Journal of Medicinal Plants Research Vol. 5(13), pp. 2879-2885, 4 July, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Simultaneous quantitation of seven anthraquinones in aqueous extract of rhubarb and analysis for absorption in cerebrospinal fluid of patients with traumatic brain injury using UPLC-PDA method

Yang Wang[1,2,3], Xi Huang[1,2,3]*, Qinghua Liang[1,2], Rong Fan[1,2,3], Feng Qin[1], Jiekun Luo[1,2], Yunhui Li[1,2], Wei Liu[1,2], Xilian Mao[1], Hui Ji[1,2,3], Zhaoqian Liu[3,4], Honghao Zhou[3,4], Lan Fan[3,4] and Lichen Gao[3,4]

[1]Institute of Integrated Traditional Chinese and Western Medicine, and Key Laboratory of Traditional Chinese Medicine Gan Organ of State Administration of Traditional Chinese Medicine, Xiangya Hospital, Central South University, Changsha 410008, China.
[2]Key Unit of the 11th Five-year Plan of SATCM in Cerebrosis, Xiangya Hospital, Central South University, Changsha 410008, China.
[3]TCM Pharmacogenetics Laboratory, Central South University, Changsha 410008, China.
[4]Institute of Clinical Pharmacology, Central South University, Changsha 410008, China.

Accepted 1 April, 2011

A simple and rapid ultra-performance liquid chromatography coupled with photo-diode array (UPLC-PDA) method was developed for determination of seven anthraquinones in aqueous extract of rhubarb (AER) and for detection of anthraquinones in cerebrospinal fluid (CSF) of patients with traumatic brain injury (TBI) after AER administration. Optimum separation was achieved on a reversed-phase column with methanol and 0.5% aqueous acetic acid as the mobile phase at a flow rate of 0.5 ml/min. Experimental results showed intra- and inter- day accuracy, precision, linear range and limits of detection were satisfactory for simultaneous determination of seven anthraquinones in AER. Good linear regression data ($r^2 > 0.9982$) were obtained for all the calibration plots within the range tested. The method was successfully used for quantitative analysis of the seven compounds in AER, and with the aid of UPLC-PDA, physcion was detected to be absorbed into CSF of TBI patient following oral administration of AER.

Key words: Quality control, ultra performance liquid chromatography, traumatic brain injury, anthraquinones, aqueous extract of rhubarb (AER), physcion.

INTRODUCTION

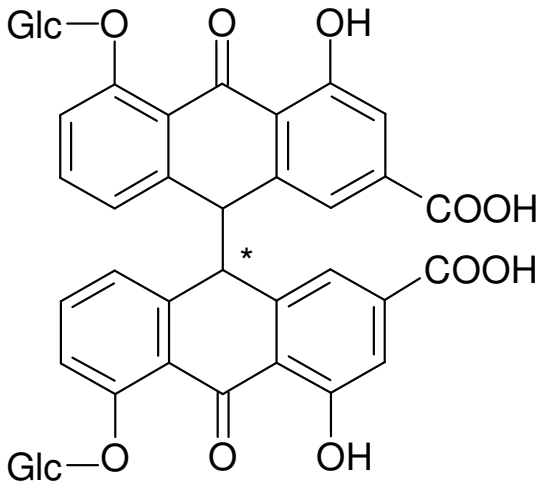
Rhubarb is one of the most popular traditional Chinese medicines (TCMs). In addition to a wide biological activities including purgation, antibacterial, antitumor and promoting blood circulation (Wang et al., 2006; Liu et al., 2005), rhubarb is also efficient in treating traumatic brain injury (TBI) recently (Gu et al., 2005, 2000; Eileen et al., 2009; Memduh et al., 2005; Shadi et al., 2009).

Among compounds in rhubarb, the pharmaceutically relevant bioactive components contain anthraquinones which are sennoside B, sennoside A, aloe-emodin, rhein, emodin, chrysophanol and physcion (the chemical structures of the seven anthraquinones are shown in (Figure 1) (Gao et al., 2009). They are used to be the basis for the quality control (QC) of rhubarb (Wang et al., 2008).

