Journal of Chromatography A, 1249 (2012) 190–200

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

A sensitive and efficient method to systematically detect two biophenols in medicinal herb, herbal products and rat plasma based on thorough study of derivatization and its convenient application to pharmacokinetics with semi-automated device

Guang Chen[a,b,c,d], Jun Li[c,d], Shijuan Zhang[a,b], Cuihua Song[c,d], Guoliang Li[a,b], Zhiwei Sun[c,d], Yourui Suo[a], Jinmao You[a,c,d,*]

[a] Key Laboratory of Adaptation and Evolution of Plateau Biota, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810001, China
[b] Graduate School of the Chinese Academy of Sciences, Beijing 100039, China
[c] The Key Laboratory of Life-Organic Analysis, Qufu Normal University, Qufu 273165, Shandong, China
[d] Key Laboratory of Pharmaceutical Intermediates and Analysis of Natural Medicine, Qufu Normal University, Qufu 273165, Shandong, China

ARTICLE INFO

Article history:
Received 2 April 2012
Received in revised form 10 June 2012
Accepted 11 June 2012
Available online 15 June 2012

Keywords:
Biophenol
Medicinal herb
Derivatization
Multivariate optimization
Artificial neural network
Pharmacokinetics

ABSTRACT

A sensitive and efficient method using a semi-automated pretreatment device, pre-column derivatization, multivariate optimization and high performance liquid chromatography with fluorescence and mass spectrometric detection was developed and validated for the systematic determination of two biophenols in four herb-related samples (medicinal herb; herbal products in tablet, capsule and oral liquid forms) and plasma samples after oral administration to rat. Only micro-sampling of 20 μL blood was needed for the analysis, and the pretreatment procedure including blood collection, derivatization by 10-ethyl-acridine-3-sulfonyl chloride (EASC) and injection to the sampling vials was efficiently finished in 10 min with no cumbersome and complicated operation. The novel application of artificial neural network (ANN) coupled with genetic algorithm (GA) to optimization of derivatization condition was executed and compared with the classical response surface methodology (RSM). The optimal condition for derivatization was validated by multi-criteria and nonparametric tests and used successfully to achieve the higher sensitivity (limit of detection: 0.6 and 0.8 ng/mL). The limit of reactant concentration (LORC) was put forward for derivatization method for the first time, and the lower values (2.0–2.7 ng/mL) provided the guarantee for the trace detection with the micro samples (<50 μL) required. The results of validation including selectivity, sensitivity, linearity, accuracy, precision, recovery, matrix effect and stability demonstrated the advantages of this method. The pharmacokinetic study of major bioactive components salidroside and *p*-tyrosol in herb *Rhodiola crenulata* and its products was more conveniently performed in 25 min. The established method could be the sensitive and efficient alternative method for the systematic detection of bioactive components in series of drug carriers from raw herb to herbal products and to blood in medical research. And the approaches of the thorough study played the guiding role in seeking a novel analytical method.

1. Introduction

Two natural biophenols salidroside and *p*-tyrosol (illustrated in Fig. 1) proved to be major bioactive constituents [1–3] of the registered and licensed herbal medicine *Rhodiola* that has been widely used in many countries [4]. The two are responsible for the efficacy of *Rhodiola* species and products for adaptogenic, transient focal cerebral ischemia and cellular antioxidant defenses [5], and the contents of them are the standard indices to estimate the quality [6]. More importantly, their existence and contents in blood are critical to understand pharmacokinetics mechanism whereby the variation of their concentrations in vivo, half-life, bioavailability and other important parameters can be achieved. Only with these data in vivo, can the absorption efficiency or activity efficiency of different drugs be compared in pre-clinical or clinical drug screening. It is noteworthy that the concentration of bioactive components after intravenous or oral doses to animal is usually rather low (ng/mL) and the number of samples is very high [7],

* Corresponding author at: Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810001, China. Tel.: +86 537 4456305; fax: +86 537 4456305.
E-mail address: jmyou6304@163.com (J. You).

0021-9673/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.chroma.2012.06.027

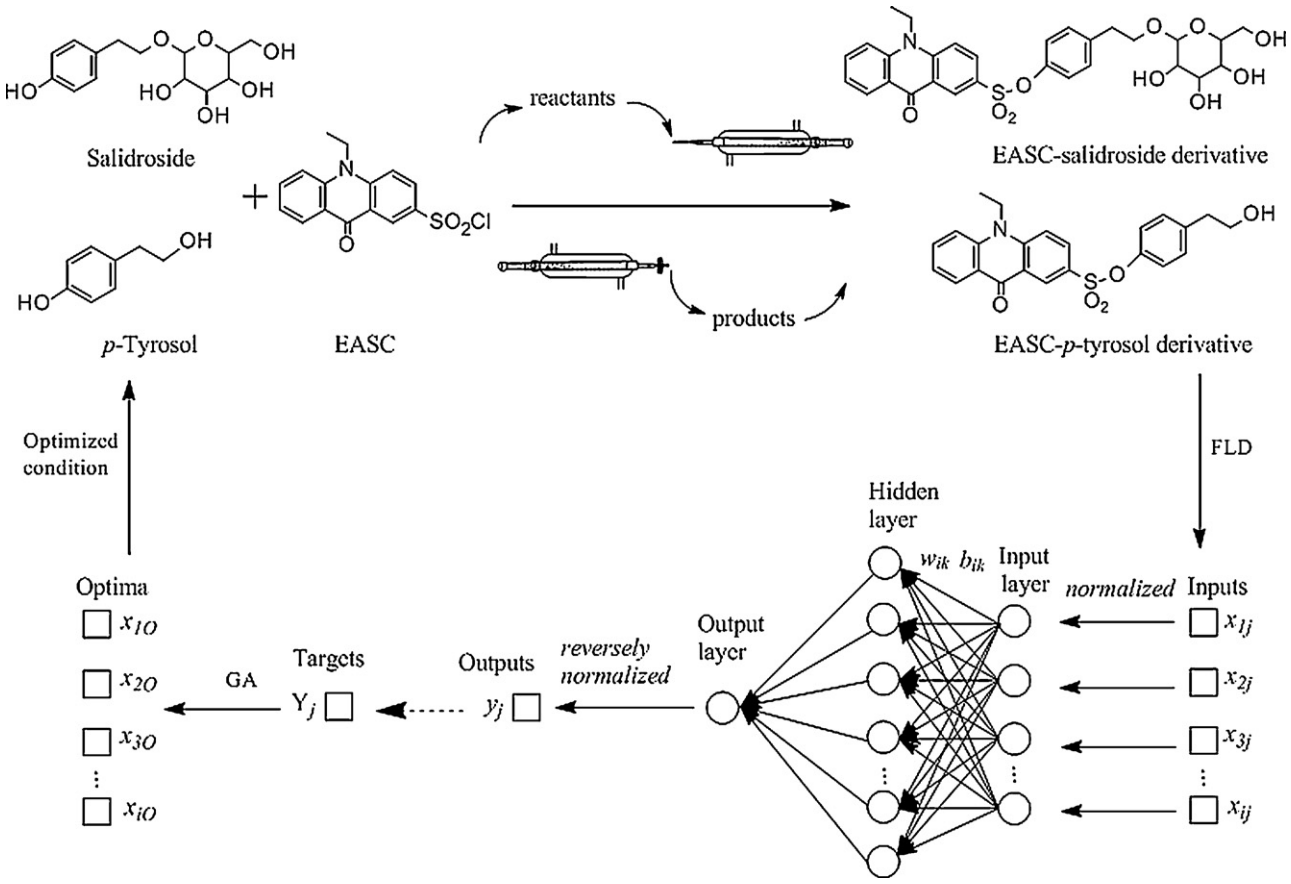

Fig. 1. Scheme of derivatization reaction between the two biophenols (salidroside and *p*-tyrosol) and fluorescence reagent 10-ethyl-acridine-3-sulfonyl chloride (EASC) with the semi-automated device, and the architecture of the back-propagation artificial neural network (ANN) with the working procedure.

so the highly sensitive and efficient method is much needed. Considering these issues, we focused on developing highly sensitive, accurate, rapid and convenient method to detect the two biophenols in the series of samples including raw herb, herbal products (tablet, capsule and oral liquid) and the rat blood samples after oral administration of the corresponding four herb-related samples.

