MS response of LA. The percent of ion suppression was <5.25% across the QC levels. No significant lot-to-lot matrix variation was observed. For the six lots of plasma spiked with 500 ng/ml of LA, the

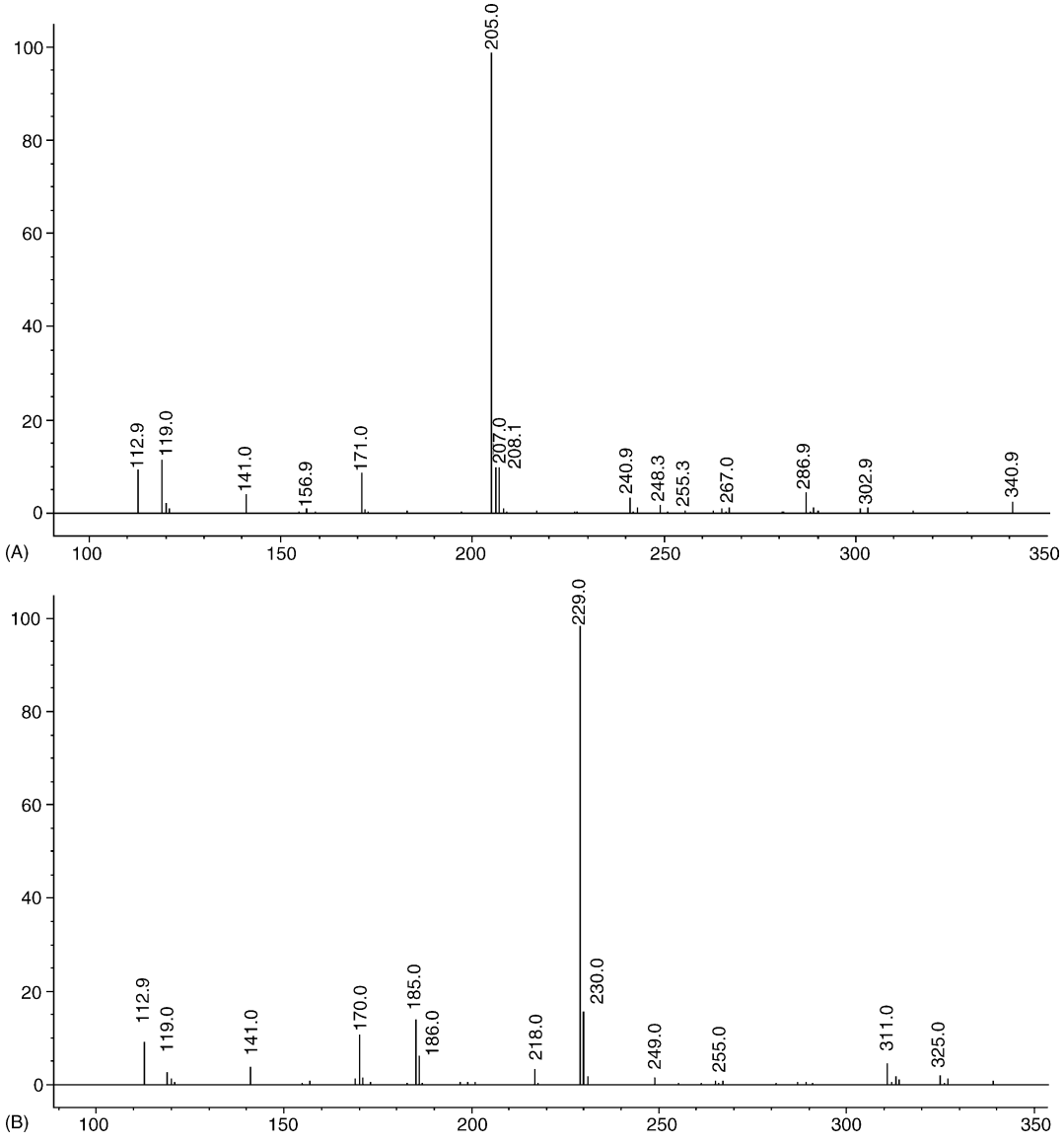

Fig. 2. Negative ion electrospray mass scan spectrum of LA (A) and naproxen (B).

interlot variation in peak area ratio (R.S.D.%) was found to be 2.93%.

3.2. Calibration and linearity

Good linearity was observed over the concentration range from 5 to 10,000 ng/ml ($r = 0.9998$–0.9999). The R.S.D. ($n = 5$) of the slope calculated with calibration curve data was 1.78%, showing a good repeatability (Table 1).

