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High-performance thin-layer chromatographic determination of six major ginsenosides in *Panax ginseng*

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Abstract

A densitometric determination of six major ginsenosides in *Panax ginseng*, separated by high-performance thin-layer chromatography (HPTLC), was optimized. Simple extraction and clean-up methods of the target constituents and the development of standardized conditions of chromatoplates with a quaternary-solvents system allowed an efficient saponins recovery from the plant material and their selective separation. After exposure of the chromatograms to thionyl chloride vapors and further heating, stable reaction products of ginsenosides, which showed absorption maxima at λ=275 nm as well as a fluorescence ($\lambda_{excitation}$=366 nm, $\lambda_{emission}$=400 nm), allowed the application of a sensitive and reproducible method for their simultaneous determination. The method was validated by spiking the ginseng extracts with pure standards.

Keywords: *Panax ginseng*; Triterpenoids; Ginsenosides

1. Introduction

According to its use in traditional Asian medicine and to its later introduction into occidental phytotherapy, Ginseng root has been at the origin of an abundance of scientific literature [1,2]. The drug content in triterpene glycosides of the damarane type, the ginsenosides, is considered at least partially responsible for its bioactivity. About 30 of these saponins have been isolated and identified. However, the individual pharmacological activities of ginsenosides Rb_1 and Rg_1 only have been clearly established. Therefore, most analytical studies on Ginseng standardization are today restricted to the identification and to the determination of the six major and commercially available ginsenosides Rb_1, Rb_2, Rc, Rd, Re and Rg_1. In addition to immunoassays which have been recently used for the determination of several ginsenosides [3,4], various applications of chromatographic methods have been devoted to such analysis. Gas chromatography (GC) features limit the drug analysis to the determination of (20*S*)-panaxadiol, (20*S*)-protopanaxatriol and oleanolic acid, the tree major sapogenins forming the ginsenosides [5]. The application of both high-performance liquid chromatography (HPLC) [6–10] and thin-layer chromatography (TLC) or high-performance thin-layer chromatography (HPTLC) is hindered by the poor sensitivity and specificity of the detection of ginsenosides whose electronic spectra show no characteristic absorption above 215 nm.

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This inconvenient has been recently limited by coupling HPLC to a MS detector [11,12] and to an evaporative light scattering detector [13]. The examination of numerous studies on the TLC detection of ginsenosides indicates that this step is generally achieved using sulphuric acid or mixtures of sulphuric acid with aromatic aldehydes such as vanillin or anisaldehyde [14–19]. In our experience, these chromogenic reagents always afford unstable colorations which limit the reproducibility and the accuracy of the final TLC determinations.

In this report, we describe the optimization of the chromatographic conditions useful for the ginsenosides TLC determination, in particular the use of thionyl chloride as the chromogenic reagent.

2. Experimental

2.1. Reagents and standards solution

The ginsenosides were supplied by Extrasynthèse (Lyon, France), and ginsenoside Rg_2 was a gift from Pharmaton (Lugano, Switzerland). All the other chemicals were of analytical-reagent grade and HPTLC plates (silicagel $60F_{254}$ and Lichrospher®) were obtained from Merck (Darmstadt, Germany).

About 4.00 mg, exactly weighted, of each ginsenoside Rb_1, Rb_2, Rc, Rd, Re and Rg_1 were dissolved together in 15 ml of methanol. This solution was fractionated and stored at −18°C in sealed vials.

2.2. Extraction and clean-up of the extracts

A 1.000-g sample of the powdered root of *Panax ginseng* C.A. Mayer or *Panax quinquefolius* L. (sieve 355) was weighted in a cellulose cartridge and extracted in a Soxtec HT2 apparatus (Tecator, Höganäs, Sweden) with 95 ml of dichloromethane (boiling period: 60 min; rinsing period 15 min; drying period: 45 min). The dichloromethane extract was discarded. The ginsenosides were then extracted from the powder during a second step, in the same cartridge, with 95 ml of 96% ethanol (boiling period: 90 min, rinsing period 90 min). The ethanol extract was evaporated to dryness under reduced pressure. The residue was added to 25 ml of water and sonicated for 5 min. The resulting aqueous solution or suspension was introduced into a separating funnel and extracted three times with 8 ml of 1-butanol; a centrifugation step may be required. The butanol extracts were combined and adjusted to 50 ml with methanol.

