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Short communication

Quantification of apolipoprotein A-I mimetic peptide D-4F in rabbit plasma by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry

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ABSTRACT

The apolipoprotein A-I mimetic peptide D-4F is a potential therapeutical agent effective in maintaining cardiovascular health. A bioanalytical assay based on high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC/MS/MS) to quantitate the D-4F amount in rabbit plasma was developed and validated. A compound with a close structure similarity to the D-4F (only one amino acid A–V altered) was used as an internal standard. Both D-4F and the internal standard were extracted by protein precipitation using acetonitrile/0.2% Triton XL 80N. The correlation coefficient of the calibration curve was 0.9991 in the range 20–40,000 ng/mL. This assay can be used for pharmacokinetic studies of the drug. Also, it may be adjusted for the quantification of other members of apolipoprotein A-I mimetic peptide family.

1. Introduction

Apolipoprotein A-I (apoA-I), the main protein of high-density lipoproteins (HDL), provides cardiovascular benefits by removing phospholipids and cholesterols via the ABCA1 pathway [1,2]. ApoA-I contains 243 amino acids with lipid-binding properties. In order to search molecules that can mimic apoA-I's effect in maintaining cardiovascular health, a series of 18 amino acid peptides have been synthesized and studied [3]. The peptides have the capability to form a class A amphipathic helix similar to those found in apoA-I which brings along a similar lipid-binding properties [4]. D-4F is an important member of this peptide family whose pharmacological effect has been well studied and documented [5,6]. In the 18 amino acids of D-4F, there are four phenylalanines and all the amino acids in this sequence are D form rather than the naturally occurring L form to enhance enzymatic stability (Scheme 1A). Even though extensive studies have been carried out to investigate the efficacy and pharmacological effect of apoA-I mimetic peptides [7–10], information regarding bioanalytical methods of these peptides is very limited. To the best of our knowledge, there is no validated bioanalytical method available for any apoA-I mimetic peptide. In this study, we developed and validated a LC/MS/MS assay to quantitate the amount of D-4F in the rabbit plasma without using expensive isotope-labeled compound as internal standard. This assay will shine further light on the apoA-I mimetic peptide research. This assay can also be adjusted for the quantification of other members in this apoA-I mimetic peptide family in different biological matrices.

2. Experimental

2.1. Chemicals and reagents

Apolipoprotein A-I mimetic peptide D-4F and the internal standard (IS) (purity >98.5% by HPLC) were synthesized by the American Peptide Company (Sunnyvale, CA). Triton XL 80N was purchased from Sigma (St. Louis, MO). Methanol (HPLC grade) and formic acid (>95% purity) were purchased from Aldrich (Milwaukee, WI). Blank rabbit plasma was obtained from Pel-Freez (Rogers, AR). The HPLC grade water was generated in-house using MilliQ system (>18 MΩ) (Millipore, Billerica, MA).

2.2. Instrumentation

A Thermo TSQ Quantum discovery triple quadruple mass spectrometer coupled with a Thermo Surveyor HPLC system (San Jose, CA) was used in this study. A stepwise gradient was employed to elute target peptides from a Waters YMC^{TM} ODS-AQ column (50 mm × 2.0 mm ID, 3 μm) (Pleasanton, CA). From time 0 to 1.5 min, 50% A (0.1% formic acid in water) and 50% B (0.1% formic acid in methanol) were flushed through the column. After 1.5 min, the gradient jumped to 95% B, 5% A in 0.01 min and this mobile

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A) D-4F Ac-DWFK**A**FYDKVAEKFKEAF-NH_2 (amino acids all in D form)

B) Internal standard Ac-DWFK**V**FYDKVAEKFKEAF-NH_2 (amino acids all in L form)

Scheme 1. Primary sequence of (A) D-4F and (B) internal standard.

phase composition was maintained for 6.5 min. Subsequently, in 0.01 min, the mobile phase composition was adjusted back to 50% A and 50% B and maintained for 5 min to equilibrate for next run. The flow rate was 0.3 mL/min and the column temperature was kept at 45 °C. The built-in divert valve on the mass spectrometer was used to conduct elute from 4.5 to 7 min to the electrospray ion source. The LC/MS/MS system was controlled by Xcalibur™ software (San Jose, CA). The electrospray source was operated in positive mode with voltage at 4.5 kV. Cone voltage was 12 V and transfer tube was set at 280 °C. Argon gas was employed as the collision gas and the pressure after optimization was 3 mTorr. The collision energy was 51 eV. During multiple reaction monitoring, the transition 578.6–119.9 was monitored for D-4F and 585.6–119.9 for the IS.

