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Utilizing column selectivity in developing a high-performance liquid chromatographic method for ginsenoside assay

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SUMMARY

A method for the determination of ginsenosides using reversed-phase high-performance liquid chromatography is described. It is demonstrated that the column selectivity can be used as a parameter in developing new methods. A study of commercial columns all packed with octadecylsilane established that these columns differ in selectivity. The method can be used for assaying ginseng raw materials, capsules, tablets and multivitamin formulations. A validation of the method is described.

INTRODUCTION

The active constituents of ginseng (*Panax ginseng* C. A. Meyer) are a complex mixture of saponins often referred to as ginsenosides. More than 30 different ginsenosides are known, but this study included only six of the most thoroughly described (Fig. 1). In ginseng plants, the amount and the composition of ginsenosides present are highly dependent on whether it orginates from the main root, the root hairs or the leaves[1]. The composition may also vary with the species, the time of harvesting and the method of preparation[2,3]. These variations in raw materials are bound to be reflected in the products available on the market. It was concluded in an American study of ginseng products that about one third of the samples did not contain any detectable ginsenosides[4]. This emphasizes the need for a quality definition for the description of ginseng products. The situation today is that the consumers have no objective way of choosing a quality ginseng product. Most product labellings specify the content in milligram of extract or milligram of root, but this does not guarantee anything. However, the pharmacological effects of the pure ginsenosides have been studied on animals. The experiments revealed that ginsenoside Rg1 possessed CNS stimulation activity and showed an anti-fatigue effect. The ginsenoside Rb1 suppressed CNS activity and showed tranquillizing properties[5–7]. Consequently, a logical labelling would be the total amount of ginsenosides and, owing to the demonstrated pharmacological effects of Rg1 and Rb1, the ratio between Rg1 and Rb1 (Rg1/Rb1).

In the last 10 years, many attempts have been made to assay ginseng by high-performance liquid chromatography (HPLC) either in the normal-phase mode or

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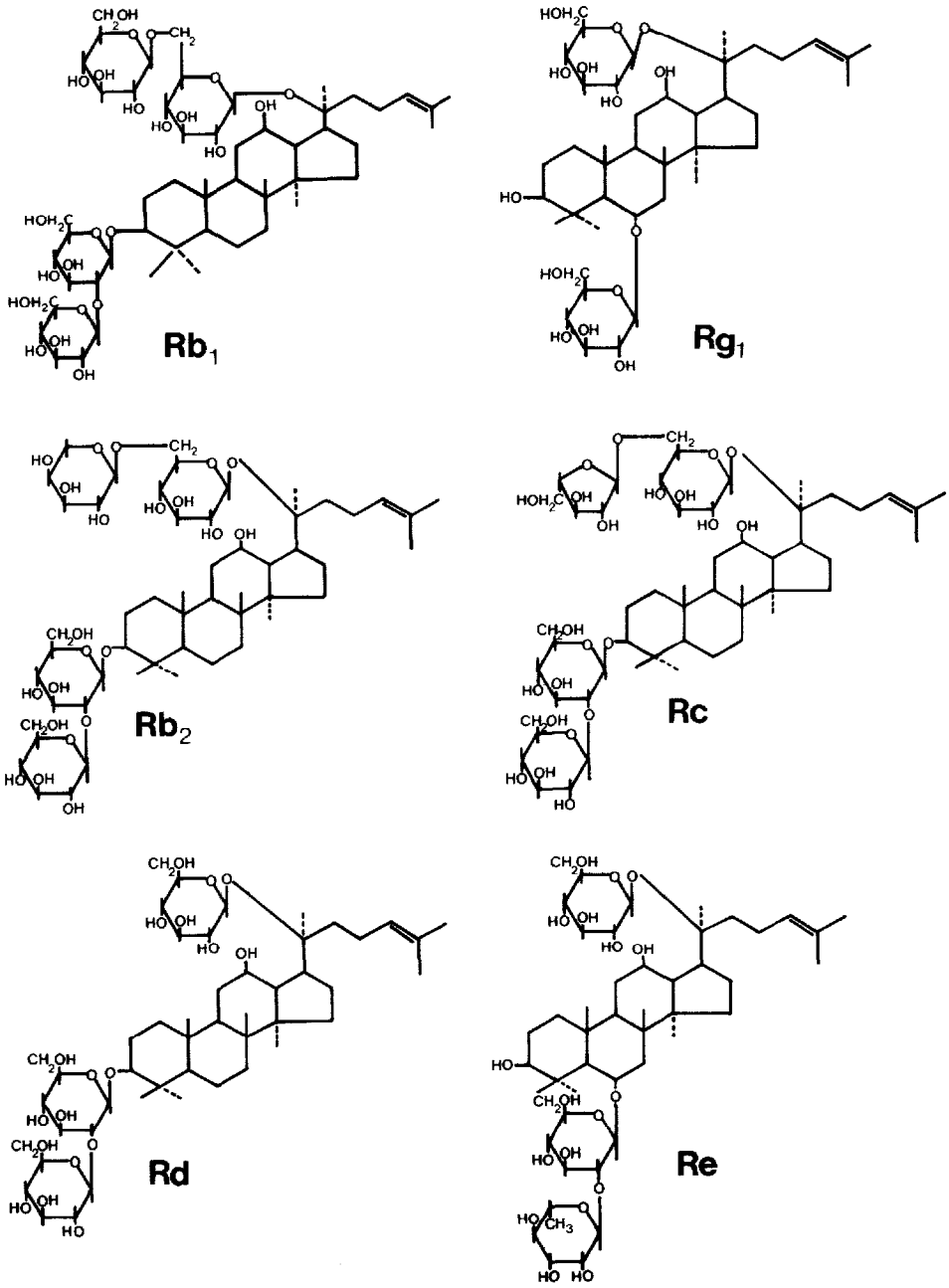

