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Short communication

HPLC analysis, semi-preparative HPLC preparation and identification of three impurities in salidroside bulk drug

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ABSTRACT

Salidroside is a bioactive compound mainly distributed in *Rhodiola* L. (Crassulaceae). It has been widely used in Chinese traditional medicine. In this paper, three impurities were found during the analysis of salidroside bulk drug. The enrichment of impurities was carried out by ODS column chromatography, using methanol–water (13:87, v/v) as eluent and the purification of impurities was achieved by semi-preparative HPLC, using methanol–water (11:89, v/v) as mobile phase, respectively. Three impurities were characterized as 4-(2-hydroxylethyl)-phenol-1-*O*-β-D-glucopyranoside, 4-hydroxyphenacyl-D-glucopyranoside and *p*-acetylphenyl-*O*-β-D-glucopyranoside by a variety of spectral data (IR, UV, MS, ^{1}H NMR, ^{13}C NMR, DEPT and 2D NMR). The simultaneous quantitative determination of salidroside and its impurities (Imp. **1**, **2** and **3**) was performed by reverse-phase HPLC method with UV detection. Specificity, linearity, sensitivity, precision and accuracy were evaluated.

1. Introduction

Salidroside (*p*-hydroxyphenylethyl-*O*-β-D-glucopyranoside), as a major bioactive phenolic glycoside of *Rhodiola* L., such as *R. rosea*, *R. sacra*, *R. crenulata*, *R. sexifolia*, etc., can preclude anoxia/reoxygenation damages upon myocardium [1], exhibits anti-neuronal apoptosis effect [2], displays antioxidant activity on lipid peroxidation [3], prevents cardiovascular disease [4], resists cancers [5], eliminates fatigue and postpones ageing [6–8]. Due to its high pharmacological values, salidroside is considered to be a drug candidate for the treatment of cardiovascular and cerebrovascular diseases.

Many analytical methods have been used to determine salidroside in herbs, pharmaceuticals and biological samples, e.g. high performance capillary zone electrophoresis (HPCE) [9], thin-layer chromatography (TLC) [10], high-performance liquid chromatography (HPLC) [11], colorimetric method [12], gas chromatography (GC) [13], high performance liquid chromatography–mass spectrometry (LC–MS) [14], and high performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) [15]. No method has been published for the simultaneous determination of salidroside and its impurities in bulk drug. The aim of this study was the identification of three impurities and their quantitative determination by HPLC together with salidroside.

2. Experimental

2.1. Materials and reagents

Salidroside bulk drug, which was isolated from *Rhodiola crenulata*, was provided by Sichuan Henxin Biomedical Co. Ltd. (Sichuan, China). Salidroside standard was supplied by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Impurity standards **1**, **2** and **3** were prepared in our laboratory by isolating them from salidroside bulk drug (purity > 98%). ODS-A (50 μm) was from YMC Co. Ltd. (Shimogyo-ku, Japan). Methanol (HPLC grade) was from Merck (Darmstadt, Germany). Deionized and purified water was prepared using a Synergy Purification System (Molsheim, France). All other reagents were of analytical grade.

2.2. Instruments and analytical conditions

Analytical HPLC was performed on a Shimadzu LC-10A system equipped with a UV detector (monitoring at 275 nm). The column was Shim-pack ODS (150 mm × 4.6 mm i.d., 5 μm; Shimadzu, Japan). Methanol–water (15:85, v/v) was used as mobile phase. Flow rate was 1.0 ml/min and the column temperature was 25 °C. The injection volume was 20 μl.

Semi-preparative HPLC was performed on a Shimadzu LC-6A system equipped with a UV detector (monitoring at 275 nm). The column was Phenomenex ODS (250 mm × 10.0 mm i.d., 8 μm; Phenomenex, USA). Methanol–water (11:89, v/v) was used as the mobile phase. Flow rate was 4.7 ml/min and

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the column temperature was 25 °C. The injection volume was 150 μl.

UV spectra were acquired on a Shimadzu UV 2450 spectrometer (200–400 nm) (Nakagyo-ku, Japan). IR spectra were recorded on a Bruker Vector 22 FT-IR spectrophotometer with KBr pellet (400–4000 cm^{-1}).