3.3. Precision, accuracy and absolute recovery

The intra- and inter-day precisions and accuracy of the assay were summarized in Table 2. The precision, presented as percentage of R.S.D. was no more than 9.97 and 5.27% for intra- and inter-day determination, respectively. The accuracy, presented as percentage of bias against the nominal concentration, ranged from −7.87 to 9.74%, and −2.85 to 1.74% for intra- and inter-day assay, respectively. The absolute recovery of LA from plasma was determined to be $98.82 \pm 5.43\%$, $95.49 \pm 7.24\%$, $97.25 \pm 3.24\%$ and $96.27 \pm 3.40\%$ at concentrations of 5, 50, 500 and 5000 ng/ml, respectively. The recovery of I.S. averaged $95.42 \pm 3.78\%$ ($n = 5$).

3.4. Sensitivity

The lower limit of quantification (LLOQ) was 5 ng/ml and the limit of detection (LOD) was 2 ng/ml for LA.

3.5. Stability

The residual percentages of LA stored in plasma at −20 °C for 30 days ranged from 97.40 to 108.50%, indicat-

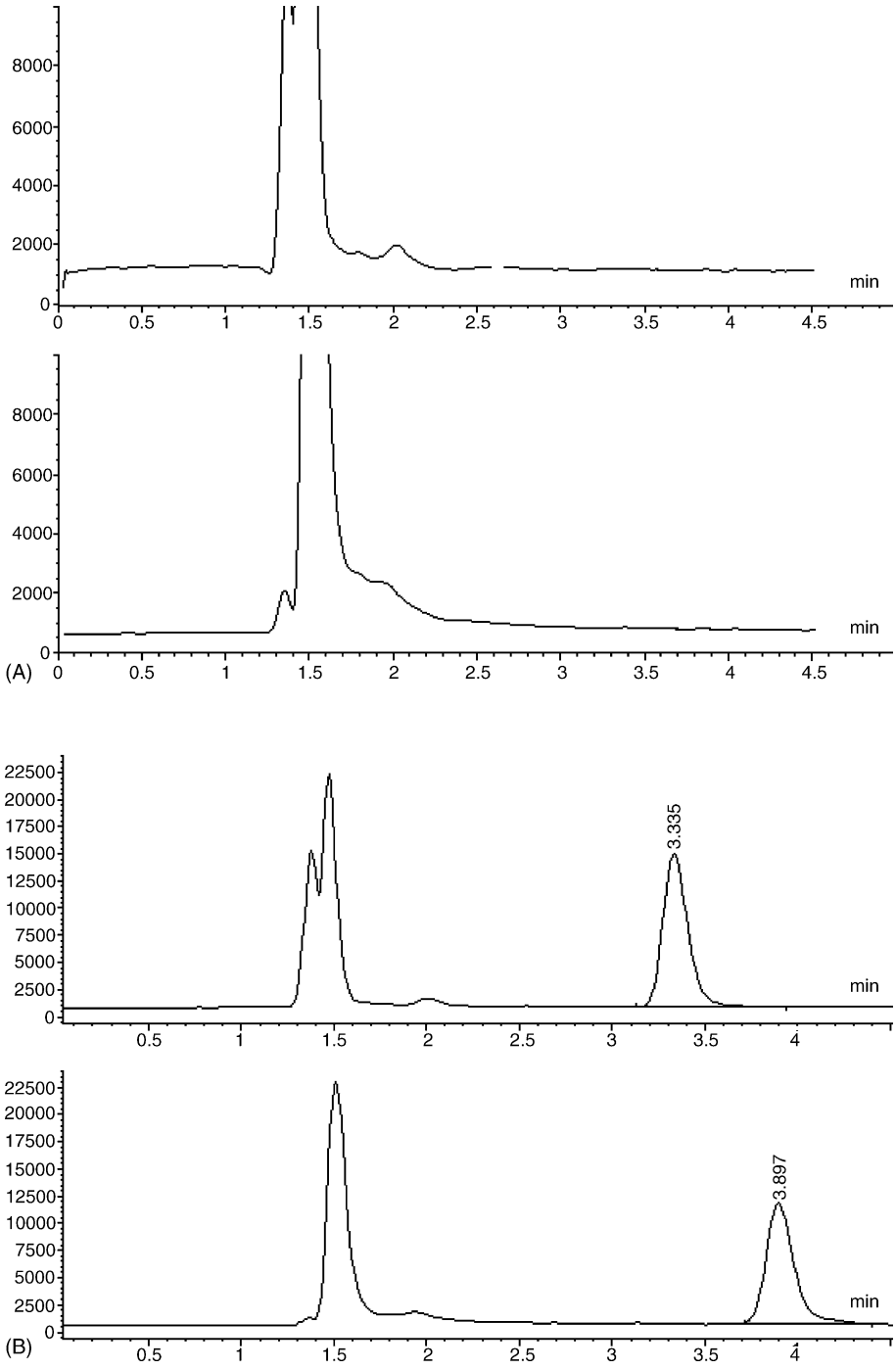

Fig. 3. Chromatograms of (A) blank plasma; (B) plasma sample spiked with 500 ng/ml of LA and I.S.; (C) plasma sample from a healthy subject following a 600 mg oral dose of LA, the plasma concentration was determined to be 399 ng/ml for LA; (D) spiked plasma sample at LLOQ (5 ng/ml).

ing no stability problems occurred. The results obtained after three freeze–thaw cycles demonstrated that 97.26–102.92% of the initial content of LA were recovered and that the analytes were stable under these conditions. LA in reconstitution solution was found to be stable for approximately 24 h since the found concentrations of analystes were within 98.60–102.50% of the initial concentrations (Table 3).

3.6. Dilution

The dilution study was also conducted to assess whether the upper concentration limit (10,000 ng/ml) can be extended. Quality control samples (in five replicates) at concentration of 20,000 ng/ml were diluted by two times with blank plasma, and the assay precision and accuracy were determined in a similar manner as described in Section 2.6.3. For LA, the

