Analytical Methods

Accepted Manuscript

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Determination of newly synthesized lipoic acid-fasudil dimer in rat plasma by LC-MS/MS and its application to pharmacokinetics study

Waner Hou[1], Guanghui Liu[1], Xiuman Sun[1], Zhiyong Xie[*2], Rongbiao Pi[2], Wei He[1], Manna Lin[1], and Qiongfeng Liao[*1]

[1] School of Chinese Materia Medica, Guangzhou University of Chinese Medicine, Guangzhou, 510006, China

[2] School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, 510006, China

*Corresponding author details:

[1] Name: Qiongfeng Liao
E-mail: liaoqf2075@yahoo.com
Tel: +86-20-39358081
Fax: +86 20 39358081
Address: School of Chinese Materia Medica, Guangzhou University of Chinese Medicine, 232 East Waihuan Road, University Town; Guangzhou 510006, P. R. China.

[2] Name: Zhiyong Xie
E-mail: xiezy2074@yahoo.com
Tel: +86-20-39943047
Fax: +86 20 3994 3047
Address: School of Pharmaceutical Sciences, Sun Yat-sen University, 132 East Waihuan Road, University Town; Guangzhou 510006, P. R. China.

Analytical Methods Accepted Manuscript

Abstract

The aim of the present work was to develop and validate an LC-MS/MS method with electrospray ionization （ESI） for the quantification of lipoic acid-fasudil dimer （L-F 001）, a novel multi-functional neuroprotective drug in rat plasma using tetrahydropalmatine as internal standard. The separation was carried out on a C_{18} column using a mobile phase consisting of acetonitrile and water (containing 5 mmol/l ammonium acetate adjusted to pH 5.0 with formic acid). The detection was performed by multiple reaction monitoring (MRM) mode via ESI source operating in the positive ionization mode. Linear calibration curve was obtained over the concentration range of 1.0-1000.0 ng/ml for lipoic acid-fasudil dimer (L-F 001). Accuracy, precision and extraction recovery of the analyte was satisfactory. The validated method was successfully applied to monitor pharmacokinetic profile of lipoic acid-fasudil dimer (L-F 001) after oral administration to rats.

Keywords: lipoic acid-fasudil dimer (L-F 001); LC-MS/MS; pharmacokinetic study

1. Introduction

In recent studies, an increasing amount of efforts have been targeted to synthesize a series of dimmers, which connected another functional molecules to the drug. Experts also found that dimeric drugs have better pharmacological activities with minimized side effects or toxicity. For example, previous studies had found that tacrin dimmers such as tacrine (n, n = 2, 3, 6)-ferulic acid (TnFA), bis (7) -tacrine (B7T) and bis (12) -hupyridone (B12H) are much more potent and selective on acetylcholinesterase

Analytical Methods Accepted Manuscript

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

61 (AChE) than tacrine[1-4]. Other studies had found that the complexation of hardly
62 soluble drug fisetin with cyclosophoraose (Cys) dimer could improve the solubility of
63 fisetin (its solubility was increased up to 6.5-fold) and increase its bioavailability[5].
64 Lipoic acid-niacin dimer (N2L) was also reported to have reduced the side effects of
65 niacin and increased its bioavailability such as significantly reducing mouse serum
66 FFA concentration and protecting effects against ARPE-19 cell damage[6].
67 Fasudil, the only clinically available Rho-associated coiled-coil protein kinase
68 (ROCK) inhibitor, has provided new insights into the treatment for central nervous
69 system （CNS） disorders, such as Alzheimer’s disease and cerebral stroke[7]. But its
70 selectivity for ROCK is limited[7]. At the same time, fasudil has a short half-life[8] and
71 low brain penetration ability[9]. Therefore, several fasudil analogs were synthesized[7,
72 10-11]. Lipoic acid, a natural antioxidant absorbed from the daily diet, has defensed in
73 brain and other tissues. The effect of lipoic acid could be attributed to its ameliorating
74 effect on the antioxidant defense systems[12-13]. In order to exert more powerful effects
75 with minimized adverse effects in the combat of CNS disorders and realize
76 antioxidative activity, as well as improve the bioavailability of fasudil, Dr R. B. Pi’s
77 team (School of Pharmaceutical Sciences, Sun Yat-sen University) synthesized a
78 novel compound: lipoic acid-fasudil dimer. (Figure 1).
79 Plenty of work proceeded to test whether the newly synthesized dimer is suitable
80 for the clinic treatment, such as ROCK selectivity, blood-brain barrier permeation,
81 vasorelaxant effects and so on. Dr R. B. Pi’s team found that L-F 001 displayed
82 milder activity against ROCK 1 (IC_{50} 1.59μM) and ROCK 2 (IC_{50} 2.10μM). At the

