Korean J. Ginseng Sci.
Vol. 12, No. 2. 164–172 (1988)

A Rapid Method of Ginsenoside Analysis in HPLC by Pretreatment through the reverse-phase minicolumn

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(Received November 19, 1988)

역상소형컬럼 전처리를 이용한 Ginsenoside의 신속정량법

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(1988년 11월 19일 접수)

Abstract

The solvent separation step in the conventional method for quantitative analysis of ginsenosides was substituted by purification through a small reverse-phase C_{18}-column resulting in the decrease of analysis time by one fourth. New method showed high recovery of total ginsenosides but low recovery in protopanaxatriol-ginsenosides. Sugars did not affect the recovery by the amount in usual root sample. Coefficient of variation in recovery of ginsenosides was lower in the rapid minicolumn method. Optimum load of ginsenosides to minicolumn was 10 to 15 mg. The rapid minicolumn method showed highly significant correlation with the solvent separation method for dried root and red ginseng. For the rapid minicolumn method a small acryl device was used for the simultaneous extraction of 8 samples. This method appeared to be beneficial in cost and for the health of analyst.

Introduction

One of the well-known biologically active compounds in Panax ginseng root is ginseng saponins. Biological activity of ginseng saponin is tested with each purified ginsenoside but quantitative analysis of ginsenosides in ginseng or ginseng products is still time-consuming and very tedious because purification step after saponin extraction is done by solvent separation.[1,2] Soldati *et al.* developed a column method (dry extraction) for solvent separation step in C_{18} column HPLC[3]. This Extrelut® column (a normal phase) method was tested with purified ginsenoside[4], and ginseng and products[5] for NH_2 column HPLC. The result was satisfact for pure ginsenosides but not for samples especially with high sugar content.

In HPLC analysis for ginsenosides C_{18}[3], NH_2 or Carbohydrate[2] columns are efficiently used. Since ginsenosides have amphiphilic with hydrophilic character and

sugars can be eliminated by water or low conc. methanol through a small reverse-phase C_{18} column, it is well expected that this column method will be effective to purify ginsenosides. The SEP-PAK C_{18}, a reverse-phase minicolumn was used for ginsenoside identification on thin-layer chromatography[6] and also in C_{18} column HPLC for pure ginsenosides, ginseng and ginseng products.[7] However the reports on precise test and comparison with solvent separation method are hardly found.

We assessed the pretreatment of sample solution through SEP-PAK® C_{18} column (Waters Associates) for recovery and variation. This new method was much rapid, stable and furthermore showed high correlation with solvent separation method for the samples of dried and red ginsengs.

Materials and Methods

SEP-PAK® C_{18} column activation and optimum elution condition: SEP-PAK® C_{18} cartridge (Waters Associations, 1.08 cm dia, and 1.3 cm length) was activated with 2 m*l* absolute methanol and then washed with 4 m*l* water to eliminate excess methanol. The purified ginsenoside mixture solution (2 m*l*) was slowly applied and then washed with 8—10 m*l* water. Ginsenosides were eluted with 8 m*l* of various methanol-water mixtures of 10% interval from 0 to 100% methanol. Each eluate was vacuum-dried at 40 °C and then chromatographed on precoated silica gel thin-layer plate (0.25 mm, Merck and Co. Inc.).

Reagents: All solvent used were HPLC grade. Sugars are purchased from Sigma Co.

Recovery test: Each ginsenoside purified in our institute was weighed and mixed together for the standard solution. This standard solution was directly injected NH_2-column HPLC for reference peak area of recovery. Aliquot of sugar mixture solution (sucrose 8.04, α-D-glucose 4.01, fructose 2.04, maltose 4.00 mg per m*l* H_2O) was added to pure ginsenosides to elucidate the interference on recovery.

Ginseng sample: Six years old fresh ginseng roots from various ginseng plantations were separated into two parts, xylem-pith and cortex-epidermis. The latter part was dried at 55 °C for 72 hours and ground to 100 mesh before crude saponin extraction. Crude Chinese red ginseng powder supplied by a dealer was ground to 100 mesh with pestle and mortar before use.

