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Journal of Pharmaceutical and Biomedical Analysis
35 (2004) 1083–1091
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Ruggedness/robustness evaluation and system suitability test on United States Pharmacopoeia XXVI assay ginsenosides in Asian and American ginseng by high-performance liquid chromatography

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Received 29 January 2004; received in revised form 7 April 2004; accepted 10 April 2004

Available online 19 June 2004

Abstract

The work of the ruggedness/robustness evaluation and system suitability tests was oriented to profound understand the practicability of using assay methods issued by United States Pharmacopoeia (USP XXVI and XXVII) for ginsenosides in Asian ginseng and American ginseng. The items chosen for the method validation included quantitative related items such as recovery of Rg_1 and Rb_1, respectively, and qualitative related items such as resolution, theoretical plate number, relative retention time of two critical-band-pairs, Rg_1/Re and Rb_1 with its neighboring peak, respectively. Totally, 16 column types were used for comparison of different vendors, different packing materials, different size, etc. and five sets of LC systems and two laboratories were involved in comparing the data of both quantitative and qualitative items. The results showed that different packing materials of columns used might significantly alters separation. The column packing material Hypersil afforded the preferable separating for the ginsenosides. No significant difference was observed from the different instrumentations and inter-laboratories. Our results suggest a modification of the system suitability test as given in USP26-NF21 and the latest version of USP27-NF22, which was not suitable for most systems. Using resolutions of Rg_1/Re and Rb_1 with its neighboring peak as critical parameters for the ginsenosides assay and omitting the relative retention time of both Rg_1/Re and Rb_1 with its neighboring peak is our suggestion for a more reasonable, yet practicable system suitability. Six typical chromatograms gain from different columns were figured out as well.

Keywords: Robustness/ruggedness; System suitability; Ginsenosides; Ginseng; USP

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doi:10.1016/j.jpba.2004.04.005

1. Introduction

Ginseng, including Asian ginseng (Oriental ginseng, root of *Panax ginseng* C.A. Mey) and American ginseng (root of *Panax quinquefolium* L.), has been traditionally used as herbal medicine for maintaining natural energy, increasing mental and physical abilities, improving mood and promoting general health and well-being [1]. In recent decades, Ginseng is increasingly consumed as a health tonic, and varieties of commercial health products such as ginseng capsules, soups, drinks and cosmetics, are widely distributed in Asian as well as in many other countries around the world [2]. It is estimated that the current world sales of various ginseng raw materials have reached about more than one billion US$ per annum. The active components and markers in Ginseng were saponins including ginsenosides Rg_1, Re, Rb_1, Rg_2, Rb_2, Rc, and Rd, etc. [3,4]. The quality control and standardization of Asian ginseng and American ginseng in most official monographs were established on the assay of those ginsenosides using chromatographic technique [5–10].

Oriental ginseng (Asian ginseng) is one of first botanical products entered the official monograph of USP24-NF19 as natural food supplement [6]. The recent editions of USP (USP26-NF21 and USP27-NF22) [7,8] record further Ginseng products, namely Asian ginseng, powdered Asian ginseng, Asian ginseng extracts, Asian ginseng tablets, American ginseng, powdered American ginseng and American ginseng extracts. The assay methods for ginsenosides in both Asian ginseng and American ginseng, as well as their extracts and finished products, have typically been developed using high-performance liquid chromatography (HPLC). The system suitability for ginseng in USP26 [7], as well as in USP27 [8], is required as following: given " the relative retention times for ginsenoside Rg_1 and ginsenoside Re are 1.0 and 1.03, respectively; the resolution, *R*, between ginsenoside Rg_1 and ginsenoside Re is not less than 0.9; the resolution, *R*, between ginsenoside Rb_1 and a neighboring minor peak, at relative retention times of 1.86 and 1.89, respectively, is not less than 1.0; the column efficiencies determined from ginsenoside Rg_1 and ginsenoside Rb_1 are not less than 17,000 and 11,000 theoretical plates, respectively; the tailing factors for the ginsenoside Rg_1 and ginsenoside Rb_1 peaks are not more than 1.0 and 1.2, respectively; and the relative standard deviation for replicate injections is not more than 4.0% determined from ginsenoside Rg_1 and ginsengoside Rb_1." It was quite infrequent to figure the system suitability of an object so much delicate and complicated. In practice, performing this assay method cannot always be unproblematic. For most chromatographic system, it is difficult to meet the above-mentioned specific requirements of the system suitability, even with state-of-the art equipment and column. Hence, the effect factors determining and requirements of system suitability evaluation for the assay yet remain decisive. Here, we report the evaluation for the robustness/ruggedness with a focus on different column types and equipment sets, and on this basis recommend practicable and reliable system suitability.