Fig. 1. The main ginsenosides, which according to their sapogenin (the aglycone part of the saponin), can be divided into two groups, the panaxatriols (Rg1 and Re) and the panaxadiols (Rb1, Rc, Rb2, Rd). The extra hydroxyl function at the panaxatriols makes them more polar than the panaxadiols. This is probably the explanation for the shorter retention times observed in reversed-phase systems for panaxatriols.

more recently in the reversed-phase mode. Previously methods such as spectrophotometry[8], gas chromatography (GC)[9] and thin-layer chromatography (TLC)[10] were employed, but with doubtful results. Spectrophotometric methods are insufficiently specific and often show large positive interferences, GC cannot be recommended for quantitative assays because of its poor reproducibility and TLC methods are too slow for routine analysis and must be regarded as antiquated. In one of the earliest HPLC methods, the saponins of ginseng were derivatized with benzoyl chloride in order to achieve greater UV sensitivity[11]. Another group of methods[12–16] uses two series of analyses, one mainly for separating Rg1 and Re and another for the remainder. More recently methods have appeared for determining all the ginsenosides using gradient elution[17–22]. However, most of them fail to separate Rg1 and Re, particularly in real samples.

Amino columns[23], ion-exchange columns[24] and columns packed with hydroxyapatite[19] have been tried, but to ensure the ruggedness of the method a C_{18} column seems to be the best choice.

In this paper we present a method in which ginsenosides are separated by a suitable reversed-phase C_{18} column and eluted with a two-step water–acetonitrile gradient with UV detection at 203 nm. The column selectivity was investigated by studying six different commericial columns under isocratic conditions.

EXPERIMENTAL

Materials

Acetonitrile was of HPLC grade (Merck, Darmstadt, F.R.G.). Water for use as a mobile phase constituent was prepared by passage through a Milli-Q water purification unit (Millipore, Bedford, MA, U.S.A.). Pure samples of ginsenosides (Rg1, Re, Rb1, Rc, Rb2 and Rd) were obtained from Sarsynthese (Merignac, France). Sep-Pak (C_{18}) cartridges were purchased from Waters Assoc. (Milford, MA, U.S.A.). The filters used were Minisart NML, pore size 0.8 μm (Sartorius, Göttingen, F.R.G.). Phosphate buffer was prepared by dissolving 3.532 g of potassium hydrogenphosphate (KH_2PO_4) (Merck) and 7.228 g of disodium hydrogenphosphate ($Na_2HPO_4 \cdot 2H_2O$) (Merck) in 1000 ml of water.

