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Short communication

Profiling and quantification of isoflavone-C-glycosides impurities in puerarin injection by liquid chromatography coupled to ESI-ion trap mass spectrometry

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ABSTRACT

An HPLC/DAD/MS^n method was established for the qualitative and quantitative analysis of the impurities in puerarin injection (PI), a widely used drug in China. The analytical HPLC was performed on an Agela RP-C18 column using 0.1% aqueous formic acid (v:v) and methanol as mobile phase. A total of nine impurities were detected and eight of them were identified as isoflavone-C-glycosides basing on their UV spectra and MS^n spectra and comparing with the literature data. An HPLC method for the assay of two common impurities in the commercial PI samples, i.e., neopuerarin A and neopuerarin B, was then established. The validation of the method, including sensitivity, linearity, precision, accuracy, was carried out. The calibration curves showed good linearity of $R^2 > 0.9999$ and LOQ (S/N = 10) were less than 3.73 ng. The precision was evaluated by intra- and inter-day assays and R.S.D. values were less than 0.94%. The average recovery rates were 97.0% and 99.5%, respectively, with R.S.D. less than 1.38%. The contents of neopuerarin A and neopuerarin B in various commercial brands of PI samples varied over the range of 0.30–1.16% and 0.42–1.66%, respectively. This is the first report on the impurities in PI.

1. Introduction

Puerarin injection (PI) is the sterilized 50% propanediol aqueous solution of puerarin (8-β-D-glucopyranosyl-7 and 4′-dihydroxyisoflavone) extracted from the Chinese herb *Pueraria lobata* (Wild.) Ohwi. It has been widely used for the treatment of coronary heart disease and angina pectoris in China. In terms of the Ch.P. regulation, the content of puerarin in PI should be within the range 90–110% to the labeled content [1]. When clinically used, the PI corresponding to 200–400 mg of puerarin is diluted with 5% glucose injection before intravenous infusion.

In recent years, the ADR (Adverse Drug Reaction) caused by PI has been rapidly increasing in terms of the reports from China state food and drug administration [2–4]. The clinical statistical investigation further confirmed the relationship between the PI and its ADR cases, e.g. acute intravascular hemolysis [5]. In this case, it is very necessary to understand the chemical composition in PI, i.e., the impurities composition, for finding out the allergen and to improve the quality control of PI. However, up to now, few chemical studies on the impurities in PI were reported. Generally, the impurities in PI are believed to be mainly the isoflavonoids imported during the extraction process of crude herb *P. lobata*, which is a rich source of isoflavone [6–8]. The structures of some isoflavones in *P. lobata* are illustrated in Fig. 1.

The purpose of this study is to develop a method using HPLC coupled to ion trap mass spectrometry for profiling the impurities in commercial brands of PI and to establish a quantitative method for the assay of their common impurities. It is the first report on the impurities in PI.

2. Experimental

2.1. Chemicals and reagents

PI samples were kindly supplied by manufacturer A (product No. 070304), and purchased from three manufacturers, namely, B (product No. 07030101), C (product No. 060307), D (product No. 070501), E (product No. 060101). All of PI samples were labeled with puerarin content of 100 mg: 2 mL. The commercial reference samples of puerarin and daidzin was kindly gifted from Jinfeng Pharmaceutics Co. Ltd., Shandong Province, and Dr. Yang Xuedong, Tianjin University. The reference sample of neopuerarin A and neopuerarin B were prepared in our lab with the purities higher than 95% measured by HPLC with area normalization method.

Methanol for HPLC analysis was of HPLC grade from Caledon (Canada). Formic acid was of analytical grade from Yuanli Chemical Plant (Tianjin, China). Water for sample preparation and HPLC analysis was purified by a Milli-Q academic water purification system (USA).

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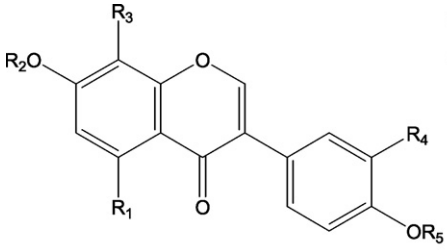

Structures of some isoflavones in the root of *Pueraria lobata*

Glc[a]: β-pyranoglucose
Glc[b]: α-pyranoglucose
Glc[c]: α-furanoglucose
Glc[d]: β-furanoglucose