Solvent separation method; It is based on Namba *et al.*[1] and slightly modified as shown in Fig. 1. HPLC condition: Instrument, Waters Associates Model 244; column, Lichrosorb® NH_2 (5μm, 25 × 0.4 cm I.D); mobile phase, acetonitrile/water/n-butanol (80/20/0.25); flow rate, 1.0 m*l*/min; chart speed, 0.5 cm/min; detection, RI (8×).

Results and Discussion

Optimum elution condition of SEP-PAK C_{18}: Ginsenosides were not appeared in the fractions below 30% aqueous methanol and over 90% as shown in Fig. 2. Thus the suitable elution condition of ginsenosides was decided to be with 8 m*l* of 85% methanol elution after washing out with 8 m*l* of 30% methanol. The elution time with

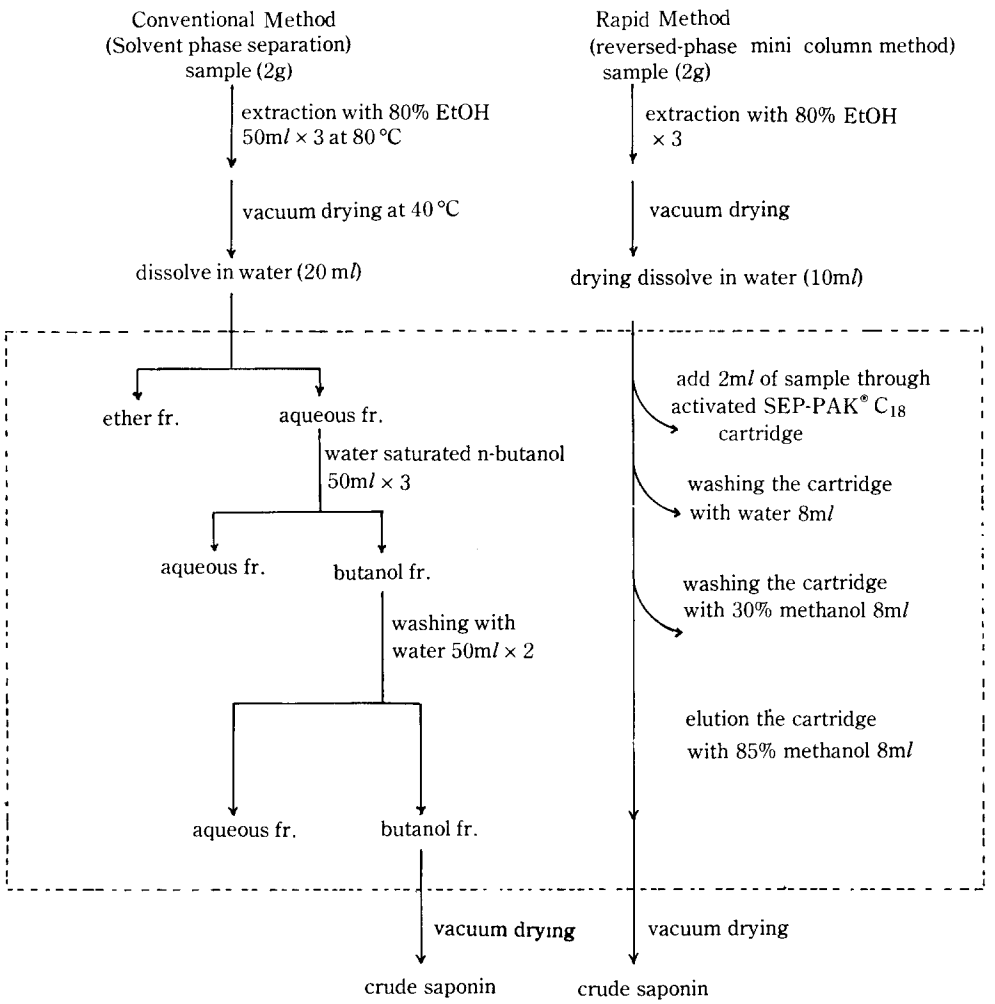

Fig. 1. Comparison between solvent separation method and rapid reversephase column method for crude ginsenosides extraction.