Several methods for determination of salidroside and *p*-tyrosol in herbs were established [6,8–12] but the limit of detection (LOD) was higher than 460 ng/mL. Among the few methods for plasma analysis, the high performance liquid chromatography–ultraviolet detection (HPLC–UV) [13,14] and the on-line solid-phase extraction integrated with HPLC-electrospray ionization tandem MS (SPE–LC–MS/MS) [15,16] was thought to be not sensitive enough to fully evaluate the pharmacokinetics in pre-clinical or clinical studies [17]; LC–MS method [17] showed the satisfactory LOD, but it needed complex pretreatment procedure including inconvenient blood collection, multiple centrifugation, evaporation and reconstitution operation to avoid matrix effects, which were not competent for large batches of biological samples in medical research. In summary, no method reported was suitable for sensitive, accurate, selective and convenient quantification of salidroside and *p*-tyrosol in biological fluids, especially for trace analysis of larger batches of micro-samples. Meanwhile, efforts should be made to find the more favorable approaches to develop a superior analytical method. Contrarily, HPLC coupled with fluorescence detector (HPLC–FLD) showed higher sensitivity and selectivity with optimized derivatization procedure [18,19]. But no report on fluorescence detection of biophenols with derivatization was found. However, tentative experiments without derivatization optimization did not show such the high sensitivity as the reported work show [20]. Consequently, it is essential to redesign and fully optimize the conditions. One of the kinetic methods, response surface methodology (RSM) [21] has been widely used to analytical sciences, and the Box–Behnken design (BBD) from RSM provides efficient derivatization design requiring fewer work [18,22–24]. But, sometimes the relationship between responses and variables cannot be fitted well, or even the model is not significant, which means the relationship may be not the simple non-linear regression but the more complex interconnection. So the other method named artificial neural network (ANN) [25] must be introduced (Fig. 1). ANN has demonstrated a superior predictive power and accuracy in data learning over the traditional RSM, and moreover ANN combined with RSM has produced more efficient procedures [25,26]. However, so far as we know no report about the application of ANN to optimization and prediction of derivatization conditions was found. Furthermore, many reports on application of ANN to analytical chemistry did not mention the method with which the optimal variables combination was obtained from the results of trained ANN [27–29]. Genetic algorithm (GA) searched the optimum by considering the global distribution and proved to be the most compatible with high-throughput combinatorial chemistry experimentation [30,31].

In pharmacokinetic study, collections (>200 μL each sample) of blood for many times within a certain time are usually indispensable [13,15–17], and always need jugular-vein cannula or other injurious methods, which are inconvenient to manipulate. Moreover, it is difficult to collect larger volume of blood for many times within several hours keeping the rat alive, not to mention the mouse Mus musculus (50 g). To overcome these difficulties, highly sensitive detection is urgently needed. In this way, the needed

micro amounts of blood can be easily and conveniently collected keeping the Mus musculus alive, which is also the added benefit of this work.

The present work described a novel method to systematically detect the two biophenols in series of carriers, with the advantages of higher sensitivity, simpler operation, shorter run-time and more convenient application, comparing with previous methods. The thorough study began with easy sample preparation and fast derivatization. After HPLC–FLD detection and APCI–MS identification, the produced responses data were used to derive the optimal condition for maximum fluorescence response with the aid of kinetic methods. The established method was applied to detect the two components in series of herb-related and rat plasma samples. To make the pharmacokinetics study more convenient, a semi-automated device and efficient pretreatment procedure were designed. In addition, with the thorough study, a guiding methodology was established for seeking a superior analytical method for detecting bioactive components in series of drug carriers from raw herb to herbal products and to blood in medical research.

2. Experimental

2.1. Materials and chemicals

The herb *Rhodiola crenulata* was collected from Yushu County, Qinghai province (elevation 4400 m, China). The tablet, capsule and oral liquid were bought from Sichuan pharmaceutical science and technology Co., Ltd. Standard salidroside and *p*-tyrosol were purchased from National institute for the control of pharmaceutical and biological products (Beijing, China). The derivatization reagent 10-ethyl-acridine-3-sulfonyl chloride (EASC) was synthesized as described in our previous work [20]. The mice (Mus musculus, 40–50 g) were bought from Shandong Lukang Pharmaceutical Co., Ltd. and were treated following the guideline approved by the institutional animal care and use committee of the National Health Research Institutes.

2.2. Instrumental and conditions

2.2.1. Instrumentation and software

The solid samples were milled by Jouyang grander (Jinan, China) and extracted with ultrasonic instrument (Ningbo, China). Analytes was separated by Agilent 1100 Series HPLC and detected by FLD (G1321A, at λ_{ex} 262 nm and λ_{em} 425 nm) and the mass spectrometer 1100 Series LC-MSD Trap-SL (ion trap) from Bruker Daltonik (Bremen, Germany). Design-Expert 8.0.6 software was used to provide experimental design, mathematical program Matlab R2010a was used to operate the artificial neural network program and genetic algorithm, and software Winnonlin 5.2. was used to process the data from pharmaceutical experiments.

2.2.2. Chromatographic conditions

Reversed-phase Akasil-C_{18} column (5 μm, 4.6 mm × 250 mm, Angela Technologies Inc., USA) with a gradient elution (0–15 min, 70% to 35% of B; 15–20 min, 35% of B, where eluent A and B were 100% acetonitrile and 5% aqueous acetonitrile (v/v), respectively) at the flow rate of 1.0 mL/min.

2.2.3. Mass spectrometric conditions

Analytes were further identified by MS operated in the positive-ion detection mode, nebulizer pressure 60 psi; dry gas temperature, 350 °C; dry gas flow, 5.0 L/min. APCI Vap temperature, 350 °C; corona current (nA) 4000 (pos); capillary voltage 3500 V), with atmospheric pressure chemical ionization (APCI) source.

2.3. Sample preparation procedures

2.3.1. Herb and products samples preparation

For three solid samples: 2 g of each powdered sample was pretreated as previous method [18]. For the oral liquid: 2 mL of oral liquid was added to a beaker, dried by nitrogen blast and then redissolved in 25 mL of pure ACN. All solutions were stored at 4 °C in darkness until use.

2.3.2. Blood samples preparation

Aliquots of 20 μL of blood were collected via tail vein puncture with the designed semi-automated device (Fig. 1) containing appropriate amounts of derivatization reagents, 50 μL of buffer solution (Na_2CO_3–$NaHCO_3$, pH = 10.16, 10^{-4} mol/mL), 10 μL of EDTA-Na_2 solution (4×10^{-6} mol/mL), 50 μL water and 200 μL ACN. Supernatant was filtered through micro membrane to sampling vials for HPLC–FLD–MS/MS analysis.

2.4. Standard solutions and quality control samples

The 50 mL mixed solution of two standard biophenols (10^{-3} mol/mL) was prepared and was divided to two aliquots. One was diluted by ACN to calibrate the analytes in four herb-related samples; the other was diluted by the plasma solution extracted from blank blood to calibrate the analytes in plasma. Dilutions of the stock solutions by ACN were performed to obtain the quality control (QC) samples (10, 100 and 1000 ng/mL) and the calibration standards within the ranges of 1.9–2280 (1.9 ng/mL, 386.7 ng/mL, 728.5 ng/mL, 1084.2 ng/mL, 1455.6 ng/mL, 1781.5 ng/mL, 2280.7 ng/mL for salidroside) and 2.5–3040 (509.3 ng/mL, 1024.4 ng/mL, 1521.32 ng/mL, 2131.5 ng/mL, 2573.5 ng/mL, 3040.3 ng/mL for *p*-tyrosol) ng/mL. The QC samples and stock solution of plasma were divided into small aliquots and stored at −20 °C in darkness until use.

2.5. Optimization of derivatization condition

2.5.1. Single variable experiments

Molar ratios from 2 to 12; temperature from 15 to 95 °C; reaction time from 1 to 15 min; concentration from 0.1 to 100 ng/mL; pH value from 8 to 13. All the experiments were performed at three low concentration levels (1.5, 2.0 and 2.5 ng/mL) according to the contents of analytes.

2.5.2. Multivariate experiments

With Box–Behnken design (BBD), 17 runs tests (1–17 runs listed in Table 1) containing combinations of the three major variables was offered. The average peak area reflecting the fluorescence response was analyzed through the fitness of a linear, quadratic or cubic equation with which the optimum were obtained. Meanwhile, the variables combinations (X_m, X_T and X_t) were delivered to a back-propagation ANN (illustrated in Fig. 1). To improve the reliability and generalization ability of the ANN, variables combinations were numbered in random order, 70%, 15%, and other 15% of which were used for ANN training, testing, and validation, respectively. Inputs were normalized to the range −1 and 1. Levenberg–Marquardt algorithm was used for training. Logistic Sigmoid and purelin transfer functions were used to construct ANN. Results from ANN were passed to GA algorithm program [32] to search the optimum. Results from single variable designs (18–52 runs in Table 1) were used to be inputs of ANN (SV-ANN). Since BBD was thought to be efficient, the experimental results from BBD were used to be inputs of ANN (i.e. BBD-ANN). To increase the generalization of the model, the total results (1–52 runs) from both

Table 1
Designs of multivariate methods, validation and optimization.