Analytical Methods Accepted Manuscript

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

83 same time, L-F 001 had 5-fold higher brain permeation over Fasudil according to in
84 vitro and in vivo blood-brain barrier permeability tests[14]. So far, pharmacokinetic
85 studies of the newly synthesized dimer still far from enough. Pharmacokinetic studies
86 were aimed at obtaining pharmacokinetic information necessary for new drug
87 development and for ensuring the appropriate use of medicine. Data obtained from
88 such studies were useful for the design and conduct of subsequent studies. So it is
89 necessary to study the pharmacokinetics of L-F 001 and develop a reliable blood drug
90 concentration monitoring method. In this study, a high performance liquid
91 chromatography-tandem mass spectrometry (LC-MS/MS) method was developed,
92 validated and successfully applied to monitor the blood concentration of L-F 001 after
93 an oral administration at 30 mg/kg to rats. It is the first method to detect the novel
94 compound in rat plasma. Furthermore, the study provides some useful information
95 about the subsequent preclinical and clinical studies on the novel compound.
96

97 2. Experimental and methods

98 *2.1 Materials and reagents*

99 Lipoic acid-fasudil dimer (L-F 001, chromatographic purity was 98.0%, purity
100 assessed by mass balance assay was 96.3%) was synthesized in Dr R. B. Pi's
101 laboratory in the School of Pharmaceutical Sciences, Sun Yat-sen University
102 (Guangzhou China). Tetrahydropalmatine (No.110726-200610, purity 98.0%) was
103 purchased from the National Institute for the Control of Pharmaceutical and
104 Biological Products (Beijing, China). HPLC-grade ammonium acetate was purchased

Analytical Methods Accepted Manuscript

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

105 from Aladdin (Shanghai, China). HPLC-grade acetonitrile and methanol purchased
106 from Merck (Darmstadt, Germany) were used for HPLC analysis and plasma sample
107 preparation. All other reagents were of analytical grade.

108 *2.2 Liquid chromatography-tandem mass spectrometry analysis*

109 *2.2.1 Equipment*

110 The LC–MS/MS system consisted of an Agilent 1260 liquid chromatograph and a
111 6460 triple quadrupole mass spectrometry with an electrospray ionization (ESI)
112 source. Data acquisition was performed with MassHunter B 05 software.

113 *2.2.2 Chromatographic Conditions*

114 Chromatographic separation was achieved at 30°C on a BDS Hypersil C_{18} column
115 (2.1 × 50 mm i.d., 2.4μm, Thermo Scientific) with a Phenomenex C_{18} guard column
116 (4 × 2.0 mm i.d.) by gradient solution with 0-1.1min， 90% mobile phase
117 A;1.1–1.2min, 90%→15% mobile phase A; 1.2-3.3min, 15% mobile phase
118 A;3.3-3.4min, 15%→90% mobile phase A, flowing at 0.25 ml/min. Eluent A was
119 water containing 5 mmol/l ammonium acetate adjusted to pH 5.0 with formic acid,
120 and B was acetonitrile. The injection volume was 5 μl. The initial 2.2 min was
121 switched to the waste. The column was fully eluted after a daily analysis cycle
122 (around 100 samples).

123 *2.2.3 Mass Spectrometric Conditions*

124 The mass spectrometer was operated in positive ion mode using multiple reaction
125 monitoring (MRM) to detect the mass transitions. High purity nitrogen served as both
126 nebulizing and drying gas. Compound-dependent parameters of the mass spectrometer

Analytical Methods Accepted Manuscript

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

127 were set as follows: drying gas flow 5 L/min; drying gas temperature 300 ^{o}C;
128 nebulizer pressure 45 psi; capillary voltage 3500V. The precursor-to-product ion pair ,
129 the collision energy (CE) and the fragmentor for L-F 001 and tetrahydropalmatine
130 were as follows: m/z 480.1→189.0 for L-F 001, (collision energy 25 eV， fragmentor
131 195 V) and m/z 356.4→192.1 for IS, (collision energy 26 eV, fragmentor 160 V). The
132 ion transitions m/z 480.1→189.0 and m/z 356.4→192.1 were selected for determining
133 L-F 001 and IS, respectively. Another ion transition m/z 480.1→161.0 (collision
134 energy 29 eV， fragmentor 195 V) for L-F 001 and m/z 356.4→176.1 (collision
135 energy 58 eV, fragmentor 160 V) for IS was used as qualitative channel to confirm the
136 identity of L-F 001 and IS.

137 *2.3 Preparation of standard solution and quality control samples*

138 Concentrated stock solution of L-F 001 was prepared by dissolving the appropriate
139 amount of the sample in acetonitrile at a concentration of 1 mg/ml. And then further
140 diluted with acetonitrile to form a series of working solutions used to prepare the
141 calibration curve. A 0.5 μg/ml stock solution of tetrahydropalmatine (IS) was also
142 prepared in acetonitrile and then diluted to obtain a working solution of 12.5 ng/ml.
143 All the solutions were stored at -20 ^{o}C.