	R_1	R_2	R_3	R_4	R_5
Daidzein	H	H	H	H	H
Daidzin	H	Glc[a]	H	H	H
Formononetin	H	H	H	H	CH_3
Genistein	OH	H	H	H	H
Genistin	OH	Glc[a]	H	H	H
Genistein 8-C-glucoside	OH	H	Glc[a]	H	H
Puerarin	H	H	Glc[a]	H	H
PG-1	H	H	Glc[a]	OH	H
PG-3	H	H	Glc[a]	OCH_3	H
Neopuerarin	H	H	Glc[b]	H	H
Neopuerarin A	H	H	Glc[c]	H	H
Neopuerarin B	H	H	Glc[d]	H	H

Fig. 1. Structures of some isoflavones in the root of *Pueraria lobata*.

2.2. HPLC analysis

An Agilent 1200 series HPLC system including a G1311A quat-pump, G1329A autosampler, G1316A column oven, G1315D diode array detector and an Agilent Chem-Station were used for HPLC analysis. The UV spectra were recorded in the scale from 190 nm to 400 nm and the effluent was monitored at 250 nm. Chromatographic separation was achieved on a Agela Venusil XBP-C18 column (4.6 mm × 250 mm, 5 μm) with Agilent C18 pre-column (4.6 mm × 12.5 mm, 5 μm) at a flow rate of 1.0 mL/min. The temperature was set at 30 °C.

The mobile phase consisted of Solvent A (0.1% aqueous formic acid, v/v) and Solvent B (CH_3OH). Gradient programs for HPLC/MS analysis were performed as follows: A:B (90:10, v/v)–A:B (68:32, v/v) at 0–12 min, A:B (68:32, v/v) isocratic for 5 min, A:B (68:32, v/v)–A:B (62:38, v/v) at 17–27 min, A:B (62:38, v/v) isocratic for 10 min. An isocratic elution with A–B = 65:35 (v/v) was used for HPLC assay.

Each PI samples of 0.5 mL (sample E for 0.25 mL) were accurately measured and diluted with water into 10 mL using volumetric flask. The diluted solutions were filtered through 0.45 μm membrane and were subjected to HPLC/MS analysis with injection of 40 μL and for HPLC assay with injection of 20 μL.

2.3. HPLC/mass analysis

The HPLC system described in Section 2.2 was online coupled to an Agilent 6310 mass spectrometer equipped with ESI interface and ion trap analyzer. Instrument control and data acquisition were performed using 6000 series Trapcontrol Version 6.1 software. The MS spectra were acquired in negative ion mode. High purity nitrogen (N_2) was used as both drying gas with a flow rate of 12 L/min and as nebulizing gas with a pressure of 60 psi. Ultrahigh pure helium (He) was used as the collision gas. The dry temperature was set at 350 °C and the capillary voltage was set at 3500 V. The mass spectra were recorded in the scale from 100 to 1000 *m/z*. MS^n data were acquired in the Auto MS^n mode. All the data were processed using DataAnalysis for 6300 series Ion Trap LC/MS Version 3.4 software.

2.4. Besson's isomerization of neopuerarin B

Neopuerarin B (1 mg) was dissolved in 4 mL of MeOH–4 M HCl (1:1) and was refluxed for 1 h. The solution was neutralized with 2 M NaOH and then extracted with *n*-BuOH for three times. The *n*-BuOH layer was combined and concentrated in vacuum. The residue was dissolved with 2 mL MeOH and then subjected to HPLC/MS analysis.

2.5. Method validation

The linearity calibration curves were made on seven experiments of the mixed reference compound ($n = 3$). The regression equation was calculated in the form of $Y = AX + B$, where Y and X were the area of peak and sample amount (mg), respectively. The precision was evaluated by the intra- and inter-day ($n = 3$) assays.

Two and half milliliters of the diluted PI sample A was spiked with 1.2 mL, 1.5 mL and 1.8 mL of the mixture reference samples of neopuerarin A (0.0181 mg/mL) and neopuerarin B (0.0225 mg/mL), respectively, each in triplicates and then diluted to 5 mL using volumetric flask. The solutions were subjected for the measurement of the recovery rate ($n = 3$).

3. Results and discussion

3.1. Optimization of chromatographic condition for HPLC/MS analysis

The chromatographic conditions of gradient program and mobile phase for the identification of the impurities in PI samples were optimized in order to acquire overall impurities peaks with good resolution within a short analysis time. Subsequently, the MS conditions were optimized in order to acquire the extensive information of the impurities. The optimized conditions were described in Sections 2.2 and 2.3. Puerarin and nine impurities were detected and the baseline separation of the major impurities was obtained.

