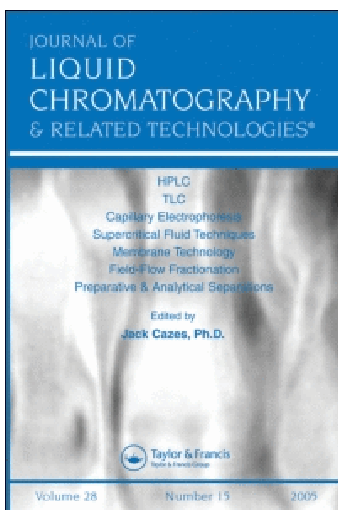
This article was downloaded by:
On: *23 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Preparative HPLC for Purification of Four Isomeric Bioactive Saponins from the Seeds of *Aesculus chinensis*

Feng Wei[a]; Lin-Yun Ma[a]; Xian-Long Cheng[a]; Rui-Chao Lin[a]; Wen-Tao Jin[b]; Ikhlas A. Khan[b]; Jian-Qiu Lu[c]

[a] Division of Chinese Materia Medica and Natural Products, National Institute for the Control of Pharmaceutical and Biological Products, State Food and Drug Administration, Beijing, People's Republic of China [b] National Center for Natural Products Research, School of Pharmacy, The University of Mississippi, MS, USA [c] Research Center of Science and Technology in Chinese Medicine, Beijing University of Traditional Chinese Medicine, Beijing, P.R. China

To cite this Article Wei, Feng , Ma, Lin-Yun , Cheng, Xian-Long , Lin, Rui-Chao , Jin, Wen-Tao , Khan, Ikhlas A. and Lu, Jian-Qiu(2005) 'Preparative HPLC for Purification of Four Isomeric Bioactive Saponins from the Seeds of *Aesculus chinensis*', Journal of Liquid Chromatography & Related Technologies, 28: 5, 763 — 773

To link to this Article: DOI: 10.1081/JLC-200048904

URL: http://dx.doi.org/10.1081/JLC-200048904

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

Journal of Liquid Chromatography & Related Technologies®, 28: 763–773, 2005
Copyright © Taylor & Francis, Inc.
ISSN 1082-6076 print/1520-572X online
DOI: 10.1081/JLC-200048904

Preparative HPLC for Purification of Four Isomeric Bioactive Saponins from the Seeds of *Aesculus chinensis*

Feng Wei, Lin-Yun Ma, Xian-Long Cheng, and Rui-Chao Lin
Division of Chinese Materia Medica and Natural Products,
National Institute for the Control of Pharmaceutical and
Biological Products, State Food and Drug Administration,
Beijing, People's Republic of China

Wen-Tao Jin and Ikhlas A. Khan
National Center for Natural Products Research, School of Pharmacy,
The University of Mississippi, MS, USA

Jian-Qiu Lu
Research Center of Science and Technology in Chinese Medicine,
Beijing University of Traditional Chinese Medicine,
Beijing, P.R. China

Downloaded At: 19:03 23 January 2011

Abstract: Four isomeric bioactive saponin compounds named escin Ia, isoescin Ia, escin Ib, and isoescin Ib were successfully isolated and purified from the crude extract of the seeds of a traditional chinese medicinal plant *Aesculus chinensis* Bge (Hippocastanaceae) by preparatory high performance liquid chromatography (Pre-HPLC). The gradient mobile phase solvent system composed of methanol–water–acetic acid was employed. An efficient large scale preparatory method was developed based on the stability investigation of escin Ia for the first time. A total amount of 5.2 g escin Ia, 2.8 g isoescin Ia, 3.8 g escin Ib, and 1.6 g isoescin Ib, separately, over 99% purity was obtained from 50 g of total saponins. The preparatory

Address correspondence to Feng Wei, Division of Chinese Materia Medica and Natural Products, National Institute for the Control of Pharmaceutical and Biological Products, State Food and Drug Administration, 2 Tiantan Xili, Beijing 100050, People's Republic of China. E-mail: hograwei@hotmail.com

purification of four isomeric saponins by Pre-HPLC was completed in 120 min in a one step separation.