Run[a]	Variables[b]			Response[c]	Predicted[d]	RE (%)[d]	Predicted[e]	RE (%)[e]	Predicted[f]	RE (%)[f]	Predicted[g]	RE (%)[g]
	X_m	X_T	X_t	(exp.)	(BBD)	(BBD)	(BBD-ANN)	(BBD-ANN)	(SV-ANN)	(SV-ANN)	(Full-ANN)	(Full-ANN)
1	8.0	85.0	9.0	101.20	98.13	-3.0350	101.2000	0.000002	80.93	-20.0319	102.85	1.6269
2	6.0	72.5	9.0	76.87	78.55	2.1831	78.8005	2.511381	85.72	11.5161	75.78	−1.4131
3	10.0	72.5	5.0	101.20	96.55	−4.5920	101.2000	0.000000	86.50	−14.5235	95.76	−5.3735
4	8.0	60.0	5.0	135.88	138.95	2.2612	135.8809	−0.000002	111.78	−17.7372	131.86	−2.9594
5	6.0	85.0	7.0	46.02	44.45	−3.4215	46.0200	−0.000062	33.78	−26.6032	46.75	1.5759
6	8.0	72.5	7.0	131.23	131.23	0.0004	131.2320	−0.000001	127.69	−2.7012	131.24	0.0075
7	10.0	72.5	9.0	99.11	88.58	−10.6238	99.1061	0.000003	132.74	33.9394	99.23	0.1221
8	8.0	72.5	7.0	131.23	131.23	0.0004	131.2320	−0.000001	127.69	−2.7012	131.24	0.0075
9	8.0	85.0	5.0	81.30	72.35	−11.0102	81.3000	0.000052	82.19	1.0957	90.09	10.8117
10	8.0	72.5	7.0	131.23	131.23	0.0004	131.2320	−0.000001	127.69	−2.7012	131.24	0.0075
11	6.0	60.0	7.0	99.98	86.38	−13.6039	99.9774	−0.000109	52.85	−47.1333	99.62	−0.3568
12	8.0	72.5	7.0	131.23	131.23	0.0004	131.2320	−0.000001	127.69	−2.7012	131.24	0.0075
13	10.0	85.0	7.0	49.95	63.56	27.2287	49.9545	0.000020	63.28	26.6846	58.64	17.3868
14	8.0	72.5	7.0	131.23	131.23	0.0004	131.2320	−0.000001	127.69	−2.7012	131.24	0.0075
15	8.0	60.0	9.0	120.94	129.90	7.4022	110.5046	−8.631384	118.81	−1.7627	109.09	−9.8023
16	10.0	60.0	7.0	118.42	120.00	1.3307	119.1247	0.593485	120.45	1.7142	130.04	9.8113
17	6.0	72.5	5.0	43.32	53.85	24.3072	43.3200	0.000064	68.53	58.1894	61.23	41.3365
18	2.0	75.0	6.0	45.86	−303.19	−761.1556	53.1275	15.853301	45.69	−0.3624	43.65	−4.8220
19	3.0	75.0	6.0	56.11	−180.67	−421.9692	79.3226	41.357499	55.89	−0.4018	52.92	−5.6912
20	4.0	75.0	6.0	71.97	−78.93	−209.6735	107.6780	49.623599	72.14	0.2437	54.39	−24.4166
21	5.0	75.0	6.0	85.61	2.05	−97.6108	127.0296	48.381386	85.72	0.1243	90.30	5.4781
22	6.0	75.0	6.0	93.57	62.25	−33.4747	133.3475	42.514018	93.58	0.0159	79.81	−14.6986
23	7.0	75.0	6.0	98.61	101.68	3.1047	128.4111	30.216314	98.63	0.0129	101.19	2.6160
24	8.0	75.0	6.0	102.56	120.33	17.3231	115.8714	12.973777	102.44	−0.1199	106.33	3.6673
25	9.0	75.0	6.0	104.59	118.22	13.0265	101.0609	−3.376431	104.33	−0.2478	104.65	0.0556
26	10.0	75.0	6.0	101.79	95.33	−6.3502	88.2824	−13.273594	101.68	−0.1080	101.64	−0.1502
27	11.0	75.0	6.0	92.29	51.67	−44.0142	78.2457	−15.219887	92.21	−0.0881	92.71	0.4500
28	12.0	75.0	6.0	79.48	−12.76	−116.0559	69.8592	−12.099950	79.48	0.0037	80.03	0.6985
29	8.0	15.0	6.0	124.68	24.97	−79.9744	108.4829	−12.991995	110.09	−11.7056	133.01	6.6764
30	8.0	25.0	6.0	128.80	76.36	−40.7126	106.4994	−17.316314	129.42	0.4769	129.09	0.2192
31	8.0	35.0	6.0	133.50	113.56	−14.9368	104.3844	−21.809512	137.23	2.7939	132.99	−0.3850
32	8.0	45.0	6.0	135.78	136.55	0.5685	104.6516	−22.926721	135.92	0.1018	137.65	1.3728
33	8.0	55.0	6.0	131.31	145.35	10.6877	107.3020	−18.285447	130.29	−0.7771	139.22	6.0230
34	8.0	65.0	6.0	118.64	139.94	17.9554	111.5082	−6.009898	118.53	−0.0940	135.86	14.5146
35	8.0	75.0	6.0	102.56	120.33	17.3231	115.8714	12.973777	102.44	−0.1199	106.33	3.6673
36	8.0	85.0	6.0	89.09	86.52	−2.8793	118.5669	33.088838	89.02	−0.0820	88.76	−0.3739
37	8.0	95.0	6.0	78.95	38.51	−51.2184	117.9363	49.377938	79.02	0.0857	75.28	−4.6503
38	8.0	75.0	1.0	90.43	15.34	−83.0316	91.2489	0.902860	90.21	−0.2490	103.61	14.5760
39	8.0	75.0	2.0	91.88	46.65	−49.2307	94.6833	3.046736	91.47	−0.4456	92.27	0.4180
40	8.0	75.0	3.0	93.78	72.80	−22.3714	99.1299	5.705685	93.39	−0.4148	89.19	−4.8943
41	8.0	75.0	4.0	96.20	93.80	−2.5026	104.5614	8.686470	95.95	−0.2648	89.28	−7.1956
42	8.0	75.0	5.0	99.17	109.64	10.5553	110.5168	11.438199	99.02	−0.1541	92.50	−6.7300
43	8.0	75.0	6.0	102.56	120.33	17.3231	115.8714	12.973777	102.44	−0.1199	106.33	3.6673
44	8.0	75.0	7.0	106.11	125.87	18.6186	118.9062	12.055883	106.03	−0.0809	123.12	16.0291
45	8.0	75.0	8.0	109.48	126.25	15.3200	118.0956	7.867481	109.54	0.0483	108.58	−0.8248
46	8.0	75.0	9.0	112.39	121.49	8.0934	113.6428	1.114561	112.66	0.2399	108.86	−3.1388
47	8.0	75.0	10.0	114.69	111.56	−2.7293	108.0517	−5.792029	115.11	0.3585	114.25	−0.3852
48	8.0	75.0	11.0	116.39	96.49	−17.0990	104.0238	−10.625836	116.77	0.3295	117.09	0.6001
49	8.0	75.0	12.0	117.56	76.26	−35.1293	102.4999	−12.809834	117.84	0.2396	120.98	2.9121
50	8.0	75.0	13.0	116.47	50.88	−56.3149	103.2260	−11.371134	118.48	1.7232	121.18	4.0412
51	8.0	75.0	14.0	116.32	20.35	−82.5090	105.8598	−8.992620	118.67	2.0189	118.42	1.8082
52	8.0	75.0	15.0	116.20	−15.34	−113.2033	110.2019	−5.161876	118.33	1.8309	119.08	2.4768

Table 1 (*Continued*)

Run[a]	Variables[b]			Response[c]	Predicted[d]	RE (%)[d]	Predicted[e]	RE (%)[e]	Predicted[f]	RE (%)[f]	Predicted[g]	RE (%)[g]
	X_m	X_T	X_t	(exp.)	(BBD)	(BBD)	(BBD-ANN)	(BBD-ANN)	(SV-ANN)	(SV-ANN)	(Full-ANN)	(Full-ANN)
Validation[h]												
AME					13.6		1.93		3.73		17.91	
CE					0.98		0.99		0.99		0.99	
MAE					−0.17		−0.46		−0.17		1.19	
RMSE					6.86		2.58		2.64		6.89	
MRE					1.08		−0.33		−0.15		1.47	
R^2					0.95		0.99		0.98		0.93	
p-value					0.679		0.624		0.99		0.65	
Optima												
BBD	8.47	60.00	6.38	148.57	147.39							
BBD-ANN	6.29	77.92	5.28	146.80			142.66					
SV-ANN	10.21	15.59	12.04	152.59					147.69			
Full-ANN	2.05	88.66	8.89	180.20							185.42	
SV	9.00	45.00	12.00	137.61								

[a] The total 52 runs of experiments were designed from BBD (1–17 runs) and single variable design (18–52 runs).