2. Experimental

2.1. Chemicals and materials

Acetonitrile (ACN), HPLC grade (Merck, Darmstadt, Germany). Milli-Q Water (Millipore, MA, USA); Reference standards of ginsenoside Rg_1, Re, Rf, Rb_1, Rb_2, Rb_3, Rg_2, Rc, and Rd were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

2.2. Standard solution

Solution (1): Two milligrams of ginsenosides Rg_1, Re, Rf, Rb_1, Rg_2, 1 mg of ginsenoside Rd, and 0.4 mg of ginsenosides Rb_2, Rb_3, Rc, accurately weighed, were dissolved in methanol in the same 10 ml volumetric flask, and diluted to volume with methanol, which was taken as the sample solution.

Solution (2): Two milligrams of ginsenosides Rg_1 and Rb_1, accurately weighed, were dissolved in methanol in the same 10 ml volumetric flask, and diluted to volume with methanol.

2.3. Equipment

A Mettler AE 200 micro/analytical balance (Mettler, OH, USA) was used. Gelman Acrodics 0.45 μm PTFE (25 mm) membrane syringe filters (Pall, NY, USA) was utilized.

Table 1
LC columns chosen for analysis

Column no.	Vendor	Type	Size (mm)	Lot number
C1	Supelco	Discovery, C_{18}, 5 μm	250 × 4.6	
C2	Phenomenex	Luna C_{18}(2), 5 μm	250 × 4.6	163197-14
C3	Phenomenex	Luna C_{18}(2), 5 μm	250 × 4.6	111570-22
C4	Phenomenex	Luna C_{18}(2), 5 μm	250 × 4.6	122053-3
C5	Phenomenex	Luna C_{18}(2), 5 μm	250 × 4.6	407299
C6	Phenomenex	Luna C_{18}(2), 5 μm	150 × 4.6	127056-10
C7	Agilent	Zorbax, SB C_{18}, 5 μm	250 × 4.6	
C8	Agilent	Zorbax, SB C_{18}, 5 μm	150 × 4.6	
C9	Hewlett–Packard	Cartridge column, ODS Hypersil, 5 μm	250 × 4.6	
C10	Hypersil	ODS Hypersil, 5 μm	250 × 4.6	
C11	Dalian Institute of Chemical and Physical Research	ODS Hypersil, 5 μm	250 × 4.6	
C12	Waters	Hypersil ODS, 5 μm	150 × 4.6	
C13	Waters	Symmetry, C_{18}, 5 μm	250 × 4.6	
C14	Eka Chemical	Kromasil, KP100-5C_{18}, 5 μm	250 × 4.6	
C15	Alltech	Alltima C_{18} 5 μm	250 × 4.6	
C16	Dikma	Diamond C_{18} 5 μm	250 × 4.6	

2.4. Columns

Totally, 16 types of LC columns were chosen to compare their performance for the analysis of gensenosides. The columns tested differed in vendors, packing materials, size, as well as batch number as listed in Table 1.

2.5. Instrumentation

Five HPLC systems were evaluated, to trace the separation of ginsenosides: (S1) System 1, Waters 2690 solvent module including quaternary pump, degaser, autosampler, column compartment, equipped with PDA 996 photo-diarray detector, and a Millennium 32 workstation for data acquiring and processing, with system suitability software to calculate the parameters of retention, peak plate number, resolution, etc. (S2) System 2, the same Waters 2690 system as S1 but equipped with a 2487 ultra-visible (UV) detector, instead of the PDA 996 photo-diarray detector. (S3) System 3, HP1100, Agilent Co., consisting of quaternary pump, degasser, autosampler, column compartment and photodiode array detector (DAD). The raw data was acquired and processed by a LC Workstation, Agilent. (S4) System 4, HP1100, Agilent Co., consisting of binary pump of high-pressure gradient system, degasser, autosampler, column compartment and UV detector. The raw data was acquired and processed by LC Workstation, Agilent. (S5) System 5, Waters 616 quaternary pump, degaser, 7725i manual inject valve, sampler, online column heater, PDA 996 photo-diode array detector, and a Millennium 32 workstation for data acquiring and processing, with system suitability software to calculated the parameters of retention, peak plate number, resolution, etc.