144 The calibration standards were prepared by spiking blank plasma (the pooled
145 plasma came from four non-administration rats of the same batch number with the
146 rats used for pharmacokinetic study) with appropriate amounts of working solutions
147 (2% of the total plasma sample volume), vortex mixing for 30 s. Then they were
148 extracted as described in the 'Preparation of plasma sample' section below. At last the

Analytical Methods Accepted Manuscript

calibration samples were made at concentrations of 1.0, 2.5, 8.0, 20.0, 50.0, 150.0, 400.0 and 1000.0 ng/ml for L-F 001. The quality control samples (QC) used in the validation and during the pharmacokinetic study had been prepared in the same way as the calibration standards with a separated stock solution: L-F 001 (2.5, 50.0, and 900.0 ng/ml).

2.4 Preparation of plasma samples

To a 30 μl of the plasma sample, a 60 μl of IS solution was added. Then the mixture was vortexed for 2 min and centrifugation at 13, 000 rpm for 10 min at 4 ^{o}C. Subsequently, transfer the supernatant liquor to centrifugation at 13, 000 rpm for 5 min at 4 ^{o}C again. Finally, 5 μl of the supernatant liquor was injected into the LC-MS/MS for analysis.

2.5 Validation of the method

The method was validated according to the currently accepted USA Food and Drug Administration (FDA) bioanalytical method validation guidance[15-17].

2.5.1 Selectivity

The selectivity of the method was tested by comparing the chromatograms of six individual rat blank samples, samples at the concentrations of lower limit of quantification (LLOQ) and the rat plasma samples collected after administration. Analytes area in blank plasma should not exceed 20% of that from samples at LLOQ.

2.5.2 Linearity and sensitivity

The calibration curve consisted of eight concentration levels. Each of these samples was prepared and assayed in triplicate on 3 separate days. The linear regression of the ratio of the areas of L-F 001 and the IS peaks versus the concentration were weighted

Analytical Methods Accepted Manuscript

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

172 with weighing factor $1/x^2$ (where x = concentration). The concentrations of the
173 analyte were determined by interpolation from the calibration curve. The LLOQ of the
174 assay, defined as the lowest concentration in the calibration curve that can be
175 measured with accuracy (relative error: RE) and precision (relative standard deviation:
176 RSD) less than 20%.

177 *2.5.3 Precision and accuracy*

178 Three validation batches, each containing six validation samples of three
179 concentration levels (low, middle and high QC level) were analyzed to assess the
180 precision and accuracy of this method on three consecutive days. Concentrations were
181 calculated with calibration curves obtained daily. The inter- and intra-day precision is
182 expressed as the relative standard deviation (RSD). Accuracy is defined as the relative
183 error (RE) and is calculated using the formula RE% = [(measured value - theoretical
184 value)] / theoretical value×100%.

185 *2.5.4 Extraction recovery and matrix effect*

186 The extraction recovery of L-F 001 from blank samples were determined by
187 comparing the areas of QC samples at low, medium and high levels (n = 6) with those
188 of spike-after-extraction samples (representing 100% recovery).

189 The matrix effect on the ionization of L-F 001 was evaluated by comparing the
190 peak areas of L-F 001 resolved in the blank sample (the final solution of blank plasma
191 after extraction and reconstitution) with that resolved in the mobile phase. Three
192 different concentration levels of L-F 001 and 12.5 ng/ml of the IS were evaluated by
193 analyzing the six samples at each level. The blank plasma used in this study were six

Analytical Methods Accepted Manuscript

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

194 different batches of blank rat plasmas. If the ratio is < 85% or >115%, an exogenous
195 matrix effect is implied.

196 *2.5.5 Carryover*

197 Carryover was tested by injecting the processed blank matrix samples sequentially
198 after injecting an ULOQ sample. The response in the first blank matrix at the
199 retention times of analyte and IS should be less than 20% of the response of a LLOQ
200 sample.

201 *2.5.6 Stability*

202 The purpose of the stability study was to evaluate the optimal storage condition of
203 samples. The stability of L-F 001 in rat plasma was investigated by analyzing stability
204 samples at three concentrations (the same levels as QC samples) (n = 6) that were
205 exposed to different conditions. And they were prepared by spiking blank plasma with
206 appropriate amounts of working solutions (2% of the total plasma sample volume) to
207 obtain the concentrations. The short-term stability was determined during storage for
208 4 hour at room temperature, the concentrations of L-F 001 in plasma deviated less
209 than ±10% from those in freshly spiked plasma. The post-treatment stability was
210 obtained by extracted samples maintained in autosampler at 4 ^{o}C for 24 h. The
211 freeze-thaw stability was evaluated after three freeze (-20 ^{o}C) - thaw (room
212 temperature) cycles on 3 consecutive days. The long-term stability was assessed after
213 the QC samples had been stored at -20 ^{o}C for 30 days.