3.2. Fragmentation analysis of isoflavone-C-glycoside and O-glycoside

According to the previous studies on the isoflavone glycosides in *P. lobata* [6–8], the glucosyl group is generally substituted on the 7/4′ position of aglycone for O-glycoside, while on the 8 position of aglycones for C-glycoside. They could be differentiated by MS/MS analysis due to their fragmentation pathways (seen in Fig. 2) [9–10]. The neutral loss of 120 amu from the deprotonated ion in negative mode is believed to be indicative of C-glycoside [9–11]. In this study, the isomeric compounds of puerarin and daidzin were selected as the representatives of isoflavone-C-glycosides and isoflavone-O-glycosides in *P. lobata*, respectively, and their fragmentation

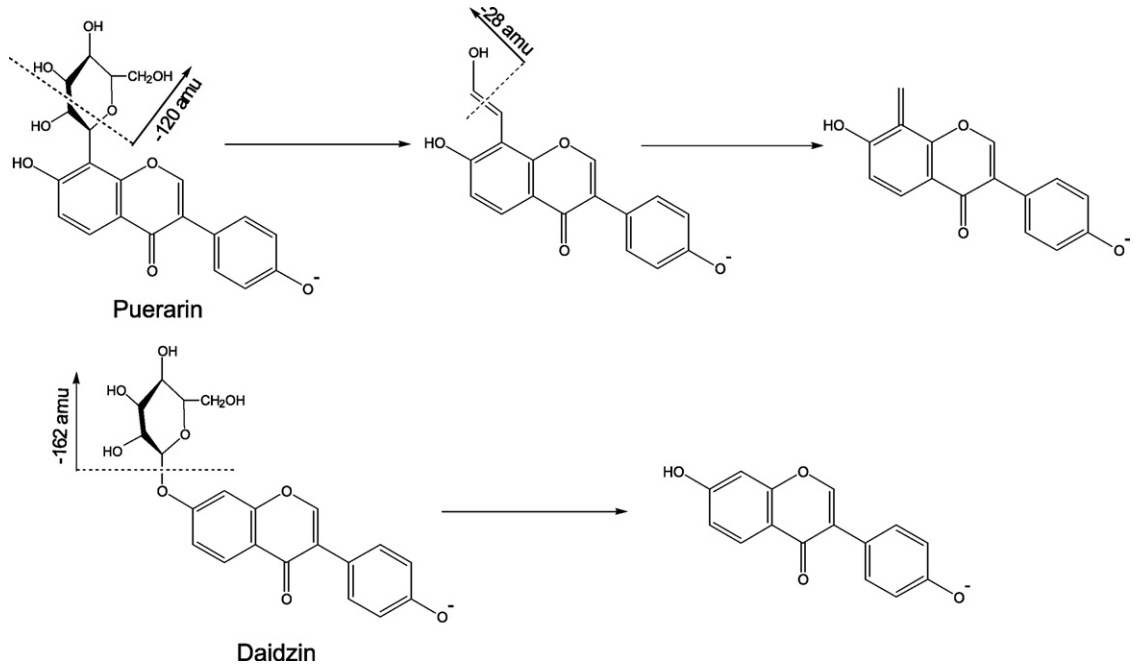

Fig. 2. Proposed fragmentation pathway of puerarin and daidzin.

pathways were studied before characterizing the impurities in PI using MS spectra. The result is consistent with the literatures.

3.3. HPLC/MS analysis and identification

The representative HPLC/MS chromatograms for PI samples were shown in Fig. 3. A total of nine impurities were detected by DAD and eight of them were characterized by MS. All of the impurities exhibited the neutral loss of 120 amu from the deprotonated molecular ion in MS^n spectra. They were therefore proposed to be the C-glycosides. Based on mass spectral and UV spectral data and comparing with the literature data [6–10,12], these impurities were identified as seen in Table 1.

For peak 1, only MS^2 spectra were acquired in this study. The $[M-H]^-$ ion at the *m/z* of 435 dissociated into product ions at the *m/z* of 315, 297, 195, 177 and 133, which were ascribed to $[M-H-120]^-$, $[M-H-120-H_2O]^-$, $[M-H-120-120]^-$, $[M-H-120-120-H_2O]^-$ and $[M-H-120-120-H_2O-CO_2]^-$, respectively. It suggested that this compound probably is a C-glycoside. However, its fragmentation behavior and the UV spectra data are significantly different from that of puerarin. The structure of this compound was not identified in this study. To our knowledge, this compound has not been reported before.