LC–MS data were obtained using a quadrupole mass spectrometer equipped with electrospray ionization (ESI) source coupled to an Agilent 1100 LC system (Palo Alto, CA, USA). The scanned m/z range was 105–1000 Da. High-resolution MS spectrum was acquired on a Mariner ESI-TOF mass spectrometer from Applied Biosystems (Foster city, CA, USA). Negative ion mode was employed.

NMR spectra were recorded on Bruker 500 MHz spectrometer (^1H, 500 MHz; ^{13}C, 125 MHz) using dimethyl sulfoxide (DMSO-d_6) as solvent and tetramethylsilane (TMS) as internal standard. The 2D NMR experiments, COSY, HSQC and HMBC were performed using standard Bruker pulse sequences.

2.3. Isolation and identification of three impurities

1 g of salidroside bulk drug was dissolved in 0.5 ml of methanol, and then subjected to chromatographic column (450 mm × 20.0 mm i.d.) which was packed with 50 g of ODS-A. The column was eluted by methanol–water (13:87, v/v) and three fractions (200 ml of fraction A, 150 ml of fraction B, and 300 ml of fraction C) were collected. Each fraction was monitored by analytical HPLC. The results showed that Impurities **1**, **2** and **3** were mainly present in fraction B. This fraction was evaporated, and then reconstituted in 0.5 ml of mobile phase to form test solution.

The above test solution was subjected to the semi-preparative HPLC system as described in Section 2.2 to get Imp. **1**, **2** and **3** (HPLC purity > 98%) which were then identified by IR, UV, MS, and NMR.

2.4. Preparation of stock and sample solution

Standard stock solution of salidroside at a concentration of 1 mg/ml was prepared by dissolving the appropriate amount of salidroside (50 mg) in 5 ml of mobile phase (methanol–water 15:85, v/v). The volume was completed to 50 ml with mobile phase.

The concentrations of the standard stock solutions of salidroside and Impurities **1**, **2** and **3** were 1 mg/ml in the mobile phase.

2.5. Validation study of analytical HPLC

2.5.1. Specificity

Specificity was tested by using the analytical HPLC method to analyze the salidroside bulk drug to demonstrate the capacity of the technique to distinguish salidroside from three impurities of the bulk drug.

2.5.2. Linearity

Calibration levels were prepared by diluting each standard stock solution with mobile phase (methanol–water 15:85, v/v) to six different concentrations (30, 125, 250, 500, 750 and 1000 μg/ml of salidroside; 1, 6, 12, 18, 24 and 30 μg/ml of Imp. **1**; 0.3, 1, 2, 3, 4 and 5 μg/ml of Imp. **2**; 0.1, 0.6, 1.2, 1.8, 2.4 and 3 μg/ml of Imp. **3**). For each compound, the external standard calibration curve (peak area vs. concentration) was generated using six data points.

