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Short communication

A sensitive LC/MS/MS bioanalysis assay of orally administered lipoic acid in rat blood and brain tissue

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ABSTRACT

A sensitive liquid chromatography tandem mass spectrometry (LC/MS/MS) bioanalytical method was developed and validated to analyze lipoic acid (LA) in rat blood and brain samples. Ten mobile phase combinations were investigated during method development. Mobile phase combination of 0.1% acetic acid (pH 4 adjusted with ammonia solution)/acetonitrile was most optimum in terms of sensitivity and peak shape of LA and the internal standard, valproic acid. Sample extraction method was explored using liquid–liquid extraction and protein precipitation methods. Protein precipitation yielded the highest recovery of the analytes from blood and brain ranging from 92 to 115%. The lower limit of quantitation (LLOQ) of LA was 0.1 ng/mL (0.485 nM) in both blood and brain while on-column lower limit of detection (LLOD) was 0.03 pg. The precision (% R.S.D.) ranged from 1.49 to 26.39% and 1.49 to 10.89% for intra- and inter-day assays, respectively. The accuracy ranged from 91.2 to 116.17% for intra-day assay and 102.68 to 114.33% for inter-day assay.

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1. Introduction

Lipoic acid (LA) is an essential cofactor for mitochondrial enzymes and a naturally occurring antioxidant. It has been explored for the treatment of many diseases such as multiple sclerosis and other chronic inflammatory diseases of the central nervous system such as Alzheimer's disease and diabetes polyneuropathy [1]. Its great potential in the prophylaxis or treatment of these diseases has sparked great interest in investigating its effects *in vivo*. As such, sensitive bioanalytical methods are needed to analyze LA in biological samples. Various bioanalytical methods of LA which included gas chromatography with flame photometric detection [2], high-performance liquid chromatography (HPLC) [3], and HPLC with fluorimetric detection [4,5] or electrochemical detection [6] had been developed. In recent years, simple, selective and highly sensitive liquid chromatography tandem mass spectrometry (LC/MS/MS) methods were developed and validated [7,8]. However, optimization of the LC/MS/MS methods during development was not systematically discussed. Moreover, the lower limit of quantitation (LLOQ) in both methods [7,8] was 5 ng/mL and this LLOQ may not be sensitive enough to allow quantitation of endogenous LA. As different mobile phases and solvent additives affect LC/MS/MS data quality [9–12], it is necessary to optimize these parameters carefully to achieve a sensitive and specific method for the analyses of both endogenous and exogenous LA. In the present study, we developed and validated a LC/MS/MS method for the bioanalysis of LA in rat blood and brain.

2. Experimental

2.1. Materials and reagents

LA and sodium valproate (internal standard, IS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals and solvents were of analytical and HPLC-grade, respectively. Water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

LC/MS/MS method development and validation were performed using an Agilent 1100 HPLC system (Agilent Technologies) interfaced with a triple quadrupole mass spectrometer equipped with Turbo Ion Spray interface (API4000, Applied Biosystems, Foster City, CA, USA). MS data acquisition and processing were performed using the Analyst Software v 1.4.2 (Applied Biosystems). All MS experiments were performed using electrospray negative ionization mode (ESI −ve).

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2.3. MS optimization

1 μg/mL each of LA and IS in methanol were infused separately into the QTRAP MS at a flow rate of 10 μL/min using a built-in microsyringe pump (Harvard Apparatus Inc., Holliston, MA, USA) for the optimization of the compound-dependent MS parameters (declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP)) using multiple reaction monitoring (MRM) experiment. The collision-activated dissociation (CAD) gas was set at 'Medium' throughout all experiments. The optimized compound-dependent parameters of LA and IS were used for all subsequent experiments. 10 μL of 250 ng/mL of LA in acetonitrile:water (50:50) was introduced into the LC/MS/MS system by flow injection analysis (FIA) for the optimization of the source-dependent MS parameters (temperature (TEM), nebulizer gas 1 (GS1), nebulizer gas 2 (GS2), ionspray voltage (ISV) and curtain gas (CUR)) for each of the ten experimented mobile phase combinations. The ten mobile phase combinations comprised solvent A: 0.1% formic acid in 10 mM ammonium acetate, 10 mM ammonium acetate, water, 0.1% acetic acid (pH 4 adjusted with ammonia solution) or 0.1% formic acid, combined with solvent B: methanol or acetonitrile. The experimented combinations of formic acid/acetonitrile and acetic acid/acetonitrile were previously used by Trivedi et al. [7] and Chen et al. [8], respectively. Isocratic conditions comprising 24–65% solvent A depending on the elution time of LA for each mobile phase combination at a flow rate of 0.7 mL/min were used. The interface heater was set to 'On' mode to maximize the ion signal and prevent contamination of the ion optics. The optimized source-dependent parameters were used for the subsequent LC/MS/MS experiments.

