ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
21 (1999) 187–192

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Short communication

Quantitative determination of ginsenosides from *Panax ginseng* roots and ginseng preparations by thin layer chromatography–densitometry

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Received 8 September 1998; received in revised form 11 December 1998; accepted 1 January 1999

Keywords: *Panax ginseng*; Ginsenosides; TLC–densitometry; Quantitative determination; Validation

1. Introduction

The major constituents and up to now the main active principles of the root of *Panax ginseng* C.A. Meyer are triterpene saponins, named ginsenosides. They are derived from the tetracyclic dammarane skeleton and are divided into two groups: (1) the ginsenosides of the Rb_1 group (or the protopanaxadiols) with 20*S*-protopanaxadiol as aglycone consists, besides ginsenoside Rb_1, mainly of ginsenoside Ra, Rb_2, Rc and Rd; (2) the ginsenosides of the Rg_1 group (or the protopanaxatriols) with 20*S*-protopanaxatriol as aglycone consists, besides ginsenoside Rg_1, mainly of ginsenoside Re, Rf and Rg_2. The roots also contain saponins derived from β-amyrine, namely ginsenoside Ro, a triglycoside of oleanolic acid. The total ginsenoside content of a 6-year-old main root varies between 0.7 and 3%. The lateral roots can contain two to three times more saponins than the main root while the slender roots can even contain up to 10 times more [1–4].

The pharmacological properties of ginsenoside Rb_1 and Rg_1 are different and sometimes antagonistic. Ginsenoside Rb_1 exhibits sedative, anticonvulsive, analgetic, antipyretic, anti-inflammatory and antipsychotic activities and it improves the gastro-intestinal motility. Ginsenoside Rg_1 shows a stimulating and antifatigue effect, and it enhances the motoric activity [5]. Therefore, it was worthwhile to use a quantitative method that can distinguish the protopanaxadiols from the protopanaxatriols.

The quantitative determination of ginsenosides could be carried out by TLC, HPLC, GLC or colorimetry. Colorimetric methods, based on the reaction of the triterpene skeleton with mineral acids, were not specific and only allowed the determination of the total ginsenoside content [4]. For GC determinations, the ginsenosides had to be hydrolyzed and derivatized. These methods

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PII: S0731-7085(99)00109-0

were cumbersome and not suitable for stability tests of pharmaceutical preparations [6,7].

HPLC analysis is usually performed on a reversed phase column. With an isocratic mobile phase system, two different methods were necessary for the determination of the panaxadiol and panaxatriol derivatives [8,9].

In a gradient system all the ginsenosides could be analyzed simultaneously but a long run time was needed for a good separation of all ginsenosides. The existing HPLC methods lack sensitivity due to the low UV maximum (203 nm) and extinction of the ginsenosides [10–12]. At 203 nm, a lot of compounds, solvents, oxygen, etc. absorb UV light, which causes drifting of the baseline.

For the quantitative determination of ginsenosides in Ginseng roots and preparations thereoff, a TLC-densitometric assay was developed which allows the simultaneous analysis of different samples in a quick, sensitive and reproducible way. The method was successfully validated for Ginseng roots and a Ginseng dry extract according to up-to-date guidelines. The method is linear and accurate ($99.6 \pm 2.4\%$ recovery) in the concentration range tested. The limit of detection for the ginsenosides is about 10 ng/spot. The method showed a precision of 2.73% for the Rb_1-group, 4.59% for the Rg_1-group and 2.88% for the determination of the total ginsenosides in Ginseng roots. For the dry extract, values of 2.41, 3.45 and 2.36% respectively were found.

2. Experimental

2.1. Chemicals

All solvents were HPLC grade from Labscan (N.V. de Bournonville, Belgium).

p-Anisaldehyde was from Sigma (Bornem, Belgium), sulfuric acid 98–97% p.a. and acetic acid glacial 100% p.a. were from Merck (Belgolabo, Overijse, Belgium). The ginsenoside standards were obtained from Roth (Polylabo, Antwerp, Belgium).