The impurities for peaks 2 and 6 were the isomeric compounds with the similar MS^n spectra, in which the consecutive neutral losses of 120 amu and 28 amu from $[M-H]^-$ of *m/z* 431 were observed. But they showed different UV spectra (seen in Fig. 3). Comparing with the literature data [6,7], peak 6 was identified as Genistein 8-C-glycoside and peak 2 was identified as PG-1.

From the MS^n spectra of peak 4, the fragmentation of $[M-H]^-$ ion at *m/z* 445 gave product ions at *m/z* of 415 $[M-H-CH_2O]^-$, 325 $[M-H-120]^-$, 310 $[M-H-120-CH_3]^-$, 297 $[M-H-120-CO]^-$, 282 $[M-H-120-CH_3-CO]^-$. It indicated a $-OCH_3$ group in the aglycone skeleton. Therefore, peak 4 was identified as PG-3 by comparing with the literature [6,7].

For peak 9, its fragmentation behavior was similar with that of puerarin. It was therefore proposed to be methylated puerarin as previous reported, the neutral loss of CH_3 amu derived from

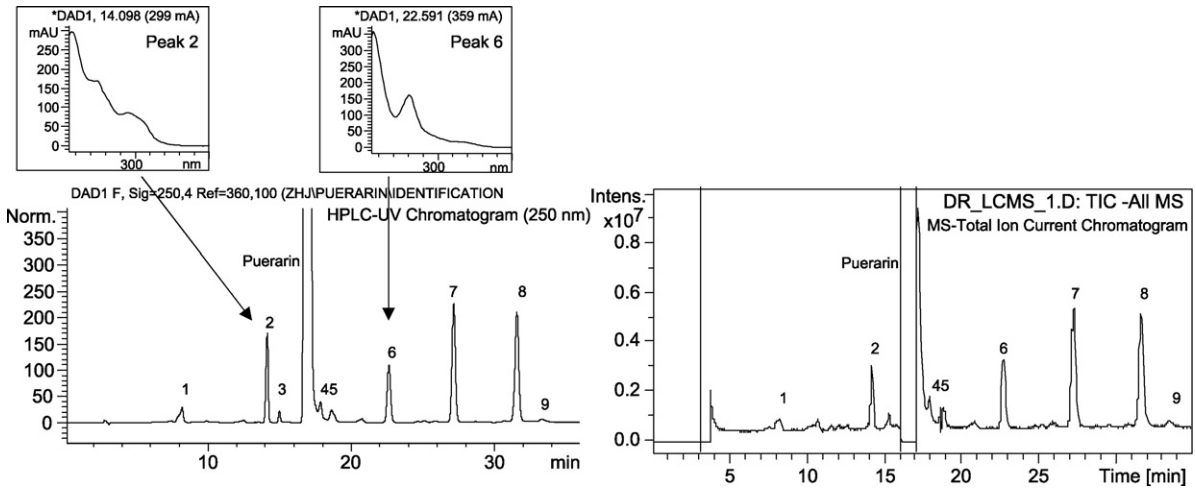

Fig. 3. The representative HPLC–UV and MS–TIC chromatogram of PI (sample B).

Table 1
Peak assignments in the HPLC–MS^n analysis of PI samples.

Peak no.	t_R (min)	$[M-H]^-$	Fragment ion m/z (MS^n spectra)	λ_{max} (nm)	Identity	A	B	C	D	E
Puerarin	16.63	415	MS^2[415]: 295, MS^3[415 → 295]: 267	250, 306	Puerarin	+	+	+	+	+
1	8.09	435	MS^2[435]: 315, 297, 195, 177, 133	260	Unknown C-glycoside compound	+	+	–	+	–
2	13.97	431	MS^2[431]: 311, 283, MS^3[431 → 311]: 283	250, 290	PG-1	–	+	+	–	+
3	14.88	–	–	–	Not identified	+	+	+	+	–
4	17.65	445	MS^2[445]: 415, 325, 310, 297, 282, MS^3[445 → 325]: 310, 297, 282	–	PG-3	–	+	–	+	+
5	18.44	415	MS^2[415]: 295, 267, MS^3[415 → 295]: 267	–	Neopuerarin	+	+	+	+	+
6	22.35	431	MS^2[431]: 311, 283, MS^3[431 → 311]: 283	260	Genistein 8-C-glycoside	+	+	+	–	+
7	26.80	415	MS^2[415]: 295, 267, MS^3[415 → 295]: 267	250, 306	Neopuerarin A	+	+	+	+	+
8	31.21	415	MS^2[415]: 295, 267, MS^3[415 → 295]: 267	250, 306	Neopuerarin B	+	+	+	+	+
9	33.08	429	MS^2[429]: 309, 281, MS^3[429 → 309]: 281	–	3′-Methylated puerarin	+	+	–	–	+