[b] Three investigated variables including: X_m, the molar ratio of EASC to total biophenols, X_T, the reaction temperature (°C) and X_t, the reaction time (min) of derivatization reaction.

[c] Responses from experiments of FLD (peak area).

[d] Predicted responses and corresponding relative error (RE (%)) from BBD (fitted with experimental responses from 1–17 runs).

[e] Predicted responses and corresponding relative error (RE (%)) from BBD-ANN (trained, validated and tested with experimental responses from 1–17 runs).

[f] Predicted responses and corresponding relative error (RE (%)) from SV-ANN (trained, validated and tested with experimental responses from 18–52 runs).

[g] Predicted responses and corresponding relative error (RE (%)) from Full-ANN (trained, validated and tested with experimental responses from 1–52 runs).

[h] Validation including AME, absolute maximum error; CE, coefficient of efficiency; MAE, mean absolute error; RMSE, root mean squared error; MRE, mean relative error; R^2, correlation of determination, and nonparametric tests (*p*-value).

BBD and single variable design were used to train the ANN model (Full-ANN).

2.5.3. Comparison of derivatizing agents

Several excellent derivatizing agents (see Table 2 for details) were investigated. Comparisons were performed to select the most appropriate derivatizing agent.

2.6. Method validation

2.6.1. Selectivity, sensitivity and linearity

Six batches of blank blood (20 μL) were collected and spiked with standard solution (20 μL 10 ng/mL) to test the endogenous interference. Sensitivity was determined by limit of detection (LOD) and limit of quantification (LOQ) at signal-to-noise ratio of 3:1 and 10:1 respectively. The limit of reactant concentration (LORC) was defined as the lowest concentration of reactants to produce detectable derivative whose concentration was equal to the concentration at the LOQ level. For further study of sensitivity, LORC was investigated by decreasing the concentration of reactants in derivatization reaction under the optimized derivatization condition. By comparing the chromatograms of blank and spiked samples, the selectivity was validated. The calibration samples of salidroside and *p*-tyrosol in plasma were prepared by adding the corresponding standard solutions with seven concentration levels (Section 2.3.1) to the semi-automated device containing 20 μL of blank blood and processed following Section 2.3.2. Each level standard was analyzed in six replicates with two replicas of the semi automated device. The calibration samples of standard salidroside and *p*-tyrosol were prepared with similar method in ACN. Calibration curves with seven concentration levels were established by linear regression of the peak area (Y) of each compound, versus the concentration (x).

2.6.2. Accuracy and precision

Experimental accuracy was calculated and the inter-day, intra-day precisions were evaluated by analyzing six replicates containing the spiked QC samples and blank plasma samples. The relative standard deviations for peak area and retention time were measured.

2.6.3. Recovery evaluation and matrix effect

Extraction recovery (ratio percentage) was calculated following the equation: recovery (%) = $S_b/S_a \times 100$ to indicate the recovery, where S_b and S_a are the peak area values of each spiked concentration before and after extraction, respectively. For plasma samples, the matrix effect was evaluated following the equation: matrix effect (%) = $S_p/S_s \times 100$, where, S_p is the peak area of sample spiked after extraction and S_s is the peak area of the standard sample reconstituted.

2.6.4. Stability

Six aliquots of QC samples at each of three concentration levels were analyzed to investigate the stability of samples. According to the practical performance, the following conditions were taken into consideration. Stability of stock solution was evaluated at room temperature for a week. Freeze–thaw stability was evaluated after two cycles of freeze (−20 °C)–thaw (room temperature, spontaneously) performance. Short-term stability was determined by analyzing QC samples at room temperature for 3 h that exceeded the usual time of samples preparation. Long-term stability was determined by analyzing the QC samples kept at the storage temperature (−20 °C) for 2 weeks. Post-preparative stability was assessed by analyzing QC samples at room temperature for 12 h. The evaluations for the stability of the two analytes in rat

Table 2
Comparison of the proposed method using different derivatizing agents with the previous methods for detection of salidroside and *p*-tyrosol in plasma samples.

Analytes	Samples (amount)	Pretreatment procedure/derivatization condition	Separation and detection	LOD[a] (original values)	Time for total run[b]	Ref./this work
Salidroside	Dog plasma (400 μL)	Venous blood; centrifuged for 5 min; dissolved, vortexed for 5 min; centrifuged for 10 min; supernatant was evaporated to dryness; residue was reconstituted; centrifugation 5 min. 10 μL injection.	HPLC–UV	250 ng/mL (0.25 μg/mL)	>31 min	[13]
Salidroside	Rat plasma (500 μL)	Eye puncture; centrifuged for 5 min; dissolved, vortexed for 2 min; centrifuged for 15 min.	HPLC–UV	150 ng/mL (0.15 mg/L)	>32 min	[14]
Salidroside	Rat plasma (500 μL)	Eye puncture; centrifuged for 5 min; vortex-mixed for 3 min and centrifuged for 10 min; supernatant was evaporated to dryness; residue was reconstituted with vortex mixing for 2 min, and the centrifugation for 5 min.	LC–MS	100 ng/mL	>38 min	[16]
Salidroside	Rat plasma (200 μL)	Jugular-vein cannula; centrifuged for 15 min; vortexed for 30 s and centrifuged for 20 min, transferred to LC.	LC/MS/MS	(LOQ[c]: 50 ng/mL)	>37.7 min	[15]
Salidroside	Rat plasma (100∼300 μL)	Oculi chorioideae vein puncture; centrifuged for 10 min; vortexed for 30 s; LLE was adopted and vortexed for 5 min; centrifuged for 10 min; upper layer was evaporated to dryness; residues were reconstituted followed by centrifugation for 10 min.	LC–ESI-MS	1 ng/mL	>42.5 min	[17]
Salidroside and *p*-tyrosol	Rat plasma (20 μL)	Tail vein puncture; drawn, dissolved, derivatized and filtered into vials for injection in 10 min with the designed semi-automated device. X_m: 2.05, X_T: 88.66, X_t: 8.89	HPLC–FLD–MS/MS.	0.6 and 0.8 ng/mL	15 minand20.5 min	EASC[d]
Salidroside and *p*-tyrosol	Rat plasma (100 μL)	Buffer solution (Na_2CO_3–$NaHCO_3$, pH = 10.16); ACN; acetic acid X_m: 7.14; X_T: 74.45; X_t: 6.37.	HPLC–FLD–MS/MS	3.7 and 4.1 ng/mL	12 and 16 min	DBCEC-Cl[d]
Salidroside and *p*-tyrosol	Rat plasma (200 μL)	Buffer solution (Na_2CO_3–$NaHCO_3$, pH = 10.16); ACN; Acetic acid; X_m: 5.47; X_T: 67.32; X_t: 7.94.	HPLC–FLD–MS/MS	64.4 and 57.2 ng/mL	13 and 19 min	DBPC-Cl[d]
Salidroside and *p*-tyrosol	Rat plasma (100 μL)	Buffer solution (Na_2CO_3–$NaHCO_3$, pH = 10.16); Water; ACN; X_m: 8.26; X_T: 75.54; X_t: 12.85	HPLC–FLD–MS/MS	7.5 and 23.8 ng/mL	16 and 23 min	ABETS[d]
Salidroside and *p*-tyrosol	Rat plasma (200 μL)	Buffer solution (Na_2CO_3–$NaHCO_3$, pH = 10.16); ACN; Acetic acid; X_m: 6.75; X_T: 80.72; X_t: 11.29	HPLC–FLD–MS/MS	120.6 and 93.8 ng/mL	16 and 23 min	BAETS[d]
Salidroside and *p*-tyrosol	Rat plasma (100 μL)	Buffer solution (Na_2CO_3–$NaHCO_3$, pH = 10.16); Water; ACN; X_m: 3.34; X_T: 90.37; X_t: 12.56	HPLC–FLD–MS/MS	5.4 and 11.2 ng/mL	17 and 24 min	PBITS[d]
Salidroside and *p*-tyrosol	Rat plasma (100 μL)	Buffer solution (Na_2CO_3–$NaHCO_3$, pH = 10.16); ACN; Acetic acid; X_m: 5.29; X_T: 40.37; X_t: 10.64	HPLC–FLD–MS/MS	7.6 and 9.2 ng/mL	16 and 23 min	DBCPC-Cl[d]
Salidroside and *p*-tyrosol	Rat plasma (200 μL)	Buffer solution (Na_2CO_3–$NaHCO_3$, pH = 10.16); ACN; DMAP; EDC·HCl X_m: 4.37; X_T: 67.87; X_t: 49.37	HPLC–FLD–MS/MS	38.9 and 51.8 ng/mL	57 and 63 min	PEBA[d]

[a] LOD, limit of detection (at the signal to noise 3:1).