The liquid chromatograph consisted of two LC-6A pumps, a SIL-6A autoinjector, an SCL-6A system controller, an SPD-6A spectrophotometric detector and a C-R5A Chromatopac integrator, all purchased from Shimadzu (Kyoto, Japan). The detector was operated at 203 nm and its output signal was recorded by the integrator. The integrator was also used for chromatographic peak-area integration, peak-height measurement and calculations derived from those measurements.

Chromatographic conditions

The HPLC column was eluted at a constant flow-rate of 1.3 ml/min. The injection volume was set at 400 μl. The separations were obtained by gradient elution using the eluents (A) water and (B) acetonitrile according to the following profile: 0–20 min, 84–82% A, 16–18% B (curve 9); 20–55 min, 82–60% A, 18–40% B (curve 0).

The columns used were as follows: 1, μBondapak TM C_{18}, 10 μm (15.0 cm × 3.9 mm I.D.); 2, LiChrosorb RP-18, 5 μm (12.5 cm × 4.0 mm I.D.); 3, Nucleosil C_{18}, 5 μm (15.0 cm × 4.6 mm I.D.); 4, Spherisorb ODS, 5 μm (15.0 cm × 4.6 mm I.D.); 5,

Techopak C_{18}, 10 μm (15.0 cm × 3.9 mm I.D.); 6, Zorbax ODS, 7 μm (15.0 cm × 4.6 mm I.D.). Column 1 was purchased from Waters Assoc., 2 from Merck and 3–6 from HPLC Technology (Macclesfield, U.K.). In these experiments no guard columns were used to ensure that nothing but the column was creating the separations. In all other instances a suitable precolumn, LiChrosorb RP-18, 7 μm (3 cm × 4 mm I.D.), was used to protect the column. After each run a 10-min wash period consisting of 95% B at 2.7 ml/min and a 10-min stabilization period consisting of 16% B at 1.3 ml/min was introduced.

Sample preparation

Ginseng tablets. Accurately weigh tablet powder corresponding to 25 mg of ginsenosides into a 250-ml volumetric flask. Add 150 ml of phosphate buffer solution, shake at 35–40°C for 15 min, cool, dilute to volume with phosphate buffer solution and mix. Apply 10 ml of this solution to a Sep-Pak cartridge, prewashed with 5 ml of methanol and 5 ml of water. Wash the Sep-Pak cartridge with 10 ml of water followed by 15 ml of 30% methanol. Elute the ginsenosides into a 50-ml round-bottomed flask with 10 ml of methanol. Evaporate to dryness under vacuum at a maximum temperature of 50°C. Dissolve the residue in water to a final ginsenoside concentration of 0.1 mg/ml.

Capsules. Weigh into a centrifuge tube an accurate amount from capsules corresponding to 10 mg of ginsenosides. Rinse the substance by extraction with 3 × 10 ml of light petroleum. Dry the residue with nitrogen, suspend the residue in 50 ml of phosphate buffer and centrifuge. Use 5 ml in the Sep-Pak procedure described for tablets.

Fluids. Dilute a volume of sample corresponding to 100 mg of extract or 10 mg of ginsenosides to 50 ml with phosphate buffer and clean 5 ml of this solution by the Sep-Pak procedure.

Standard solution. Weigh an accurate amount of standard extract corresponding to 10 mg of ginsenosides in a 100-ml volumetric flask. Dissolve in phosphate buffer solution and dilute to volume. If necessary a short warming period of 5 min to a temperature of 40°C could be accomplished before dilution to volume. Apply 10 ml of this final solution in the Sep-Pak procedure.