2.6. Inter-laboratory evaluation

Two laboratories (L1), laboratory 1, analytical lab, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai, China (L2), laboratory 2, Shanghai R&D Center for Standardization of traditional Chinese medicines, Shanghai, China, have been involved in this evaluation research, and the instrumentation used was the same set of Agilent 1100 with quaternary pump and DAD detector.

2.7. Chromatographic condition

According to the assay method of ginsengs in USP, the chromatography was performed using a gradient of water (eluent A) and acetonitrile (eluent B). The initial condition was eluent B (18%) for the first 10 min, and gradient elution from 18 to 40% B in the following 40 min, followed by keeping 40% B from 50 to 80 min. The flow rate was 1 ml/min. The total analysis time

was 90 min, including column stabilization. The UV detector was set at 203 nm, and the injection volume was 10 μl.

2.8. Procedures

For comparison of the columns effects, using the LC elution system as described above, 16 types of columns were used in series to run the separation on the same System S1. Column C9 was chosen to carry the separation out on the five different Systems S1 to S5 individually, while in two different laboratories, the two columns of C1 and C9 were used for ginsenosides separation. For each individual condition, 10 μl of solution (1) and solution (2) were injected separately. All data were collected and summarized.

2.9. Calculation

The recoveries of Rg_1 and Rb_1 were determined, respectively, with Rg_1 and Rb_1 standards (solution 2) as the reference solution standards and the mixture standards (solution 1) as the sample solution. Secondly, the system suitability tests including the theoretical plate number of Rg_1 and Rb_1, the resolution of Rg_1 from Re, Rb_1 from its neighboring peak, the relative retention time of Rg_1 to Re, Rb_1 to its nearest peak, the selectivity of Rg_2 and Rb_1, the tailing factors for Rg_1 and Rb_1, and the capacity factor (k') of Rd were calculated.

3. Results and discussion

3.1. Columns effects

The data of 16 types of LC columns were given in Table 2, and typical chromatograms from six different columns were shown in Fig. 1. The efficiency of these chosen columns on the separation of ginsenosides was significantly varied as follows.

The six typical chromatograms (Fig. 1) revealed differences not only in the resolution of two critical-band-pair peaks of Rg_1/Re, and Rb_1 with the nearest peak, but also in the selectivity between Rb_1 and Rg_2, etc. The distribution and retention times of the peaks of the nine selected ginsenosides on the chromatograms differed from each other as well.

The recoveries of Rg_1 and Rb_1 were calculated and taken as the indicators of the accuracy and system suitability of the chosen analytical conditions. The recoveries of Rg_1 and Rb_1 ranged from 89 to 199% and from 87 to 218%, respectively. The nominal confidence ranges of the recovery percentage of Rg_1 and Rb_1 was $100 \pm 2.13\%$, respectively, gotten from USP26 system suitability R.S.D.% 4.0% of Rg_1 and Rb_1 peak area with t-tests under confidence level $\alpha = 0.05$ and experimental number $n = 16$. The recoveries of both Rg_1 and Rb_1 from seven columns met or nearly met the requirements of $100 \pm 2.13\%$ while the other nine columns failed. The statistical evaluation of these recoveries of Rg_1 and Rb_1 generated from two column groups (Fig. 2) demonstrated that they significantly differed from each other ($P < 0.001$). The results revealed different type columns used might lead to quite variation accuracy obtained in the assay of ginsenosides.

The packing materials were shown to be the dominating factor to effect the recoveries and separation of Rg_1 and Rb_1, as well as other ginsenosides. Among the chosen columns, the packing material of Hypersil was the most preferable for the separation of ginsenosides. Waters Symmetry and Agilent Zorbax columns were the second choice regarding their separation efficiency.

The type of packing material also affected the selectivity of Rg_2 and Rb_2 (Table 2). The selectivity of more than 1.0 and less than 1.0 demonstrated the peak of Rg_2 in the front of or back of the peak of Rb_1.