2.4. Mobile phase optimization

Triplicate LC/MS/MS analyses of a mixture containing 250 ng/mL of LA and 500 ng/mL of IS in acetonitrile:water (50:50) were performed consecutively using each of the investigated mobile phase combinations. All chromatographic separations were performed using a Luna C_{18} 3 μm 50 mm × 2 mm i.d column (Phenomenex, Torrance, CA, USA) at a column temperature of 60 °C and mobile phase flow rate of 0.7 mL/min. The LC gradient used in the experiment was unique to each of the ten tested mobile phase combinations. All LC methods involved the following set-up: a linear gradient from a range of 15–60% solvent B to 75–95% solvent B (0–1.90 min), isocratic at 95% solvent B (1.90–2.50 min) and isocratic at 15–60% solvent B (2.51–5.00 min). The peak width, signal-to-noise (S/N) ratio and integrated peak areas of LA and IS were documented for each of the ten mobile phase combinations. Selected combinations were analyzed statistically using the paired *t*-test (GraphPad Prism 4, San Diego, CA, USA).

2.5. Sample extraction optimization

The extraction efficiency of LA and IS from blood and brain was explored using (a) protein precipitation (PP) using acetonitrile, and liquid–liquid extraction (LLE) using (b) *tert*-butyl methyl ether (MTBE), (c) ethyl acetate (EA) and (d) dichloromethane (DCM). Brain homogenate was prepared by homogenizing the brain tissues with an equal weight of water. For the extraction by PP, 50 μL of blood was diluted with 50 μL of water. The diluted blood was then spiked with 50 μL each of 500 ng/mL LA and 250 ng/mL IS. 300 μL of acetonitrile was added and the mixture was vortex-mixed at high speed for 2 min. After centrifugation for 20 min at 13,000 rpm and 4 °C, the supernatant was transferred into a HPLC vial and 10 μL was injected into the LC/MS/MS for analysis. This extraction procedure was repeated for the brain samples by substituting the 100 μL of blood:water (1:1) mixture with 50 μL of brain homogenate. For the extraction by LLE, 50 μL of water was added to 50 μL of blood and the diluted blood was spiked with 50 μL each of 100 ng/mL LA and 50 ng/mL IS. 1250 μL of MTBE, EA or DCM was then added and the mixture was vortex-mixed at high speed for 30 min. The mixture was centrifuged for 15 min at 13,000 rpm and 4 °C. 1190 μL of the supernatant was transferred into a clean Eppendorf tube and evaporated to dryness for 20 min at 40 °C under a gentle flow of nitrogen gas (TurboVap LV, Caliper Life Science, Hopkinton, MA, USA). The residue was reconstituted with 100 μL of 40% acetonitrile in water, vortex-mixed for 2 min, sonicated for 5 min and then centrifuged for 10 min at 13,000 rpm and 4 °C. 70 μL of supernatant was transferred into a HPLC vial and 10 μL was injected for LC/MS/MS analysis. This extraction procedure was repeated for the brain samples by substituting 50 μL of blood with 50 μL of brain homogenate. Each extraction method for each sample matrix was performed in triplicates. A control mixture prepared by adding 50 μL each of 500 ng/mL LA and 250 ng/mL IS to 400 μL of acetonitrile:water (40:60) was used to calculate the recovery of LA and IS from blood (PP and LLE) and brain (LLE). For the calculation of recovery from brain using PP, the same control mixture was prepared except that volume of acetonitrile:water (40:60) was reduced to 350 μL due to the differences of volume in sample preparation of blood and brain by PP. Percentage recovery of LA and IS was calculated using the formula:

$$\text{Percentage recovery} = \frac{\text{Peak Area}_{\text{sample}}}{\text{Peak Area}_{\text{control}}} \times 100\%$$