There were many QC analytical methods about crude drug of rhubarb established including high-performance liquid

*Corresponding author. E-mail: tcmhuangx59@163.com Tel: +86 731 84327222. Fax: +86 731 84328386.

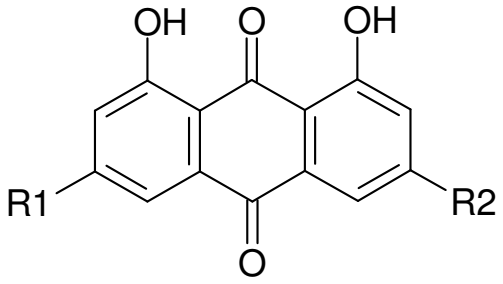
A



1 Sennoside B,* = erythro

2 Sennoside A,* = threo

B



No.	Compound	R_1	R_2
3	Aloe-emodin	H	CH_2OH
4	Rhein	H	COOH
5	Emodin	OH	CH_3
6	Chrysophanol	H	CH_3
7	Physcion	OCH_3	CH_3

Figure 1. Chemical structure of the seven anthraquinones in rhubarb.

chromatography (HPLC), thin-layer chromatography (TLC), capillary electrophoresis (CE), liquid chromatography-mass spectrometry (LC-MS) and fluorescence detection (He et al., 2009; Jin et al., 2007; Lin et al., 2006; Junko et al., 2007; Subash et al., 2005; Singh et al., 2005; Ye et al., 2007), but none involved its aqueous extract apart from our previous work (Ren et al., 2009). Aqueous extract of herb is a direct dosage form for drinking by patient which is more significantly determined in clinical application. Meanwhile, the results of recent basic and clinical studies showed that rhubarb exerted neuroprotective effect (Gu et al., 2000, 2005), but which absorbed bioactive compounds (ABCs) are present in CSF of TBI patients after aqueous extract of rhubarb (AER) treatment remains unknown.

In this study, we aimed to develop a simple and rapid

ultra-performance liquid chromatography coupled with photo-diode array (UPLC-PDA) method to simultaneously determine the seven anthraquinones in AER, and to explore which anthraquinone was present in CSF of TBI patient.

EXPERIMENTAL

Material and reagents

The raw drug of rhubarb was purchased from the Pharmacy of Xiangya Hospital, Hunan Province, PR China. The voucher specimen was deposited in Laboratory of Ethnopharmacology, Xiangya Hospital, Central South University. The crude drug was also authenticated by the herbal medicine botanist Professor Hu ZH, Department of Botanical Anatomy of Northwest University in China.

Reference compounds including sennoside B, sennoside A, aloe-emodin, rhein, emodin, chrysophanol and physcion (purity > 98%) were supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Analytical grade methanol from Tedia Company Inc. (OH, USA), acetic acid from Sinopharm Chemical Reagent Company (Shanghai, China), and triple-distilled water from silica glass equipment in this laboratory were used for preparation of the mobile phase. All other reagents were of analytical grade.

Chromatography

Chromatographic separation was performed on an Acquity UPLC BEH C_{18} column (100 × 2.1 mm i.d., 1.7 µm) (Waters Corporation, Milford, USA) using an Acquity ultra performance liquid chromatography (UPLC) system equipped with an Acquity photo-diode array detector (Waters, Milford, USA). Raw data was acquired and processed by use of Empower software. The mobile phase consisted of 0.5% aqueous acetic acid (solvent A) and methanol (solvent B). The gradient elution was: 0 to 12 min, 18% B; 12 to 14 min, 24 to 37% B; 14 to 18 min, 37 to 40% B; 18 to 21 min, 40 to 70% B; 21 to 25 min, 70 to 100% B. The components were quantified based on peak areas at their maximum wavelength in the UV spectrum. The flow rate was kept constant at 0.5 ml/min during the analysis, the column temperature was held at 45°C, the operating temperature was maintained at 25°C and the sample volume injected was 3 µl.