[b] Time for total run: the total run time including sample pretreatment, separation and detection of analytes.

[c] LOQ, limit of quantification; X_m, molar ratio of agent to analyte; X_T, temperature; X_t, reaction time.

[d] Data in this work with the present pretreatment procedure; EASC, 10-ethyl-acridine-3-sulfonyl chloride; DBCEC-Cl, 2-[2-(dibenzocarbazole)-ethoxy] ethyl chloroformate; DBPC-Cl, 1-[1,2,5,6-dibenzocarbazol-9-yl]propan-2-yl chloroformate; ABETS, 2-(2-(anthracen-10-yl)-1 H-benzimidazol-1-yl) ethyl-*p*-toluenesulfonate; BAETS, 2-(5-benzoacridine)ethyl-*p*-toluene sulfonate; PBITS, 2-(2-(pyren-l-yl)-1 H-benzo[d]imidazole-l-yl)ethyl-4-methylbenzenesulfonate; DBCPC-Cl, 2-[2-(7H-dibenzo [a,g]-carbazol-13-yl)] isopropyl chloroformate; PEBA, 5-(2-oxo-2-(piperazin-1-yl)ethyl)benzo[b]acridin-12 (5H)-one; DBCEIC, 2-((2-methylnaphthalen-1-yl)(1-methy-Inaphthalen-2-yl)amino)ethyl 1 H-imidazole-1-carboxylate.

plasma were the ratio percentage of the detected concentration and the concentration of QC samples.

2.6.5. Application and pharmacokinetic study

The method was applied to detect the contents of two biophenols salidroside and *p*-tyrosol in samples before and after the oral administration. When detecting the solid herb-related samples, the weighed 3 g of each powdered sample was divided to six aliquots, each one of which is extracted with 15 mL of ethanol–acetonitrile (50:50, v/v), then prepared following the description in Section 2.3.1. Blood samples were collected at 5, 10, 30, 60, 90, 120, 240 min from 6 healthy rats after oral administration (solid herb and products: 50 mg/kg, oral liquid: 17.6 mL/kg) and prepared following the Section 2.3.2.

3. Results and discussion

3.1. Pretreatment procedure development

During the preparation of plasma sample, blood was collected with the modified injector (Fig. 1) containing anticoagulant, EASC, buffer solution and ACN, with the heated water circulating. After reaction, the supernatant plasma solution was transferred to sampling vials after filtration with polymer filter. Afterward, excessive ACN were continuously added to the obtained solution, no more precipitate or suspended substance was observed, indicating that plasma samples would not produce precipitate when they were eluted by the mobile phase consisting of ACN and water in HPLC–FLD–MS. The pretreatment procedure including blood collection, derivatization and injection into via was more conveniently finished within 10 min than usual. Thus, large batches of samples for detection of bioactive components in pre-clinical or clinical studies could be efficiently prepared with this semi-automated derivatization extraction method.

3.2. Optimization of derivatization condition

3.2.1. Single variable optimization

No matter what a simple or complicated system, the influence of single variable should be thoroughly studied before any optimization. The usual solvents acetonitrile (ACN), acetone, chloroform, dichloromethane (DCM), ethyl acetate (EA), *N*,*N*-dimethylformamide (DMF) and dimethylsulfoxide (DMSO) were investigated. Acetonitrile showed the best fluorescence response and meanwhile it was one constituent of the mobile phase, which demonstrated that acetonitrile had the superiority to be the co-solvent for the derivatization reaction, as it had the advantages to be extractants and mobile phase. To get the higher derivatization response and reaction rate, several basic catalysts were investigated to optimize derivatization reaction of phenolic hydroxyl group with reagents. The addition of NaOH, K_2CO_3, $NaHCO_3$ and Na_2CO_3 led to the obvious decrease in fluorescence responses due to hydrolyzation at higher pH value. Hence the buffer solution Na_2CO_3–$NaHCO_3$ (pH = 10.16, 10^{-4} mol/mL) was investigated in detail. As a result, the highest response occurred in the system with the addition of 50 μL of Na_2CO_3–$NaHCO_3$ buffer solution (pH^* = 10.16) and no obvious decrease was observed within 48 h. Constant fluorescence intensity was achieved at the 9-fold molar ratio. The effect of temperature on derivatization reaction was investigated from 15 to 95 °C. The maximum responses was observed at 75 °C. The reaction times were investigated and the optimal range from 6 to 8 was obtained.

3.2.2. Multivariate optimization

Multivariate optimization methods were introduced to get the optimal combination of the three variables (X_m, X_T and X_t), while

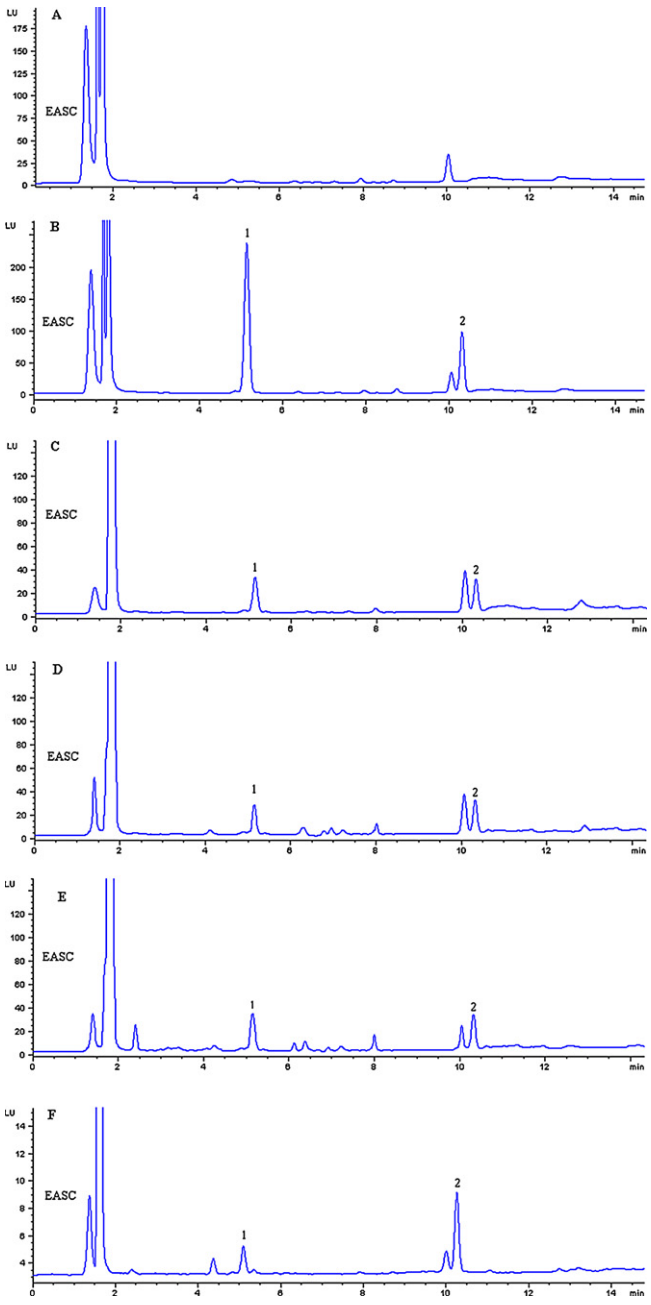

Fig. 2. The representative chromatograms for blank plasma (A), spiked blank plasma with standard (B), and plasma samples after oral administration of *Rhodiola crenulata* root (C), tablet (D), capsule (E) and oral liquid (F).

the initial concentration was set to be 2 ng/mL. The responses, corresponding combinations variables, validation and predicted optima were listed in Table 1. Multi-criteria (see Table 1 for details) and nonparametric tests (*p*-value with Wilcoxon rank sum method) were used to reflect the accuracy of the model and to indicate the best model [33,34]. With the results from corresponding runs of experimental designs, the four models showed good correlation ($R^2 > 0.9$) and coefficient of efficiency (approximately to 1). The absolute values of MRE were less than 1.5%, indicating that the fitting or learning processes of the four models were fully operational and the predicted responses could be correlated with the experimental responses [35]. The four groups of the *p*-value greater than 0.05 demonstrated that the differences between the experimental and predicted value were not statistically significant and the four models could simulate the statistic characteristic of predicted