Keywords: Preparatory HPLC, *Aesculus chinensis*, escin Ia, isoescin Ia, escin Ib, isoescin Ib, stability studies

INTRODUCTION

Escin (Aescin), the major active principle from the seeds of Aesculus plants (Hippocastanaceae), has shown satisfactory evidence for a clinical significant activity in chronic venous insufficiency (CVI), hemorrhoids, and post-operative edema.[1,2] Phytochemical investigation shows that the seeds of genus Aesculus plants contain many triterpenosides and flavoloids.[3–6] HPLC-MS analysis has demonstrated that escin was mainly composed of four isomeric triterpenoid saponins named escin Ia, isoescin Ia, escin Ib, and isoescin Ib, respectively.[7] Their chemical structures are shown in Fig. 1.

In China, several genus aesculus medicinal plants such as *A. chinensis* Bge., *A. chinensis* Bge. Var. checkiangensis (Hu et Fang) Fang, and

Downloaded At: 19:03 23 January 2011

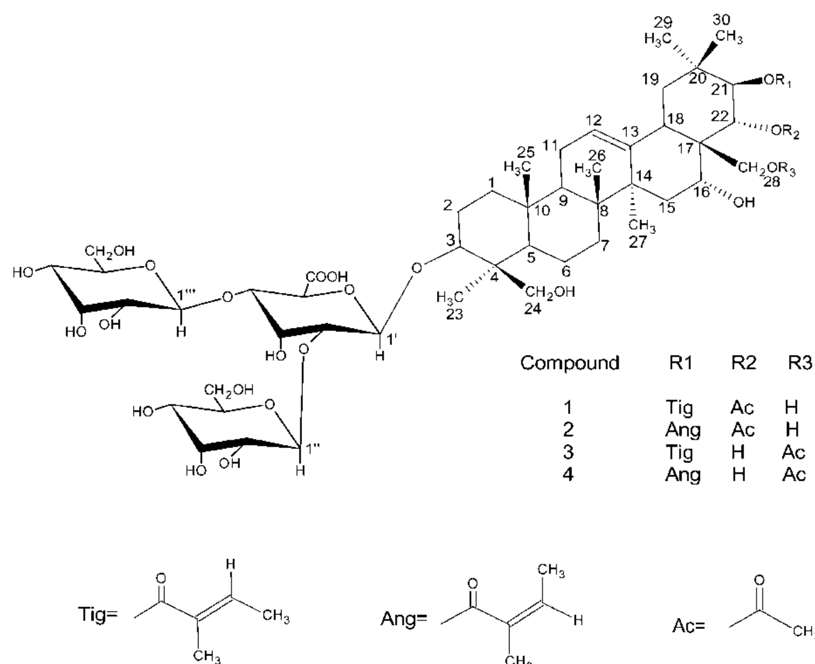


Figure 1. Structures of compounds escin Ia (1), escin Ib (2), isoescin Ia (3), and isoescin Ib (4).

A. wilsonii Rehd are widely distributed. According to the theory of traditional chinese medicine, their dried ripe seeds (Suoluozi) have been used as a carminative, stomachic, and analgesic for the treatment of distention and pain in chest and abdomen.[8] The saponin mixture (escin) extracted from the seeds of chinese aesculus plants are almost the same in constituents as that extracted from the seeds of Europe horse chestnut tree (*A. hippocastanum* L.).[9] At present, the finished products containing escin are commercially manufactured and widely used as anti-inflammatory, anti-edema, capillary protective drugs both in Europe and Asia countries.[10,11]

In view of the wide biological activities and broad clinical use, the preparation of four main saponins with high purity has been of much interest to pharmaceutical chemists in the area of new drug development or quality control of escin related products. Although many saponin constituents of escin have been characterized and their activities have been investigated, the scale preparation of the main saponins were merely reported.[12,13] The preparatory separation and purification of escin Ia, isoescin Ia, escin Ib, and isoescin Ib from plant materials by conventional methods is tedious and usually requires multiple chromatography steps, such as normal column chromatography (CC) and thin-layer chromatography (TLC).[14,15] Moreover, to obtain highly pure escin Ia, isoescin Ia, escin Ib, and isoescin Ib is very difficult, because of their unstable properties and irreversible adsorption on normal Silica G, and existing HPLC methods are not suitable for large-scale isolation of these compounds. Pre-HPLC has been successfully applied to the analysis and separation of various natural products.[16] Recently, we have developed a new preparatory reverse phase HPLC method based on the stability studies of escin Ia. Our primary experiments have demonstrated that this condition is most suitable for large scale preparatory isolation and purification from crude extracts of aesculus plants, at a relatively low cost and high efficiency. So far, although there are several reports about HPLC analysis of escin, no report has been published on the scale isolation and purification of the four compounds. Moreover, little is known about the stability of escin under different processing conditions such as pH and temperature. The purpose of this study, therefore, was to develop a method for the isolation and purification of escin Ia, isoescin Ia, escin Ib, and isoescin Ib by pre-HPLC based on the stability studies.