The ginsenosides isolation from *Panax ginseng* involved either liquid or dry extracts, according to their composition, ethanol distillation under reduced pressure and, after dissolution in water or dilution with water, the partition with 1-butanol.

2.3. HPTLC

The standard and tests solutions were applied to the HPTLC silicagel plate (Merck, Darmstadt, Germany) at 12 mm of the lower edge using an automatic TLC Sampler III apparatus (Camag, Muttenz, Switzerland) as bands of 10 mm length. Samples of 0.4, 1.1, 1.8 and 2.5 μl of the standards mixture solution, each spotted in duplicate, and 2 μl of the test solution spotted in quadruplicate were applied. This last volume value could be modified according to the ginsenosides concentration in the drug.

The plates were placed in a 20×20 cm TLC tank containing about 110 ml of the mobile phase prepared by mixing 1,2-dichloroethane–100% ethanol–methanol–water, 56.8:19.2:19.2:4.80 (v/v%) and developed at 4°C over a path of 10 cm. The tank and the mobile phase were maintained at 4°C for at least 30 min before chromatography and the mobile phase was added just before the chromatography (unsaturated conditions); the development time ranged from 70 to 75 min. After chromatography, the plates were heated at 50°C on a heating plate for 20 min. The chromatograms were then exposed at room temperature for 30 min to vapors of thionyl chloride, which had been introduced into a covered glass dish (about 25×23 cm) preliminary saturated for 20 min with the reagent. The reagent volume (about 50 ml) was adjusted to obtain the complete covering of the dish bottom. Two glass rods positioned in the dish at each end of the chromatoplate maintained the thin-layer at about 6 mm of the reagent surface. The chromatoplates were then heated at 110°C for 30 min on a heating plate and, finally, left to stand at ambient

temperature for at least 30 min before the densitometric measurements.

The ginsenosides (R_F values: Rb_1=0.34, Rb_2=0.40, Rc=0.45, Re=0.53, Rd=0.60 and Rg_1=0.67) were quantified by UV absorbance at 275 nm in the reflectance mode using a densitometer (TLC Scanner III controlled by CATS 3 software, Camag, Muttenz, Switzerland). The parameters used were: deuterium lamp; optical slit dimension: 5×0.10 mm; scanning speed: 5 mm s^{-1}. Alternatively, the chromatograms were scanned using fluorescence–reflection detection in the following conditions: monochromator wavelength of 366 nm (mercury lamp) and a K400 cut off filter without modification of the other settings. The calculations were based on the area determination of ginsenosides peaks separated from the four external standards mixtures and from the test solution using a second order polynomial regression.

2.4. Calibration studies

Calibration curves were determined using seven ginsenosides concentrations over the range 0.5–5.0 μg, each applied in duplicate and 12 ginsenosides concentrations over the range 0.15–0.75 μg, applied once on the HPTLC plates. Quantification was achieved either by absorbance or by fluorescence.

2.5. Determination of the limit of detection (LOD) and of quantification (LOQ)

These parameters were calculated using a Validation Method Manager (v. 1.6.2., Merck-Clevenot, France). The noise levels were determined with the CATS 4.3 software.

2.6. Determination of the inter-assay precision

The precision was determined from the standard deviation of six assay runs carried out by two analysts working independently (two assays per day) achieved with the same Ginseng sample extract (absorbance measurements).

2.7. Determination of the accuracy

This parameter was determined on the basis of the recovery. A ginseng extract solution was added to the ginsenosides standards solution to give 1:1, 3:1 and 1:3 final ratios and the recovery of each ginsenoside was determined (absorbance measurements).

3. Results and discussion

The extraction and clean-up efficiencies were checked at each step of the method by determination of the target constituents by the proposed method. It could be assumed that the ginsenosides content was extracted from the powder by ethanol and from the crude extractive solution by 1-butanol without degradation and with a yield higher than 98%.

