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HPLC analysis of liquorice triterpenoids — applications to the quality control of pharmaceuticals*

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Abstract: A reversed-phase HPLC method is proposed for the separation of five liquorice triterpenoids, 18 β glycyrrhetic acid (β GA), 18 α glycyrrhetic acid (α GA), 24-hydroxy-18 β-glycyrrhetic acid (24-OH-β GA), α and β liquiritic acid (α and β LA), with potentially different biological activities. The method has been developed by studying the influence of the type of stationary phase, pH, amine modifier and organic modifier on the resolution of the five compounds. The optimized chromatographic conditions were then successfully applied to the analysis of α- and β-GA in pharmaceutical preparations (toothpaste and creams) on a reversed-phase Phenomenex Ultracarb 5 ODS (30) column (150 × 4.6 mm i.d.), using as the mobile phase acetonitrile–THF–0.010 M dioctylammonium phosphate buffer (pH 6.5) (25:20:55, v/v/v) at a flow-rate of 0.8 ml min^{-1}. SPE methods with diolic and C18 sorbents were developed to isolate and concentrate the analytes and to enhance the sensitivity for the determination of α-GA as an impurity in the β-GA preparations. The method was found to be reliable and suitable for the quality control of β-GA preparations.

Keywords: *Liquorice triterpenoids; 18 β glycyrrhetic acid; 18 α glycyrrhetic acid; HPLC; SPE; pharmaceutical preparations.*

Introduction

The roots of liquorice (licorice) contain a complex mixture of triterpene saponins, the most abundant of which is glycyrrhizic acid (I) (GZ), a diglucuronide of 18 β-glycyrrhetinic acid (II) (β-GA) which is obtained from (I) by acid hydrolysis. β-GA and GZ are the most used liquorice saponins in pharmaceutical and cosmetic products owing to their many biological properties including anti-inflammatory, anti-ulcer and anti-allergic activity. Recently β-GA has been investigated as a tumour protective agent [1]. Their mineralocorticoid-like effects and the inhibition of the metabolic enzymes for adrenocorticosteroids have also been studied, because of their structural similarity to steroids [2].

Other isomers of β-GA like 18 α-glycyrrhetic acid (α-GA), 18 α and β liquiritic acid (α and β-LA) as well as derivatives like 24-hydroxy-18 β-glycyrrhetic acid (24-OH-β-GA) are present in liquorice roots [3].

β-GA and α GA present different biological activities and physicochemical properties [4]; the latter is a more potent anti-inflammatory isomer [5] but has a weaker antihepatotoxic activity [6], demonstating how the different stereochemistry is important for biological activity.

Therefore, it was considered useful to develop a reversed-phase HPLC chromatographic method to separate simultaneously the four stereoisomers and 24-OH-β-GA and to determine α- and β-GA in pharmaceutical formulations.

The method has been developed by studying the influence of the type of stationary phase, pH, amine modifier, organic modifier on the resolution of the five compounds. The optimised chromatographic conditions were then successfully applied to the analysis of α- and β-GA in pharmaceutical preparations (toothpaste and creams) and to the purity assessment of some raw materials. SPE methods with diolic and C18 sorbents were developed to isolate and concentrate the analyte and to increase sensitivity for the determination of α-GA as an impurity in the β-GA preparations.

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Experimental

Materials

18 β glycyrrhetic acid (β GA), 18 α glycyrrhetic acid (α GA), 24-hydroxy-18 β-glycyrrhetic acid, α and β liquiritic acid (α and β LA) were kindly given by Indena (Milan, Italy). Testosterone, methylhydroxyprogesterone acetate (internal standards), lignocaine hydrochloride and hydrocortisone acetate were supplied by the Sigma Chemical Company (Italy).

Quinuclidine, 1,8-diaminooctane (DAO), triethylamine and 10-camphorsulphonic acid were obtained from Aldrich (Italy). Analytical grade orthophosphoric acid (85% w/w), perchloric acid and potassium dihydrogen phosphate were purchased from Farmitalia C. Erba (Italy).