2.3. Sample preparation

Stock solutions of D-4F and IS were prepared by dissolving 10 mg of D-4F peptide powder in 10 mL of 1% acetic acid solution to reach 1000 μg/mL. The IS solution was further diluted in 1% acetic acid to produce 20 μg/mL concentration. D-4F was diluted using blank rabbit plasma to a series of calibration standards, ranging from 20 to 40,000 ng/mL. A 270-μL aliquot of each D-4F plasma standard was mixed with 30 μL from IS solution (20 μg/mL), after which 300 μL of 0.2% Triton XL 80N in ACN was added. After vortexing for 30 s, the mixture was centrifuged at 13,000 rpm for 10 min and the supernatant analyzed using LC/MS/MS. A series of QC solution were prepared separately in the same manner at concentrations 50, 200, 2000 and 20,000 ng/mL and analyzed. The injection volume for each solution was 25 μL.

3. Results and discussion

3.1. Method development

The quality of internal standard (IS) plays a critical role in the quantitative determination using LC/MS/MS. In this study, the IS design was closely aligned with the D-4F amino acid sequence by switching an alanine (A) in D-4F to a valine (V) in IS. Given the relatively long sequence of D-4F and the hydrophobic property of both A and V, the IS will have similar physical properties compared with D-4F. Thus, the IS could mimic closely the behavior of D-4F both in sample preparation process and in chromatographic system. Under the selected chromatographic conditions, D-4F and IS co-elute with each other, which could minimize the matrix effect from endogenous interference.

The mass transitions in MRM mode were obtained as m/z 578.6 (4+) to 119.9 (immonium ion of phenylalanine, F) for D-4F and m/z 585.6 (4+) to 119.9 (immonium ion of F) for IS (Fig. 1). The quadruple charged species were chosen as precursor for both D-4F and IS because these ions show the highest intensity under acidic mobile phase condition. In the selection of product ions, we found that it is more efficient to select the immonium ion of F compared with b ions and y ions which may be explained as both D-4F and IS contain four phenylalanines, and also the higher internal energy in quarterly charged species will lead to more complete fragmentation.

All parameters of MS under electrospray positive mode were optimized by flow injection analysis. The optimum capillary voltage was 4.5 kV for both D-4F and IS. The highest ion counts for both MRM transitions stated above were obtained under cone voltage at 12 V, collision energy at 51 eV and the Argon collision gas pressure at 3.0 mTorr.

Generally, in plasma protein crash method for sample clean up and compound extraction, a higher ACN content (normally >3×) is preferred as a cleaner background could be achieved and the matrix effect in MS could be minimized. However, in our study, a minimal ACN content was used (1×), as both D-4F and IS have a better solubility in aqueous condition than in organic phase. Also, under this extraction condition, no obvious endogenous inference and matrix effect was observed, indicating that this approach is satisfactory. In the extracting ACN solution, Triton XL 80N was added to 0.2% (v/v) level which improves the recovery of D-4F from sample loss in the sample preparation process due to adsorption and retains the calibration linearity within a wide dynamic range.

3.2. Specificity

The chromatographic and MS conditions used could potentially cause problems in the assay specificity for the short retention time and the relatively high energy used in CAD. Any phenylalanine-containing peptide eluting at this retention time will generate interference. To access the specificity of this assay, the blank rabbit plasma samples and the plasma samples spiked with D-4F and IS were compared. As shown in Fig. 2A, no interference from any endogenous compounds were observed at the retention time of target compound and IS in MRM, indicating that this method is capable of the differentiation and quantification of the analyte in the presence of other components in the sample such as endogenous matrix components, and decomposition products.

3.3. Calibration

Calibration standards were prepared at seven different concentrations using blank plasma: 20, 100, 500, 1000, 5000, 10,000, 40,000 ng/mL, and 5 injections were made for each standards. The calibration curve was established using linear through zero fitting of the data. The calibration curve equation is $y = 0.8509x$, where y represents the peak area ratios of D-4F to IS and x represents the concentration of D-4F in plasma. The correlation coefficient was 0.9991, indicating a good linearity across the wide calibration range. Based on S/N > 10, RSD < 15% and the carryover from ULOQ to LLOQ < 20% of the LLOQ peak area, the LLOQ was determined as 20 ng/ml.