The chromatographic conditions selection was based on the results of our previous study [17]. The development length (10 cm) was higher than that usually recommended for HPTLC plates in order to reduce the bands overlapping of the very complex chromatogram. The mobile phase composition was slightly modified and the solutions were applied as bands at 12 mm from the lower edge to improve the ginsenosides separation. One demixion solvent front was observed on the plates at R_F 0.85 but no demixing of the mobile phase occured at 4°C. In comparison with the results obtained with most other mobile phases, the resolution between ginsenosides Rd and Re was markedly increased and, as previously observed [17], an inversion of the R_F values of ginsenosides Rd and Re occurred. No resolution improvement was reached when the HPTLC plates were replaced with the more recently commercially available Lichrospher® plates. Resolution variations of the ginsenosides were observed with the selected quaternary-solvents system. This variability was related to the temperature development conditions and the development was therefore achieved at 4°C. These conditions highly improved the reproducibility of the ginsenosides separations; the C.V.% of the R_F values ranged from 1.31 to 2.03 as determined from the R_F measurements from ten different chromatograms obtained over a period of 1 month. Chromatoplates drying was achieved on a heating plate to obtain the most favorable vertical concentration profiles of compounds inside the sorbent layer and the most reproducible results as recently demonstrated by Vovk et al. [20].

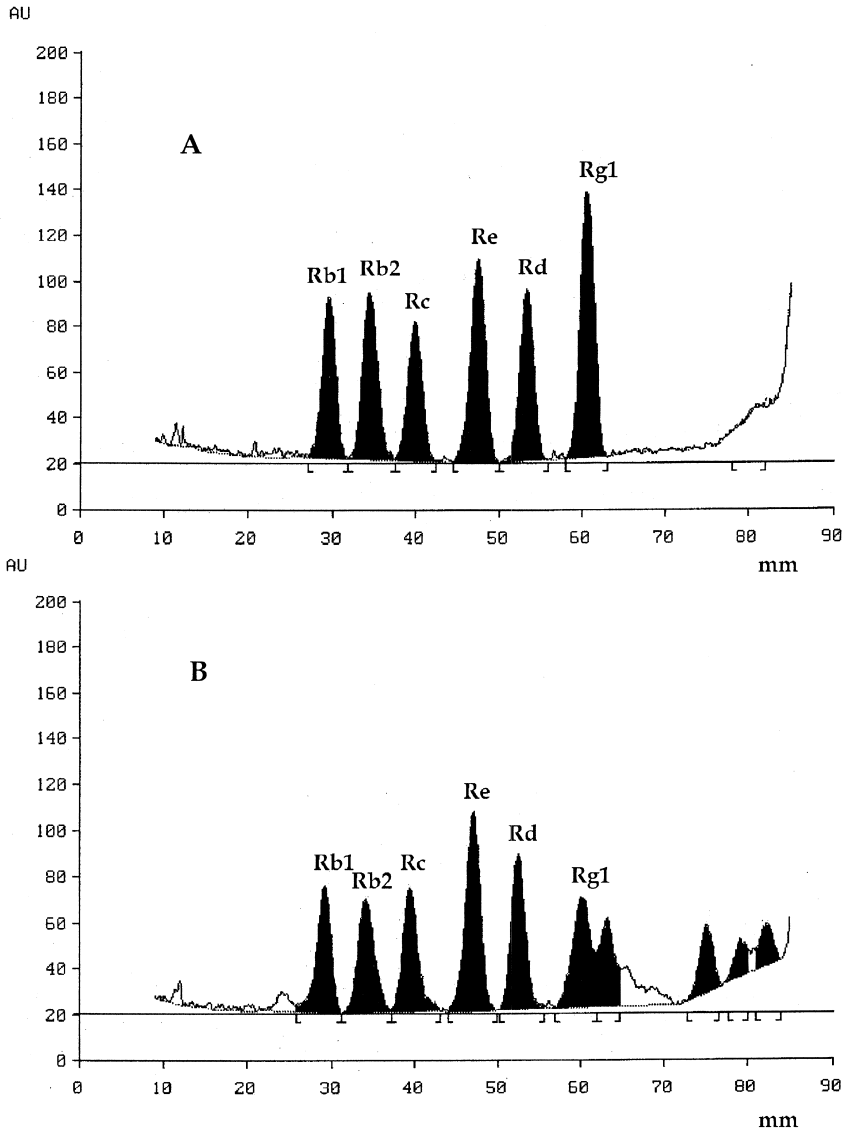

Fig. 1. Chromatographic profiles of the ginsenosides standards solution and of an extract of *Panax ginseng*.