85% methanol was about 10 to 30 minutes. This elution time is much shorter than the 80 minutes in the case of Extrelut® column[4] indicating that reverse-phase C_{18} mini-column method is more efficient.

Recovery: The percent recovery of each ginsenoside was shown in Table 1. Average recovery of ginsenosides was 100.5 ± 3.9% and that of total was 99%. In C_{18} column HPLC the recovery of pure ginsenoside was reported as 100% but no data was shown.[7] In our recovery experiment coefficient of variation of recovery was 1.3% for the rapid minicolumn method but 3.0% for the solvent separation method.

The addition of sugar mixture to pure ginsenosides did little affect the recovery of ginsenosides at the concentration of sugar to ginsenoside ratio 2.4 that is slightly

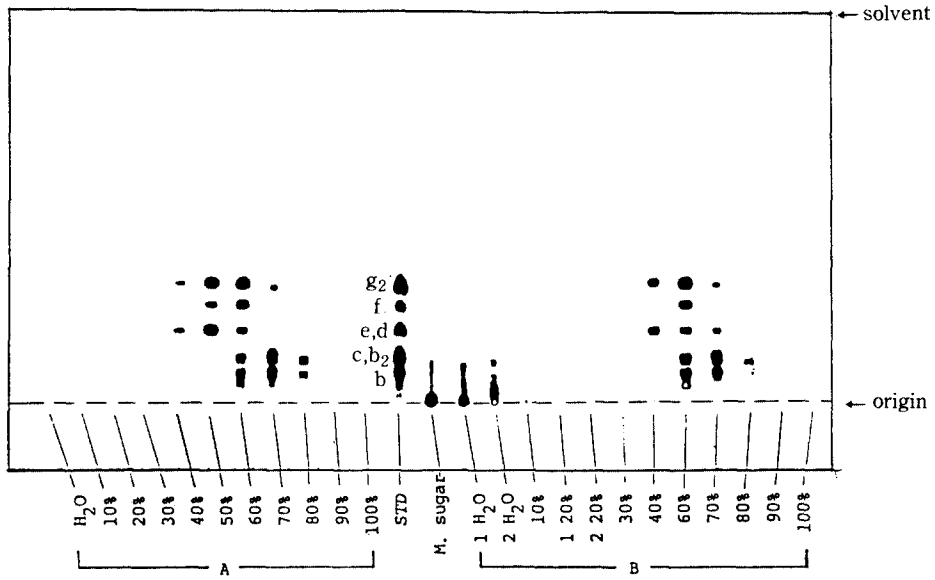

Fig. 2. Thin-layer chromatogram of eluted fractions of pure ginsenoside mixture (A) and pure ginsenoside mixture plus sample (B) through SEP-PAK C_{18} by aqueous methanol. Each fraction was eluted by the noted percent of aqueous methanol in sequence. Developing solvent; chloroform/methanol/water (65:35:10, lower phase), spray reagent; Liebermann-Buchard reagent

Table 1. Recovery of pure ginsenosides treated through SEP-PAK C_{18} column

(%)

Rg_2	Rg_1	Rf	Re	Rc	Rb_2	Rb_1	PT	PD	Total*
98.7	101.6	104.7	106.5	93.8	99.2	99.0	101.9	97.0	98.8

*sum of ginsenosides

higher than that of 6 year old ginseng. Sugar was eluted into washing water before aqueous methanol elution as shown in Fig. 2. Amino acids were also eluted with sugars.[6] This rapid minicolumn method will be applicable to most ginseng samples without interference of sugar.

Recovery was affected by loading amount of ginsenosides as shown in Fig. 3. Optimum loading amount showing over 95% recovery appeared to be 10 to 15 mg as total ginsenosides.