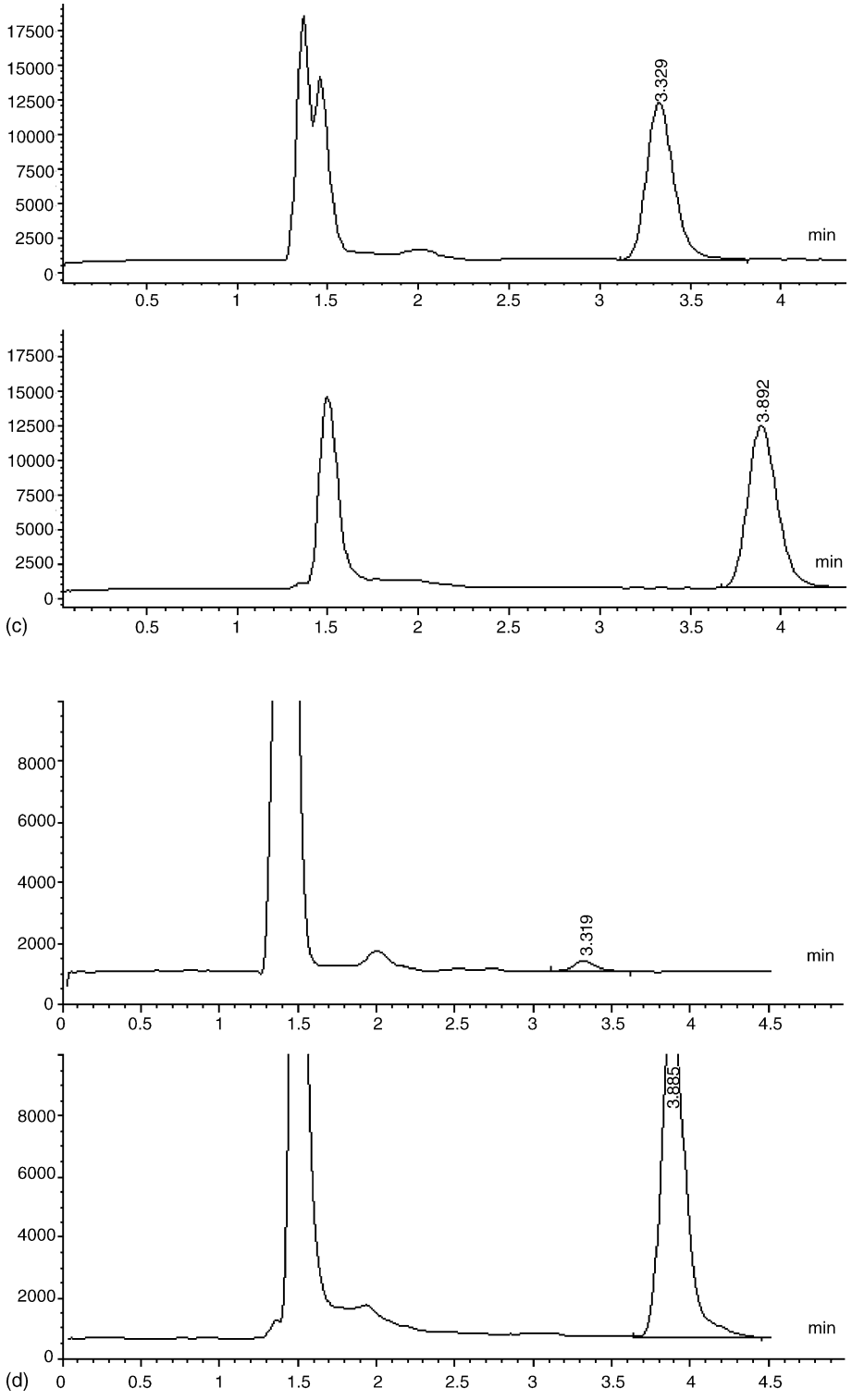

Fig. 3. (*Continued*).

R.S.D. was 3.27% and Bias (%) was +2.76%. The results suggested that samples whose concentrations were greater than the upper limit of the standard curve could be re-analyzed by appropriate dilution.

3.7. Application to clinical study

The assay method was used in a clinical pharmacokinetic study of LA in 10 healthy subjects. The study was

Table 1
Inter-day precision in the slope and intercept of standard curves

Days	Slope	Intercept	Correlation
1	0.002246	0.004738	0.9999
2	0.002215	0.005177	0.9999
3	0.002174	0.004632	0.9999
4	0.002239	0.006232	0.9999
5	0.002157	0.004988	0.9998
Mean ± S.D.	0.002206 ± 0.00003935	0.005153 ± 0.0006395	0.9998
R.S.D. (%)	1.78	12.41	

Table 2
Intra- and inter-day precision and accuracy of LA spiked in human plasma

Nominal concentration (ng/ml)	Parameters	Intra-day					Inter-day
		Day 1 ($n=5$)	Day 2 ($n=5$)	Day 3 ($n=5$)	Day 4 ($n=5$)	Day 5 ($n=5$)	5 Days ($n=25$)
5	Mean	4.99	5.03	4.82	5.32	4.61	4.95
	S.D.	0.32	0.24	0.48	0.17	0.20	0.26
	R.S.D. (%)	6.41	4.77	9.96	3.20	4.34	5.27
	Bias (%)	−0.20	0.60	−3.60	6.40	−7.80	−1.00
50	Mean	51.23	54.87	52.74	48.72	46.80	50.87
	S.D.	4.32	2.57	3.24	2.58	3.21	3.19
	R.S.D. (%)	8.43	4.68	6.14	5.30	6.86	6.39
	Bias (%)	2.46	9.74	5.48	−2.56	−6.40	1.74
500	Mean	504.2	494.4	484.6	509.9	486.8	496.0