The C_{18ec} SPE columns (500 mg/6 ml) were from Machery-Nagel (Filter Service, Eupen, Belgium). The HPTLC Silica gels F_{254} (0.2-mm layer thickness, 10×20 cm) were obtained from Merck, and prewashed in the TLC solvent system after which they were dried at 120°C for 30 min.

2.2. Equipment

All solutions were spotted with a Desaga TLC-applicator AS 30 (4 mm width). A Desaga densitometer CD60 was used to scan the plates at 535 nm (slit: 0.1×3 mm; remission mode). The plates were sprayed with a Merck TLC-sprayer.

2.3. TLC conditions

2.3.1. Detection reagent

p-Anisaldehyde (0.5 ml) was successively mixed with 10 ml glacial acetic acid, 85 ml methanol (MeOH) and 5 ml sulfuric acid.

2.3.2. TLC assay

According to the ginsenoside concentration 1–2 μl of the sample solution were applied in duplicate. Of the standard solution 2, 3, 4 and 5 μl were spotted in duplicate (15 mm from the edge). Plates were developed to 6 cm in chloroform–ethyl acetate–methanol–water (15:40:22:9, v/v/v/v), dried, sprayed and heated at 120°C for about 5 min. The TLC plate was covered with a glass plate and the remission absorption was measured at 535 mm.

Using the data-pair technique the protopanaxadiols (Ra, Rb_1, Rb_2, Rc, Rd) were calculated as Rb_1 and the protopanaxatriols (Re, Rf, Rg_1, Rg_2) as Rg_1.

The following Rf values were obtained: Ra 0.25, Rb_1 0.30, Rb_2 0.35, Rc 0.39, Re 0.43, Rd 0.49, Rg_1 0.53, Rf 0.56, Rg_2 0.60.

2.4. Sample preparation

2.4.1. Standard solutions

Stock solutions for Rb_1 and Rg_1 were prepared in MeOH at a concentration of 400 and 250 μg/ml, respectively.

Standard solution was prepared by a 10-fold dilution of the stock solutions.

2.4.2. Sample preparation

2.4.2.1. Roots and root preparations. Pulverized roots (5 g) or an equivalent amount, accurately weighted, were refluxed three times with 60 ml MeOH 70% (v/v) during 30 min. The combined extracts were evaporated to about 50 ml and diluted to 100.0 ml with water. A 5.0-ml aliquot of this solution was brought onto a C_{18ec} SPE column (prewashed with 10 ml MeOH and 10 ml water). After washing with 10 ml water and 10 ml MeOH 30% (v/v), the ginsenosides were eluted with 20 ml MeOH and diluted to 25.0 ml with MeOH.

2.4.2.2. Liquid extracts, dry extracts and corresponding preparations. These were dissolved in water in a concentration equivalent to about 1.5 mg/ml total ginsenosides and worked up as in Section 2.4.2.1.

3. Results and discussion

Different TLC solvent systems have been described in the literature [1,4]. A solvent system based on that of Xie and Yan [13] was selected because not only could the protopanaxadiols and protopanaxatriols be simultaneously determined, but also the different ginsenosides in each group were well separated. The disadvantage of this method is that it is very cumbersome: the TLC plate is pre-equilibrated at 47% relative humidity, the mobile phase (chloroform–ethyl acetate–methanol–water (15:40:22:9, v/v/v/v)) has to equilibrate overnight at 10°C and the lower phase is used. For detection the plate is dipped into 5% sulfuric acid in ethanol, heated at 105°C for 1 min, and then dipped into liquid paraffin/hexane (1:2) and detected under UV 366 nm. Compared with this method the one presented here is quick