214 *2.6 Application*

215 *2.6.1 Application to pharmacokinetic study*

Analytical Methods Accepted Manuscript

216 Six male Sprague-Dawley rats (200 ± 20 g) were supplied by the Center of
217 Experimental Animals, Sun Yat-sen University (Guangzhou, China, Certificate No.
218 SCXK 20110029). The rats were housed in standard cages at room temperature with
219 free access to food and water until 12 h prior to experiments. The pharmacokinetic
220 study was approved by the Animal Ethics Committee of Sun Yat-sen University.
221 Rats were administrated an oral dose of 30 mg/kg of L-F 001 solution (L-F 001
222 solution was prepared in Cremophor EL/Ethanol (10/90, v/v) at 3 mg/mL)， and the
223 oral dose based on the previous pharmacology experiment by Dr R. B. Pi's team.
224 Blood samples of approximately 0.1 ml were collected in heparinized centrifuge tubes
225 at 0, 5, 15, 30, 60, 90, 120, 180, 240, 360, 480 and 720 min after a single oral
226 administration. Then the samples were centrifuged at 8000 rpm for 10 min. The
227 separated plasma samples were frozen in polypropylene tubes at -20 ^{o}C until analysis.

228 *2.6.2 Data analysis*

229 Non compartmental pharmacokinetic analysis of concentration time data was
230 performed using Winnonlin 5.0.1 software. The pharmacokinetic parameters, such as
231 maximum plasma concentration (C_{max}) and time of maximum concentration (T_{max}),
232 were obtained directly from the plasma concentration-time plots. The elimination rate
233 constants (k) were determined by linear regression analysis of the logarithmic
234 transformation of the last three or four data points of the curve. The elimination
235 half-life ($t_{1/2}$) was calculated using the following equation: $t_{1/2}$ = 0.693/k. The area
236 under the plasma concentration-time curve up to the last time (t) (AUC_{0-t}) was
237 determined using the trapezoidal rule. An unpaired Student's t test was used for

Analytical Methods Accepted Manuscript

238 comparisons with SPSS 13.0.

239 3. Results and discussion

240 *3.1 LC-MS/MS optimization*

241 In the positive ion full scan mode, the protonated molecular ion $[M + H]^+$ for L-F
242 001 and tetrahydropalmatine were the most abundant ions. Then the operation
243 parameters, including fragmentor and collision energy, were optimized by Masshunter
244 Optimizer software. The MS/MS product ion spectra of L-F 001 and IS are shown in
245 Figure 2. Under the product ion scan mode, the most intensive product ions observed
246 were m/z 480.1→189.0 for L-F 001 and m/z 356.4→192.1 for IS. So they were
247 selected for determination of L-F 001 and tetrahydropalmatine. For confirming the
248 identity of L-F 001 and IS, another ion transition m/z 480.1→161.0 for L-F 001 and
249 m/z 356.4→176.1 for IS were used as qualitative channel.

250 In optimizing the chromatographic conditions, the pH of the mobile phase and the
251 concentration of ammonium acetate were explored. Mobile phase of pH 5.0 with
252 formic acid was found to be the best as mobile phase to obtain a good peak shape. So
253 the pH 5.0 mobile phase was adopted. It was found that the concentration of
254 ammonium acetate (5, 10, 15 mmol/l) did not significantly affect the sensitivity and
255 retention time of the analyte. So the concentration of 5 mmol/L adjusted to pH 5.0
256 with formic acid was selected as the mobile phase in the end.

257 *3.2 Selection of internal standard*

258 It is necessary to use an internal standard (IS) to get high accuracy when LC is
259 equipped with MS as the detector. Tetrahydropalmatine was selected because its

Analytical Methods Accepted Manuscript

appropriate retention action, high ionization efficiency, less endogenous interference and it has no significant influence on the determination of L-F 001.

3.3 Method validation

3.3.1 Selectivity

Figure 3 demonstrated typical chromatograms of the blank plasma sample (A), the blank plasma sample spiked with L-F 001 at LLOQ with IS (B), and the test plasma sample obtained after administration of L-F 001 to the rats (C). No significant interference by endogenous entities was observed under the current chromatographic and MS condition. The running time of each injection was only 6 min. The retention time of L-F 001 and IS was 4.14 min, and 4.13 min, respectively.

3.3.2 Linearity and sensitivity

The calibration curve acquired for L-F 001 exhibited a good linear response within the range of concentrations from 1.0 to 1000.0 ng/ml (R2 of calibration curves in all inter-run cases were > 0.99). The LLOQ of L-F 001 was 1.0 ng/ml, and the concentration measured was 1.06±0.06. The RSD of precision was within 5.70% while the RE of accuracy was within 5.85%.