The validity of the Sep-Pak procedure was checked by injecting the solution not applied in the Sep-Pak procedure and compared it with that cleaned by the Sep-Pak procedure. The standard extract was standardized by the use of the pure ginseng standards. Before injecting sample or standard solutions into the liquid chromatograph, all solutions were filtered through a 0.8-μm pore size filter.

RESULTS AND DISCUSSION

The results from testing the six types of columns are summarized in Tables I and II. From these tables and the chromatograms shown in Fig. 2 it can be seen that the columns differ in selectivity, efficiency and separation of impurities.

The selectivity α can be affected by the mobile phase and by the column packing material. The effects of the stationary and mobile phases on selectivity are not directly related, but we cannot expect to differentiate the column selectivity from the solvent

TABLE I

COLUMN SELECTIVITY UNDER ISOCRATIC CONDITIONS (21% ACETONITRILE, 1.3 ml/min)

t_0 = Time in minutes for unretained molecules to move from the point of injection to the detector; k'_{Rg1} = capacity factor for ginsenoside Rg1; k'_{Re} = capacity factor for ginsenoside Re; α = selectivity ($\alpha = k'_{Re}/k'_{Rg1}$).

Parameter	*μBondapak*	*LiChrosorb*	*Nucleosil*	*Spherisorb*	*Techopak*	*Zorbax*
t_0	0.793	0.628	0.935	0.880	0.896	0.818
k'_{Rg1}	16.18	21.68	21.06	21.25	15.09	13.51
k'_{Re}	18.79	24.46	23.48	24.67	17.17	14.38
α	1.16	1.13	1.11	1.16	1.14	1.06

selectivity in the described system. The solvent selectivity cannot be considered to be constant for several reasons. The concentration profile through the column will be a function of the column dimensions and the packing material. The packing material affects the mobile phase in a number of ways. Eddy diffusion is caused by velocity differences between various paths that the solvent will follow during passage through the porous bed. Film resistance close to the surfaces of the particles exists for laminar flow. In the pores of the particles, regions with stagnant solvent will be present. All this results in a certain degree of back-mixing. As a consequence of the different column dimensions and packing materials, none of the six columns tested were actually exposed to the same elution profile. A reasonable way to find an expression for the column selectivity is to test the columns under isocratic conditions. This will make the mobile phase composition constant and eliminate all solvent selectivity variations.

We chose to carry out an isocratic test with acetonitrile–water (21:79) as the mobile phase. The sample was a solution consisting of a mixture of Rg1 and Re standard. The column void time is an important factor in calculating the selectivity. The void time was carefully examined by injecting unretained molecules. For this

TABLE II

ESSENTIAL DATA FOR COLUMN PERFORMANCE

N = Number of theoretical plates calculated for Rg1 in the isocratic test by the equation $N = 5.54 (t_{Rg1}/W_{1/2})^2$; α = separation selectivity between Rg1 and Re in the isocratic test; R = resoluton between Rg1 and Re calculated assuming that the peaks in the gradient elution system were isosceles triangles.

Column	*N*	*α*	*R*	*Separation of impurity a (Fig. 2) from Rb1*	*Separation of impurity b (Fig. 2) from Rb2*
μBondapak	1500	1.16	0.9	Poor	Poor
LiChrosorb	3600	1.13	1.4	Usable	Good
Nucleosil	4800	1.11	1.0	Good	Good
Spherisorb	4100	1.16	1.1	Usable	Usable
Techopak	1700	1.14	1.0	Poor	Poor
Zorbax	3300	1.06	0.8	Poor	Good