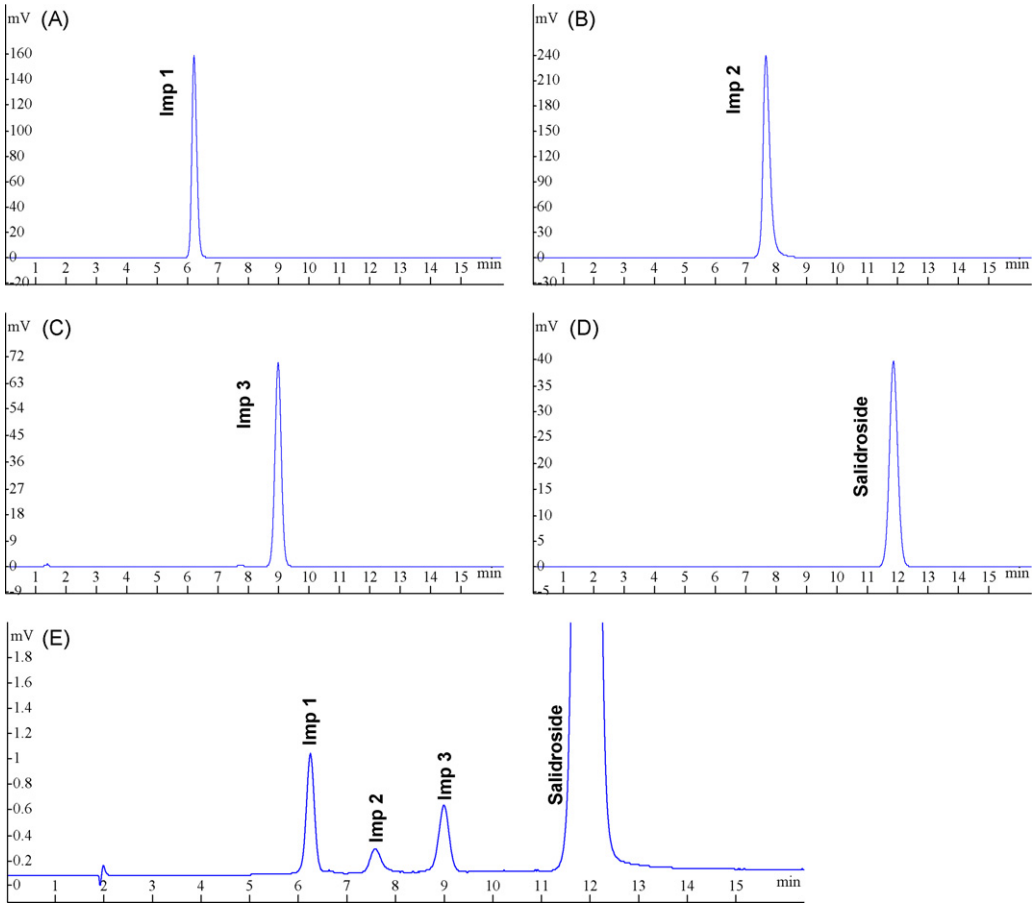


Fig. 1. Analytical HPLC chromatograms: (A) Imp. **1**, (B) Imp. **2**, (C) Imp. **3**, (D) salidroside and (E) salidroside bulk drug. Experimental conditions as in Section 2.2.

2.5.3. Limit of detection/quantitation

The LOD (S/N = 3) and LOQ (S/N = 10) of the method were determined by injections of each standard solution at the LOD and LOQ concentrations.

2.5.4. Precision

The precision was tested by performing intra- and inter-day multiple injections of each standard solution (250 μg/ml of salidroside, 30 μg/ml of Imp. **1**, 4 μg/ml of Imp. **2**, and 3 μg/ml of Imp. **3**), and then checking the %R.S.D. of retention times and peak areas. Ten injections were performed each day for three consecutive days.