3.3.3 Precision and accuracy

Table 1 summarized the intra- and inter-day precision and accuracy of L-F 001 at three concentration levels (low, medium and high). The intra- and inter-day precision was less than 9.90% for each QC level. The accuracy, determined from QC samples, was within 5.72-9.52% for each QC level. These results were within the acceptance criteria, and indicated that the method was accurate, reliable, and reproducible.

Analytical Methods Accepted Manuscript

1 2 3

282 *3.3.4 Extraction recovery and matrix effect*

283 The extraction recovery and matrix effect of L-F 001 and IS are shown in Table 2.
284 It demonstrated that at three concentration levels of L-F 001, all the extraction
285 recoveries were between 95.14% and 99.48% (RSD%: 4.26-7.49); an endogenous
286 matrix effect is implied if the ratio is less than 85% or greater than 115%. In the
287 current article, the mean absolute matrix effect values obtained for L-F 001 were
288 within 98.20 and 99.79% (RSD: 2.80-8.10), demonstrating that there was no
289 significant matrix effect. And the extraction recovery and matrix effect of IS were
290 98.40±8.55 and 98.07±3.15, respectively.

291 *3.3.5 Carryover*

292 Carryover was tested by injecting blank plasma sample sequentially after injecting
293 an ULOQ sample. The response in the blank matrix at the retention times of L-F 001
294 was 4.3 % of the LLOQ. At the same time, we did not find the carry-over of IS. So the
295 carryover was within the acceptable range.

296 *3.3.6 Stability*

297 In our study, stability samples (at three levels) subject to short-term storage,
298 post-treatment storage, three freeze-thaw cycles, and long-term storage were
299 examined for their stabilities, and the results were summarized in Table 3. As can be
300 seen from Table 3, there were no significant reduction (RE% between 0.54% and
301 11.56%) in the assay concentrations at any QC level following the above conditions.
302 This demonstrated that the newly synthesized compound had a good stability under
303 the four conditions in this method and there were no stability related problems during

Analytical Methods Accepted Manuscript

the routine analysis of samples for the pharmacokinetic study.

3.4 Application of analytical method in pharmacokinetic studies

The LC-MS/MS method was successfully applied to the pharmacokinetic study of L-F 001 following oral administration at a dose of 30 mg/kg. The blood concentration-time profile of L-F 001 is shown in Figure 4 and the determined pharmacokinetic parameters are summarized in Table 4. From the results, the mean maximum concentration of L-F 001 found in plasma after oral administration was reached at 0.67±0.38 h, and the C_{max} was 651.11±380.40 ng/ml, $t_{1/2}$ was 1.29 ± 0.79 h. The pharmacokinetic parameters revealed an $AUC_{0\rightarrow t}$ of 805.47±286.08 ng/ml. This is the first report of pharmacokinetic studies of L-F 001 following an oral administration in rats. For L-F 001 is a new compound which has potential use for the treatment of CNS disorders, data obtained from such studies were useful for the design and conduct of subsequent studies.

4. Conclusion

A sensitive and selective LC-MS/MS method for the determination of L-F 001 in rat plasma using multiple reaction monitoring (MRM) mode via electrospray ionization (ESI) source operating in the positive ionization mode was developed and validated for the first time. The developed method had accepted sensitivity (LLOQ:1.0 ng/ml), selectivity, precision, accuracy in a running time of 6 min. Immediate sample preparation of the one-step protein precipitation showed great simplicity and efficiency and ensured better chemical stability when analysing a large number of plasma samples. And in our study, this method was successfully applied to

Analytical Methods Accepted Manuscript

the pharmacokinetic study of L-F 001 in rats after an oral administration to rats.

Notes:

The authors declare no competing financial interest.