	S.D.	12.4	8.52	6.30	7.28	11.3	11.0
	R.S.D. (%)	2.46	1.72	1.30	1.43	2.32	2.20
	Bias (%)	0.84	−1.12	−3.12	1.98	−2.64	−0.81
5000	Mean	4920	4918	4830	4881	4738	4857
	S.D.	228	142	55	47	78	76
	R.S.D. (%)	4.63	2.89	1.14	0.96	1.65	1.52
	Bias (%)	−1.60	−1.64	−3.38	−2.38	−5.24	−2.85

Table 3
Stability of LA in plasma

Nominal concentration (ng/ml)	Determined concentration of stability sample (mean ± S.D., ng/ml)		
	Storage stability (−20 °C, 30 days) ($n=5$)	Freeze–thaw stability (−20 to 5 °C) ($n=5$)	Autosampler stability (25 °C, 24 h) ($n=5$)
5	4.87 ± 0.30	5.08 ± 0.27	4.93 ± 0.12
50	54.25 ± 3.79	51.46 ± 3.82	51.25 ± 1.27
500	509.2 ± 11.2	486.3 ± 11.0	496.2 ± 7.2
5000	5053 ± 43	4930 ± 83	5032 ± 23

Table 4
Pharmacokinetic parameters of LA in 10 health volunteers after oral administration 600 mg of LA

Volunteer number	C_{max} (μg ml^{-1})	t_{max} (min)	K_e (min^{-1})	$t_{1/2}$ (min)	AUC_{0-t} (min μg ml^{-1})	$AUC_{0-\infty}$ (min μg ml^{-1})
1	3.99	90	0.0435	15.90	192.4	192.6
2	3.51	90	0.0316	21.92	144.8	145.1
3	2.08	60	0.0226	30.65	153.3	160.2
4	2.19	45	0.0320	21.60	171.6	173.1
5	4.47	60	0.0236	29.35	273.7	275.4
6	3.61	45	0.0262	26.38	231.3	233.1
7	4.49	60	0.0310	22.29	199.7	199.7
8	2.80	45	0.0267	25.96	212.4	216.5
9	3.34	90	0.0355	19.48	156.0	156.3
10	2.59	60	0.0352	19.66	152.8	152.8
Mean	3.31	65	0.0308	23.32	188.8	190.5
S.D.	0.87	19	0.0064	4.66	41.6	41.7