2.5.5. Accuracy

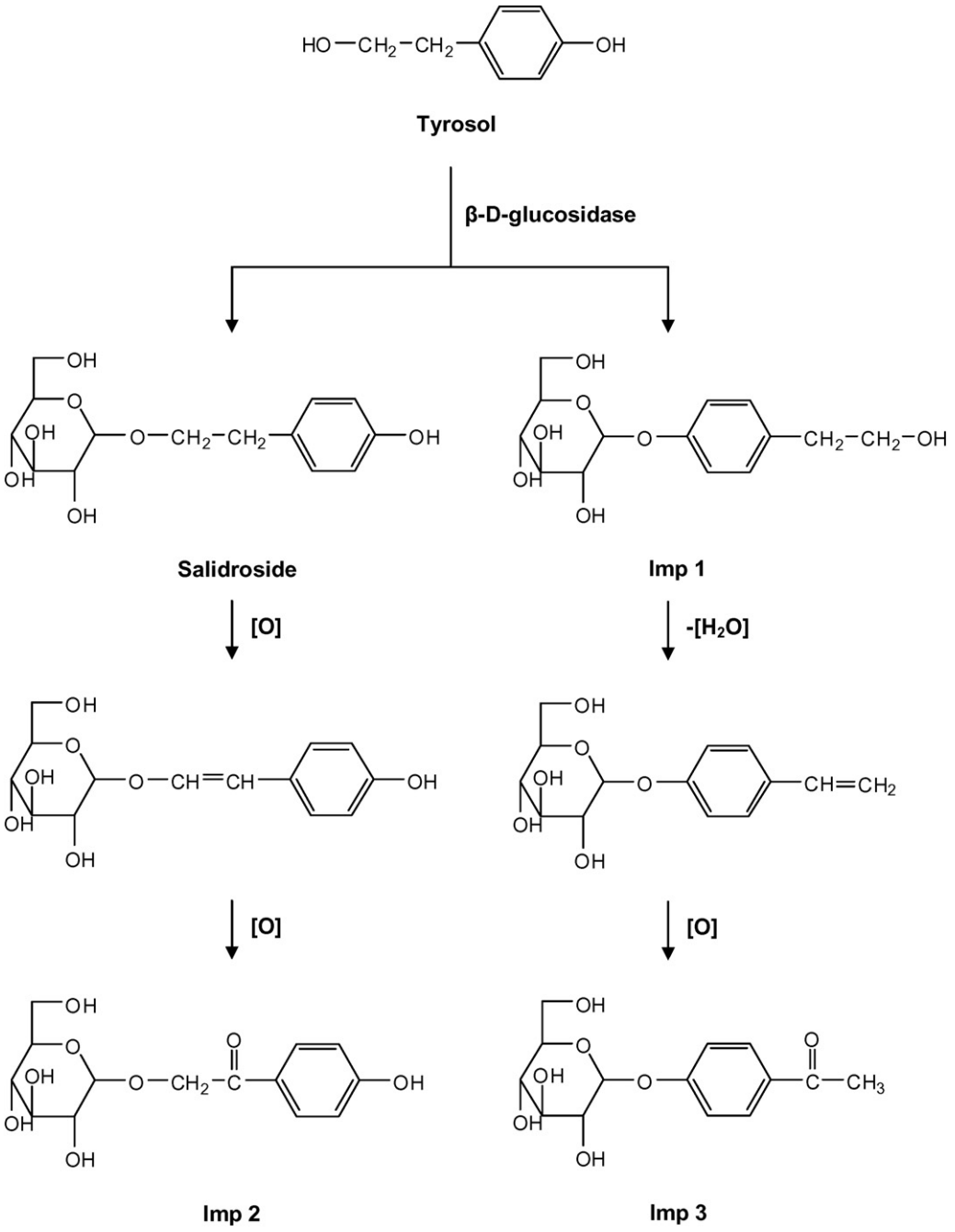
Accuracy of the methods was determined by the standard addition technique. Known amounts of reference standard compounds in a range of low, medium and high concentrations were added to preanalyzed sample of salidroside bulk drug and analyzed under the optimized conditions. Addition experiments for each concentration were performed in triplicate and the accuracy was calculated as the % of analyte recovered. Three analyses per concentration were performed and mean ± S.D. was determined.

2.5.6. Robustness

Robustness of the method was determined by introducing small changes in certain chromatographic parameters and expressed in terms of %R.S.D.

2.6. Analysis of salidroside bulk drug

The sample solution was prepared by diluting the sample stock solution with mobile phase to a nominal concentration of



Scheme 1. Proposed biosynthetic pathway of impurities.

Table 1
Statistical analysis for the calibration curves of impurities and salidroside[a].

Compound	Linearity range (mg/ml)	Slope (a)	Intercept (b)	r^2
Imp **1**	0.0010–0.0300	2×10^6 (±0.011)	−49.071 (±1.012)	0.9994
Imp **2**	0.0003–0.0050	5×10^6 (±0.003)	163.66 (±0.914)	0.9990
Imp **3**	0.0001–0.0030	2×10^7 (±0.007)	58.623 (±0.388)	0.9993
Salidroside	0.0300–1.0000	3×10^6 (±0.002)	14948 (±0.877)	0.9999

[a] For each curve the equation is $y = ax + b$, where y is the peak area, x is the concentration of the analyte (mg/ml), a is the slope, b is the intercept and r^2 is the correlation coefficient. Standard error (S.E.) values are given in parenthesis. The P value was <0.0001 for all calibration curves.