References

1. L. Zhang, H. Yu, W. Li, M. Cheung, Y. Pang, Z. Gu, K. Chan, Y. Wang, Z. Zuo, Y. Han. *International Journal of Pharmaceutics*, 2008, **357**, 85-94.
2. H. Yu, W. Li, K. K. Kan, J. M. Ho, P. R. Carlier, Y. Pang, Z. Gu, Z. Zhong, K. Chan, Y. Wang, Y. Han. *Journal of Pharmaceutical and Biomedical Analysis*. 2008, **46**,75-81.
3. H. Zhang, H. Mak, W. Cui, W. Li, R. Han, S. Hu, M. Ye, R. Pi, Y. Han. *Neurochemistry International*. 2011, **59**, 981-988.
4. R. Pi, X. Mao, X. Chao, Z. Cheng, M. Liu, X. Duan, M. Ye, X. Chen, Z. Mei, P. Liu, W. Li, Y. Han. *Public library of science one*. 2012, **7**, 1-8.
5. D. Jeong, J. M. Choi, Y. Choi, K. Jeong, E. Cho, S. Jung. *Carbohydrate Polymers*. 2013, **97**, 196-202.
6. R. Pi, Y. Jiang, X. Cao, P. Liu, Y. Huang, M. Yao and X. He. Preparation process and medicinal composite of nicotinic acid and its derivatives. CN patentCN102532114A, 2012
7. M. Chen, A. Liu, Y. Ouyang, Y. Huang, X. Chao, R. Pi. *Expert Opin Investig Drugs*. 2013, **22**, 537-50
8. H. Chen, Y. Lin, M. Han, et al. *Journal of Pharmaceutical and Biomedical Analysis*. 2010, **52**, 242-8
9. T. Ishida, Y. Takanashi, H. Kiwada. *Biological and Pharmaceutical Bulletin*. 2006, **29**, 397-402
10. D. Lavogina , K. Kalind , J. Bredihhina , M. Hurt , A. Vaasa , M. Kasari , E. Enkvist , G. Raidaru , A. Uri . *Bioorganic & Medicinal Chemistry Letter.* 2012, **22**, 3425-30
11. P. Ray , J. Wright, J. Adam, S. Boucharens , D. Black , A. R. Brown , O. Epemolu, D. Fletcher, M. Huggett, P. Jones, S. Laats, A. Lyons, J. D. Man, R. Morphy, B. Sherborne, L. Sherry, N. V. Straten, P. Westwood, M. York. *Bioorganic & Medicinal Chemistry Letter*. 2011, **21**, 1084-8
12. T. B. Moraes, F. Zanin, A. D. Rosa, A. D. Oliveira, J. Coelho, F. Petrillo, M. Wajner, C. S .Dutra-Filho. *Journal of the Neurological Sciences*. 2010, **292**, 89–95
13. K. A. Kaplan, F. Odabasoglu, Z. Halici, M. Halici, E. Cadirci, F. Atalay, O. Aydin, A. Cakir. *Journal of Food Science*. 2012, **77**, H224-30.
14. M. Chen, Q. Liu, A. Liu, M. Tan, Z. Xie, A. Uri, Z. Chen, G. Huang, Y. Sun, H. Ge, P. Liu, M. Li, X. Li, S. Wen, R. Pi. RSC Adv. 2014, 4, 37266-37269
15. V. P. Shah, K. K. Midha, S. Dighe, I. J. McGilveray, J. P. Skelly, A. Yacobi, T. Layloff, C. T. Viswanathan, C. E. Cook, R. D. McDowall, K. A. Pittman, S. Spector, *Journal of Pharmaceutical Sciences*, 1992, **81**, 309-312.
16. V. Shah, K. Midha, J.A. Findlay, H. Hill, J. Hulse, I. McGilveray, G. McKay, K. Miller, R. Patnaik, M. Powell, A. Tonelli, C. T. Viswanathan, A. Yacobi, *Pharmaceutical Research*, 2000, **17,** 1551-1557.

Analytical Methods Accepted Manuscript

17. Food and Drug Administration. Guidance for Industry: Bioanalytical Method Validation, US Department of Health and Human Services, FDA, Center for Drug Evaluation and Research, Rockville, MD, 2001.

List of Tables:

Table 1. Intra-day and inter-day accuracy and precision of L-F 001 in rat plasma at low, medium and high concentration levels.

Spiked concentration (ng/mL)	Intraday (n = 6)			Interday (n = 18)		
	Concentration measured (Mean ± SD, ng/ml)	Accuracy (%)	Precision (%)	Concentration measured (Mean ±SD, ng/ml)	Accuracy (%)	Precision (%)
2.5	2.68±0.22	7.10	9.10	2.72±0.25	8.70	9.90
50.0	54.76±3.52	9.52	1.66	54.71±3.46	9.41	6.70
900.0	951.45±27.77	5.72	3.44	954.64±31.18	6.07	1.35

SD: Standard deviation.

Analytical Methods Accepted Manuscript

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

399
400
401
402 Table 2. The extraction recovery and matrix effect of L-F 001 in rat plasma at low,
403 medium, high concentration levels (n = 6).