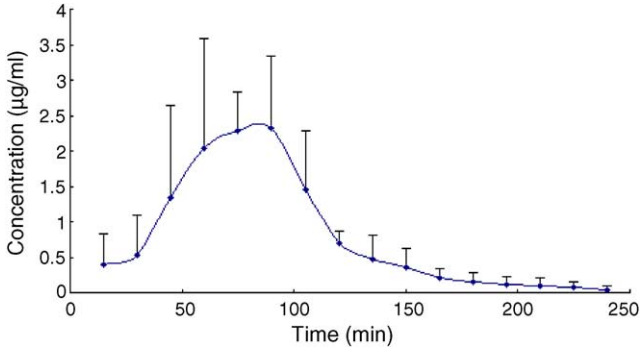

Fig. 4. Plasma concentration–time profiles of LA in a healthy subject following a 600 mg oral dose of LA.

approved by the Clinical Research Ethics Committee of the Shanghai Zhongshan Hospital. All subjects provided written informed consent prior to participating in the study. The subjects received a 600 mg oral dose of LA after an overnight fast of 10 h. Blood samples (2.5 ml) for assay of plasma concentration of LA were collected at the time of 0, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 195, 210, 225 and 240 min after oral administration of the medicals. After centrifugation at 3000 × *g* for 10 min, the separated plasma was stored at −20 °C and analyzed within 1 month. The mean plasma concentrations versus time profile of LA following a single 600 mg oral dose in 10 subjects were shown in Fig. 4. The pharmacokinetic parameters of the LA were listed in Table 4. The values were in good agreement with those reported previously [9–12].

4. Discussion

Various methods have been developed for the determination of LA. An overview of chromatographic methods has been published by Kataoka [13]. Since LA is an organic acid with large polarity, it is very difficult to separate LA from plasma by the liquid–liquid extraction, so all the previous papers described a solid phase extraction to extract the LA from plasma [1–9]. In this study we utilized a simple one-step protein precipitation method, which provided satisfactory extraction efficiency. Among the various precipitating reagents tested (i.e. methanol, acetonitrile, 10% perchloric acid, and 10% trichloroacetic acid), perchloric acid and trichloroacetic acid were found to cause low extraction efficiency of LA (only 8%). However, clean and sharp peaks of LA and IS were observed when using methanol and acetonitrile as the precipitating reagent. When compared to methanol, acetonitrile resulted in much cleaner aqueous supernatant after deproteination. Two volumes of acetonitrile were enough to remove proteins from the sample.

Although naproxen is not chemically related to LA, it was chosen as an internal standard for its stability in both stock solutions and prepared samples. Its chromatographic retention time and peak symmetry were similar to those of the LA. Naproxen also showed similar solubility and recovery in sample preparation steps including acetonitrile deproteination.

At neutral or alkaline pH of the mobile phase, where both LA and I.S. are deprotonated, the silicabased reversed-phase chromatographic separation is inefficient. Acetic acid was added to lower the pH of the mobile phase. Acetonitrile used in sample preparation was also used in the mobile phase as organic modifier. Methanol could also be used in the mobile phase, but better ESI results were obtained with acetonitrile. Additionally, the pH from 3.5 to 4.5 causes no interfering peaks in the retention times of both compounds and exceptional improvement in the chromatographic peak shapes. Hence, an eluent consisting of acetonitrile–0.1% acetic acid (pH 4, adjusted with ammonia solution) (65:35, v/v) appeared to be most appropriate for the LC–MS analysis of LA from both mass spectrometric and chromatographic points of view. Furthermore, the chromatographic elution of the analyte and the IS could be obtained in less than 5.0 min.

5. Conclusion

A novel method for the quantitative determination of LA in human plasma has been established, which is specific, accurate, precise, and can cover a very broad concentration range. Moreover, the method has a LLOQ of 5 ng/ml and proved to be superior in sensitivity and speed of analysis to the analytical methods reported previously. The sample pretreatment procedure is based on a simple one-step protein precipitation method, thereby eliminating the need of solid phase extraction, column switching procedures, and/or the use of large volumes of plasma for sample clean-up. In conclusion, the method is suitable for clinical pharmacokinetic studies and is advantageous over existing methods due to its simplicity, short turn-around time and good sensitivity.

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