Table 2
Intra- and inter-day precision data for retention time (t_R) and peak area of impuries and salidroside.

Compound	Intra-day precision (n = 10, mean)						Inter-day precision (n = 30, mean)	
	Day 1		Day 2		Day 3		t_R (min)	R.S.D. (%)
	t_R (min)	R.S.D. (%)	t_R (min)	R.S.D. (%)	t_R (min)	R.S.D. (%)		
Imp. **1**	6.22	0.05	6.22	0.14	6.24	0.15	6.23	0.22
Imp. **2**	7.71	0.11	7.78	0.18	7.80	0.06	7.76	0.23
Imp. **3**	8.97	0.06	8.96	0.05	8.97	0.15	8.97	0.18
Salidroside	11.85	0.13	11.85	0.17	11.84	0.15	11.85	0.33
	Area (uv s)	R.S.D. (%)	Area (uv s)	R.S.D. (%)	Area (uv s)	R.S.D. (%)	Area (uv s)	R.S.D. (%)
Imp. **1**	67,873	0.83	67,891	0.79	67,911	0.67	67,892	1.19
Imp. **2**	22,375	1.17	22,300	1.21	22,401	0.98	22,359	1.95
Imp. **3**	68,172	0.66	68,272	0.89	68,133	0.73	68,192	1.04
Salidroside	7,75,342	0.34	7,75,288	0.51	7,75,987	0.22	7,75,539	0.47

250 μg/ml. Five batches of salidroside bulk drug were analyzed by analytical HPLC. The amounts of salidroside and impurities were calculated by comparing the peak areas with calibration curves.

2.7. Statistical analysis

Statistical analysis was performed with Microsoft Excel 2003 and Microcal origin 6.0.

3. Results and discussion

3.1. Analytical HPLC optimization

The HPLC system, previously used for the determination of salidroside in *R. crenulata* (C_{18} column, mobile phase: methanol–water 20:80, v/v) [16] was not suitable for separation of salidroside and its impurities. In the present study appropriate separation was obtained using Shim-pack ODS column (150 mm × 4.6 mm i.d., 5 μm) and methanol–water 15:85 (v/v) at a flow rate of 1.0 ml/min. On the basis of the UV spectra of the analytes 275 nm was selected as the analytical wavelength.

Using the system described above, the chromatogram of salidroside bulk drug is shown in Fig. 1E. It can be seen that a good separation was achieved within 15 min. The retention times for Impurities **1**, **2** and **3** and salidroside were 6.2, 7.7, 9.0 and 11.8 min, respectively.

3.2. Confirmation of three impurities

Impurity **1** (80 mg): white, amorphous powder; UV (H_2O) λ_{max} (log ε) 219 (4.19), 270 (3.28) nm; IRν_{max} (KBr) 3404, 3030, 1613, 1514, 1234, 1088, 1045, 827 cm^{-1}; ^1H NMR, ^{13}C NMR, DEPT and 2D NMR data (see supplementary material); ESI-MS m/z 299.1 $[M-H]^-$; *anal.* C 55.84%, H 6.68%, calcd for $C_{14}H_{20}O_7$, C 55.99%, H 6.71%. The spectral data are consistent with icariside D_2 data [17].

Impurity **2** (20 mg): white, amorphous powder; UV (H_2O) λ_{max} (log ε) 219 (4.94), 280 nm; IRν_{max} (KBr) 3386, 2917, 1677, 1604, 1517, 1444, 1406, 1382, 1246, 1075, 1040, 838 cm^{-1}; ^1H NMR, ^{13}C NMR, DEPT and 2D NMR data (see supplementary material); HR-ESI–MS m/z 313.09132 $[M-H]^-$, calcd for $C_{14}H_{17}O_8$, 313.09179. The ^1H, ^{13}C NMR spectra data are in agreement with a compound described in Ref. [18].