Preparation of standard solutions

The standard stock solutions of the seven components were directly prepared in methanol. Working standard solutions containing the seven compounds were prepared and diluted with methanol to appropriate concentration ranges to establish calibration curves. The standard stock solutions and working solutions were all prepared in dark brown calibrated flasks and stored at 4°C. The calibration curves were prepared with at least six appropriate concentrations. Empower software was used to prepare the standard curves from the peak area of each compound. The contents of these constituents in the test samples were calculated using the regression parameters obtained from the standard curves.

Preparation of sample solutions

Thirty gram of raw rhubarb was crushed into small pieces and the crude drug was soaked in 360 ml distilled water (1:12, *w/v*) for 30 min under room temperature with occasional stirring. The rhubarb was extracted twice by refluxing in boiling water for 10 min (Wang and Chen, 1990). The water extraction was then cooled and filtered through a five-layered bandage. The filtrate was concentrated and lyophilised. The yield of lyophilized powder of rhubarb was about 24.8% (*w/w*). The freeze dried powder was stored under 4°C until use. The sample injection volume for UPLC analysis was 3 µl. The samples were filtered through 0.22 µm film before UPLC analysis.

Method validation

In order to evaluate the accuracy of the proposed methods, a recovery test was performed by adding known amounts of reference standard solutions to the sample of AER before extraction, followed by analysis using the proposed method. Three concentrations of accurately determined amounts of the seven standard substances were used to spike the drug, and then extracted and analyzed as described in the previously paragraph. The percentage of recovery was calculated according to the formula: recovery (%) = (total amount after spiking – original amount in sample) /spiked amount × 100%.

Detection of Anthraquinones in CSF of TBI patients

The protocol was approved by Medical Ethics Committee of Xiangya Hospital, Central South University, China. The clinical trial was performed in accordance with the Declaration of Helsinki and informed consent was obtained from patients and close relatives before CSF sampling.

Five patients experienced severe TBI and they were consecutively eligible for enrollment from emergency ward (Xiangya Hospital, Central South University, China) after evacuation of hematoma. The patients were treated with AER at a dose of 0.5 g/kg after the 10th day of evacuation of hematoma. All subjects received standard care and treatment for their injuries. CSF samples were drawn before AER administration and at approximately the 4th hour after administration, respectively. CSF (5 ml) was then centrifuged at 3000 × g for 15 min at 4°C to obtain clear CSF. They were stored at -70°C until use.

The supernatant was evaporated under nitrogen at 37°C. A 6 ml volume of diethyl ether (included 50µl 1 M HCL) was added to each dry extract and vortexed for 60 s before being centrifuged at 15000 × g for 15 min at 4°C. The upper layer was evaporated to dryness under room air. The residues were reconstituted with 100µl of pure methanol after abstraction of diethyl ether and were then centrifuged at 15000 × g for 15 min at 4°C. Clear supernatant filtered through a 0.22 µm film. 3 µl of filtrate was injected into UPLC for analysis.

RESULTS AND DISCUSSION

Chromatography

In this study, an UPLC method was successfully developed to analyze seven components in AER. The selected monitoring wavelengths for these components were their maximum absorption wavelengths. With the PDA, UV spectra of the bioactive constituents could be compared with those of the authentic standards. The desired compound from the products was identified by comparing both the retention times and UV spectra with