2.6. Method validation

Lower limit of quantitation (LLOQ) was determined based on the criteria that: (1) the analyte response is at least five times of baseline noise, and (2) can be determined with precision of 20% and accuracy of 80–120%. The concentrations of LA experimented to determine the LLOQ were 0.01, 0.1, 1, 2, 5 and 10 ng/mL. On-column lower limit of detection (LLOD) was determined based on the criterion that the analyte response is at least three times of baseline noise. The concentrations of LA explored for LLOD determination ranged from 0.1 pg/mL to 1 ng/mL. The absolute recovery and percentage ion suppression/enhancement was determined at 5, 100 and 5000 ng/mL of LA in blood and brain samples. For each concentration, three sets of samples in six replicates were prepared: (1) LA and IS spiked into blood and brain samples before protein precipitation (PP) extraction, (2) LA and IS spiked into PP-treated blank blood and brain extracts, and (3) LA and IS spiked into PP-treated blank solvent. The peak areas of LA of *set 1* samples were compared to those of *set 2* to calculate absolute recovery.

3. Results and discussion

3.1. Analytical method optimization

Both LA and IS possess carboxylic acid moieties (Fig. 1A and B) which can be easily ionized to carboxylate ions, therefore all MS experiments were conducted in the ESI −ve mode. Product ion scan of LA (*m*/*z* 205.0) displayed clear and abundant product

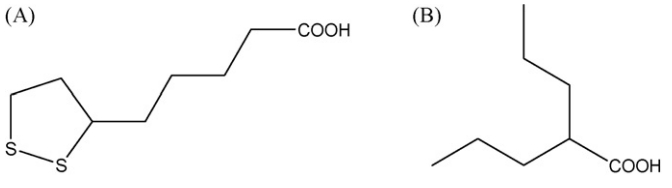

Fig. 1. Chemical structures of (A) LA and (B) IS.