3.4. Accuracy (extraction recovery) and precision

To evaluate the accuracy and precision of this method, QC samples prepared in blank rabbit plasma separately at four levels, 50, 200, 2000 and 20,000 ng/mL were assessed. Each sample was injected five times to evaluate the precision. The accuracy was obtained by comparing the mean recovery with theoretical values. The data indicated that the accuracy was ranging from 105.3% to 96.7% and the repeatability was below 4.0% RSD. The results met the acceptable specification for bioanalysis of D-4F.

3.5. Stability

The studies were performed to gain information on the stability of standard and sample solutions under defined storage conditions and to assure that the solutions are stable enough to allow for delays such as instrument breakdowns or overnight analyses

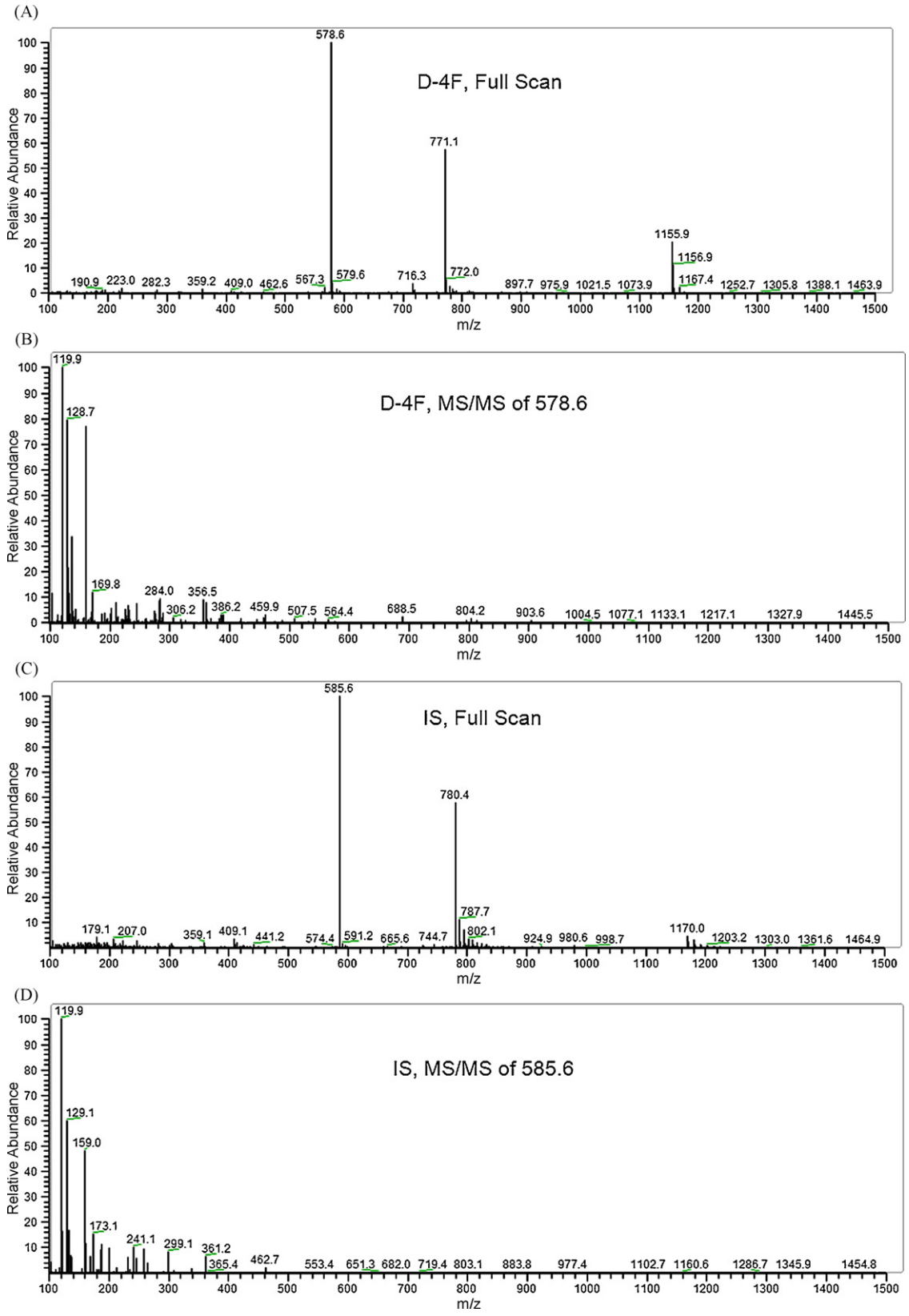

Fig. 1. Mass spectra of D-4F and IS. (A) Full scan spectrum of D-4F; (B) MS/MS spectrum of precursor ion *m/z* 578.6 of D-4F; (C) full scan spectrum of IS;. (D) MS/MS spectrum of 585.6 of IS.

using auto-samplers. The stability of stock solutions was evaluated over a 30-day period at 4 °C for long term storage and 1 day at room temperature for short term storage. In addition, the stability of stock solutions was determined after three freeze–thaw cycles. There was no significant loss in D-4F as shown among different concentration levels indicating the satisfactory stability of D-4F (Table 1). In the initial method development without adding surfactant into the extracting buffer, poor stability was observed.