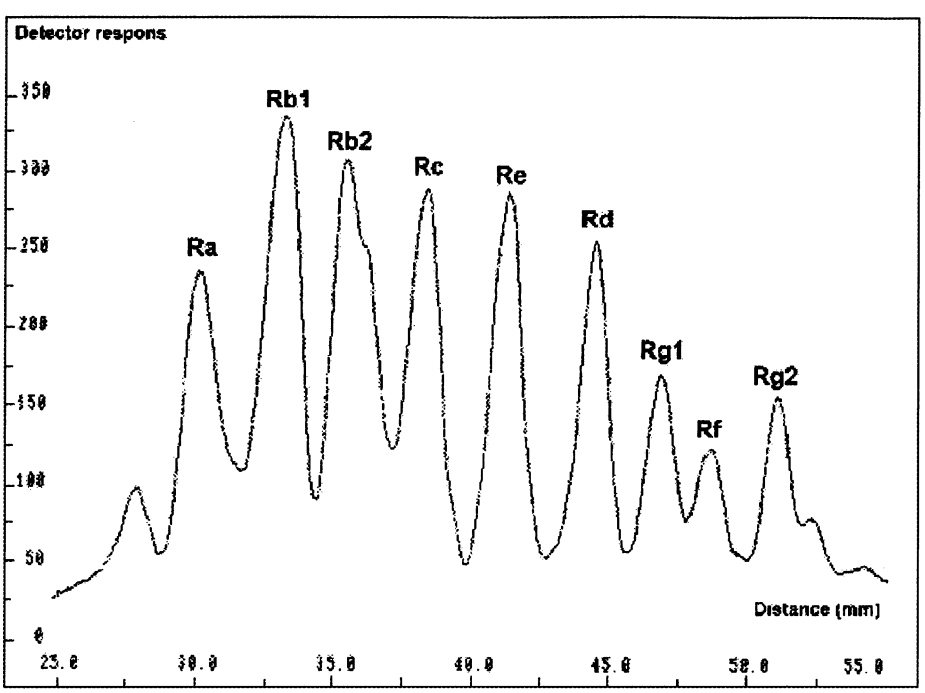

Fig. 1. Chromatogram of a Ginseng dry extract.

Table 1
Linearity[a]

Parameter	Standard		Dry extract			
	Rb_1	Rg_1	$Ra+Rb_1+Rb_2+Rc$	Re	Rd	$Rg_1+Rf+Rg_2$
$a \pm s_a$	52.71 ± 8.08	42.13 ± 10.86	184.57 ± 38.65	37.10 ± 5.82	26.90 ± 6.38	39.15 ± 8.96
$b \pm s_b$	2.507 ± 0.047	2.63 ± 0.075	69.71 ± 2.12	11.37 ± 0.32	9.12 ± 0.35	14.01 ± 0.49
r	0.9990	0.997	0.997	0.998	0.996	0.996
VC (%)	1.2	2.0	1.2	1.6	2	1.9
ANOVA	NS	NS	NS	NS	NS	NS

[a] s_a, standard error of a; s_b, standard error of b; r, correlation coefficient; VC, variation coefficient of the residuals; ANOVA, analysis of variance according to Ref. [14]; NS, not significant.

and straightforward, and a complete validation was carried out.

A typical chromatogram of a ginseng dry extract is shown in Fig. 1.

3.1. Method validation

3.1.1. Linearity

Notwithstanding the linearity could be verified for each analysis because a series of standards was always applied from which the calibration function could be calculated, the linearity was examined by applying different standards four times (Rb_1: 92.6–231.5 ng/spot; Rg_1: 78.2–195.5 ng/spot) or four different solutions of ginseng dry extract (11.82–23.64 μg extract/spot). The calibration curve, area versus concentration, was calculated according to the least squares method ($y = a + bx$) for the different ginsenosides. The results of the evaluation of the linearity are presented in Table 1.

3.1.2. Detection limit (LOD)

The detection limit calculated according to Kaiser ($LOD = 3 \times Se/b$, with Se being the standard deviation of the residuals and b the slope of the calibration curve) was 7 ng/spot for Rb_1 and 9 ng/spot for Rg_1 [15]. By analysing a blank and measuring the maximum height (h_{max}) of this signal over a distance of 20 times the width of the standard peak at half height, the detection limit could be calculated as follows: $LOD = 3 \times h_{max} \times R$, with R being the response factor: quantity/signal (expressed as height) [16]. For both Rb_1 and Rg_1, this resulted in a LOD of about 10 ng/spot.