Analytes	Spiked concentration (ng/mL)	Extraction recovery (%,mean±SD)	RSD(%)	matrix effect (%,mean±SD)	RSD(%)
L-F 001	2.5	95.14±7.49	7.87	99.79±8.09	8.10
	50.0	99.48±6.56	6.59	99.36±3.15	3.17
	900.0	99.06±4.26	4.31	98.20±2.74	2.80
IS	12.5	98.40±8.55	8.69	98.07±3.15	3.21

404 SD: Standard deviation; RSD: Relative standard deviation.
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434

Analytical Methods Accepted Manuscript

Table 3. Stability of L-F 001 in rat plasma (n = 6)

Conditions	L-F 001		
	mean ± SD (ng/ml)	RSD (%)	RE%
short term stability (at room temperature for 4 h)			
2.5	2.59 ± 0.15	2.72	3.13
50.0	52.98 ± 2.63	4.96	5.96
900.0	938.68 ± 10.16	1.08	4.30
long term stability (at -20°C for 30 days)			
2.5	2.61 ±0.13	5.18	4.29
50.0	51.08 ± 1.06	2.06	2.16
900.0	904.89 ± 3.54	0.39	0.54
three freeze-thaw cycles			
2.5	2.79 ± 0.25	8.99	11.56
50.0	52.37 ±1.91	3.64	4.74
900.0	932.73 ± 32.64	3.50	3.64
post-preparative stability(at -4 ^{o}C for 24 h)			
2.5	2.61 ± 0.23	8.98	4.22
50.0	52.15 ± 0.75	1.43	4.30
900.0	943.16± 14.43	1.53	4.80

SD: Standard deviation; RSD: Relative standard deviation; RE: Relative error.

Analytical Methods Accepted Manuscript

1
2
3 461
4 462
5 463
6 464
7
8 465 Table 4. Pharmacokinetic parameters of L-F 001 in rats after single oral administration (n
9 466 = 6).

Pharmacokinetic parameters	Experimental group (n=6) mean ± SD
$t_{1/2}$(h)	1.29±0.79
Ke(h^{-1})	0.71±0.36
C_{max}(ng/m)	651.11±380.40
T_{max}(h)	0.67±0.38
$AUC_{(0-t)}$ (ng/ml·h)	793.63±287.07
$AUC_{(0-\infty)}$ (ng/ml·h)	805.47±286.08

467 SD: Standard deviation.

468
469
470
471
472
473
474
475
476
477
478
479
480

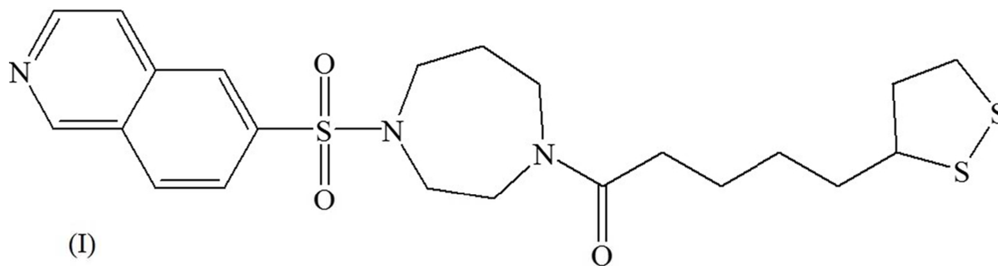
10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

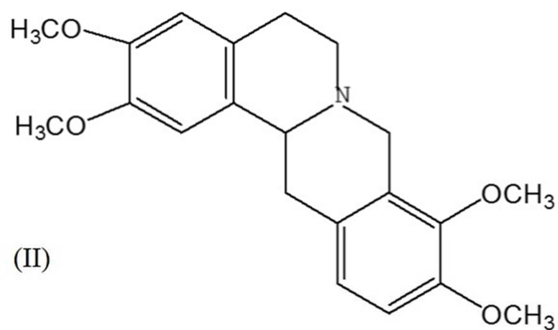
Analytical Methods Accepted Manuscript

1
2
3
4 481
5
6 482
7
8
9 483
10
11 484 **Legends to Figures:**
12
13 485 Figure 1. Chemical structures of lipoic acid-fasudil dimer (L-F 001, I);
14 486 tetrahydropalmatine (IS, II)
15 487
16
17 488 Figure 2. Product ion mass spectra of L-F 001 and IS: (a) L-F 001; (b)
18 489 tetrahydropalmatine (IS)
19 490
20
21 491 Figure 3. Chromatograms of the two compounds in plasma: (A) blank plasma, (B)
22 492 blank plasma spiked with L-F 001 at LLOQ, (C) plasma obtained 30 min
23 493 after a single oral administration of L-F 001 (I = L-F 001, II = IS).
24 494
25
26 495 Figure 4. Mean plasma concentration-time profiles for L-F 001 in rats after a single oral
27 496 dose of 30 mg/kg.
28 497
29 498
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Analytical Methods Accepted Manuscript