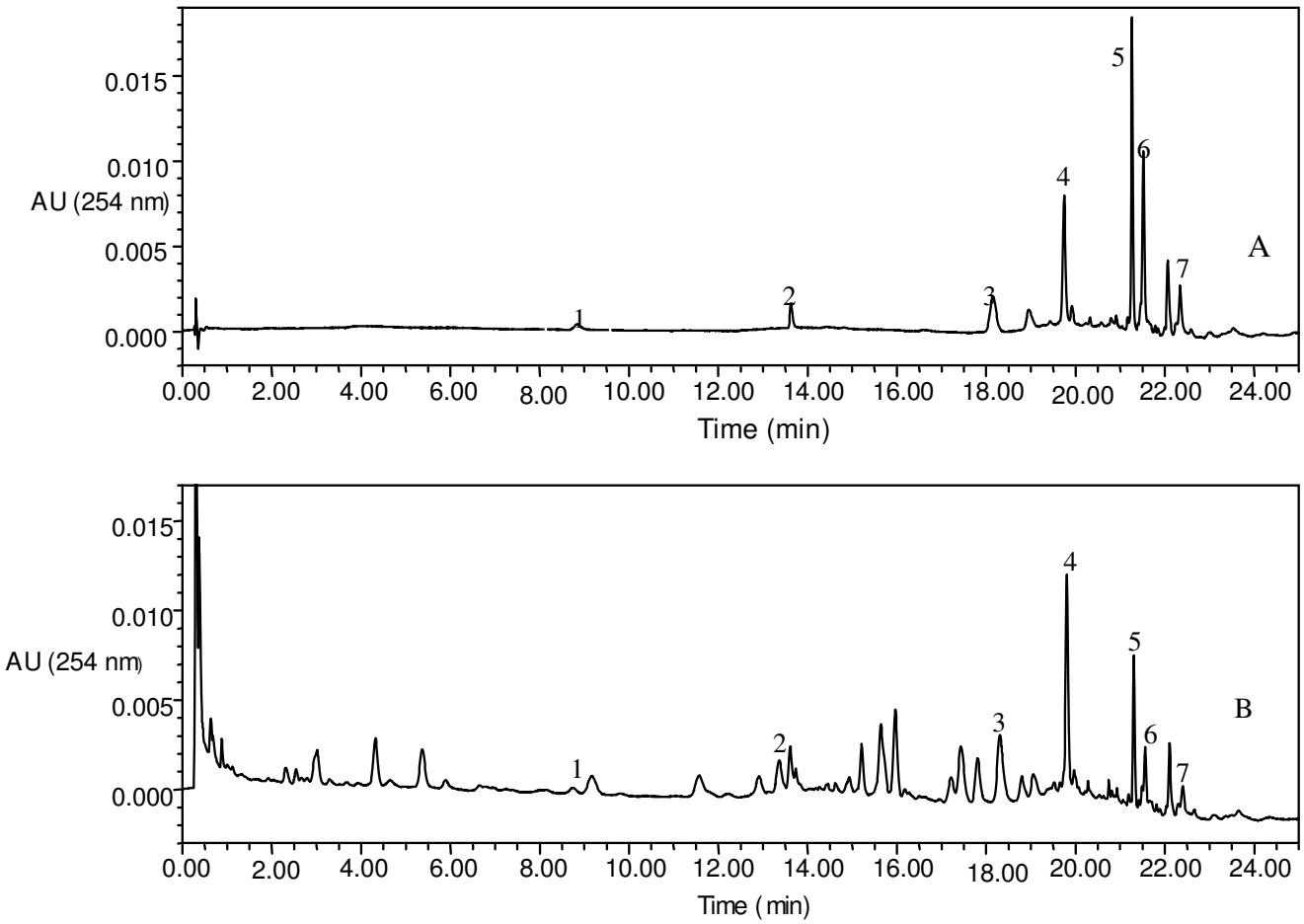


Figure 2. Typical chromatograms of the standard mixture (A) and water extract of rhubarb (B) at 254 nm. (1) sennoside B; (2) sennoside A; (3) aloe-emodin; (4) rhein; (5) emodin;(6) chrysophanol; (7) physcion.

Table 1. Linear regression data, LOD and LOQ of investigated compounds.