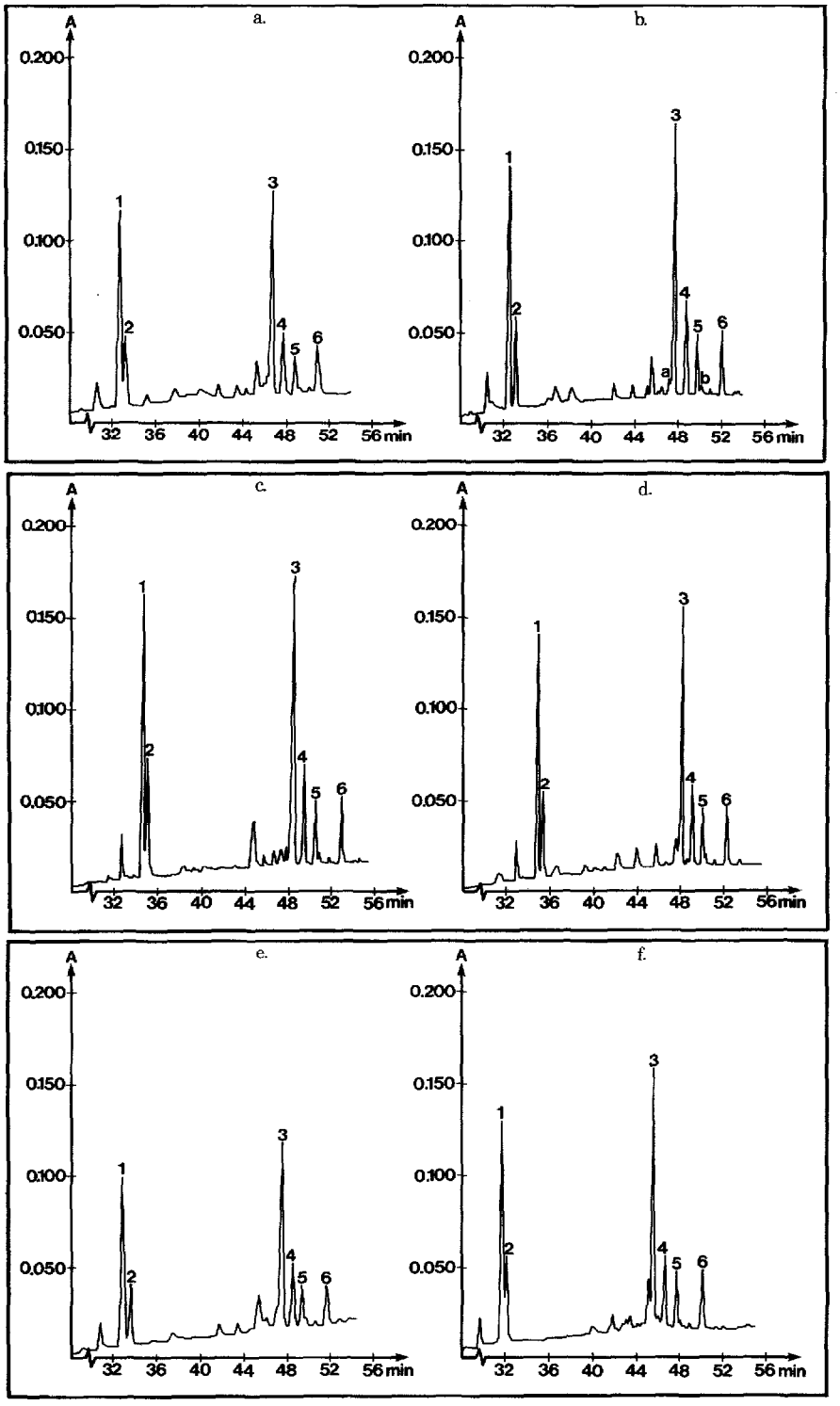

Fig. 2. Chromatograms for the performance of the columns in the gradient elution system. Peaks: 1 = Rg1; 2 = Re; 3 = Rb1; 4 = Rc; 5 = Rb2; 6 = Rd. The letters a and b in (b) refer to impurities often present close to Rb1 and Rb2, respectively. (a) μBondapak; (b) LiChrosorb; (c) Nucleosil; (d) Sperisorb; (e) Techopak; (f) Zorbax.

purpose, we used a sample of sodium nitrite as recommended[25]. The response was measured at both 355 and 260 nm. The peak-height ratio was then compared with that obtained for sodium nitrite solution measured at the same two wavelengths with an ordinary spectrophotometer. The results in Table I show pronounced differences in the values of the selectivity α. These differences must reflect the impact of the column on the selectivity.