Impurity **3** (50 mg): white, amorphous powder; UV (H_2O) λ_{max} (log ε) 265 (3.67) nm; IRν_{max} (KBr) 3364, 3061, 2969, 1661, 1604, 1580, 1511, 1425, 1390, 1366, 1247, 1088, 1037, 841 cm^{-1}; ^1H NMR, ^{13}C NMR, DEPT and 2D NMR data (see supplementary material); HR-ESI–MS m/z 297.09151 $[M-H]^-$, calcd for $C_{14}H_{17}O_7$, 297.09688. The ^{13}C NMR spectral data agree with picein data [19].

3.3. Biosynthetic pathway of impurities

Impurities **1**, **2**, and **3** were firstly reported in salidroside bulk drug, which was isolated from *Rhodiola crenulata*. They have similar molecular structures with salidroside as shown in Scheme 1. As previously suggested, tyrosol, the aglycon of salidroside, is biosynthesized through the relatively well-characterized shikimic acid pathway. A molecule of glucose is then transferred by the β-D-glucosidase (or possibly by the UDP-glucosyltransferase) to the hydroxyethyl group of tyrosol to form salidroside [20]. The proposed biosynthetic pathway of the impurities is depicted in Scheme 1. The two methylenes in salidroside is dehydrogenated to form a double bond and then oxidized to form Imp. **2**. A molecule of glucose may be transferred by the β-D-glucosidase to the phenolic hydroxyl group of tyrosol to form Imp. **1**. Then Imp. **1** (icariside D_2) is dehydrated and oxidized to form Imp. **3** (picein).

Table 3
Accuracy of impurities and salidroside.

Excess of analyte (%)	Recovery (%)			
	Imp. **1**	Imp **2**	Imp. **3**	Salidroside
80	99.8	99.2	99.5	100.2
100	100.1	98.7	98.9	99.9
120	99.4	99.0	98.3	99.6

Table 4
Results obtained by analytical HPLC analysis of salidroside bulk drug.

Batch no.	Salidroside		Imp. **1**		Imp. **2**		Imp. **3**	
	Content (%)	R.S.D. (%)	Content (%)	R.S.D. (%)	Content (%)	R.S.D. (%)	Content (%)	R.S.D. (%)
1	96.96	0.54	1.47	0.79	0.43	1.92	1.12	0.89
2	96.84	0.23	1.47	0.64	0.43	1.74	1.13	0.83
3	96.90	0.28	1.46	0.67	0.43	1.83	1.13	0.72
4	97.00	0.51	1.46	1.01	0.43	1.95	1.12	1.02
5	96.93	0.21	1.47	0.98	0.43	1.88	1.13	0.99

3.4. Evaluation of validation data

3.4.1. Specificity

All peaks in Fig. 1, are adequately resolved. The retention times of all the isolated impurities are in good agreement with those of impurities in the bulk drug. No significant interference was observed at the retention times of salidroside and its impurities.

3.4.2. Linearity

For each target compounds, linear regression analysis was performed by the external standard method. The validation parameters of each calibration curve (slope (a), intercept (b), standard error of slope, standard error of intercept and correlation coefficient (r^2)) are shown in Table 1. A linear response for the peak areas versus concentrations over the range tested was observed for both salidroside and its impurities with a correlation coefficient of 0.999 or better.

3.4.3. Limit of detection/quantitation

The LOD of salidroside, Impurity **1**, **2** and **3** were 100, 50, 10 and 10 ng/ml, respectively, while the LOQs were 10,000, 300, 100, 80 ng/ml, respectively.

3.4.4. Precision

Detailed results of the precision are shown in Table 2, which lists the intra- and inter-day %R.S.D. values of retention times and peak areas. The values of intra- and inter-day %R.S.D. for both retention times and peak areas are not more than 2%.

3.4.5. Accuracy

The accuracy of the methods, assessed as % recovery for low, medium and high concentrations of salidroside and impurities are reported in Table 3. The recovery rates obtained were close to 100%. Based on the results of the recovery test, this method was accurate.

3.4.6. Robustness

For the robustness of analytical HPLC method, variations in flow rate of mobile phase (±10%), column temperature (±2 °C) and detection wavelength (±2 nm) and their dependence on retention times and peak areas were evaluated. %R.S.D. were ≤1.5 and they were within the limit required for HPLC analysis.