Sample analysis: Chromatogram of ginsenosides in root sample by NH_2 column HPLC with the rapid minicolumn method showed no difference from that with the solvent separation method (Fig. 4). Appearance of nonsaponin peaks were same in both methods.

Relationship between the rapid minicolumn method and the conventional solvent separation method by using dried root samples was shown in Table 2. As shown in Fig. 5 for total ginsenosides each ginsenoside showed highly significant correlation ($P = 0.001$) between two methods except comparatively low Rb_2 ($P = 0.01$) and Rd ($P = 0.05$). The lower correlation coefficients in case of Rb_2 and Rd were due to high

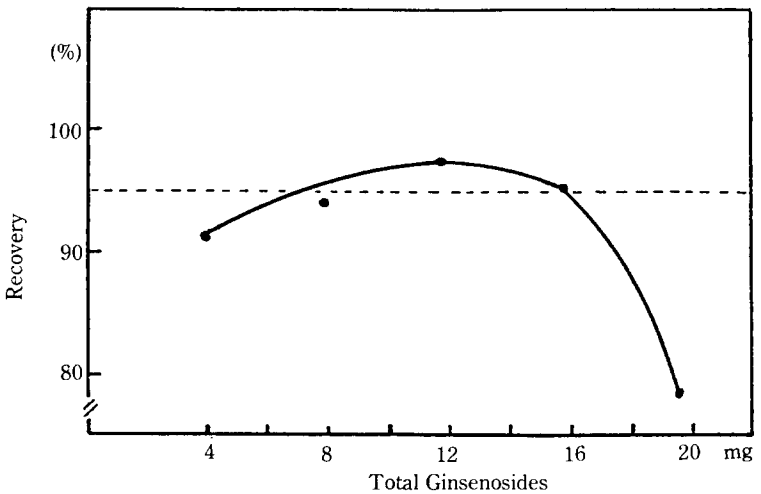

Fig. 3. Recovery of total ginsenosides after rapid treatments according to loading amounts.

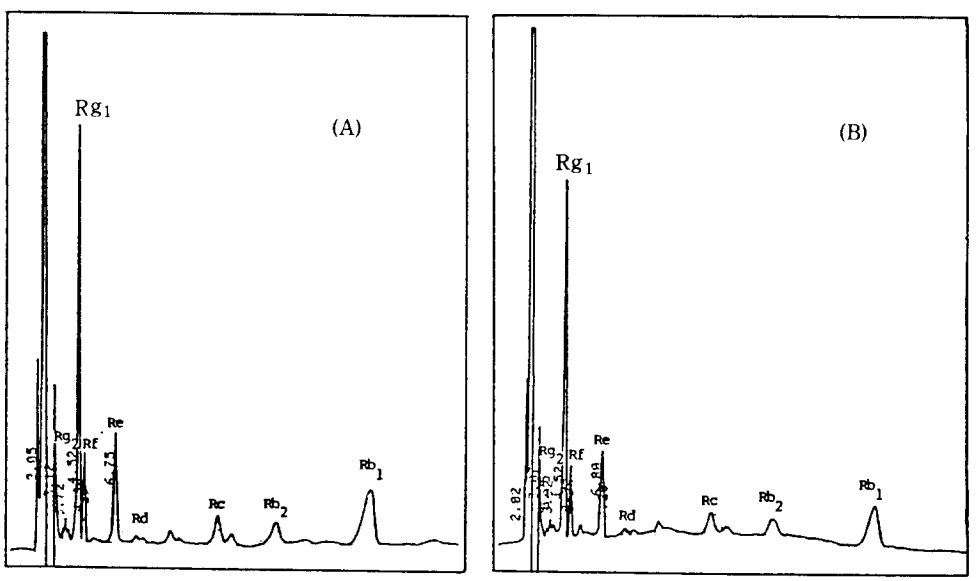

Fig. 4. HPLC chromatograms of ginseng saponins in cortex part purified by rapid preparation(A) and solvent phase separation(B). Chromatographic conditions: column, Lichrosorb NH_2 (5 um, 25 × 0.4cm I.D.); mobile phase, acetonitrile/water/n-butanol (80/20/0.25); flow-rate, 1ml/min; chart speed, 0.5cm/min; detection, RI (8X). Sample amount is different in A and B.

coefficient of variation of percent recovery (extraction ratio × 100) of the rapid minicolumn method to the conventional method (Table 3). Such difference between two methods may likely arise from the reading error of peak due to nonsaponin neighbor peak in Rb_2 and too small peak by small amount in Rd.

