Journal of Pharmaceutical & Biomedical Analysis
Vol. 9, Nos 10–12, pp. 805–809, 1991
Printed in Great Britain

0731–7085/91 $3.00 + 0.00
Pergamon Press plc

Chiral separation of glycyrrhetinic acid by high-performance liquid chromatography*

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Abstract: An HPLC separation of the 18α and 18β diastereomers of glycyrrhetinic acid (GA) has been developed. The separation was achieved with a chiral column (Merck ChiraSpher, Darmstadt, Germany) and a methanol–water (65:35, v/v) mobile phase. The separation is excellent, having a resolution factor of 2.3. The HPLC method is efficient and accurate for the optical purity determination of 18α- and 18β-GA samples. The run time is less than 30 min. Quantitation is linear with a lower detection limit of 0.2% for one isomer in the presence of the other.

Addition of 0.2% acetic acid to the water in the mobile phase improved the ruggedness of the system without affecting the resolution of 18α- and 18β-GA. This modification also allowed partial separation of 18α- and 18β-glycyrrhizin, precursors of the GA isomers.

Keywords: *Chiral separation; HPLC; 18α- and 18β-glycyrrhetinic acid; 18α- and 18β-glycyrrhizin.*

Introduction

Glycyrrhetinic acid (GA), a triterpenoid, is the aglycone of glycyrrhizin (G) which exists naturally in roots of licorice plants. GA has many biological activities including anti-inflammatory, anti-ulcerative, anti-allergic, anti-viral, and anti-AIDS [1–5]. Recently, it has been investigated as a tumour chemopreventive agent [6–9]. GA is obtained by acid hydrolysis of G [10]. The naturally occurring G is the 18β isomer. The 18β isomers of GA and G (β-GA, β-G) can be isomerized to their α isomers (α-GA, α-G) under alkaline conditions [11]. Because the biological activities of α- and β-GA are different [12], an accurate and efficient method to assess the optical purity of GA samples is desirable.

Many methods based on HPLC [13–15], GLC [16], immunoassay [17, 18] have been reported for the analysis of GA. Reports of the separation of α-GA and β-GA are rare. Only two GLC [19, 20] and one HPLC [21] methods have been reported for the separation of the GA isomers. The GLC methods require silyl or methyl derivatization and is cumbersome. The HPLC separation reported by Tisse *et al.* [21] was accomplished with an ODS column using methanol–water–acetic acid (76:20:4, v/v/v) as the mobile phase. The separation of α- and β-GA was excellent with a resolution factor of 6.6. However, our attempts to repeat Tisse *et al.*'s HPLC separation with various ODS columns gave no resolution for the isomers. Changing the acetic acid (from 2 to 6%) or methanol (from 65 to 90%) composition in the mobile phase gave no improvement in the resolution. Because of the current interest in β-GA as a cancer chemopreventive agent, a more rugged HPLC assay for its optical purity is needed.

This paper presents an efficient and accurate HPLC assay for the optical purity of α- and β-GA samples. Factors affecting the ruggedness of the method and the application of the method to α- and β-G samples is also discussed.

Experimental

Samples

18α-Glycyrrhetinic acid (α-GA) and 18β-glycyrrhetinic acid (β-GA) were received from Sigma Chemical Co. (Poole, UK), Aldrich Chemical Co. (Milwaukee, WI, USA) and MacAndrew & Forbes Co. (Camden, NJ, USA). Monoammonium salts of 18α-glycyrrhizin (α-G), and 18β-glycyrrhizin (β-G) were received from MacAndrew & Forbes Co. Their identities and organic purities (>98%) were confirmed with optical rotation, NMR and HPLC. The chemicals were used without

*Presented at the "Third International Symposium on Pharmaceutical and Biomedical Analysis", April 1991, Boston, MA, USA.

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further purification. Sample solutions (1 mg ml^{-1}) were prepared by dissolving individual chemical as well as mixtures of each isomeric pair in methanol (for GA) or water (for G).

Reagents

Methanol (Mallinckrodt, New York, USA) was ChromAR HPLC grade, water was HPLC Milli-Q filtered, and acetic acid (glacial, Baker Chemicals, Deventer, The Netherlands) was reagent grade.