EXPERIMENTAL

Apparatus

The Pre-HPLC instrument employed in the present study is a Beckman analysis product (Beckman Instruments Inc. CA, USA). The HPLC system was

Downloaded At: 19:03 23 January 2011

composed of two Beckman HPLC 125P pumps, a preparatory C_{18} column (300×50 mm I.D., particle size 5 μ, Waters, USA), a Beckman 166P UV detector and a Gold Nouveau chromatography data system (Beckman). For analytical HPLC, waters separation products (two Waters HPLC 510 pumps, Millennium chromatography data system and a 996 detector) were used, and an ODS column (YWG C_{18} 150×4.6 mm I.D. 5 μ, U.S.A.) was employed.

Reagents

Ethanol, acetonitrile, ethyl acetate, chloroform, methanol were of HPLC grade. Ammonium hydroxide, acetic acid, citrate phosphate buffer, and hydrochloric acid were all of analytical reagent grade and obtained from Fisher (Fisher Scientific, NJ, USA). Water was purified in a Milli-Q water purification system from Millipore. The dried seeds of *A. chinensis* Bge. were collected at Luoyang, Henan Province, P.R. China, in Septermber 1998. The voucher specimens are deposited at the Herbal Museum of National Institute for the Control of Pharmaceutical and Biological Products, State Food and Drug Administration (Beijing, P.R. China).

Downloaded At: 19:03 23 January 2011

Preparation of Sample Solution

The powdered seeds (20 Kg) were extracted three times with 15 L 50% EtOH at room temperature. After removal of the solvent in *vacuo*, the extract (3.6 Kg) was further partitioned between H_2O and EtOAc to give H_2O-soluble and EtOAc-soluble fractions. The H_2O-soluble (2.8 Kg) fraction was subjected to a D-101 macroreticular resin column and eluted successively with H_2O, 30% EtOH, 70% EtOH, and 95% EtOH, giving four fractions. The third fraction (70% EtOH eluate) was evaporated to dryness by freeze-dry yielding 490 g of crude total saponin (escin). The sample solution was prepared by dissolving a quantity of 50 g escin in 500 mL mixture solution of water-methanol (5 : 1 (v/v)) solvent system used for Pre-HPLC separation.

Separation Procedure

The Pre-HPLC column was first entirely equilibrated with initial mobile phase (methanol-0.1% acetic acid aq. (20 : 80)) at a flow rate of 20 mL/min. After equilibrium was established in the column, 20 mL of the sample solution containing 2.0 g of total saponin was injected, and a gradient mobile phase solvent system was applied (Table 1). The effluent was continuously monitored at 210 nm and the chromatography was recorded. Each peak

Table 1. Gradient mobile phase elution timetable of Pre-HPLC

Time (min)	Methanol (%)	0.1% Acetic acid, aq. (%)
0	20	80
10	20	80
60	30	70
110	40	60
120	60	40

fraction was collected according to the chromatography and determined by HPLC.

Downloaded At: 19:03 23 January 2011

HPLC Analysis and Identification of Pre-HPLC

The total saponins from the seeds of *A. chinensis* seeds, escin Ia, isoescin Ia, escin Ib, isoescin Ib (standard), and each main Pre-HPLC peak fraction were analyzed by a analytical HPLC. The separation was performed with an isocratic elution using acetonitrile–0.1% acetic acid aq. (33 : 67 (v/v)) at a flow rate of 1.0 mL/min, column temperature at 28°C, and the effluent was monitored at 210 nm with DAD. The components were confirmed from their retention times and UV-Vis spectra from 200 to 360 nm against the standards. Routine sample calculations were made by comparison of the peak area with that of the standard reference substances of the four compounds.

Stability Investigation of Escn Ia

Most oleanane type saponins, which have two or more big hydroxyl substitutions at adjacent positions such as at C-21, C-22, and/or C-28 position, are very unstable. Escin are such saponins that are very sensitive to environment conditions. The stability of escin is affected by temperature, pH, oxygen, and light. As temperature and pH are main parameters influencing its stability, special care was taken to study the effect of these parameters.