Thionyl chloride in combination with stannic chloride (Noller reagent) has been used in the past for the detection of resins constituents separated by paper chromatography. The use of thionyl chloride vapors alone as chromogenic reagent afforded many advantages. During the plates immersion or spraying, it enables to suppress, the ginsenosides diffusion into the reagent solution and/or irregular spreading and to avoid the resulting lower reproducibility. In addition, the ginsenosides chromatograms exposure to the reagent vapors afforded after heating brownish derivatives whose stability allowed densitometric determinations over several days. Even 1 year after the treatment, the quantification of plates stored at −18°C remained possible. Typical chromatographic profiles of the ginsenosides standards and of a

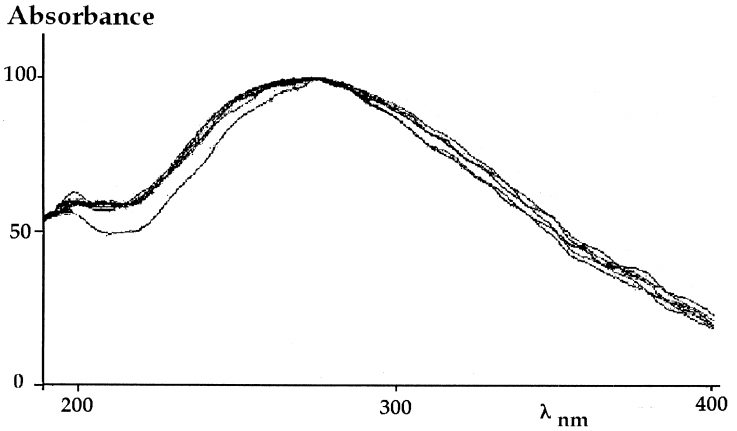

Fig. 2. Superimposed electronic spectra of ginsenosides Rb_1, Rb_2, Rc, Rd, Re and Rg_1 after reaction with thionyl chloride and heating.

ginseng extract are presented in Fig. 1. After reaction, the electronic spectra of the different ginsenosides measured in situ showed no significant difference (Fig. 2).

The reaction mechanism between ginsenosides and thionyl chloride is still unclear. Comparison experiments carried out with unsaturated terpenoids and terpenoids showing an hydroxyl group suggested that the reagent reacts only with the hydroxyl function and leads to addition products; in addition, thionyl chloride could also dehydrate the ginsenosides structure.

The calibration curves were determined from two concentrations scales. Changing from linear regression to polynomial regression for the quantitative analysis avoided dilution steps and guaranteed more accurate results when Ginseng extracts from different sources were analyzed. In these conditions, the *r* values of the calibration curves were always higher than 0.995 (Table 1).

In comparison with other detection methods, the LOD and the LOQ values were very low. The calculated LOD values for both absorbance and fluorescence measurements and for all ginsenosides were 10 ng/10 mm. In comparison with the absorbance measurements, the sensitivity observed in the fluorescence mode were higher but the noise level was increased. Chromatograms treatment with hexadecane or polyethylene glycol-4000 slighty improved the sensitivity by a factor of 1.5. For routine

Table 1
The *r* values of the calibration curves of ginsenosides standards

Ginsenosides	*r* values (second order polynomial regression)			
	Absorbance at 275 nm		Fluorescence	
	0.10–5.0 μg Concentration scale	0.15–0.75 μg Concentration scale	0.10–5.0 μg Concentration scale	0.15–0.75 μg Concentration scale
Rb_1	1	0.999	0.999	0.999
Rb_2	0.999	0.999	0.999	0.996
Rc	0.999	0.999	0.999	0.995
Re	0.999	0.998	0.999	0.996
Rd	0.999	0.997	0.999	0.998
Rg_1	0.999	0.997	0.999	0.997