Different batches of columns of the same size packed with the same packing material, produced by the same vendor, e.g. Phenomenex C2 to C5, did not effect the separation significantly, with a similar distribution of peaks and a relative standard deviation percentage (R.S.D.%) of 4.5 and 1.9% for the recovery of Rg_1 and Rb_1, respectively. Results from using the same packing material and columns produced by different vendors such as Hypersil C9, C10, C11, and C12, also illustrated a similar separation of ginsenosides with a similar peak distribution and R.S.D.% for recovery of Rg_1 and Rb_1, 1.1% and 0.4%, respectively. In addition, the size of columns had a little effect on the separation, since the column volume was different and gradient elution was used. Summarily, the packing material of the column was the only important factor influencing the resolution and selectivity of ginsenosides.

Table 2
The data of ginsenosides separation by 16 different LC columns

Column	Vendor	RC% (Rg_1)	RC% (Rb_1)	N (Rg_1) (×1000)	N (Rb_1) (×1000)	Rs (Rg_1)	RRT (Rg_1/Re)	Rs (Rb_1/)	RRT (Rb_1/n)	Sele (Rg_2/Rb_1)	TF (Rg_1)	TF (Rb_1)	k' (Rd)
C1	Supelco	97.68	217.9	170.0	406.0	1.54	1.015	0.10	1.000	1.000	0.99	1.13	23.05
C2	Phenomenex	93.56	98.10	134.1	265.5	0.30	1.007	4.23	1.034	1.035	1.02	0.90	23.90
C3	Phenomenex	93.07	101.1	148.6	393.4	0.50	1.007	4.76	1.034	1.035	0.97	0.89	23.95
C4	Phenomenex	96.82	102.2	130.6	269.2	0.50	1.005	4.31	1.035	1.037	0.96	0.84	24.30
C5	Phenomenex	102.5	98.92	150.2	374.9	0.40	1.008	4.17	1.031	1.031	0.89	0.85	24.06
C6	Phenomenex	97.76	101.9	103.3	279.4	1.20	1.013	0.60	1.006	1.006	0.98	0.88	29.47
C7	Agilent	89.60	87.67	138.4	410.1	1.04	1.010	1.91	1.013	1.013	0.96	1.03	21.81
C8	Agilent	101.2	122.5	92.3	254.2	1.14	1.015	1.92	0.983	0.983	0.91	0.87	28.25
C9	HP Cart[a]	99.51	99.64	47.1	166.4	1.62	1.028	3.08	1.030	0.889	0.98	0.91	23.41
C10	Hypersil	97.52	99.16	88.4	209.2	0.60	1.012	1.31	1.013	0.987	1.29	0.79	23.62
C11	Dalian[b]	100.0	100.0	109.5	305.0	1.91	1.024	3.91	1.030	0.874	1.08	0.91	25.09
C12	Waters	99.22	99.26	73.2	237.1	1.65	1.024	2.06	1.017	0.983	0.98	1.23	27.91
C13	Waters	98.81	98.99	116.1	276.6	1.19	1.015	3.28	1.026	1.022	1.03	0.87	22.86
C14	Eka Chemical	93.24	98.88	100.5	136.8	0.40	1.007	1.37	1.012	1.012	0.92	1.08	23.91
C15	Alltech	199.3	100.2	131.0	291.5	0.00	1.000	2.38	1.019	1.019	1.07	0.93	24.80
C16	Dikma	199.3	99.89	37.33	309.9	0.00	1.02	2.06	1.017	1.017	0.95	0.95	23.28
Minimum		89.60	87.67	37.33	136.80	0.00	1.00	0.10	0.98	0.87	0.89	0.79	21.81
Maximum		199.30	217.90	170.00	410.13	1.91	1.028	4.76	1.04	1.04	1.29	1.23	29.47
Mean		109.94	107.89	110.66	286.57	0.87	1.01	2.59	1.02	1.00	1.00	0.94	24.60
R.S.D.%		31.9	27.9	33.4	28.0	70.2	0.8	54.7	1.4	4.8	9.4	12.5	8.6
USP SST		100 ± 2.13[c]	100 ± 2.13[c]	>17	>11	>0.9	>1.03	>1.0	>1.016		<1.0	<1.2	
Pass number		6	13	16	16	8	0	14	9		11	15	
Pass percentage (%)		37.5	81.2	100	100	50	0	87.5	56.2		68.8	93.8	