Percent recovery by the rapid minicolumn method to the conventional method was 93.4 for total ginsenosides (Table 3). The low recovery was due to the recovery of protopanaxatriol ginsenosides(PT) since the recovery of PT was consistently lower in

Table 2. Simple correlation between rapid minicolumn method and solvent separation with dried ginseng cortex-epidermis.

	Rg_1	Rf	Re	Rd	Rc	Rb_2	Rb_1	PT	PD	Total
r	0.944***	0.952***	0.966***	0.750*	0.961***	0.871**	0.963***	0.924***	0.976***	0.959***
Coefficient	0.815	0.947	0.934	0.608	0.893	0.612	0.960	0.866	0.853	0.844
Intercept	0.416	-0.176	-0.065	0.074	0.359	0.891	0.373	-0.072	1.811	2.146

n = 9, ***, **, *; significant at p = 0.001, 0.01 and 0.05

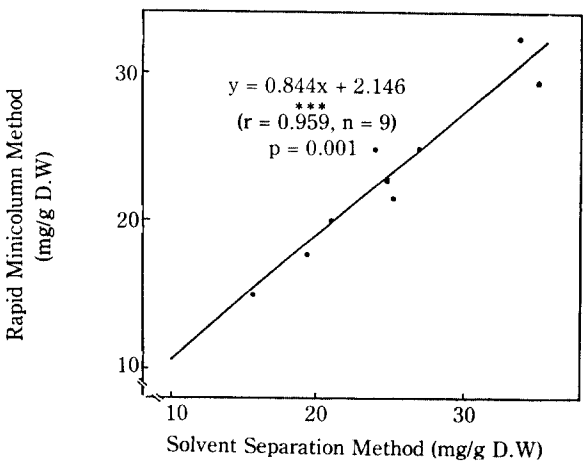

Fig. 5. Relationship between rapid minicolumn method and solvent separation method for total ginsenosides of the dried root samples.

Table 3. Percent recovery of ginsenosides by rapid minicolumn method to solvent separation with dried ginseng cortex-epidermis (%)

Sample number	Rg_1	Rf	Re	Rd	Rc	Rb_2	Rb_1	PT	PD	Total
1	75.1	80.5	89.2	129.7	128.7	166.6	91.2	79.8	115.8	95.5
2	86.0	91.0	91.7	52.0	100.5	86.3	99.6	88.3	96.9	91.7
3	71.4	71.7	85.6	81.1	97.0	97.9	101.8	74.7	100.0	85.7
4	77.7	74.7	85.4	64.0	94.6	86.0	90.6	79.1	90.3	84.6
5	83.3	82.3	83.3	54.1	104.2	124.2	105.6	83.1	108.5	93.1
6	79.0	84.7	89.7	111.2	109.7	83.6	107.4	82.7	101.0	93.0
7	101.5	98.5	107.9	113.8	101.9	94.2	115.8	102.9	107.6	105.0
8	88.5	92.6	93.8	97.3	105.5	100.4	99.4	91.8	100.9	95.6
9	88.8	94.7	97.0	89.5	111.9	86.5	91.6	91.6	102.4	96.3
average	83.5	85.6	91.5	88.1	106.0	102.9	101.8	86.0	102.6	93.4
CV(%)	10.2	10.1	7.8	29.6	9.1	24.7	7.3	9.4	6.7	6.1
average content (mg/gd.w)	7.88	2.02	3.95	0.33	2.34	2.46	6.42	13.85	11.55	25.39