HPLC

The HPLC system consisted of a Waters Model 6000A pump, a Rheodyne Model 7125 valve injector, a Schoeffel Model SF770 UV monitor, and a Dynamic Solution Maxima data station.

Duplicate 10 μl aliquots of each sample solutions were chromatographed with a Merck ChiraSpher, 250 × 4.6 mm analytical column using a mobile phase of methanol–water (65:35, v/v) at 0.5 ml min^{-1}. The water component of the mobile phase contained 0.0–0.4% acetic acid. Ultraviolet detection was at 254 nm with 0.04 AUFS sensitivity setting. Peak retention time and area measurements were made by the Maxima data station.

Results and Discussion

The ODS column used by Tisse *et al.* [21] which gave excellent resolution for α-GA and β-GA has an effective theoretical plate number (N) of only 2500. The Chemcosorb and Phenomenex ODS columns that we used to reproduce Tisse *et al.*'s work had much larger N (>5000) values, but did not provide separations for α- and β-GA. The stereo-selectivity of Tisse *et al.*'s system, therefore, was not due to the reverse-phase C_{18} stationary phase but most likely due to interaction of the analytes with residual silanols in the column. The structures of α- and β-GA (Fig. 1) do not suggest significant difference in their affinity to ODS. However, the adsorption interaction of the residual silanols to the polar hydroxyl, carbonyl and carboxylate groups in the isomers will be different. The more linear and proximal arrangement of these three groups in the α isomers would favor a stronger adsorption to the unbonded silica than the β isomer. If the ODS column has sufficient unbonded silica, normal phase adsorption chromatography would become significant and result in a longer

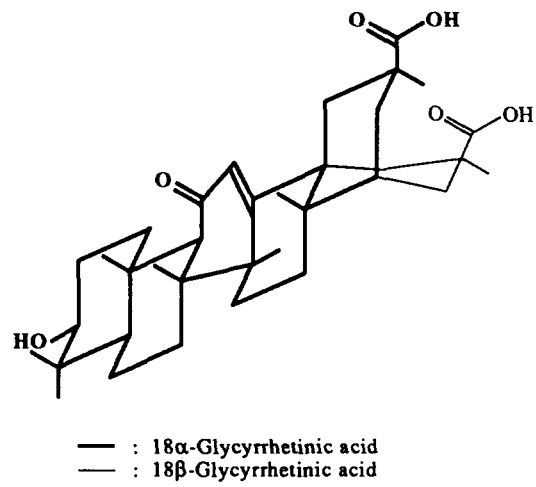

Figure 1
Structures of glycyrrhetinic acids, 18α-GA and 18β-GA (– – – 18α-GA, – – – 18β-GA).

retention (t_R) for the α isomer than for the β isomer as is observed in Tisse *et al.*'s chromatogram. Since the amount of unbonded silica in an ODS column varies with its type, brand and age, separation of α- and β-GA by ODS columns will be difficult to reproduce. Therefore, a different approach is warranted.

Although a silica column could be used, we decided to develop the separation of α- and β-GA with chiral columns. Efforts with cyclodextrin columns (Cyclobond II, Astec, Whippany, NJ, USA) yielded no separation. The desired resolution was finally achieved with the ChiraSpher column whose chiral stationary phase is optically active polyacrylamide with *S*-phenylalanine residues. Figure 2 presents the separation of the two isomers by a ChiraSpher column using methanol–water (65:35, v/v) as the mobile phase. The t_R for α-GA and β-GA are 24.6 and 20.1 min, respectively. The separation is baseline and has a resolution factor (R_s) of 2.3. Quantitation of one isomer in the presence of the other isomer is linear as depicted by Fig. 3. The lower detection limit of one isomer in the other isomer is 0.2%. The optical purity of several GA samples, as determined by this HPLC assay, is shown in Table 1.