To make it clear why the selectivity is so important, we can consider the approximate expression for the resolution:

$$R_s = 1/4[(\alpha - 1)/\alpha][k'/(1 + k')]\sqrt{N}$$

To improve the resolution, columns with better efficiency are not a very powerful tool, beause the resolution is a function of the square root of the number of theoretical plates. The resolution is far more sensitive to changes in selectivity, especially when α becomes close to unity. In Table II, the most important factors are summarized in order to judge which column should be used. The resolution between Rg1 and Re, actually obtained in the gradient elution system does not seem to fit perfectly with the data for efficiency and selectivity obtained in the isocratic column test. In the gradient elution system the selectivity is also affected by the solvent selectivity. Variations in solvent selectivity in the gradient elution test of the columns must be a result of different elution profiles. Changes in elution profiles can be caused either by different column dimensions or differences in the packing material. Columns 3 and 4 have the same dimensions and size of packing material, but the values for the resolution in Table II suggest that there is a difference in solvent selectivity. This change in solvent selectivity, which must be related to the nature of the packing material, also seems to occur if we compare columns 1 and 5. In gradient elution systems, changes in the elution profiles affect the solvent selectivity, and because the resolution is a function of the selectivity it is not possible to predict from an isocratic column selectivity test which column under the gradient elution conditions will give the best resolution. However, in general, the column with the best selectivity in an isocratic test is to be preferred.

From the values for the column selectivity and the number of theoretical plates in Table II, the best resolution should be achieved with column 4. Instead, we see that column 2 gives a better resolution. This means that for column 4 an even better resolution of Rg1 and Re should be possible by using another elution profile.

The resolution of Rg1 and Re is not the only aspect to be considered in judging the suitability of the columns for the ginsenoside assay. Often impurities near Rb1 and Rb2 can be observed in the chromatograms. These impurities might well be ginsenosides. Table II includes a qualitative grading of the ability of the columns to separate these impurities. Column 3 is the best for this purpose, but column 2 is almost as good.

Summarizing all this information, column 2 is the preferred choice, and this is also confirmed by the visual impression of the chromatograms in Fig. 2.

Method validation

Identification. The ginsenosides in the samples were identified by comparing the retention times of pure ginsenoside standards with the retention times obtained in the sample chromatograms. Further, the elution order of the ginsenosides was the same as reported with other reversed-phase methods.

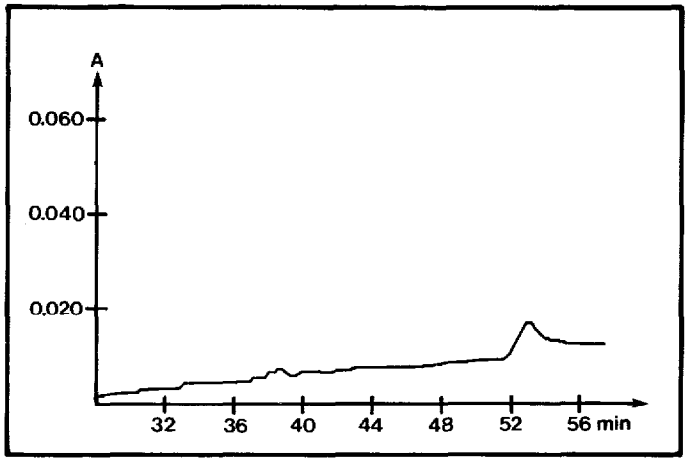

Fig. 3. Background chromatogram for the gradient elution system. The sample was a tablet containing vitamins and minerals, but no ginseng, degraded under accelerated conditions. Column: LiChrosorb RP-18, 5 μm (12.5 cm × 4.0 mm I.D.).