RC%: recovery percentage; N: theoretical plate; Rs: resolution; RRT: relative retention time; Rs (Rb_1/): resolution of Rb_1 with a neighboring peak; RRT (Rb_1/n): relative retention time of Rb_1 with a neighboring peak; Sele (Rg_2/Rb_1): selectivity of peaks Rg_2 and Rb_1; TF: USP tailing factor; k' (Rd): capacity factor (k') of Rd; USP SST: the nominal of system suitability in USP; pass number: the numbers meet the requirements of USP system suitability.

[a] HP cart: Hewlett–Packard cartridge column.

[b] Dalian: Institute of Dalian Physical and Chemical Research, Liaoning, China.

[c] The value 100 ± 2.13 calculated as the equation of confidence range, $\mu = X \pm t_{1-\alpha/2}\,(\text{S.D.})/n^{-1/2}$, where $\alpha = 0.05$, $n = 16$, average recovery $X = 100\%$, S.D. $= 4$ (from USP, R.S.D.% $= 4$).

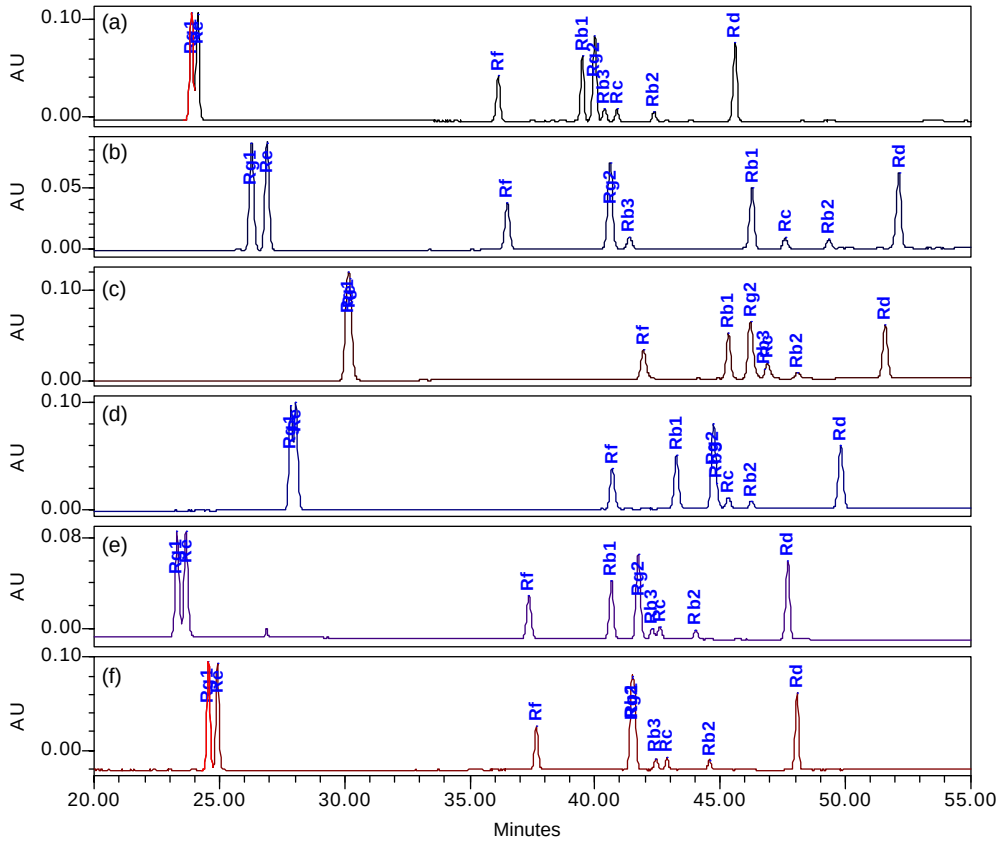

Fig. 1. Six typical chromatograms of HPLC tracing to ginsenosides by different columns (System 1, gradient elution as USP26, UV detector at 203 nm; (a) C7 Agilent Zorbax; (b) C11 Dalian Hypersil; (c) C15 Alltech Alltima; (d) C2 Phenomenex Luna; (e) C13 Waters Symmetry; (f) C1 Supelco Discovery; all column size 250 mm × 4.6 mm, 5 μm).