Components	Regression equation	Correlation coefficient (r^2)	Linear range (µg/ml)	LOD (µg/ml)	LOQ (µg/
Sennoside B	6080.76*x*-233.65	0.9994	0.255-16.304	0.077	0.238
Sennoside A	7738.58*x*-79.82	0.9995	0.177-11.286	0.045	0.167
Aloe-emodin	22565.5*x*+1652.13	0.9986	0.198-12.666	0.05	0.171
Rhein	19413.4*x*-102.03	0.9997	0.21-26.858	0.061	0.191
Emodin	16109*x*+9342.86	0.9984	0.285-36.428	0.048	0.16
Chrysophanol	16099.2*x*+1945.86	0.9996	0.386-24.642	0.068	0.193
Physcion	8822.88*x*+303.92	0.9964	0.268-17.142	0.034	0.135

In the regression equation $y = ax + b$, x refers to the concentration (µg/ml), y indicates the peak area, and r^2 is the correlation coefficient of the equation. LOD, limit of detection. LOQ, limit of quantification.

those of the authentic standard. The analyte was further confirmed by spiking the actual sample with the standard. Under the proposed analytical conditions, the seven marker constituents were sufficiently resolved, and there was no interference from other components in the matrix. Typical chromatograms of the authentic standards and the AER recorded at 254 nm are depicted in (Figure 2).

Method validation and quantification

Table 1 shows the linearity, LOD and LOQ of each

Table 2. The precision data of UPLC method.

Components	Nominal concentration (μg/ml)	Precision			
		Intra-day (n = 5)	R.S.D (%)	Inter-day (n = 3)	R.S.D (%)
		Mean ± SD (μg/ml)		Mean ± SD (μg/ml)	
Sennoside B	0.51	0.44±0.023	5.23	0.465±0.019	4.09
	1.019	0.818±0.045	5.50	0.892±0.087	9.75
	4.076	4.241±0.312	7.36	4.092±0.261	6.38
Sennoside A	0.353	0.325±0.018	5.54	0.324±0.018	5.55
	1.411	1.26±0.086	6.82	1.382±0.058	4.19
	5.643	5.47±0.074	1.35	5.256±0.387	7.36
Aloe-emodin	0.396	0.389±0.031	7.97	0.398±0.037	9.29
	1.584	1.477±0.109	7.38	1.717±0.073	4.25
	6.333	6.41±0.106	1.65	5.97±0.291	4.87
Rhein	0.84	0.824±0.017	2.06	0.787±0.015	1.90
	3.358	3.077±0.069	2.24	3.333±0.139	4.17
	13.429	13.414±0.216	1.61	13.283±0.349	2.62
Emodin	1.139	1.139±0.055	4.82	1.326±0.022	1.66
	4.554	4.316±0.104	2.40	4.905±0.077	1.57
	18.214	17.09±0.472	2.76	17.134±1.029	6.01
Chrysophanol	0.771	0.864±0.074	8.56	0.769±0.067	8.71
	3.081	3.243±0.198	6.11	3.235±0.121	3.74
	12.321	10.343±0.422	4.08	10.819±0.539	4.98
Physcion	0.536	0.519±0.048	9.25	0.52±0.032	6.15
	2.143	1.935±0.109	5.63	2.021±0.104	5.15
	8.571	8.298±0.367	4.42	8.29±0.176	2.12

component determined. The LOD and LOQ were determined as signal-to-noise (S/N) ratio of 3 and 10, respectively. All calibration curves of seven analytes exhibited good linear regression within test ranges under the established chromatographic conditions. The relative standard deviation (RSD) was considered to be a measurement of precision and accuracy. Precision was measured by intra- and inter- day variability. Intra- and inter- day precisions were determined by analyzing standard solutions at three concentrations during a single day and on three different days, respectively. As shown in (Table 2), the overall intra- and inter- day variations were less than 10% for all the 7 analytes. All these data revealed that the described method had an accepted degree of precision. The accuracy tests were carried out using a recovery test. Recovery of all seven tested bioactive compounds was within the range of 96.6 to 104.5%, with an RSD of between 0.59% and 4.8% (n = 3). These values indicated that the method showed the reliability and accuracy for the measurement of these components. The stability test was performed with sample solutions placed under 4℃ and these were analyzed at 0, 24, and 48 h. The RSD values of the peak area were no more than 7.15%, respectively. The solution was therefore considered to be stable for at least 48 h at 4℃. Representative chromatograms of the authentic standards and extracts of rhubarb are shown in (Figure 2) and the quantity of each compound identified is summarized in (Table 3).