Specificity. Ginseng placebo tablets containing vitamins and minerals were checked for chromatographic background. The tests were performed on both fresh placebo and placebo degraded under accelerated conditions (6 months at 40°C and 75% relative humidity). No chromatographic interference was observed (Fig. 3).

Detection limit. At a 2:1 signal-to-noise ratio, the limit of detection was determined to be 20 ng/ml for all six ginsenosides. This is equivalent to 0.1% of the nominal concentration of 20 μg/ml. The low detection limit was a consequence of the large injection volume of 400 μl. A much smaller volume would not have ensured an adequate distance from the detection limit.

Precision. The values of the relative standard deviation for the six ginsenosides were found to be in the range 2.4–4.6%. For the total ginsenoside content the

TABLE III

DATA FOR LEAST-SQUARES REGRESSION ANALYSIS OF THE CALIBRATION GRAPH: $Y = SX + I$

Y = Response in mV s; X = concentration in μg/ml; S = slope; I = intercept; C = correlation coefficient; SDS = estimate of standard deviation of slope; SDI = estimate of standard deviation of intercept; SDM = estimate of standard deviation of single measurement.

Parameter	*Rg1*	*Re*	*Rb1*	*Rc*	*Rb2*	*Rd*
S	82.1	15.9	61.9	54.1	45.2	69.8
I	34.1	−5.9	−25.3	−10.4	−14.1	−56.3
C	0.9998	0.9994	0.9998	0.9998	0.9997	0.9994
SDS	0.72	0.25	0.57	0.53	0.47	1.07
SDI	30.5	9.6	23.1	21.6	18.7	43.6
SDM	53.9	16.9	40.8	38.2	32.9	77.0

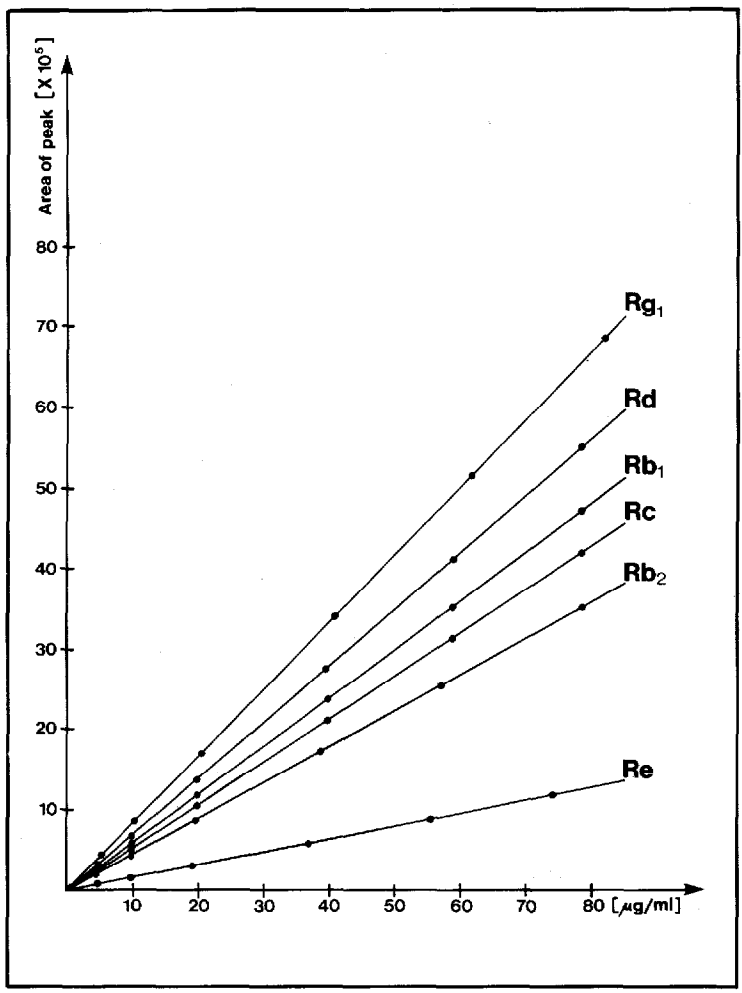

Fig. 4. Calibration graphs for the ginsenosides under gradient elution conditions. The units on the ordinate are $\mu V \cdot s$. Column as in Fig. 3.

reproducibility of the method was calculated by assaying eight replicates of the same batch. In this instance the relative standard deviation was estimated to be 2.7%.