The analytical data obtained totally from 16 different types of columns and the requirements of system suitability in USP26 are compared in Table 2. As nominal, the numbers of meeting the requirements and passed percentage were given in Table 2. In 100% of the cases the theoretical plate number of both Rg_1 and Rb_1 accessed the requirements while 100 and 87% of relative retention times of both Rg_1/Re and Rb_1 with its neighboring peak did not meet the suitability limits. These findings suggested that the requirement given in USP26-NF21 for the relative retention times of ginsenosides were unreasonable and unnecessary. Furthermore, the threshold for the theoretical plates of ginsenoside Rg_1 and Rb_1 (17,000 and 11,000, respectively) were insufficient to demonstrate the column efficiency for the purposed separation of ginsenosides.

The resolutions (Rs) of both Rg_1 with Re and Rb_1 with its neighboring peak were varies with different columns. The data of Table 2 show that in some of the chosen columns, the resolutions of Rg_1/Re was as high as 1.91 (C11) and 1.65 (C12), respectively, and the resolutions of Rb_1/*n* (neighboring minor peak) were even 3.91 (C11) and 2.06 (C12), respectively. Fig. 3 shows the dependency of the recovery of Rg_1 on the resolution of Rg_1/Re. It can be concluded that ideal recovery is obtained for a resolution of more than 1.0. The similar tendency was found for the relationship of recovery of Rb_1 with the resolution of Rb_1 to its nearest peak. According to our experience, the resolution requirement of 0.9 between Rg_1/Re in USP is too low to realize the baseline separation and accurate quantitation of Rg_1 and Re, which were found in equivalent amounts in Asian ginseng.

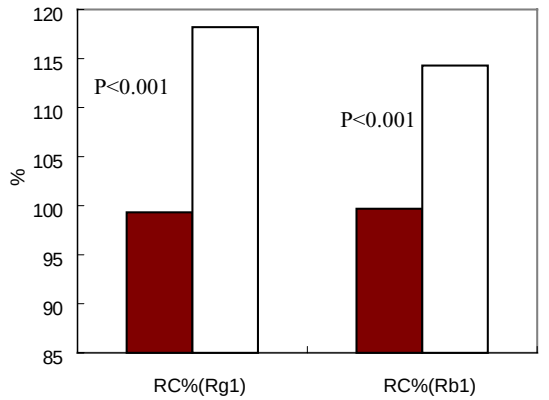

Fig. 2. Diagram of the recovery percentage of ginensosides Rg_1 and Rb_1, respectively, for different column types divided by two groups of "ideal" and "not ideal" recovery. Black: "ideal recovery" group including columns C5, C6, C9, C10, C11, C12, and C13. Blank: "not ideal recovery" group including columns C1, C2, C3, C4, C7, C8, C14, C15, and C16.

Conclusively it should be suggested that the resolution between Rg_1/Re and between Rb_1 and the neighboring minor peak is required to not less than 1.0 and 1.1, respectively, for assay of Rg_1 and Rb_1, two marked critical components in ginseng and the related products. The relative retention times of Rg_1/Re and Rb_1/neighboring peak were seriously effected by the HPLC systems used, and most importantly, are not directly related to the resolution and the column

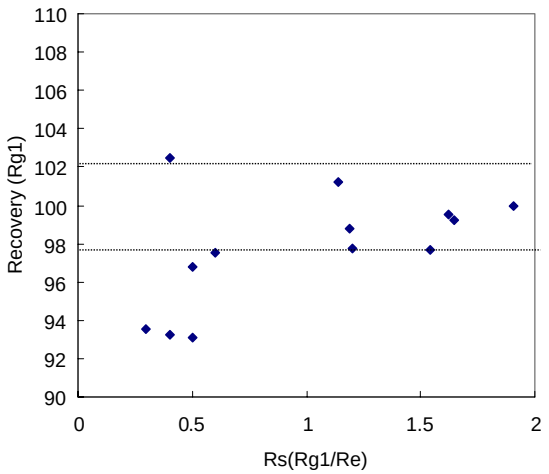

Fig. 3. Plot of the recovery percentage of Rg_1 as a function of resolution of Rg_1/Re.