Thermal and pH Stability of Escin Ia

The stability of escin Ia was studied on the purified escin Ia (over 99% in purity, and 1 mg/mL in water). An extremely accelerated experiment was designed to study the effects of different temperatures, and the samples,

which were kept refrigerated at 4°C before being treated, were incubated in a water bath at 0, 20, 30, 40, 50, 60, 80, and 100°C for 60 minutes, respectively. After each treatment, the sample tubes were returned to room temperature, and the contents of escin Ia in these samples were determined by HPLC, and its conversion rate under different temperatures was calculated. The HPLC condition was the same as mentioned before.

To ascertain the effects of pH on the degradation of escin Ia, aqueous solutions (1 mg mL^{-1} in water) at different pH values (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11) were obtained in citrate-phosphate buffers, and was adjusted with hydrochloric acid or ammonium hydroxide when needed. After 30 min at room temperature (25°C), the samples were then subjected to HPLC analysis by the same method mentioned above, and the degradation rate of escin Ia in different pH values were calculated.

RESULTS AND DISCUSSION

The total saponin (escin) from the seeds of *A. chinensis* was first analyzed by HPLC, which indicated that it contained many compounds among which escin Ia, isoescin Ia, escin Ib, and isoescin Ib represented a major component accounting, over 80% of the total saponins based on HPLC peak area percentage (Fig. 2).

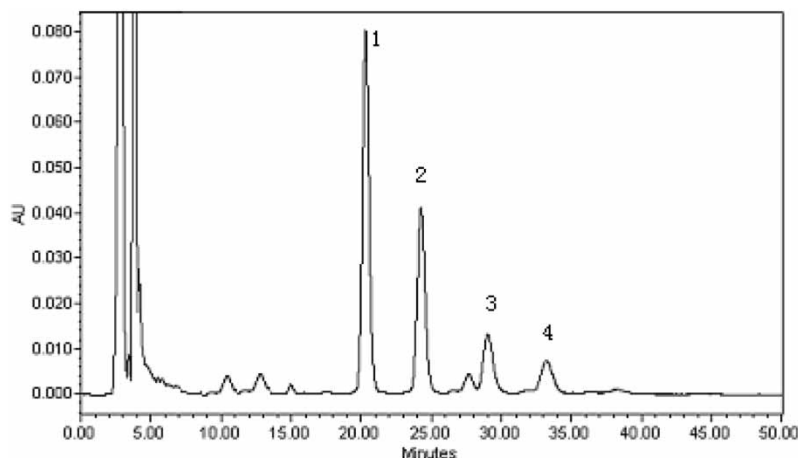


Figure 2. HPLC chromatography of escin (the total saponin extracts) from the seeds of *A. Chinensis.* 1: escin Ia. 2: escin Ib. 3: isoescin Ia. 4. isoescin Ib. HPLC conditions: reversed-phase Symmetry C_{18} column (150 × 4.6 mm I.D., 5 μ); column temperature, 28°C; mobile phase, acetonitrile–0.1% acetic acid aq. (67 : 33 (v/v)); flow rate, 1.0 mL/min; diode array detection at 210 nm; injection volume, 10 μL.

Downloaded At: 19:03 23 January 2011

Selection of Mobile Phase Solvent System

Escin Ia, isoescin Ia, escin Ib, and isoescin Ib are all chemical isomeric glycuronide saponins. Little is known about the use of methanol–water–acetic acid for the scale separation of these compounds by Pre-HPLC. Using this solvent system and gradient procedure, escin Ia, isoescin Ia, escin Ib, and isoescin Ib could be separated from each other, and from other compounds. But, the flow rate of the mobile phase was relatively low, so that resulted in an excessively broad peak with long elution time. The chromatography of total saponin from the seeds of *A. chinensis* by Pre-HPLC separation, along with the HPLC chromatography of purified escin Ia (1), isoescin Ia (2), escin Ib (3), and isoescin Ib (4) are summarized in Fig. 3.