QC assessment for commercial herbal products to patients should be more suitable than the crude herb. However, all the compounds detected came from crude rhubarb apart from our previous work (He et al., 2009; Jin et al., 2007; Lin et al., 2006; Junko et al., 2007; Subash et al., 2005; Singh et al., 2005; Ye et al., 2007; Ren et al., 2009).

Most of the compounds in rhubarb extracted by chloroform, phenol, acetone or sulphuric acid were not determined in the aqueous extract because of their liposolubility. The determination of four anthraquinones in aqueous extract of rhubarb granula was developed by HPLC in our past study (Ren et al., 2009). In present study, UPLC is better than HPLC in speed, resolution, and sensitivity of analysis, especially for time saving and

Table 3. The contents of seven components in WER (n = 3).

Components	Contents (μg/ml)	R.S.D (%)
Sennoside B	4.493±0.18	4.01
Sennoside A	10.813±0.711	5.55
Aloe-emodin	11.397±0.452	3.96
Rhein	20.223±0.708	3.5
Emodin	9.787±0.302	3.09
Chrysophanol	3.101±0.167	5.38
Physcion	5.407±0.322	5.96

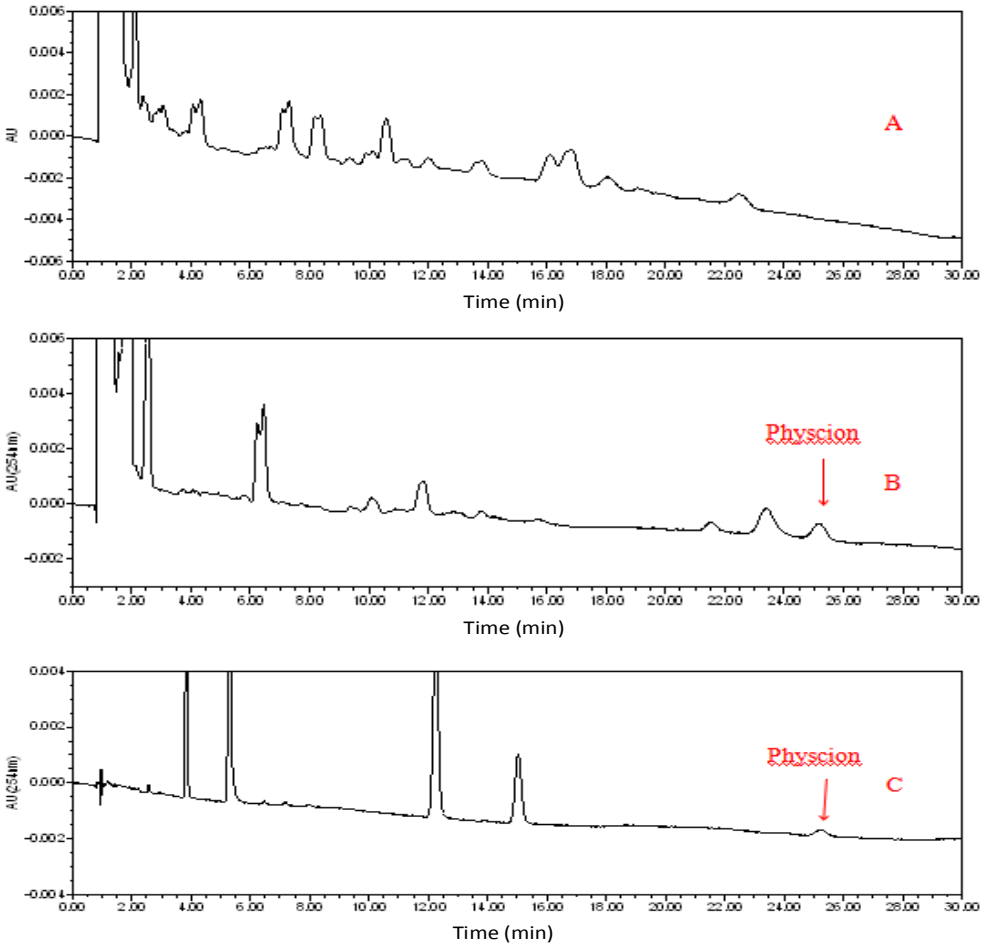


Figure 3. Typical chromatograms for the determination of physcion in CSF samples: (A) chromatogram of the pre-treated CSF sample; (B) chromatogram of the post-CSF sample; (C) chromatogram of standard solution. Time of physcion appearance is depicted by an arrow.

solvent consumption (Wu et al., 2008). The seven anthraquinones in AER were well separated within 23 min by UPLC (Figure 2), while retention time needed approximately 70 min by HPLC method (Gao et al., 2009). From Tables 1 to 2, the LOD (0.034 to 0.077 μg/ml), LOQ (0.135 to 0.238 μg/ml), the overall intra- and inter-day variations (< 10%) and all recovery (> 90%) indicated that the developed method was rapid, simple, sensitive, accurate and valid. This method is more useful than previous methods for analysis of rhubarb.

Application to detection of anthraquinone in CSF samples

Chromatograms of pre- and post- treated CSF are shown in (Figure 3). The comparison made through analysis of standard solutions with the UPLC-PDA method. The

results indicated that although physcion content ranked No. 5 among seven anthraquinones of rhubarb (Table 3), only physcion was detected in 5 ml CSF of TBI patient under the chromatographic conditions employed. Previous research reported that five anthraquinones including physcion were detected in CSF of rats following oral administration of rhubarb (5 g/kg) (Ding et al., 2003). However, their concentrated CSF volume (5 ml) from several rats was too high to be obtained from a rat whose total CSF volume was only about 0.58 ml (Lai et al., 1983).