Notes: +: detected and –: not detetcted.

the O–CH_3 cleavage is a characteristic fragmentation in methoxylated flavonoids [13] and have been observed in many methoxyl isoflavonoids, such as formononetin, 3′-methoxydaidzein, 3′-methoxyformononetin, glycitein, biochanin [9–10,12,14] and also for peak 4 in present study. However, it was absent for peak 9. Thus, peak 9 was deduced not containing methoxyl group in the aglycone and was tentatively identified as 3′-methylated puerarin.

Peaks of 5, 7 and 8 were the common impurities in PI samples, especially for peaks 7 and 8, which appeared as the main impurities. All of them exhibited the neutral loss of 120 amu and were proposed to be C-glycosides isomers of puerain. Such compounds have not been reported yet. The compounds for peaks 7 and 8 were then isolated in our lab and their structures were elucidated as 8-C-β-glucofuranosyl-7, 4′-dihydroxyiso flavone and 8-C-α-glucofuranosyl-7, 4′-dihydroxyisoflavone by spectral analysis, named neopuerarin B and neopuerarin A, respectively [15]. Neopuerarin B was hydrolyzed using Besson's isomerization method [16,17] described in Section 2.4. The HPLC/MS analysis of the hydrolyzed products using conditions described in Section 2.2 showed that the isomerization of neopuerarin B yielded puerarin, neopuerarin A and the compound for peak 5 (seen in Fig. 4). Thus, peak 5 is identified as puerarin 8-C-α-glucopyranoside, named neopuerarin.

Peak 3 appeared in very low-level content from the UV chromatogram detected at 250 nm. Due to the lack of UV spectra and MS data, it is not identified in this study.

3.4. Method validation

On the basis of chromatographic condition for HPLC/MS analysis, an isocratic HPLC method as described in Section 2.2 was established for the assay of neopurarin A and neopuerarin B in PI samples. The representative chromatogram was shown in Fig. 5.

As described in Section 2.5, the method validation was performed. Good linearity with $R^2 > 0.9999$ were achieved for neopuerarin A and neopuerarin B within the content range of 0.40–31.80 μg/mL and 0.50–39.60 μg/mL, respectively. The limits of quantitation (LOQ) were 3.73 ng and 3.30 ng, respectively. The R.S.D. was less than 0.66% for intra-day assays and 0.94% for inter-day assays, respectively. The average recovery rates were 97.0% and 99.5%, respectively. The results are listed in Tables 2–4.

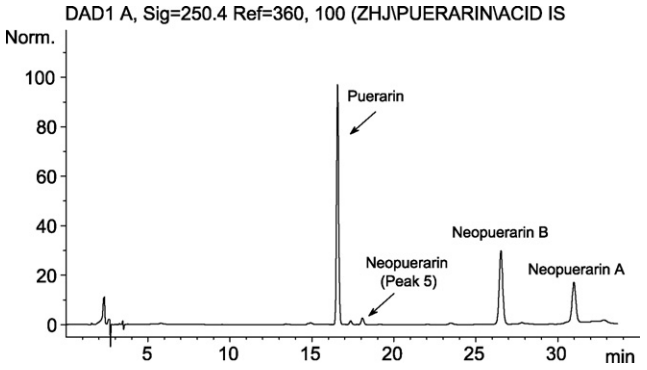

Fig. 4. HPLC–UV chromatograms at 250 nm of isomerized neopuerarin B.