Table 1
Stability of stock solutions.

Stock conditions	Found conc. (ng/mL) (SD) [%RSD]/theoretical conc. (ng/mL)			
	50	200	2000	20,000
Long term (1 month at 4 °C)	48.1 (1.38) [2.87]	218.8 (5.41) [2.47]	2083.8 (33.73) [1.62]	21,183.5 (263.04) [1.24]
Short term (24 h at room temp.)	47.4 (2.73) [5.75]	217.2 (2.68) [1.23]	2083.5 (39.25) [1.88]	20,595.9 (129.64) [0.63]
Freeze–thaw (three cycles)	49.6 (3.19) [6.43]	208.2 (13.03) [6.25]	2088.6 (40.81) [1.95]	21,170.3 (152.64) [0.72]

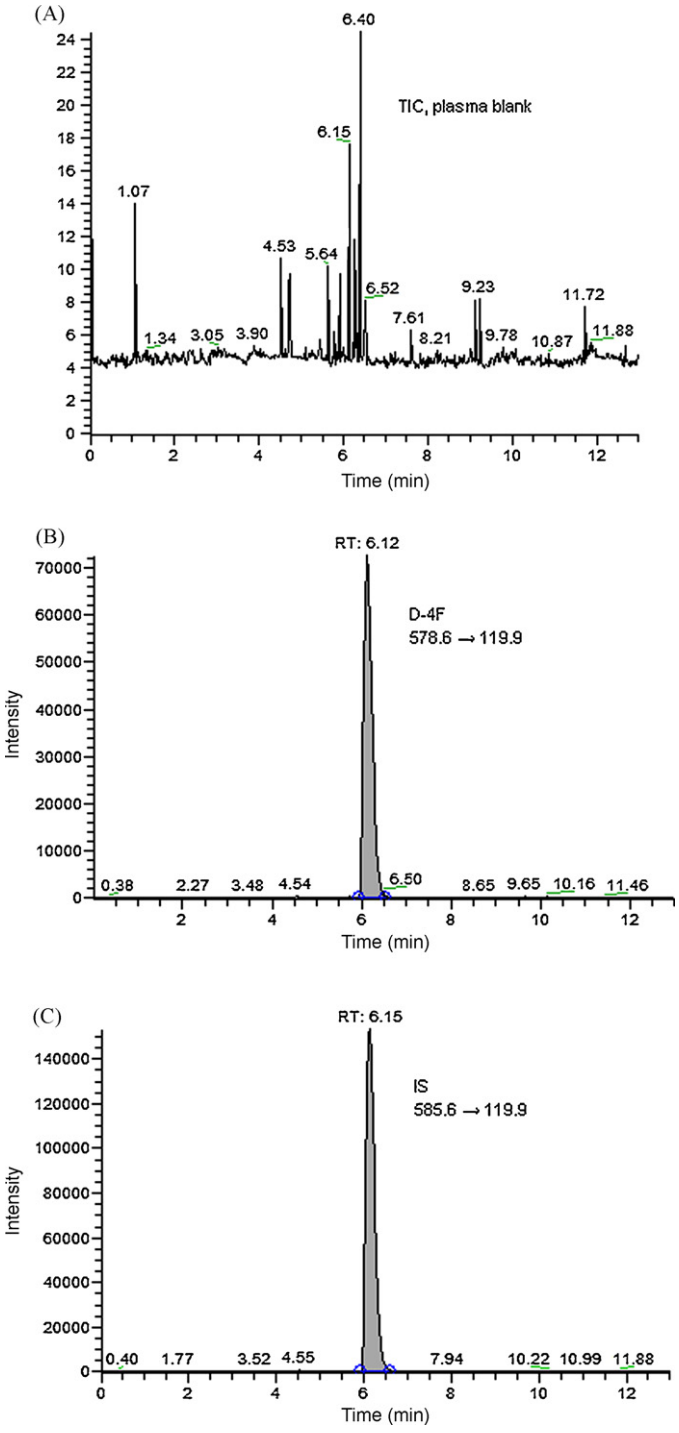

Fig. 2. Representative chromatograms of plasma blank, D-4F and IS. (A) TIC obtained from both transitions (578.6–119.9 and 585.6–119.9) from plasma blank after extraction; (B) representative chromatogram of D-4F from 1000 ng/mL standard plasma solution after extraction; (C) representative chromatogram of internal standard in plasma sample after extraction.