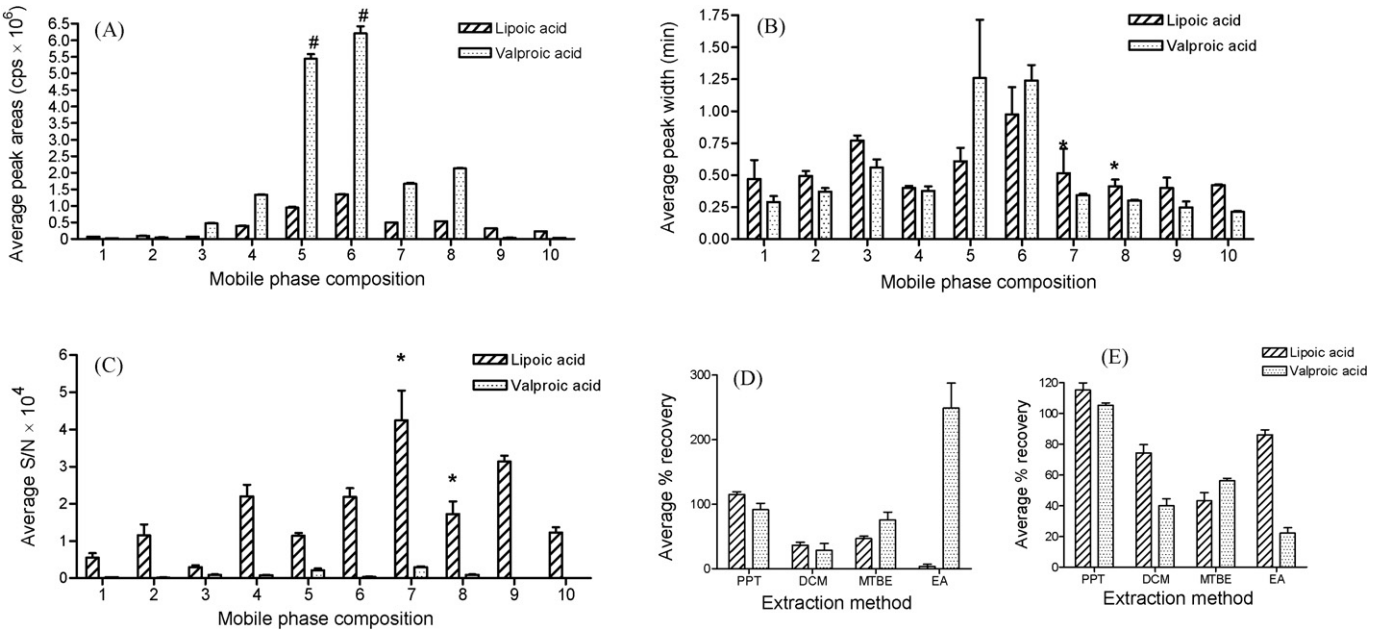

Fig. 2. Comparison of the average (A) peak area, (B) peak width and (C) signal-to-noise ratio amongst the ten different mobile phase combinations: (1) 0.1% formic acid in 10 mM ammonium acetate/acetonitrile, (2) 0.1% formic acid in 10 mM ammonium acetate/methanol, (3) 10 mM ammonium acetate/acetonitrile, (4) 10 mM ammonium acetate/methanol, (5) water/acetonitrile, (6) water/methanol, (7) 0.1% acetic acid (pH 4)/acetonitrile, (8) 0.1% acetic acid (pH 4)/methanol, (9) 0.1% formic acid/acetonitrile and (10) 0.1% formic acid/methanol. # indicates peak splitting. * indicates differences that are not statistically significant ($P > 0.05$) between mobile phase combinations 7 and 8. Comparison of the extraction efficiency of LA and IS by PPT (protein precipitation), DCM (LLE using DCM), MTBE (LLE using MTBE) and EA (LLE using EA) from (D) blood and (E) brain.

Table 1
(A) Optimized MS parameters for the detection of LA and VA (internal standard, IS) using mobile phase combination 7. (B) Intra- and inter-day precision and accuracy of LA spiked in rat brain.

Parameter	Value				
(A)					
Curtain gas, psi	15				
Ionspray voltage, V	−4500				
Temperature, °C	600				
GS 1, psi	55				
GS 2, psi	60				
EP, V	−4				
CXP, V	−1				
DP for LA, V	−30				
DP for VA, V	−42				
CE for LA, V	−14				
CE for VA, V	−10.5				
Nominal concentration (ng/mL)	Parameters	Intra-day			Inter-day
		Day 1 ($n = 6$)	Day 2 ($n = 3$)	Day 3 ($n = 3$)	3 days ($n = 12$)
(B)					
0.1	Mean accuracy (%)	111.35	102.83	93.87	102.68
	S.D.	29.38	8.21	12.51	8.74
	R.S.D. (%)	26.39	7.98	13.33	8.51
0.5	Mean accuracy (%)	110.82	110.93	91.20	104.32
	S.D.	10.11	5.79	3.28	11.36
	R.S.D. (%)	9.12	5.22	3.60	10.89
1	Mean accuracy (%)	108.88	108.03	111.80	109.57
	S.D.	8.71	4.44	7.07	1.98
	R.S.D. (%)	8.00	4.11	6.32	1.80
10	Mean accuracy (%)	115.75	105.83	110.20	110.59
	S.D.	2.61	2.23	5.34	4.97
	R.S.D. (%)	2.26	2.11	4.85	4.49
50	Mean accuracy (%)	116.17	114.03	112.80	114.33
	S.D.	1.90	2.47	2.95	1.70
	R.S.D. (%)	1.63	2.16	2.61	1.49
100	Mean accuracy (%)	112.98	108.93	114.33	112.08
	S.D.	3.76	1.63	1.75	2.81
	R.S.D. (%)	3.33	1.49	1.53	2.51