Table 2
Inter-assay precision of the HPTLC determination of ginsenosides (absorbance measurements)

Ginsenosides	Coefficient of variation (%)
Rb_1	4.9
Rb_2	4.5
Rc	3.2
Re	8.3
Rd	7.2
Rg_1	2.8
Total ginsenosides	3.3

controls, absorbance measurements were found more convenient as the fluorescence mode required a surface treatment of the plates to eliminate particles affording interference during the determination. The calculated LOQ for both absorbance and fluorescence measurements ranged from 30 to 50 ng/10 mm; however, 100 ng was considered to be the practical lower limit of quantification. These values are comparable to those obtained with HPLC using light scattering detection [13] or using UV detection at 203 nm as described in more conventional methods affording a poor selectivity.

The results of the precision study are presented in Table 2. The coefficient of variation calculated for each ginsenoside ranged from 2.8 to 8.3% and for the total ginsenosides, 3.3%. This last lower value could be explained by small differences occurring from plate to plate during the peaks integration process of the very complex chromatograms.

The accuracy study results are presented in Table 3. They showed that the recovery values were satisfactory over a wide range of ginsenosides concentrations.

The ratios between the concentrations of different ginsenosides afforded valuable data for the differentiation of *Ginseng* spp. [9], particularly the differentiation between *Panax ginseng* C.A. Mayer (Korean ginseng) and *Panax quinquefolius* L. (American ginseng) [12,21]. In addition, the chromatograms profiles showed evident differences around the Rg_2 and Rf bands whose identification was confirmed by direct comparison with the standards (Fig. 3).

Finally, these results were also found to be useful in specifying the root part used for the extracts preparation, as the ginsenoside content varies greatly according to the biosynthesis localization, i.e., rootlets and roots.

4. Conclusions

High-performance thin-layer densitometry after detection with thionyl chloride is an attractive alternative for the simultaneous determination of the six major ginsenosides in *Panax ginseng* roots with regard to the reproducibility, accuracy and selectivity. This powerful method could be widely applied to the analysis of various ginseng extracts. Additional advantages over known methods are related to the simplicity of extraction, the low detection and

Table 3
Determination of the accuracy

Ginsenosides	Recovery (%)		
	Ratio ginseng extract solution /standards solution: 1:1 (v/v)	Ratio ginseng extract solution /standards solution: 1:3 (v/v)	Ratio ginseng extract solution /standards solution: 3:1 (v/v)
Rb_1	102	100	90
Rb_2	100	94	89
Rc	103	106	115
Re	95	102	103
Rd	96	97	97
Rg_1	98	98	98
Total ginsenosides	99.0	99.5	98.7