It was certain that the anthraquinones in less than 0.58 ml CSF were difficult to be detected according to their limit of detection. Actually, it was difficult to obtain more than 5 ml human CSF which was only a small percentage of total CSF volume in a patient because of ethics and informed consent (Tsunoda et al., 2002; Mardini et al., 2005). Among five anthraquinones from 5 ml CSF of rats, only physcion was much easier to be detected while the contents of other four were very low (Ding et al., 2003), which indirectly supported the determination of only physcion in 5 ml of patient's CSF in the present study.

It is well-known that the herbal drug the patients take is the herbal products. The bioactive components should be water-soluble. Though the contents of products of TCMs must be much lower than the raw herbal drugs, we consider that it is more significant to determine absorbed bioactive components of the products of herbal drugs for quality control assessment. The interesting results may help to reveal physcion whose content was lower than other four anthraquinones in AER, could exert neuroprotective effect by passing though blood-brain barrier into human brain, and to elucidate the pharmacological foundation of anthraquinone from rhubarb in treatment of TBI. And further study should be done.

Conclusion

A simple and rapid ultra-performance liquid chromatography coupled with photo-diode array (UPLC-PDA) method has been developed for simultaneous determination of seven anthraquinones of AER. The method enables identification and quantification of anthraquinones in one run, with acceptable linearity, precision, and accuracy, for quality control purposes and furthermore for detection of anthraquinone in CSF of TBI patient to help reveal pharmacological foundation of anthraquinone in treatment of TBI.

ACKNOWLEDGEMENTS

This work was supported by the fund for Key Laboratory of Traditional Chinese Medicine Gan of SATCM, by the Major Research plan of the National Natural Science Foundation of China (Nos. 30572339; 81072967), by the Administration of Traditional Chinese Medicine of Hunan Province, China (No. 2010113), and was partly supported by the Program for Innovative Research Team in University.

REFERENCES

Ding MY, Ma SW, Liu DL (2003). Simultaneous Determination of Hydroxyanthraquinones in Rhubarb and Experimental Animal Bodies by High-Performance Liquid Chromatography. Anal. Sci., 19: 1163-1165.