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

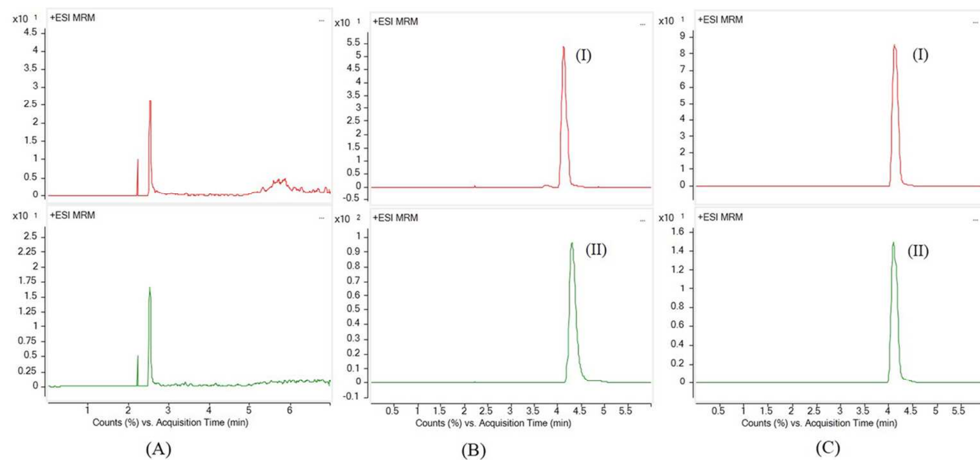




114x74mm (300 x 300 DPI)

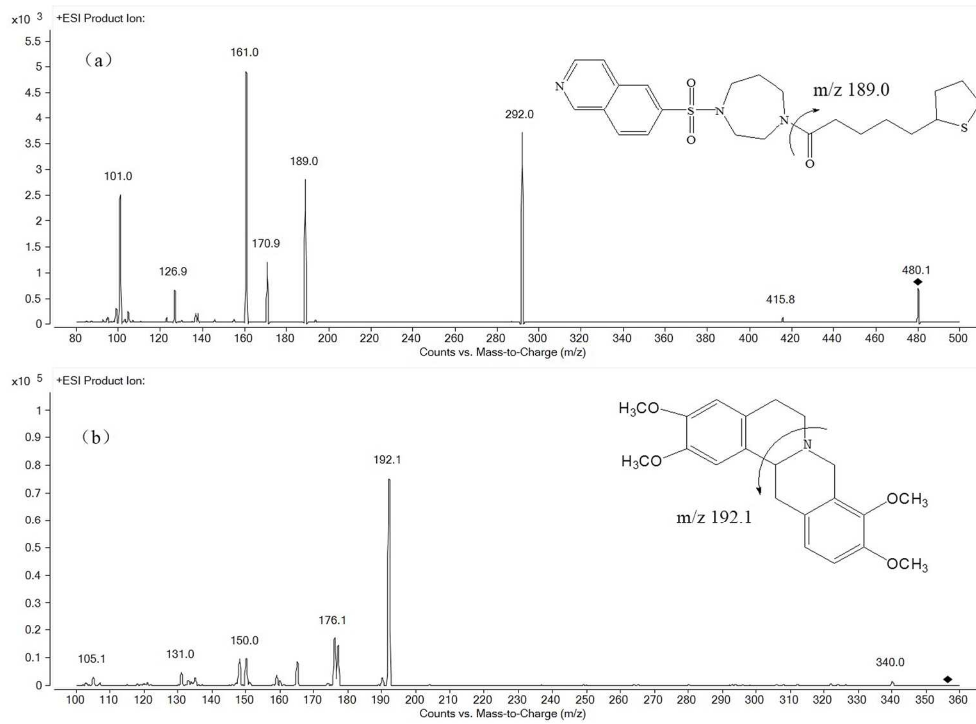
Analytical Methods Accepted Manuscript

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



123x57mm (300 x 300 DPI)

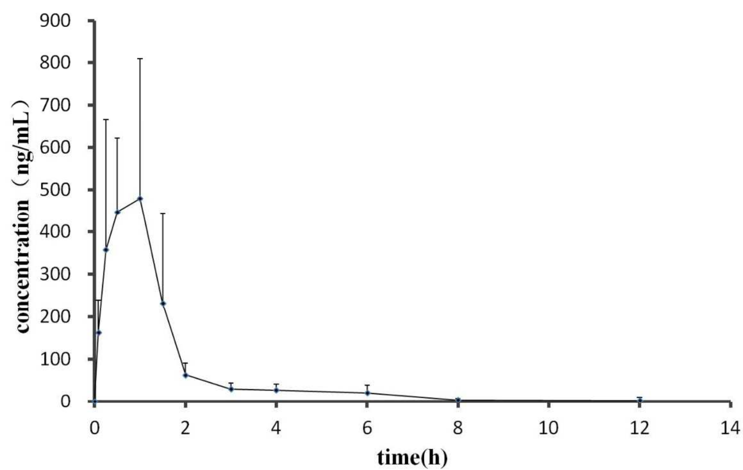
Analytical Methods Accepted Manuscript



180x130mm (300 x 300 DPI)

Analytical Methods Accepted Manuscript

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



Analytical Methods Accepted Manuscript