Separation of Escin Ia, Isoescin Ia, Escin Ib, and Isoescin Ib by Pre-HPLC

A 50 g quantity of the crude extract (containing 80% of saponin) was dissolved in water–methanol (5 : 1 (v/v)) to get a sample solution, which contained about 100 mg/mL saponins. Then, every 20 mL sample solution was injected and purified by Pre-HPLC using gradient methanol–0.1% acetic acid aq. as the solvent system. Although the retention time was a little long, four isomeric saponins were well separated and the total separation time was 120 min (Fig. 3). After each run, methanol was used to wash the column. Based on the HPLC analysis and the elution curve of the Pre-HPLC, peak 1, 2, 3, and 4 corresponded to escin Ia, isoescin Ia, escin Ib, and isoescin Ib, respectively. The eluates were separated repeatedly by Pre-HPLC to yield escin Ia 5.2 g (99.7% in purity), isoescin Ia 3.8 g (99.5% in purity), escin Ib 2.8 g (99.3% in purity), and isoescin Ib 1.6 g (99.1% in purity). The purity of the compounds was analyzed by HPLC [acetonitrile-0.1% acetic acid aq. (67 : 33 (v/v)); flow rate, 1.0 mL min^{-1}; column temperature, 28°C]. The HPLC chromatography of pure compounds is shown in Fig. 3.

Stability Studies of Escin Ia

Escins are composed of many similar oleanane type saponins in structures, which have a tiglical acyl group or an anglic acyl group at C-21position, and an acetyl group at C-22 or C-28 position. Fig. 4 shows that escin Ia can be converted to isoescin Ia when temperature is high than 40°C. As can be seen, escin Ia is stable when storage temperature is between 0°C and 40°C, and the conversion rate of escin Ia to isoescin Ia gets higher with the increase of temperature. To ascertain the effect of pH on the stability of escin Ia, aqueous solutions at different pH values (1, 2, 3, 4, 5, 6, 7, 8, 9,

Downloaded At: 19:03 23 January 2011

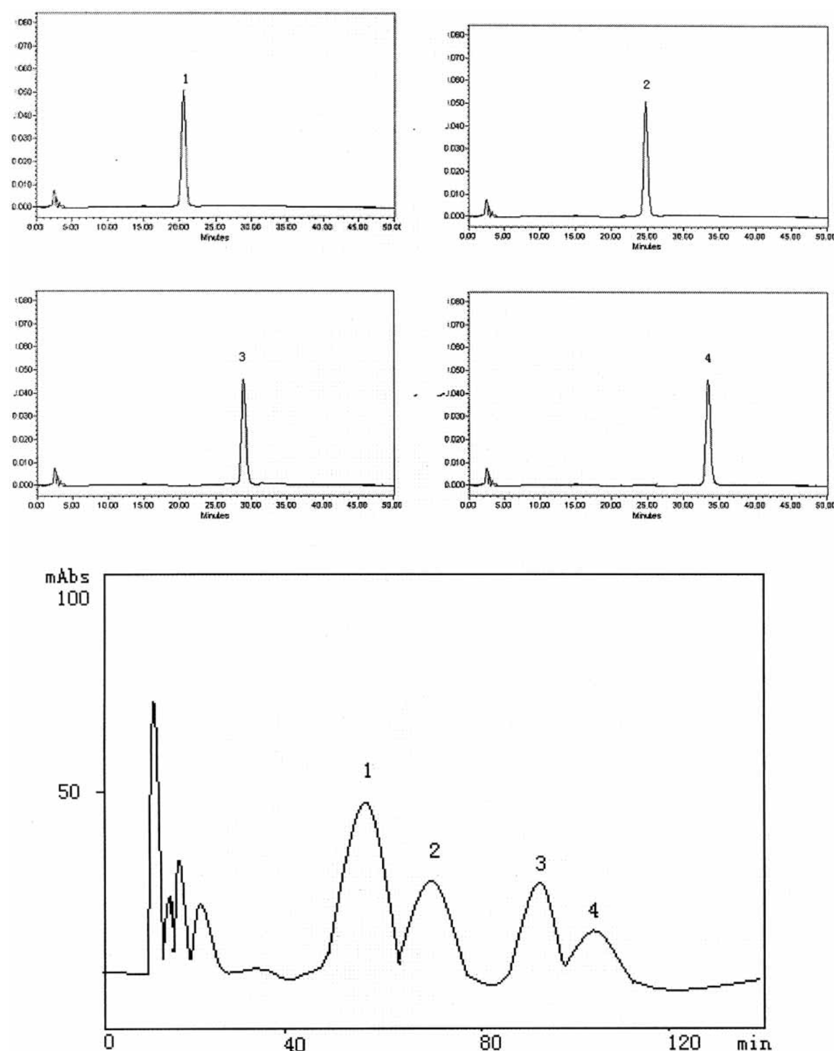


Figure 3. Pre-HPLC chromatography of escin and HPLC chromatography of four purified isomeric saponins from the seeds of *A. chinensis.* 1: escin Ia. 2: escin Ib. 3: isoescin Ia. 4. isoescin Ib.