ions at m/z 171.0. However, the product ion scan of IS (m/z 142.9) yielded minimal low abundance product ions (m/z 71.1 and 80.0). Hence, transitions of m/z 205.0–171.0 and m/z 142.9–142.9 were used in the MRM experiments to quantitate LA and IS, respectively. Comparisons of the average peak areas, widths and signal-to-noise (S/N) ratios amongst the ten mobile phase systems are shown in Fig. 2A–C. In general, mobile phase combinations with the addition of weak acids resulted in better peak shapes and higher S/N ratios for both LA and IS. This observation may be due to the conjugate bases of the weak acids aiding in deprotonation of the analyte and IS. This is consistent with the use of weak acids as solvent A by both Trivedi et al. [7] and Chen et al. [8] in their analysis of LA by LC/MS/MS. Formic acid ($pK_a = 3.75$) yielded poorer sensitivity when compared to acetic acid ($pK_a = 4.75$). This might be due to the lower pK_a of formic acid resulting in a less basic formate ion and therefore, a lower degree of ionization of the two acidic analytes. Although combinations 5 and 6 appeared to yield the highest average peak area, peak splitting occurred. Such poor peak shape may result in inaccurate quantitation and is not desirable. Combinations 7 and 8 yielded the next highest average peak areas with combination 7 resulting in the highest S/N ratio compared to all other combinations. The difference between the average S/N ratios and peak widths of combinations 7 and 8 were not statistically significant ($P > 0.05$). Among the ten mobile phase combinations, combination 7 consisting of 0.1% acetic acid (pH 4 adjusted with ammonia solution) (solvent A) and acetonitrile (solvent B) was considered the best combination due to its high S/N ratio, considerable average peak area, optimum peak shape and small average peak width. The optimized MS parameters for the detection of LA and IS using mobile phase combination 7 are summarized in Table 1A. All optimized parameters for both analyte and IS were similar except for DP and CE. The elution conditions for combination 7 were: linear gradient 40–95% solvent B (0–1.90 min), isocratic at 95% solvent B (1.90–2.50 min) and isocratic at 40% solvent B (2.51–5.00 min). Based on the retention time of LA from the previous method, the elution conditions were recalculated and adjusted to shorten the run time for sample analyses. For all subsequent experiments, the elution conditions were: linear gradient 40–84% solvent B (0–1.50 min), isocratic at 84% solvent B (1.50–2.00 min) and isocratic at 40% solvent B (2.01–3.50 min).

3.2. Sample extraction optimization

Comparisons of the extraction efficiency by PP using acetonitrile and LLE using MTBE, DCM and EA from blood and brain samples are shown in Fig. 2D and E, respectively. In both blood and brain samples, extraction of LA and IS by PP yielded the highest efficiency ranging from 92 to 115%. LA was not extracted efficiently by LLE as compared to PP and this may be attributed to the greater polarity of LA resulting in its poor partitioning into non-polar solvents. Extraction of the IS from the blood samples by LLE using EA yielded an unusually high efficiency of 248%. This observation was probably due to an ion enhancement effect. As the extraction of LA from blood using the same LLE method with EA showed very poor efficiency, we concluded that this condition was not optimum. Sample extraction by PP was most optimal and was employed in all subsequent sample preparations.

3.3. Method validation

The LLOQ of LA was 0.1 ng/mL (0.485 nM) in both blood and brain while on-column LLOD was 0.03 pg. The absolute recovery of LA from blood ranged from 47 to 52% and from brain, 56 to 66%, across the experimented three concentrations. The average absolute recovery of IS was 69% from blood and 74% from brain. The regions of signal suppression or enhancement were investigated to ensure that it did not interfere with the signal of LA and the IS. Under this chromatographic condition, the retention time of LA and IS was 0.70 min and 1.33 min, respectively and the signals were clearly resolved from the regions of ion suppression and enhancement (data not shown). The intra- and inter-day precisions and accuracy of the method are summarized in Table 1B. The precision (% R.S.D.) ranged from 1.49 to 26.39% and 1.49 to 10.89% for intra- and inter-day assay, respectively. The accuracy ranged from 91.2 to 116.17% for intra-day assay and 102.68 to 114.33% for inter-day assay. This method was successfully employed to analyze LA in blood and brain pharmacokinetic (PK) samples and enabled the detection of endogenous levels of LA [13]. This sensitive, accurate and precise LC/MS/MS method will play an important role in the determination of the basal endogenous levels of LA in the biological system.

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