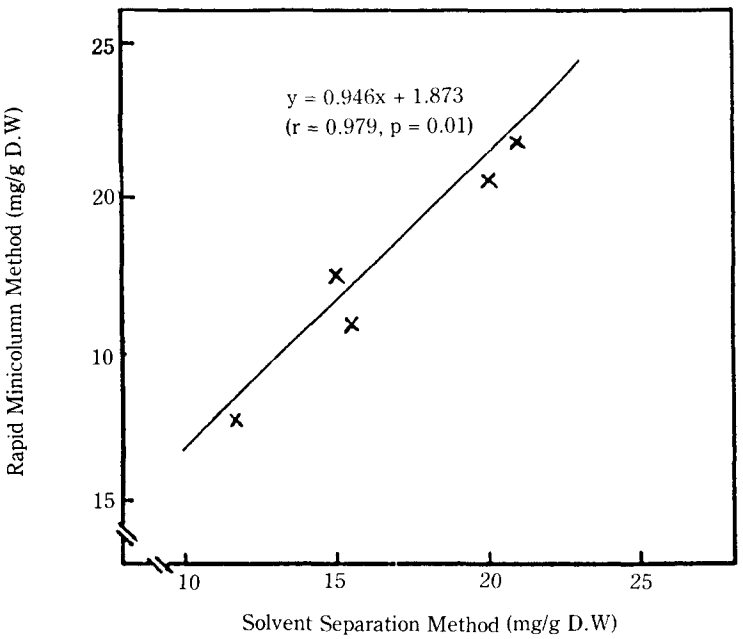

Fig. 6. Relationship between rapid minicolumn method and solvent separation method for total ginsenosides of the red ginseng samples.

most samples while Rd recovery was not consistent (Table 3).

For the red ginseng samples the rapid minicolumn method showed similar relation with the conventional method (Fig. 6, Table 4) as it was for the dried ginseng. The recovery of PT was lower than protopanaxadiol too (Table 5). The lower percent recovery of PT was shown also in the case of pure saponin (Table 6) although the degree was smaller than that in samples. When pure ginsenosides were added to sample extract, the percent recovery decreased more (Table 6). In this experiment with thin layer the trace of saponin appeared in the washing water, 10% and 20% methanol eluate with sugars although this is not seen in Fig. 2. It was not due to overloading. From above facts it seems that there is some PT loss in the eluate below 30% methanol. It is also possible that such loss may be stimulated by unknown substance from root sample solution. Thus 25% methanol washing and elution with 90% methanol will decrease the probable loss of PT and Protopanaxadiol ginsenosides.

Table 4. Correlation between rapid minicolumn method (RM) and solvent separation (SS) with tap root of red ginseng.

	Rg_2	Rg_1	Rf	Re	Rd	Rc	Rb_2	Rb_1	PT	PD	Total
r	0.945^{**}	0.996^{****}	0.872^{*}	0.958^{**}	0.926^{**}	0.930^{**}	0.965^{***}	0.964^{***}	0.979^{***}	0.988^{***}	0.985^{***}
Coefficient	0.626	0.848	1.156	0.642	0.696	0.953	1.186	1.389	0.840	1.112	0.980
Intercept	0.087	0.021	- 0.230	0.799	0.411	0.189	- 0.032	- 0.924	0.517	0.213	1.087

n; 5, ****, ***, **, *; significant at p = 0.001, 0.01, 0.05 and 0.1

Table 5. Percent recovery of ginsenosides by rapid minicolumn method to solvent separation with tap root of red ginseng

(%)