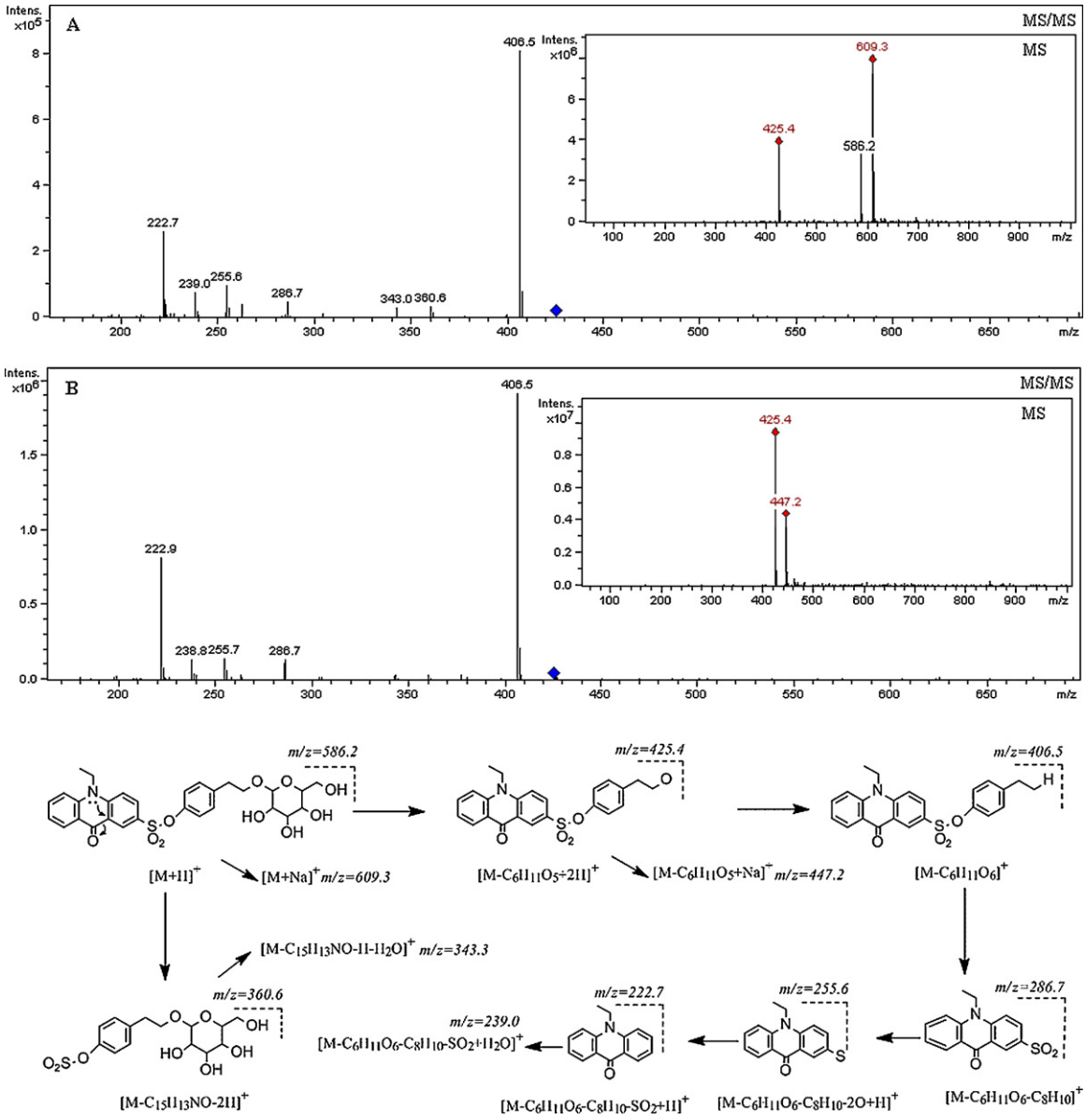

Fig. 3. The MS and MS/MS spectra for salidroside (A) and *p*-tyrosol (B) with the cleavage mode.

responses at the 95% confidence level [33]. Thus, the four models proved to be able to produce predicted values correlating well with experimental data, and the BBD-ANN and SV-ANN with the lower absolute values of AME, MAE, RMSE, MRE and the higher R^2 seemed to be better than other models. The BBD and BBD-ANN were constructed from the results of 1–17 runs of experiments with the lower MRE of 1.08 and −0.33 respectively. However they produced the higher relative error when used to predict the other 35 runs of experiments. It could be seen from this that the relationship between responses and combination of variables was extremely complex and the optimized value were the local optimum rather than the global optimum. The SV-ANN model constructed by 18–52 runs of experiments produced better responses with lower relative error when used to predict the other top 17 runs. Obviously, Full-ANN model showed the best generalization ability. Consequently, the four models were all used to predict the optimum for their predominance in respective variable range. Though the Full-ANN method predicted an optimum with the larger error, it offered the maximum optimal value (185.42) among the four methods. These should be attributed to the reason that BBD, BBD-ANN and SV-ANN methods predicted the local optimum in the narrower range of variables, but the Full-ANN searched the global optimum in the wider variable range including the complementary variables combination from Box–Behnken and single variable designs. The artificial neural network showed robustness with the model based on larger amounts of experiment data. The other probable reason was that the variable ranges in BBD method were not suitably selected for this work and therefore the generalization ability was observed not strong. Large amounts of tentative experiments and designs were required in order to get the most appropriate variable ranges for BBD, but that might be more time-consuming than to get the artificial neural network model with the single variable experiments. As a result, four models predicted the derivatization conditions leading to more sensitive responses than response (137.61) from single variable experiments. The variable combination (X_m: 2.05; X_T: 88.66; X_t: 8.89) from Full-ANN method was applied as optimized derivatization condition for the following experiments.

3.2.3. Comparison of derivatizing agents

The comparison of several derivatizing agents was listed in Table 2. Results indicated that EASC was most likely to be suitable for seeking such a sensitive and efficient method in view of its

higher sensitivity, easier and faster operation. The reason might be caused by the conjugation of nitrogen and oxygen atoms with the π–π planar structure, which improved the fluorescence responses. Moreover, the required solvent phase for DBCEC-Cl, DBCEC-Cl, BAETS and DBCPC-Cl is the acetic acid, which was unfavorable to the fluorescence response due to the protonation of nitrogen or oxygen atom in their substructures. The solvents for PEBA and DBCEIC are not favorable to both fluorescence response and pretreatment of blood samples. Therefore, the EASC was chosen as the most appropriate derivatizing agent for the sensitive and efficient analysis of the series of samples.

3.3. Detection and identification method development

Analytes were detected at 5.2 min and 10.2 min respectively (Fig. 2). The five-min interval was designed to avoid the potential matrix interference from samples. Though free salidroside or *p*-tyrosol could be detected by ESI/MS [9,15–17], it was recommended that the isolated EASC-biophenol derivatives were identified with APCI mode (Fig. 3). The EASC-salidroside derivative produced intense peak at *m/z = 586.2* ($[M+H]^+$). Due to formation of the dipolar ion caused by isomerization [20], the 10-ethyl-acridine substructure of fragments exhibited stronger ion current responses and was easy to catch water molecule or ions ($[M+Na]^+$ appeared at *m/z* = 609.3). Remarkably, the formation of $[M—C_{15}H_{13}NO—2H]^+$ (*m/z = 360.6*) and subsequent $[M—C_{15}H_{13}NO—H—H_2O]^+$ (*m/z = 343.3*) produced by losing the water molecule due to the multi-hydroxyl substructure was the special identification of EASC-salidroside derivatives.

3.4. Method validation

3.4.1. Selectivity and sensitivity

The clear peaks of the two analytes were observed at relatively fixed retention time and interfering was not found (Fig. 2), which showed the excellent specificity. As expected, the method provided the higher sensitivity with the lower LOD (0.6 ng/mL for salidroside and 0.8 ng/mL for *p*-tyrosol) than those reported in previous work [11,15–17,36,12,37–40]. The LORC values of 2.0, 2.2, 2.6 and 2.7 ng/mL provided the guarantee for the trace detection of blood samples by pre-column derivatization method.

3.4.2. Calibration curve and linearity correlation

Calibration curves were prepared daily and showed good linearity in corresponding ranges: 1.9–2280 ng/mL for salidroside, 2.5–3040 ng/mL for *p*-tyrosol. The coefficient of estimation (r^2) were greater than 0.9994 and the RSD of the retention time and peak area were lower than 0.04% and 1.31%, respectively, which demonstrated that external standard calibration could be applied for quantitative purposes [41].