Table 3
The data of the ginsenosides separation by five different LC systems

System	Column	RC% (Rg_1)	RC% (Rb_1)	N (Rg_1) (×1000)	N (Rb_1) (×1000)	Rs (Rg_1)	RRT (Rg_1/Re)	Rs (Rb_1/)	RRT (Rb_1/n)	Sele (Rg_2/Rb_1)	TF (Rg_1)	TF (Rb_1)	k' (Rd)
S1	C9	99.51	99.64	47.1	166.4	1.62	1.028	3.08	1.03	0.89	0.98	0.91	22.91
S2	C9	100.50	99.78	48.2	170.2	1.58	1.027	3.09	1.03	0.89	0.97	0.92	22.89
S3	C9	101.00	99.74	57.1	186.9	1.66	1.024	3.11	1.02	0.89	0.98	0.95	22.44
S4	C9	99.77	99.84	50.1	170.5	1.67	1.024	3.10	1.02	0.89	0.98	0.93	22.28
S5	C9	98.23	99.15	42.5	146.4	1.47	1.026	2.86	1.03	0.89	0.89	1.15	23.16
Mean		99.80	99.63	48.99	168.08	1.60	1.026	3.05	1.03	0.89	0.96	0.97	22.73
R.S.D.%		1.06	0.28	10.86	8.61	5.06	0.17	3.47	0.44	0.00	4.10	10.35	1.60

S, mean system; C, mean column. Others same as in Table 2 footnotes.

Table 4
Comparison of the ginsenosides separation by two individual laboratory

Lab	Column	RC% (Rg_1)	RC% (Rb_1)	N (Rg_1) (×1000)	N (Rb_1) (×1000)	Rs (Rg_1)	RRT (Rg_1/Re)	Rs (Rb_1/)	RRT (Rb_1/n)	Sele (Rg_2/Rb_1)	TF (Rg_1)	TF (Rb_1)	k' (Rd)
L1	C1	98.68	217.9	170.0	406.0	1.54	1.015	0.10	1.00	1.00	0.99	1.13	22.55
	C9	99.51	99.64	47.1	166.4	1.62	1.028	3.08	1.03	0.89	0.98	0.91	22.91
L2	C1	99.15	199.6	178.2	399.5	1.51	1.016	0.1	1.00	1.00	0.98	1.06	22.65
	C9	99.32	99.26	49.1	170.4	1.58	1.024	3.05	1.03	0.89	0.97	0.89	22.88

L, laboratory; C, column. For others abbreviations, as in Table 2 footnotes.

efficiency. So, we suggest that it is reasonable to omit these items in system suitability requirements in USP or/and other Pharmacopoeia and regulations.

3.2. Different LC system

A tolerance test was conducted by five sets of HPLC systems, using the same column and chromatographic condition shown in Table 3. No significant deviation was found between these HPLC instruments, except of S5, the Waters 616-pump system, which gave a slight change due to a higher dwell volume comparing to the other four systems.

3.3. Reproducibility from Inter-laboratories

This validation assay was conducted by different operators in two individual laboratories using two types of columns, C1 and C9. The results suggested that all of the quantitative and qualitative parameters using same column were in good agreement, while the discrepancies were found using various column in both laboratories for both columns C1 and C9. It can be concluded that the method for ginsenosides separation is reproducible in different laboratories, by different operators under the condition of the same type of column and similar LC systems (Table 4).

4. Conclusion

Robustness/ruggedness evaluation is one part of method validation, especially for the method widely used in different environmental. Significant difference in accuracy as recovery was generated from using different types of LC columns for assay ginsenosides in ginseng. The Hypersil column is preferable for the separation of ginsenosides in Asian ginseng and American ginseng, and Zorbax SB or Waters Symmetry could also be used as back-up choice.

System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns, and is used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. So, the limits of system suitability given would be in a suitable range and guarantee the system operation smoothly for target analysis. It might be necessary to deliberate

the system suitability on assay of USP26 for ginsenosides in Asian ginseng and American ginseng from this study of ruggedness/robustness evaluation.

Acknowledgement

This research was financially supported by the Key Subject of Shanghai Education Committee and State Administration of TCM, China.

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