Linearity. The linearity of the response *vs.* concentration curve for each of the six ginsenosides was investigated in the range 0–80 μg/ml. Regression analysis data are given in Table III and the calibration graphs for the ginsenosides are shown in Fig. 4. The calibration graphs had correlation coefficients very close to unity, and the intercepts were not statistically significantly different from zero at the 95% confidence level.

Accuracy. The accuracy of the Sep-Pak procedure was checked by comparing the results obtained from extract solutions applied in the Sep-Pak procedure with those from the same solutions not applied in the Sep-Pak procedure. Setting the result for

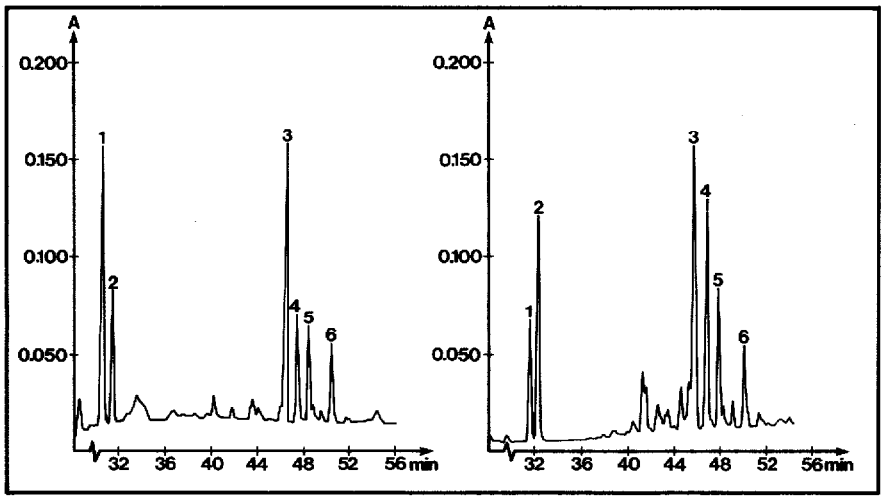

Fig. 5. Left: chromatogram obtained from a sample of Ginozin Combi (trade-name for a Ferrosan, Soeborg, Denmark, tablet containing 2 mg of ginsenosides, eleven vitamins and eight minerals). Right: chromatogram for a sample of Gericomplex (trade-name for a capsule produced by Pharmaton, Lugano, Switzerland, containing ginseng extract corresponding to 200 mg of root of *Panax ginseng* C. A. Meyer, ten vitamins and six minerals). The two chromatograms illustrate well that the profiles of ginsenosides in various products can be very different. Both analyses were performed with the gradient elution programme. Column as in Fig. 3. Peak numbers as in Fig. 2.

total ginsenosides for the extract solution not applied in the Sep-Pak procedure to 100%, the recovery for the Sep-Pak procedure was found to be 98.9% with calculated relative standard deviation of 4.2% (n = 41).

The accuracy of the method, including the Sep-Pak procedure, was substantiated by assaying production samples of ginseng tablets. The recovery was found to be 98.4% of theory for Ferrosan products, with a standard deviation of 5.0% (n = 7).

CONCLUSION

This method for assaying ginsenosides has the advantage of dealing with all the ginsenosides in question in a single run, with a good separation of all the compounds. Especially the resolution of Rg1 and Re, which has been a major drawback in many earlier methods, is acceptable (Fig. 5). The results emphasize that it is important which type of C_{18} column is chosen. The six columns tested here showed considerable differences in column selectivity. In developing new methods this fact can be utilized and should be thought of as a possible optimizing parameter together with the solvent selectivity and the number of theoretical plates.

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