The validation data highlighted the suitability of the proposed HPLC method for the simultaneous analysis of salidroside and its impurities.

3.5. Analysis of salidroside bulk drug

The optimized method was applied to the analysis of salidroside bulk drug. Data are reported in Table 4. The results indicated that the proposed HPLC method can be used to monitor the quality of the bulk drug. The chromatographic conditions allow the simultaneous analysis of salidroside and impurities within a short analysis time, without the ion-pair reagents and gradient elution, which were traditionally used.

4. Conclusion

A simple and straightforward HPLC method with UV detection has been successfully developed to determine salidroside and its impurities in salidroside bulk drug. In this work, three impurities in salidroside bulk drug were identified as icariside D_2, 4-hydroxyphenacyl-D-glucopyranoside and picein, respectively, the analogues of salidroside from *R. crenulata*. A possible biosynthetic pathway of the three impurities was proposed and will likely contribute to the prediction of their toxicity. Specificity, linearity, sensitivity, precision and accuracy were discussed. The method should be applicable with minor modification for drug quality control and assurance in future investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2008.12.025.

References

[1] Y.C. Ye, Q.M. Chen, K.P. Jin, S.X. Zhou, F.L. Chai, P. Hai, Acta Pharmacol. Sin. 14 (1993) 424–426.
[2] W.S. Zhang, L.Q. Zhu, F.L. Niu, R.C. Deng, C.X. Ma, Chin. J. Chin. Mater. Med. 29 (2004) 459–462.
[3] Y. Zhang, Y.G. Liu, J. Chin. Med. Mater. 28 (2005) 794–796.
[4] A. Bonanome, A. Pagnan, D. Caruso, A. Toia, A. Xamin, E. Fedeli, Nutr. Metab. Cardiovasc. Dis. 10 (2000) 111–120.
[5] S.N. Udintsev, V.P. Schakhov, Eur. J. Cancer 27 (1991) 1182.
[6] P.T. Linh, Y.H. Kim, S.P. Hong, J.J. Jian, J.S. Kang, Arch. Pharm. Res. 23 (2000) 349–352.
[7] M.W. Lee, Y.A. Lee, H.M. Park, S.H. Toh, E.J. Lee, H.D. Jang, Arch. Pharm. Res. 23 (2000) 455–458.
[8] V. Darbinyan, A. Kteyan, A. Panossian, E. Gabrielian, G. Wikman, H. Wagner, Phytomedicine 7 (2000) 365–371.
[9] H.L. Wang, Y.F. Ming, Y.L. Li, Anal. Lab. 24 (2005) 40–41.
[10] X.Y. Xu, B.M. Li, L.Y. He, Chin. J. Pharm. Anal. 16 (1996) 272–273.
[11] H. Zhao, L.Y. Jia, C.S. Sun, J. Shengyang Pharm. Univ. 22 (2005) 23–25.
[12] C.G. Deng, D.J. Sun, N.S. Du, Chin. Tradit. Pat. Med. 17 (1995) 36–37.
[13] S.L. Kang, J. Wang, J. Zhang, Chin. J. Chin. Mater. Med. 23 (1998) 365–366.
[14] A. Tolonen, A. Hohtola, J. Jalonen, J. Mass Spectrom. 38 (2003) 845–853.
[15] Y.W. Chang, H.T. Yao, S.H. Hsieh, T.J. Lub, T.K. Yeh, J. Chromatogr. B 857 (2007) 164–169.
[16] Y. Mao, Y. Li, N. Yao, J. Pharm. Biomed. Anal. 45 (2007) 510–515.
[17] T. Miyase, A. Ueno, N. Takizawa, Phytochemistry 28 (1989) 3484–3485.
[18] H. Pan, L.N. Lundgren, Phytochemistry 39 (1995) 1423–1428.
[19] A.D. Roger, V.H. Lucia, J.V. Arnold, Phytochemistry 25 (1986) 1201–1204.
[20] L.L. Shi, L. Wang, Y.X. Zhang, Y.J. Liu, Chin. Bull. Life Sci. 20 (2008) 287–290.