Sample number	Rg_2	Rg_1	Rf	Re	Rd	Rc	Rb_2	Rb_1	PT	PD	Total
1	60.2	89.6	103.5	97.5	102.0	105.1	115.7	115.6	90.8	111.7	104.2
2	79.9	80.5	84.8	96.1	84.5	94.9	123.9	126.2	86.0	112.4	102.5
3	76.0	86.0	89.8	120.1	139.4	116.9	113.8	122.5	94.5	120.6	110.3
4	87.9	86.4	72.1	118.8	142.8	121.7	140.8	101.8	94.2	118.0	108.2
5	101.9	85.4	108.4	114.1	143.8	82.6	92.9	114.6	93.6	104.1	98.6
Average	81.2	85.6	91.7	109.3	122.5	104.2	117.4	116.1	91.8	113.4	104.8
CV (%)	16.9	3.4	14.2	9.5	20.0	13.7	13.2	7.2	3.5	5.0	4.0
Average content (mg/gd.w.)	0.55	3.29	1.01	1.86	1.00	2.28	2.44	4.21	6.71	9.93	16.64

Table 6. Percent recovery of ginsenosides by rapid minicolumn method to solvent separation method with pure saponin mixture (PSM), PSM plus sugar mixture and PSM plus sample solution.

(%)

		Rg_2	Rg_1	Rf	Re	Rc	Rb_2	Rb_1	PT	PD	Total
Pure saponin	I	90.7	78.1	93.3	92.2	105.4	110.0	89.3	85.9	99.8	94.5
mixture	II	96.8	85.6	91.4	94.2	114.5	117.4	104.4	90.3	111.3	103.0
(PSM)	aver.	93.7	81.9	92.4	93.2	110.0	113.7	96.9	88.1	105.6	98.8
PSM plus	I	104.3	82.3	82.7	95.4	106.1	116.2	106.1	88.3	109.0	100.8
sugar	II	98.1	77.2	79.7	90.0	99.9	116.2	82.3	83.4	102.7	95.2
mixture	aver.	101.2	79.8	81.2	92.7	103.0	116.2	94.2	85.9	105.9	98.0
PSM plus	I	88.4	73.7	79.5	80.3	100.5	104.0	105.6	77.6	103.7	92.0
sample	II	79.1	62.9	69.3	71.5	93.7	99.0	93.5	67.6	95.1	82.7
soln.	aver.	83.8	68.2	74.4	75.9	97.1	101.5	99.6	72.6	99.4	87.4

Ginseng samples from various plantations in 1987 were analyzed by this way and the results were good enough the interpretation of the effect of growth conditions on saponin content comparing with the results of previous years (unpublished data).

The volume of washing solution may affect the recovery. We used 8 to 10 m*l* as it is in the use of SEP-PAK® C_{18} cartridge for sample pretreatment in organic compound analysis. For ginsenosides 30 m*l* was used.[7] High recovery of PT in Table 1 may be due to pipetting error. In this case pooling of 0.2 m*l* each ginsenoside solution was done separately for the minicolumn treatment and for the direct injection to HPLC.

For the rapid minicolumn method, an acryl device was used to hold 8 minicolumns and 8 test tubes to collecting eluates under the controlled vacuum.

The rapid minicolumn method is beneficial not only in analysis time (one fourth) but also in cost. The minicolumn appeared to be reusable once more according preliminary test. The rapid minicolumn method gives the safe environment by minimizing the exposure to harmful solvents.

요 약

인삼사포닌 정량분석의 상법 purification 과정을 역상소형 C_{18} 컬럼 전처리로 대체할 수 있었으며, 분석시간은 1/4로 단축되었다. 이 방법을 사용하면 total ginsenoside의 회수율은 높으나 protopanaxatriol계 ginsenoside들의 회수율은 약간 낮았으며, 根中 농도 정도의 糖類는 ginsenoside 회수율에 영향을 미치지 않았다. Ginsenoside 회수율의 변이계수는 상법의 경우보다 작았으며 이 방법에 사용되는 ginsenoside의 적정량은 10~15mg이었다. 역상소형컬럼방법은 건삼과 홍삼시료처리시에도 상법과 고도의 유의성을 나타냈다. 신속 역상소형컬럼 방법을 실제로 이용하기 위하여 8점의 시료를 동시에 처리하는 소형아크릴 장치를 사용하였다.

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