10, and 11) were also tested at room temperature (25°C). As show in Fig. 5, escin Ia is very sensitive to low or high pH value, and the highest stability was observed between pH 3 and pH 5. However, according to HPLC results (shortened), the degradation products from escin Ia were not the only ones, and further studies are required to ascertain their structures.

Downloaded At: 19:03 23 January 2011

Downloaded At: 19:03 23 January 2011

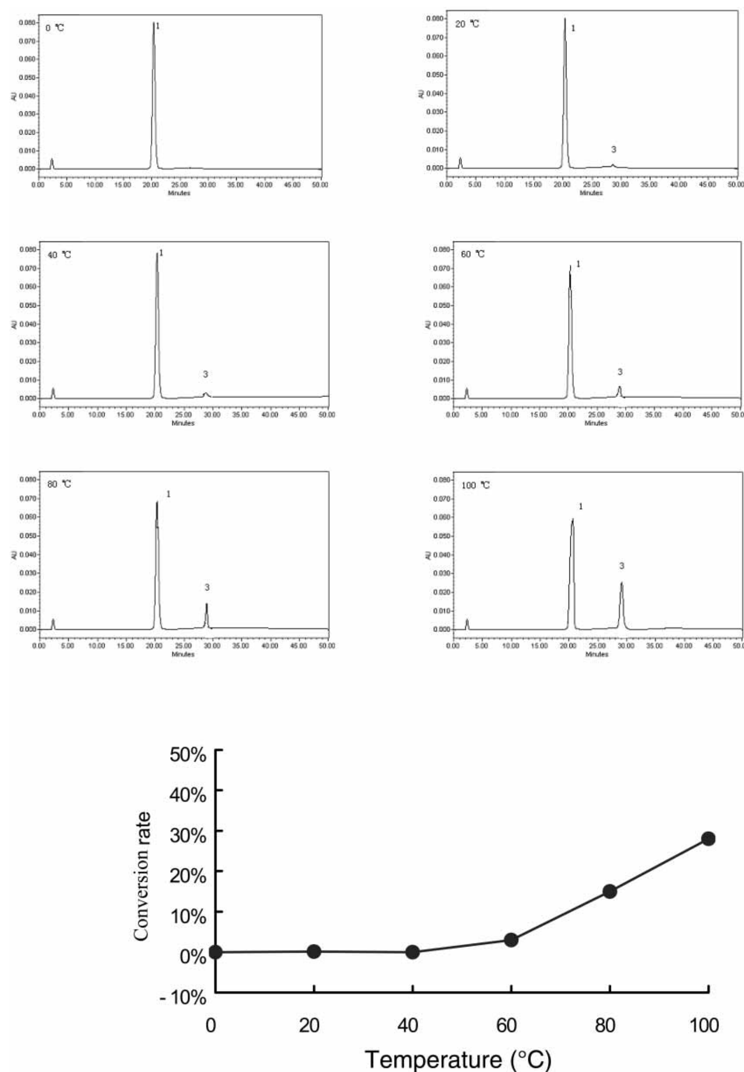


Figure 4. Specificity of the chromatographic method for the assay of escin Ia after the samples were treated 1 hr. under accelerated conditions (0, 20, 40, 60, 80, and 100°C) in water bath, and the correlation of temperature to conversion rate of escin Ia. 1: escin ia, 3: isoescin Ia.