3.1.3. Precision

Since four different samples could be applied on one plate, the analysis was repeated on four different days. In this way, 16 values were obtained to calculate the intermediate precision [17] in addition to the repeatability [18]. Normally, the protopanaxadiol derivatives were calculated as a group and expressed as Rb_1 and the protopanaxatriols as Rg_1, as shown in Table 2 for the results of ginseng root. For the ginseng dry extract (Table 3), the concentration of all the ginsenosides was given separately and as a group. These results showed that the standard deviations for the individual components were much greater than those for the groups and the total content. This was due to the inferior separation within the groups in comparison to the separation between the groups. A single factor (ANOVA, $\alpha = 0.05$) showed that there was no significant difference between the results on different days for the Rb_1 group, the Rg_1 group and the total ginsenosides.

Table 2
Precision, Ginseng roots

Parameter	Rb_1 group	Rg_1 group	Total
Concentration (% w/w)	3.663	0.915	4.578
Repeatability (VC[a])	2.73	4.59	2.88
Intermediate precision (VC)	2.73	4.59	2.88
ANOVA[b]			
F ($F_{critical} = 3.490$)	0.492	0.577	0.497
P-value	0.72	0.58	0.69

[a] VC, variation coefficient.
[b] ANOVA, analysis of variance according to Ref. [14].

Table 3
Precision, Ginseng dry extract

Parameter	Ra	Rb_1	Rb_2	Rc	Rd	Rb_1 group	Re	Rf	Rg_1	Rg_2	Rg_1 group	Total
Concentration (% w/w)	0.624	1.123	0.877	0.775	0.474	3.873	0.601	0.142	0.240	0.284	1.267	5.140
Repeatability (VC[a])	4.22	3.45	5.59	3.38	4.27	2.41	3.60	5.04	5.16	4.95	3.45	2.36
Intermediate precision (VC)	4.22	6.26	8.22	3.71	4.27	2.41	3.60	5.04	5.16	4.95	3.45	2.36
ANOVA[b]												
F ($F_{critical} = 3.490$)	0.03	10.15	5.63	1.82	0.95	0.48	0.33	0.98	0.18	0.38	0.062	0.12
P-value	0.97	0.0049	0.026	0.22	0.42	0.63	0.72	0.41	0.84	0.69	0.94	0.89

[a] VC, variation coefficient.
[b] ANOVA, analysis of variance according to Ref. [14].

3.1.4. Accuracy

Accuracy was examined by analyzing eight times a test mixture of seven ginsenosides (Rb_1, Rb_2, Rc, Rd, Re, Rf and Rg_1). The recovery was $99.6 \pm 2.4\%$ ($t = 0.47 < t_{(0.05;\ 7)} = 2.365$) for the Rb_1 group and $99.1 \pm 2.9\%$ ($t = 0.88 < t_{(0.05;\ 7)} = 2.365$) for the Rg_1 group. The extraction efficiency was investigated by repeating the extraction a fourth time. The yield of ginsenosides in this extract was only 0.4% of the ginsenoside content obtained after the first three successive extractions.

4. Conclusion

The newly developed TLC–densitometric method could be used for the quantitative determination of protopanaxadiols, protopanaxatriols and total ginsenosides. The method was thoroughly validated on ginseng root and ginseng dry extract. Besides the quantitative analysis of the ginsenoside groups, the method could also be used for the fingerprinting of the different ginsenosides in e.g. pharmaceutical stability studies.

The described TLC-densitometric method is selective compared to the colorimetric method, is quick and straightforward with regard to GC and is much more sensitive than the existing HPLC-methods.

Acknowledgements

S. Apers is a research assistant of the Fund for Scientific Research (FWO, Flanders, Belgium).

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