Switching to surface-deactivated container in sample preparation demonstrated similar results. However, by adding Triton XL 80N, the stability data fell into a satisfactory range. This indicates that the poor recovery is from the sticky nature of this peptide which can cause sample loss during storage and preparation. Freshly adding internal standard cannot compensate the sample loss, which could be explained as the binding of the D-4F to the container was fortified with longer period of storage. Surfactants have been helpful to improve the recovery of extremely hydrophobic analytes including both small molecules and macromolecules [11–14]. In this study, Triton XL 80N demonstrated good capability to recover samples loss due to strong non-specific binding. Using Triton XL 80N is novel in biological sample preparation, which could be useful to researchers to recover samples with sticky nature. Triton XL 80N did not show negative effect on column life, as the column performs consistently after more than a thousand injections.

3.6. Application

The method was successfully applied to in-house preclinical research and D-4F concentrations in rabbit plasma were successful quantified. The plasma concentrations versus time profile and pharmacokinetic parameters of D-4F have been successfully obtained.

4. Conclusion

In summary, the assay was developed and validated for the quantitative determination of D-4F in rabbit plasma using LC/MS/MS with electrospray interface. This method is easy to perform by using non-isotope-labeled internal standard. In the sample preparation process, surfactant was added into acetonitrile which minimizes the sample loss during sample storage and peptide extraction. This assay will provide useful information in the development of LC/MS/MS-based methods to quantitate other peptides in apoA-I mimetic peptide family in various biological matrices.

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References

[1] H.B. Brewer Jr., A.T. Remaley, E.B. Neufeld, F. Basso, C. Joyce, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 1755.
[2] Y. Zhang, I. Zanotti, M.P. Reilly, J.M. Glick, G.H. Rothblat, D.J. Rader, Circulation 108 (2003) 661.
[3] G.M. Anantharamaiah, J.L. Jones, C.G. Brouillette, C.F. Schmidt, B.H. Chung, T.A. Hughes, A.S. Bhown, J.P. Segrest, J. Biol. Chem. 260 (1985) 10248.
[4] G.M. Anantharamaiah, Methods Enzymol. 128 (1986) 627.
[5] M. Navab, G.M. Anantharamaiah, S. Hama, D.W. Garber, M. Chaddha, G. Hough, R. Lallone, A.M. Fogelman, Circulation 105 (2002) 290.
[6] M. Navab, G.M. Anantharamaiah, S.T. Reddy, S. Hama, G. Hough, V.R. Grijalva, A.C. Wagner, J.S. Frank, G. Datta, D. Garber, A.M. Fogelman, Circulation 109 (2004) 3215.
[7] B.J. Van Lenten, A.C. Wagner, G.M. Anantharamaiah, M. Navab, S.T. Reddy, G.M. Buga, A.M. Fogelman, Curr. Atheroscler. Rep. 11 (2009) 52.
[8] Q. Zhang, L. Liu, X.Y. Zheng, Int. J. Cardiol. (2008).
[9] M. Navab, G.M. Anantharamaiah, S.T. Reddy, B.J. Van Lenten, A.M. Fogelman, Curr. Drug Targets 9 (2008) 204.

[10] C.R. White, G. Datta, Z. Zhang, H. Gupta, D.W. Garber, V.K. Mishra, M.N. Palgunachari, S.P. Handattu, M. Chaddha, G.M. Anantharamaiah, Curr. Atheroscler. Rep. 10 (2008) 405.
[11] C. Chen, L. Bajpai, N. Mollova, K. Leung, J. Chromatogr. B 877 (2009) 943.
[12] Y. Xu, L. Du, M.J. Rose, I. Fu, E.J. Woolf, D.G. Musson, J. Chromatogr. B 818 (2005) 241.
[13] N. Nagaraj, A. Lu, M. Mann, J.R. Wisniewski, J. Proteome Res. 7 (2008) 5028.
[14] E.I. Chen, D. Cociorva, J.L. Norris, J.R. Yates III, J. Proteome Res. 6 (2007) 2529.