3.4.3. Accuracy and precision

In Table 3 the intra- and inter-day accuracy of salidroside in QC sample ranged from −2.55% to −1.56% and from −2.96% to −2.46%, respectively. The intra- and inter-day accuracy of salidroside in rat plasma ranged from −2.90% to 0.40% and from −3.04% to 0.78%, respectively. The intra-day precisions were within acceptance criteria.

3.4.4. Recovery evaluation and matrix effect

The recoveries of the two analytes in rat plasma at three examined concentrations levels were in the range from 96.95% to 104.76% with RSD less than 5%, and the corresponding matrix effects ranged from 94.69% to 100.79% with RSD less than 5%. The results indicated that the extraction recoveries of analytes in rat plasma samples were precise and reproducible, and there were no significant matrix interference from the concomitants in

Table 3
The intra- and inter-day accuracy and precision and stability of the two biophenols at three concentration levels.

Biophenols	Spiked	Intra-day ($n = 6$)			Inter-day ($n = 6$)			Evaluation[c] (±SD %) for stability				
	(ng/mL)	Measured (ng/mL) ± SD	Accuracy (RE%)	Precision (RSD %)	Measured (ng/mL) ± SD	Accuracy (RE %)	Precision (RSD %)	Stock solution	Freeze–thaw	Short-term	Long-term	Post- preparative
Salidroside[a]	10	9.75 ± 0.14	−2.55	1.25	9.75 ± 0.12	−2.46	0.49					
	100	97.47 ± 1.12	−2.53	1.31	97.22 ± 1.23	−2.78	0.82					
	1000	984.43 ± 6.16	−1.56	1.41	970.38 ± 17.77	−2.96	3.68					
Salidroside[b] in rat plasma	10	10.04 ± 0.53	0.40	4.97	10.08 ± 0.47	0.78	1.43	102.17 ± 2.32	105.68 ± 4.35	103.59 ± 3.53	103.96 ± 3.72	103.45 ± 2.51
	100	97.22 ± 1.51	−2.78	1.95	96.23 ± 1.92	−3.77	2.26	98.69 ± 2.54	104.91 ± 3.77	98.78 ± 3.63	103.35 ± 4.57	97.74 ± 2.67
	1000	970.99 ± 8.71	−2.90	1.44	969.56 ± 14.46	−3.04	1.81	98.69 ± 2.73	97.67 ± 2.74	102.66 ± 2.16	101.83 ± 1.99	96.88 ± 2.73
p-Tyrosol[a]	10	9.73 ± 0.10	−2.73	0.93	9.77 ± 0.11	−2.29	1.89					
	100	97.93 ± 1.03	−2.07	1.43	97.42 ± 1.36	−2.58	1.11					
	1000	971.32 ± 13.95	−2.87	1.32	970.14 ± 12.18	−2.99	0.53					
p-Tyrosol[b] in rat plasma	10	9.65 ± 0.14	−3.52	1.95	9.67 ± 0.18	−3.31	1.27	98.74 ± 2.48	107.29 ± 4.76	105.21 ± 4.12	105.85 ± 4.88	97.39 ± 3.45
	100	97.23 ± 1.57	−2.77	1.40	97.26 ± 1.28	−2.74	0.07	98.94 ± 1.05	105.52 ± 1.05	103.83 ± 1.95	105.87 ± 5.42	97.67 ± 2.84
	1000	1017.99 ± 34.63	1.80	3.89	1009.72 ± 37.42	0.97	1.80	98.17 ± 3.42	107.49 ± 3.42	98.82 ± 2.06	105.30 ± 3.19	98.01 ± 3.39

[a] Three concentration levels of analytes in QC samples.
[b] Three concentration levels were added to the blank rat plasma samples.
[c] The evaluations for the stability of the two analytes in rat plasma were the percent ratio of the detected concentration to the concentration of QC samples.

Table 4
The pharmacokinetic parameters (±SD) of representative salidroside and the four samples, following intravenous and oral administration with dose of 50 mg/kg (12.5 mg/kg, i.e. 17.6 mL/kg for oral liquid) ($n = 6$).

Parameter[a]	Unit	Standard salidroside IV	Standard salidroside PO	*Rhodiola crenulata* PO	Tablet PO	Capsule PO	Oral liquid PO
k_{el}	1/min	0.0330 ± 0.005	0.0128 ± 0.012	0.0077 ± 0.025	0.0111 ± 0.0038	0.0108 ± 0.0051	0.0115 ± 0.0047
$t_{1/2}$	min	20.9 ± 2.1	54.0 ± 3.8	89.9 ± 7.8	62.5 ± 6.3	64.2 ± 5.1	60.5 ± 3.4
T_{max}	min	5 ± 0.6	30 ± 1.9	30 ± 4.7	30 ± 3.1	30 ± 4.5	30 ± 3.9
C_{max}	ng/mL	21409.66 ± 754.19	4986.5 ± 254.19	197.13 ± 61.54	1746.92 ± 985.21	1672.15 ± 794.78	420.53 ± 55.69
AUC_{0-t}	min ng/mL	730484.28 ± 2154.58	380002.28 ± 1733.92	21719.12 ± 997.64	154529.9 ± 453.35	147009.82 ± 656.32	35665.51 ± 188.65
$AUC_{0-\infty}$	min ng/mL	746410.59 ± 2279.59	400032.88 ± 1968.75	26035.83 ± 735.92	165401.58 ± 316.58	157911.56 ± 408.55	38467.34 ± 216.24
V_d	mL/kg	2028.25 ± 351.81	9741.70 ± 768.37	249246.46 ± 3548.26	27277.96 ± 2159.61	29367.44 ± 2465.91	28376.41 ± 2060.35
CL	mL/min/kg	66.98 ± 11.43	124.98 ± 20.69	1920.43 ± 451.13	302.29 ± 89.58	316.63 ± 82.56	324.95 ± 61.54
MRT_{0-t}	min	26.27 ± 8.62	59.54 ± 17.62	79.28 ± 31.94	68.27 ± 19.63	68.59 ± 23.45	63.05 ± 17.62
$MRT_{0-\infty}$	min	28.92 ± 9.02	72.48 ± 20.52	127.44 ± 31.37	85.49 ± 23.92	86.82 ± 25.46	82.30 ± 19.59
F	%	–	53.59	3.48	22.16	21.16	20.61

[a] The parameters were k_{el} (first-order elimination rate constant), $t_{1/2}$ (half life), T_{max} (peak time), C_{max} (peak concentration), AUC_{0-t} (area under curve from 0 to last time), $AUC_{0-\infty}$ (area under curve from 0 to infinite time), V_d (apparent volume of distribution), CL (clearance), MRT_{0-t} (mean residence time from 0 to last time), $MRT_{0-\infty}$ (mean residence time from 0 to infinite time) and F (bioavailability $F = [AUC_{0-\infty}(PO)/dosage(PO)]/[AUC_{0-\infty}(IV)/dosage(IV)]$).

Fig. 4. The mean plasma concentration–time profile of representative analyte (salidroside) after intravenous (IV) and oral administration of standard salidroside (A), oral administration of herb and tablet (B) and oral administration of oral liquid and capsule (C).

these examined samples. The results were caused by the intense fluorescence response that reduced the interference from the concomitants or made the interfering signal insignificant. On the other hand, the interferences might be removed by the sample pretreatment process.

3.4.5. *Stability*

Results in Table 3 showed that the freeze and thaw stability, short-term stability, long-term stability and post-preparative stability were all within the range from 96.88 to 107.49%, indicating that the analytes were stable enough to be handled in the experiments.

3.4.6. *Application and pharmacokinetic study*

Using the developed method, samples were analyzed to obtain the contents and the pharmacokinetic parameters (Fig. 4 and

Table 4), which was imperative for the quality control of herbal products, and new dosage forms of traditional herbal medicine. Among the four samples the k_{el} of salidroside in *Rhodiola crenulata* was the smallest (0.0077 ± 0.025 1/min) and the half life was the largest (89.9 ± 7.8 min). The peak concentrations (C_{max}) of salidroside in tablet sample (1746.92 ± 985.21 ng/mL) was larger than others. Correspondingly, the tablet showed the largest AUC_{0-t} and $AUC_{0-\infty}$. The raw herb had the longer half-life but lower C_{max}, and contrarily its products led to the shorter half-life but higher C_{max}, indicating that the absorption of herb-related products was faster and further than that of the raw herb. Meanwhile, the results of *R. crenulata* showed the largest V_d, CL, MRT_{0-t} and $MRT_{0-\infty}$, which might indicate the sustained release effect of raw herb. The bioavailability of tablet was higher than that of other three samples.