3.5. Sample analysis

The isocratic HPLC method was applied to the assay of neopuerarin A and neopuerarin B in five commercial brands of PI samples. The contents for neopuerarin A and neopuerarin B were calculated and the results are listed in Table 5. The mass fractions of neopuerarin A and neopuerarin B in PI samples were calculated within the range of 0.30–1.16% and 0.42–1.66%, respectively. It indicated that the biological safety evaluation of these impurities is very necessary to insure the clinical safety. Moreover, an obvious difference of impurities contents among these five brands of PI samples could be seen. For PI sample D, the content of neopuerarin A and neopuerarin B were 1.16% and 1.66%, respectively,

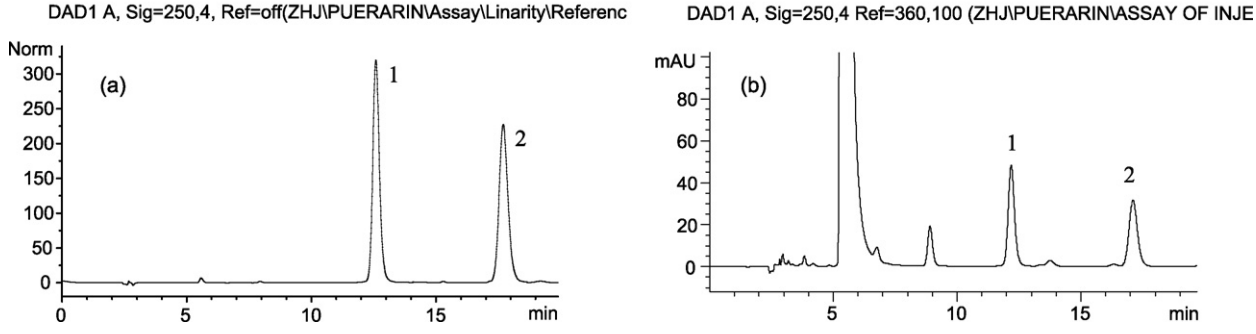

Fig. 5. HPLC–UV chromatograms at 250 nm of (a) reference samples and (b) PI sample A.

Table 2
Linearity calibration curve factors and LOD of neopuerain A and neopuerain B.

Compound	Slope (A/10)	Intercept (B)	R^2	Linear range (μg/mL)	LOQ (ng)
Neopuerain A	8.4036	3.7682	0.9999	0.40–31.80	3.73
Neopuerain B	7.0829	0.2951	1.0000	0.50–39.60	3.30

Table 3
Intra- and inter-day precisions in the assay of neopuerain A and neopuerain B.

Compound	Intra-day (n = 9)		Inter-day (n = 9)	
	Calculated amout (μg)	R.S.D. (%)	Calculated amout (μg)	R.S.D. (%)
Neopuerain A	0.247 ± 0.002	0.66	0.246 ± 0.001	0.59
Neopuerain B	0.183 ± 0.001	0.56	0.182 ± 0.002	0.94

Table 4
Recoveries of neopuerain A and neopuerain B.

Compound	Added amount (ng)	Calculated amount (ng)	Recovery (%)	R.S.D. (%)
Neopuerain A	21.6	21.0 ± 0.4	97.2	2.04
	27.0	26.3 ± 0.1	97.4	0.54
	32.4	31.2 ± 0.3	96.3	0.66
Neopuerain B	27.0	27.0 ± 0.6	100.0	2.05
	33.8	33.8 ± 0.3	100.0	0.82
	40.5	39.9 ± 0.2	98.4	0.44

Table 5
Contents of neopuerain A and neopuerain B in different brands of PI samples.

PI sample	Content (mg/mL)		Mass fraction (%)	
	Neopuerarin A	Neopuerarin B	Neopuerarin A	Neopuerarin B
A	0.18	0.25	0.36	0.50
B	0.22	0.25	0.44	0.50
C	0.15	0.21	0.30	0.42
D	0.17	0.22	0.34	0.44
E	0.58	0.83	1.16	1.66

much higher than that for other PI samples. As a result, it is necessary to improve the quality control of PI by limiting the impurities level.

4. Conclusion

This paper described an HPLC/MS method for profiling and quantitative analysis of the impurities in puerarin injection. A total of nine impurities were detected and eight of them were identified as isoflavone-C-glycosides based on their MS^n spectra and UV data. An isocratic HPLC method was then established for assay of the main and common impurities in various commercial brands of PI. The method presented a good sensitivity, repeatability and accuracy. The assay results indicated an obvious difference of impurities contents among various commercial brands of PI.

This study is the first report on the impurities in puerarin injection. The result would provide the chemical support for the further biological safety evaluation of the impurities in PI and for the improvement of quality control of PI.

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