Eileen MW, Vicente G, Arthur I, Katherine H, Stephanie B, Yang W, Susan C, Peter DL (2009). Brain tissue oxygen and outcome after severe traumatic brain injury: A systematic review. Crit. Care Med., 37: 2057-2063.

Gao XY, Jiang Y, Lu JQ, Tu PF (2009). One single standard substance for the determination of multiple anthraquinone derivatives in rhubarb using high-performance liquid chromatography-diode array detection. J. Chromatogr. A., 1216: 2118-2123.

Gu JW, Hiroshi H, Mitsue T, Takashi A (2005). Effects of emodin on synaptic transmission in rat hippocampal CA1 pyramidal neurons *in vitro*. Neuropharmacol., 49: 103-111.

Gu JW, Zhang X, Zhou F, Wen AD, Qin SY, Yi SY, Chen YJ, Li X (2000). Rhubarb extracts in treating complications of severe cerebral injury. C.M.J., 113: 529-531.

He DX, Chen B, Tian QQ, Yao SZ (2009). Simultaneous determination of five anthraquinones in medicinal plants and pharmaceutical preparations by HPLC with fluorescence detection. J. Pharm. Biomed. Anal., 49: 1123-1127.

Jin W, Wang YF, Ge RL, Shi HM, Jia CQ, Tu PF (2007). Simultaneous analysis of multiple bioactive constituents in *Rheum tanguticum Maxim. ex Balf.* by high-performance liquid chromatography coupled to tandem mass spectrometry. Rapid Commun. Mass Spectrom., 21: 2351-2360.

Junko K, Izumi M, Norihiro K (2007). Simultaneous determination of anthraquinones in rhubarb by high-performance liquid chromatography and capillary electrophoresis. J. Chromatogr. A, 1145: 183-189.

Lai YL, Smith PM, Lamm WJE, Hildebrandt J (1983). Sampling and analysis of cerebrospinal fluid for chronic studies in awake rats. J. Appl. Physiol., 54: 1754-1757.

Lin CC, Wu C, Lin TC, Sheu SJ (2006). Determination of 19 rhubarb constituents by high-performance liquid chromatography-ultraviolet-mass spectrometry. J. Sep. Sci., 29: 2584-2593.

Liu LH, Fan LY, Chen HL, Chen XG, Hu ZD (2005). Separation and determination of four active anthraquinones in Chinese herbal preparations by flow injection capillary electrophoresis. Electrophor., 26: 2999-3006.

Mardini S, See LC, Lo LJ, Salgado CJ, Chen YR (2005). Intracranial space, brain, and cerebrospinal fluid volume measurements obtained with the aid of three-dimensional computerized tomography in patients with and without Crouzon syndrome. J. Neurosurg., 103: 238-246.

Memduh K, Bayram C, Fehmi O, Ahmet D, Recep S, Irfan A, Namik D (2005). Does melatonin protect or treat brain damage from traumatic oxidative stress?. Exp. Brain Res., 163: 406-410.

Ren P, Qin F, Huang X, Zhu ZY (2009). Simultaneous LC Analysis of Aloe-Emodin, Rhein, Emodin, and Chrysophanol in *Rhizoma Rhei-Type* Preparations. Chromatographia, 70: 1515-1517.

Shadi H, Fabiola F, Nicole C, Bruno P, Michel P, Catherine ML, Mehrnas JT (2009). Minocycline effects on cerebral edema: Relations with inflammatory and oxidative stress markers following traumatic brain injury in mice. Brain Res., 1291: 122-132.

Singh NP, Gupta AP, Sinha AK (2005). High-performance thin layer chromatography method for quantitative determination of four major anthraquinone derivatives in Rheum emodi. J. Chromatogr. A., 1077: 202-206.

Subash CV, Narendra PS, Arun KS (2005). Determination and locational variations in the quantity of hydroxyanthraquinones and their glycosides in rhizomes of *Rheum emodi* using high-performance liquid chromatography. J. Chromatogr. A., 1097: 59-65.