The above chiral separation was reproducible with insignificant day-to-day variation. Increasing the methanol composition in the mobile phase decreased the t_R of both isomers, and *vice versa*. In both cases, R_s was unaffected. The chiral selectivity of the column is easily affected by the monoammonium salts of α-G and β-G, precursors of GA. Not only the

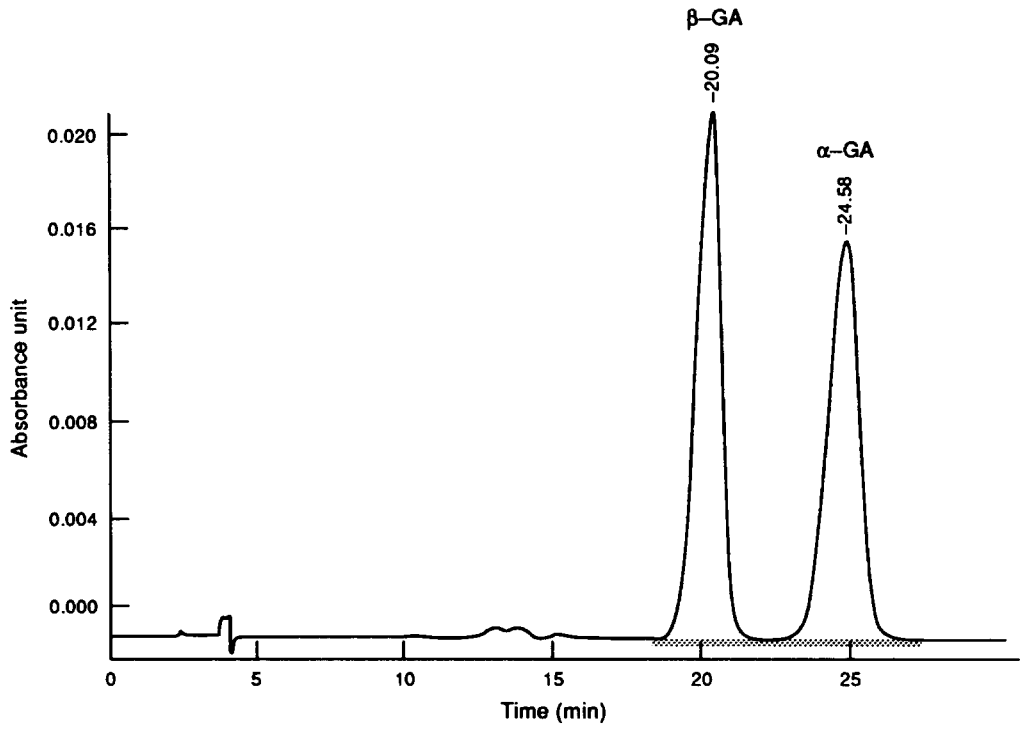

Figure 2
HPLC chromatogram of a mixture of 18α-GA and 18β-GA. Column: Merck ChiraSpher, 250 × 4.6 mm. Mobile phase: methanol–water (65:35, v/v) at 0.5 ml min^{-1}. Detection: UV at 254 nm.

Table 1
Optical purity (%) of 18α- and 18β-glycyrrhetinic acids (GA)

Sample	Manufacturer (Lot number)	αGA	β-GA	σ	*n*
α-GA	Sigma (43F-0100)	99.4	0.6	0.1	3
	MacAndrew*	99.3	0.7	0.1	3
β-GA	Sigma (85F-0048)	0.6	99.4	0.1	3
	Aldrich	0.8	99.2	0.1	3
	MacAndrew†	5.2	94.8	0.1	3
	MacAndrew (80209)	4.0	96.0	0.1	3

The relative optical purity (%) was determined by HPLC with a ChiraSpher column and mobile phase of methanol–water (65:35, v/v) at 0.5 ml min^{-1}.
*Prepared by acid hydrolysis of 18α-G, monoammonium salt, (MacAndrew lot 2231N006).
†Prepared by acid hydrolysis of 18β-G, monoammonium salt (MacAndrew lot 2809N365).