CONCLUSION

Using Pre-HPLC, we were able to purify four isomeric saponins such as escin Ia, isoescin Ia, escin Ib, and isoescin Ib, which are the main bioactive

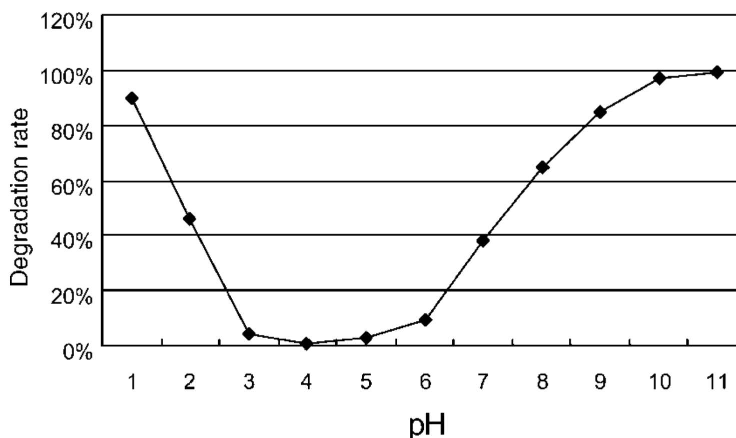


Figure 5. Influence of pH on the stability of escin Ia at 25°C.

Downloaded At: 19:03 23 January 2011

components from the seeds of *A. Chinensis*, with methanol–0.1% acetic acid aq. gradient solvent system as mobile phase. The purity of escin Ia, isoescin Ia, escin Ib, and isoescin Ib could be increased to 80% after only a one-step separation, and after three times of repeated chromatographical purification the purity of each component could reach 99%. The overall results indicate that Pre-HPLC is a fast and efficient technique to prepare pure bioactive isomeric saponins from the seeds of *A. Chinensis.* In addition, stability studies showed that escin Ia could be easily affected by heat exposure and extreme pH conditions, however, it is relatively stable when temperature is below 40°C, and pH value is in the range of 3–5. This is, therefore, very useful for the proper process and storage of escin containing products. However, the other factors such as light, oxygen, and humidity that may influence the stability of escin Ia need further investigation.

ACKNOWLEDGMENT

This research was supported by Ministry of Science and Technology of P.R. China, Grant No. 99-929-01-27.

REFERENCES

1. Bombardelli, E.; Morazorri, P. Fitoterapia **1996**, *L67*, 483.
2. Sda, H.; Li, Y.; Murakami, T.; Ninomiya, K.; Yoshikawa, M. Biol. Pharm. Bull. **1997**, *20*, 1092.

3. Yoshikawa, M.; Murakami, T.; Yamahara, Y.; Matsuda, H. Chem. Pharm. Bull. **1998**, *46*, 1764.
4. Yoshikawa, M.; Murakami, T.; Yamahara, J.; Matsuda, H.; Yamashina, J.; Murakami, N.; Kitagawa, I. Chem. Pharm. Bull. **1996**, *44*, 1454.
5. Wei, F.; Ma, S.C.; Ma, L.Y.; But, P.H.; Lin, R.C.; Khan, I.A. J. Nat. Prod. **2004**, *67*, 650.
6. Wei, F.; Ma, L.Y.; Ma, S.C.; Lin, R.C. Chinese Chem. Letts. **2002**, *13*, 59.
7. Griffini, A. Fitoterapia **1997**, *XVIII*, 520.
8. Pharmacopoeia Commission of P. R. C., *Pharamacopoeia of the People's Republic of China*. (Vol. I, English edition 1997); Chemical Industry Press: Beijing, 1997; 207.
9. Yang, X.W.; Zhao, J.; Cui, Y.Y.; Liu, X.H.; Chao, M.M.; Hattori, M.; Zhang, L.H. J. Nat. Prod. **1999**, *62*, 1510.
10. Cesare, R.S. Pharmacol. Res. **2001**, *44*, 183.
11. Matsuda, H.; Li, Y.; Murakami, T.; Ninomiya, K.; Yoshikawa, M. Biol. Pharm. Bull. **1997**, *20*, 1092.
12. Matsuda, H.; Li, Y.; Murakami, T.; Ninomiya, K.; Araki, N.; Yoshikawa, M.; Yamahara, J. Bioorg. Med. Chem. Lett. **1997**, *7*, 1611.
13. Konoshima, T. J. Nat. Prod. **1986**, *49*, 650.
14. Wei, F.; Ma, L.Y.; Jin, W.T.; Ma, S.C.; Han, G.Z.; Khan, I.A.; Lin, R.C. Chem. Pharm. Bull. **2004**, *in press*.
15. Pietta, P. J. Chromatogr. **1989**, *478*, 259.
16. Hubner, G.; Wray, V.; Nahrstedt, A. Planta Medica **1999**, *65*, 635.

Received October 19, 2004
Accepted November 22, 2004
Manuscript 6518

Downloaded At: 19:03 23 January 2011