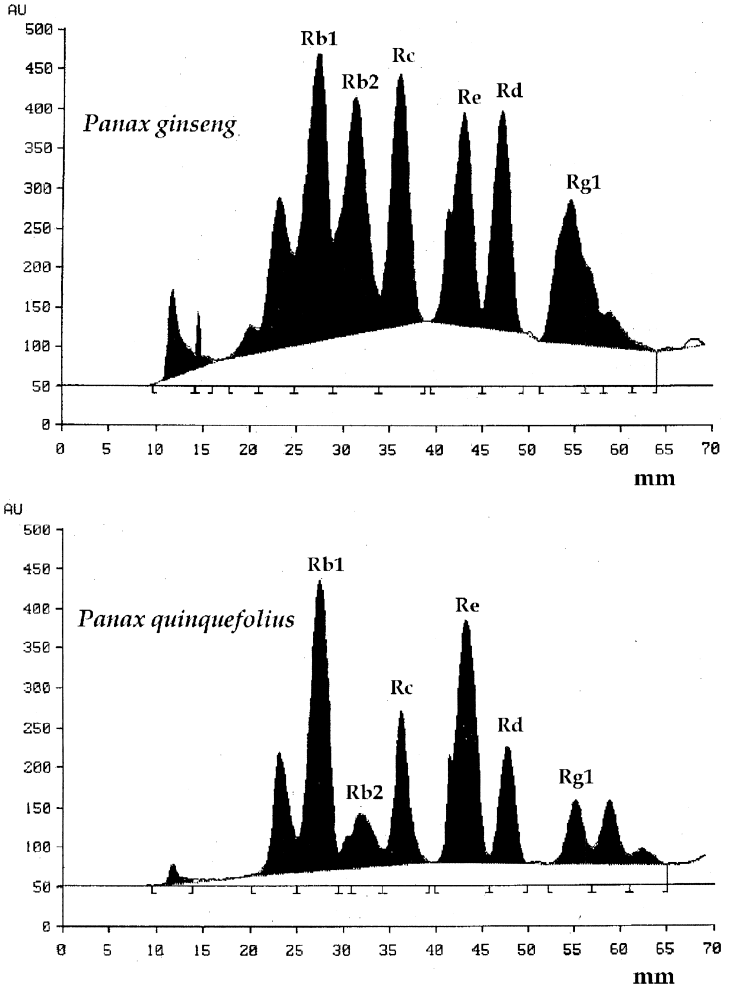

Fig. 3. Comparison of the HPTLC profiles of extracts of two *Panax* spp. roots extracts.

quantification limits without a derivatization step. The method is suitable for quality assurance of the drug and related extracts.

References

[1] J.A. Duke, Ginseng: A Concise Handbook, Reference Publications, Inc, Algonac, Michigan, 1989.
[2] V. Schulz, R. Hänsel, V.E. Tyler, Rational Phytotherapy, Springer, Berlin, 1997.
[3] S.R. Yoon, J.J. Nah, S.K. Kim, S.C. Kim, K.Y. Nam, D.W. Jung, S.Y. Nah, Chem. Pharm. Bull. 46 (1998) 1144.
[4] H. Tanaka, Y. Shoyama, Phytomedicine 5 (1998) 397.
[5] J.F. Cui, Europ. J. Pharm. Sci. 3 (1995) 77.
[6] P. Pietta, P. Mauri, J. Chromatogr. 356 (1986) 212.
[7] H. Kanazawa, Y. Nagata, E. Kurosaki, Y. Matsuhima, J. Chromatogr. 632 (1993) 79.
[8] K. Samukawa, H. Yamashita, H. Matsuda, M. Kubo, Yakugaku Zasshi 115 (1995) 241.
[9] W.A. Court, J.G. Hendel, J. Elmi, J. Chromatogr. A 755 (1996) 11.
[10] M.P. Gomez-Serranillos, O.M. Palomino, E. Carretero, A. Villar, M.A. Cases, Fitoterapia 68 (1997) 533.

[11] R.B. van Breemen, C.-R. Huang, Z.-Z. Lu, A. Rimando, H.H.S. Fong, J.F. Fitzloff, Anal. Chem. 67 (1995) 3985.
[12] X.M. Wang, T. Sakuma, E. Asafuadjaye, G.K. Shiu, Anal. Chem. 71 (1999) 1579.
[13] M.I. Park, J.H. Park, S.B. Han, Y.G. Shin, I.H. Park, J. Chromatogr. A 736 (1996) 77.
[14] X. Di, W. Wu, Y. Sun, Chinese J. Chromatogr. (Sepu) 12 (1994) 173.
[15] Y. Zhu, K. Yan, G. Tu, Chinese J. Pharm. Anal. 9 (1989) 5.
[16] P. Xie, Y. Yan, J. High Resol. Chromatogr. 10 (1987) 607.
[17] M. Vanhaelen, R. Vanhaelen-Fastré, J. Chromatogr. 312 (1984) 497.
[18] A. Koch, M. Müller, in: 46th Annual Congress of the Society for Medicinal Plant Research, Vienna, 1998, Poster.
[19] A. Koch, E. Hahn-Deinstrop, in: 46th Annual Congress of the Society for Medicinal Plant Research, Vienna, 1998, Poster.
[20] I. Vovk, M. Franko, J. Gibkes, M. Proseck, D. Bicanic, J. Plan. Chromatogr. 11 (1998) 379.
[21] D.-Q. Dou, W.-B. Hou, Y.-J. Chen, Planta Med. 64 (1998) 585.