3.4.7. Comparison with the reported methods

To justify the superiority of the present method, comparison with the previous analysis methods for the two biophenols in plasma samples was listed in Table 2. Remarkably, HPLC–UV [13,14] and LC–MS [15,16] methods were very disadvantageous in the sample amount ($\geq$200 μL), detection limits (> 10 ng/mL) and the pretreatment procedure, which were not sensitive enough to fully evaluate the pharmacokinetics. It was only the LOD of 1 ng/mL from LC–ESI-MS method [17] that was comparable to but higher than LOD of this work (0.6 ng/mL). However, the pretreatment procedure of 100–300 μL blood including inconvenient oculi chorioideae vein puncture, multiple centrifugation, vortex, evaporation and reconstitution, was cumbersome and low-efficient as the routine operation, which was not suitable for large batches of blood samples analysis in pre-clinical or clinical studies for medical research. In this work, the whole procedure of pretreatment could be efficiently and conveniently finished in 10 min and the total run time was no more than 15 min for salidroside, which was more rapid than previous methods [13–17].

4. Conclusion

The novel method with the elaborated semi-automated pretreatment device, the pre-column derivatization, multivariate optimization and the fluorescence detection with MS identification techniques was developed and validated for the systematical detection of two biophenols in series of carriers. The developed method not only proved to be successful to detect the content of two biophenols, but also showed the robustness for pharmacokinetic study, such as higher sensitivity, better accuracy, micro-amount of blood collection, easier pretreatment and shorter run-time. The robustness was attributable to the thorough study on optimized conditions, one of which, derivatization conditions was obtained from the multivariate calibrations using kinetic methods. The BBD, BBD-ANN, SV-ANN and Full-ANN methods validated by multi-criteria and nonparametric tests proved to be favorable for optimization, and Full-ANN coupled with GA method showed the better predictive power with the global optimum. The method itself played the important role in investigating the pharmacokinetics mechanism and provided the sensitive, efficient and convenient alternative for simultaneous analysis of the biological samples with trace content of bioactive components. And the approaches of the thorough study could serve as guiding methodology for seeking a novel analytical method for medical research.

Acknowledgments

This work was supported by the 100 Talents Program of the Chinese Academy of Sciences (no. 328), Key Laboratory of Bioorganic Analysis Shandong Province, Key Laboratory of Pharmaceutical Intermediates and Analysis of Natural Medicine Shandong Province, the research project fund (XJ201102) of Qufu Normal University, and the undergraduate research-training programs (2011A026).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2012.06.027.

References

[1] C.Y. Dai, C.L. Liu, Y.Q. Xu, L.Y. Ren, Food Sci. 30 (2009) 165.
[2] O.H. Lee, Y.I. Kwon, E. Apostolidis, K. Shetty, Y.C. Kim, Phytother. Res. 25 (2011) 106.
[3] Y.C. Ma, X.Q. Wang, F.F. Hou, J. Ma, M. Luo, S. Lu, P. Jin, A. Chen, I. Xu, A.V. Patel, J. Pharm. Biomed. Anal. 55 (2011) 908.
[4] S.K. Hung, R. Perry, E. Ernst, Phytomedicine 18 (2011) 235.
[5] R. Di Benedetto, R. Varě, B. Scazzocchio, C. Filesi, C. Santangelo, C. Giovannini, P. Matarrese, M. D'Archivio, R. Masella, Nutr. Metab. Cardiovasc. Dis. 17 (2007) 535.
[6] Y. Mao, Y. Li, N. Yao, J. Pharm. Biomed. Anal. 45 (2007) 510.
[7] R.N. Rao, P.K. Maurya, D.D. Shinde, S. Khalid, J. Pharm. Biomed. Anal. 55 (2011) 282.
[8] X. Han, T. Zhang, Y. Wei, X. Cao, Y. Ito, J. Chromatogr. A 971 (2002) 237.
[9] Y. Peng, J. Luo, Q. Lu, X. Chen, Y. Xie, L. Chen, W. Yang, S. Du, J. Pharm. Biomed. Anal. 49 (2009) 828.
[10] A. Tolonen, A. Hohtola, J. Jalonen, J. Mass Spectrom. 38 (2003) 845.
[11] S. Cui, X. Hu, X. Chen, Z. Hu, Anal. Bioanal. Chem. 377 (2003) 370.
[12] B. Chen, L. Zhang, G. Chen, Electrophoresis 32 (2011) 870.
[13] Y. Mao, X. Zhang, G. Lu, J. Sep. Sci. 30 (2007) 3218.
[14] L. Zhang, X.H. Liu, Y.Y. Song, Q.F. Shen, X.H. Wang, L. Chen, Chin. Pharm. J. 44 (2009) 935.
[15] Y.W. Chang, H.T. Yao, S.H. Hsieh, T.J. Lu, T.K. Yeh, J. Chromatogr. B 857 (2007) 164.
[16] J. Zhang, X. Chen, P. Wang, L. Huo, Z. Shen, X. Guo, W. Cheng, K. Bi, Chromatographia 67 (2008) 695.
[17] S. Yu, L. Liu, T. Wen, Y. Liu, D. Wang, Y. He, Y. Liang, X. Liu, L. Xie, G. Wang, J. Chromatogr. B 861 (2008) 10.
[18] G. Li, X. Zhang, J. You, C. Song, Z. Sun, L. Xia, Y. Suo, Anal. Chim. Acta 688 (2011) 208.
[19] M. Yoshitake, H. Nohta, H. Yoshida, T. Yoshitake, K. Todoroki, M. Yamaguchi, Anal. Chem. 78 (2006) 920.
[20] J. You, H. Zhao, Z. Sun, L. Xia, T. Yan, Y. Suo, Y. Li, J. Sep. Sci. 32 (2009) 1351.
[21] M.A. Bezerra, R.E. Santelli, E.P. Oliveira, L.S. Villar, L.A. Escaleira, Talanta 76 (2008) 965.
[22] G. Li, J. You, Y. Suo, C. Song, Z. Sun, L. Xia, X. Zhao, J. Shi, Food. Chem. 125 (2011) 1365.
[23] G.L. Li, J.M. You, C.H. Song, L. Xia, J. Zheng, Y.R. Suo, J. Agric. Food Chem. 59 (2011) 2972.
[24] Z. Sun, J. You, C. Song, L. Xia, Talanta 85 (2011) 1088.
[25] G. Hanrahan, Anal. Chem. 82 (2010) 4307.
[26] S. Busam, M. McNabb, A. Wackwitz, W. Senevirathna, S. Beggah, J.R. Van der Meer, M. Wells, U. Breuer, H. Harms, Anal. Chem. 79 (2007) 9107.
[27] B. Jancic, M. Medenica, D. Ivanovic, S. Jankovic, A. Malenovic, J. Chromatogr. A 1189 (2008) 366.
[28] F. Ruggieri, A.A. D'Archivio, G. Carlucci, P. Mazzeo, J. Chromatogr. A 1076 (2005) 163.
[29] K. Novotna, J. Havlis, J. Havel, J. Chromatogr. A 1096 (2005) 50.
[30] R. Leardi, J. Chemom. 15 (2001) 559.
[31] H. Tamaki, H. Kita, S. Kobayashi, Proceedings of IEEE International Conference on Evolutionary Computation, 1996, p. 517.
[32] L. Cséfalvayová, M. Pelikan, I. Kralj Cigic, J. Kolar, M. Strlic, Talanta 82 (2010) 1784.
[33] R. Modarres, Hydrol. Earth. Syst. Sci. 13 (2009) 411.
[34] W.J. Conover, Practical Nonparametric Statistics, Wiley, New York, 1980.
[35] A.C. Cabrera, J.M. Prieto, Food Chem. 118 (2010) 141.
[36] W. P-Hong-lun, L. Yu-lin, C. Shi-long, S. Yourui, M. Yong-fei, Anal. Lab. 24 (2005) 40.
[37] D. Wang, L. Shi, J. Hu, Pharm. Care Res. 6 (2006) 118.
[38] P.T. Linh, Y.H. Kim, S.P. Hong, J.J. Jian, J.S. Kang, Arch. Pharmacol. Res. 23 (2000) 349.
[39] L. Shi, Y. Ma, Z. Cai, Biomed. Chromatogr. 12 (1998) 27.
[40] P. Jiangnan, C. Hao, M. Chengyu, Chin. J. Pharm. Anal. 4 (1995) 21.
[41] M. Piriyapittaya, S. Jayanta, S. Mitra, N. Leepipatpiboon, J. Chromatogr. A 1189 (2008) 483.