G isomers were not retained (t_R = 2.5 min); just four injections of G onto the column caused deterioration in column efficiency for subsequent separation of the GA isomers. The GA peaks were broadened and less retained, resulting in loss of resolution. Fortunately, the separation of α-GA and β-GA was restored simply by washing the column with the mobile phase (methanol–water, 65:35, v/v) containing a small amount (0.01%) of acetic acid. It appeared that the ammonium ions or the G ions adhered to the chiral sorbent and caused a loss of column selectivity for the GA isomers.

In order to improve on the ruggedness of the HPLC system for the chiral separation of α-GA and β-GA samples which may contain the precursor α-G and β-G, the mobile phase was modified to include acetic acid. Table 2 lists the effect of acetic acid on the retention (t_R) and resolution (R_s) of GA and G isomers. The column was pre-conditioned with each mobile phase for 30 min before chromatography. The t_R and R_s for the GA isomers were much reduced after four injections of mono-ammonium glycyrrhizin (MAG) but was immediately restored with just 0.01% acetic

Table 2
Effect of acetic acid in the mobile phase on the retention and resolution of 18α and 18β isomers of glycyrrhetinic acid (GA) and glycyrrhizin (G)

% HOAc in the water of mobile phase*	Retention, t_R (min)					
	α-GA	β-GA	R_s†	α-G	β-G	R_s‡
0.0 (pre-MAG)§	23.0	19.5	2.0	2.5	2.5	0.0
0.0 (post-MAG)‖	17.2	15.4	1.1	2.5	2.5	0.0
0.01	26.7	22.0	2.3	5.0	4.0	0.2
0.1	26.5	21.8	2.4	8.5	7.4	0.7
0.2	26.6	21.5	2.4	8.9	7.8	0.7
0.4	26.3	21.8	2.4	9.2	8.0	0.7

*Methanol–water (65:35, v/v).
†Resolution factor between α-GA and β-GA.
‡Resolution factor between α-G and β-G.
§Before any injections of monoammonium glycyrrhizin (MAG) on the ChiraSpher column.
‖After four injections of MAG on the ChiraSpher column.

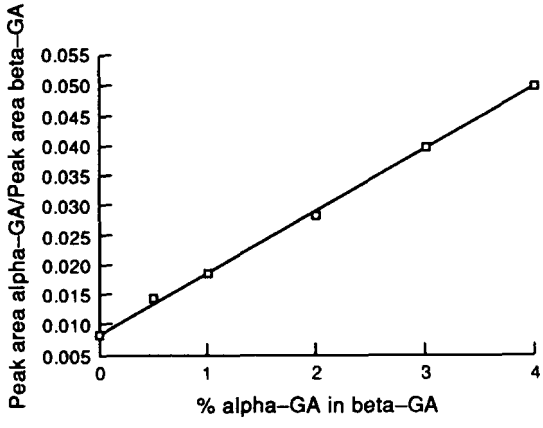

Figure 3
Linearity plots of optical purity assessment of α-GA in β-GA. The plot follows the linear equation of $y = 0.0105x + 0.0083$, $r = 0.9995$.

acid added to the water in the mobile phase. Further addition of acetic acid to the mobile phase had no additional effect on the t_R and R_s of the GA isomers. Being a salt, the MAG had no retainment on the column until acetic acid was added to the mobile phase. The t_R for both MAG isomers increased with the amount of acetic acid in the mobile phase until 0.2% acetic acid was added to the water. Presumably, MAG was converted to the free acid by the mobile phase and 0.2% of acetic acid was needed in the water to completely suppress the ionization of MAG. The R_s for the glycyrrhizin isomers remained constant at 0.7 during the entire addition of acetic acid to the mobile phase.

Conclusion

A rugged chiral separation of 18α- and 18β-glycyrrhetinic acid (GA) by HPLC has been developed. The system employs a Merck ChiraSpher column and a mobile phase of methanol–water containing 0.2% acetic acid (65:35, v/v). Detection is by UV at 254 nm. The system is accurate and sensitive in optical purity assessment of GA isomers. It can detect at least 0.2% of one isomer in the presence of the other isomer. The system also gives a 0.7 resolution to the isomers of glycyrrhizin (G), the precursors of GA.

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[Received for review 29 April 1991; revised manuscript received 21 May 1991]