For the solid phase extraction, 2.8 ml (500 mg) 2 OH diol Bondelut (Varian) cartridges were conditioned by rinsing with 6 ml of methylene chloride–n-hexane (20:80, v/v); 2.8 ml (500 mg) C18 Octadecyl Bondelut (Varian) cartridges were conditioned by rinsing with 6 ml of methanol, followed by 6 ml of 0.15 M ammonium acetate (pH 4.2). Extraction was carried out using a Baker-10 SPE system connected to a water aspirator.

Methanol, acetonitrile, tetrahydrofuran (THF) were of HPLC grade from Promochem (Germany); double-distilled water filtered through a 0.45-μm filter was used to prepare all solutions and buffers.

Phosphate buffer solutions (0.01 M) (pH 3.0, 4.0, 5.0, 6.3, 7.0) were perpared by mixing potassium dihydrogen phosphate and phosphoric acid or sodium hydroxide solutions in the proportions required to give the desired pH.

Quinuclidinium, 1,8-diammoniumoctane, triethylammonium (5–30 mM), phosphate and perchlorate solutions (pH 6.5) were prepared by dissolving the molar quantity of amine in double-distilled water and adjusting the pH to the desired values with phosphoric or perchloric acid.

Triethylammonium 10-camphorsulphonate (pH 7.0) solution was prepared by dissolving 10-camphorsulphonic acid (10 mmoles) in water (1000 ml) and adjusting the pH to the desired valued with triethylamine.

Ammonium acetate buffer (0.15 M) (pH 4.2) was prepared by mixing 1 ml of ammonium hydroxide (32% w/w) with 1.5 ml of glacial acetic acid in a 100-ml volumetric flask and diluting to volume with water.

The liquorice triterpenoids standard solutions (60 μg ml^{-1}) were prepared in methanol; the internal standard stock solutions of testosterone (0.1 mg ml^{-1}) and methylhydroxyprogesterone acetate (0.2 mg ml^{-1}) were also prepared in methanol.

Apparatus

The solvent delivery system was a quaternary HP 1050 Ti Series pump equipped with a Reodyne Model 7125 injection with a 20-μl sample loop. The eluates were monitored by a diode-array detector HP1050 connected to a HP 3396II integrator. The detector wavelength was 248 nm (reference wavelength 350 nm) with the integrator attenuation set at 4 or 1.

Chromatographic conditions

Separation of liquorice triterpenoids. Separation of the five liquorice triterpenoids (18 α- and β-GA, α- and β-LA, 24-OH-β-GA) was performed isocratically at ambient temperature in the reversed-phase mode on three different C18 packings: 5-μm Hypersil C18 column (250 × 4.6 mm i.d.) using as mobile phase acetonitrile–THF–0.010 M potassium phosphate buffer (pH 6.3) (27:13:60, v/v/v) at a flow-rate of 0.8 ml min^{-1}; C18 Phenosphere 5 BDS 90 Å column (250 × 4.6 mm i.d.) using acetonitrile–THF–0.010 M potassium phosphate buffer (pH 6.3) (25:20:55, v/v/v) at a flow-rate of 1 ml min^{-1}; Phenomenex Ultracarb 5 ODS (30) column (150 × 4.6 mm i.d.), using acetonitrile– THF–0.015 M dioctylammonium phosphate buffer (pH 6.5) (25:20:55, v/v/v) at a flow-rate of 0.8 ml min^{-1}.

Separation of 18 α and β-GA was also performed on: a C8 5-μm Shandon MOS Hypersil column (250 × 4.5 mm i.d.) using–THF–0.010 M dioctylammonium phosphate buffer (pH 6.5) (28:9:63, v/v/v), at a flow-rate of 1.2 ml min^{-1}; and on a Phenomenex Ultracarb 5 ODS (30) column (150 × 4.6 mm i.d.) using methanol–THF–quinuclidinium phosphate 30 mM (pH 6.5) (55:15:30, v/v/v) at a flow-rate of 1 ml min^{-1}.

Analysis of pharmaceutical formulations. To determine the content of α- and β-GA in the pharmaceutical formulations reported in Table 2, routine analyses were carried out isocratically at ambient temperature on a reversed-phase Phenomenex Ultracarb 5 ODS (30)

column (150 × 4.6 mm i.d.) using as mobile phase acetonitrile–THF–0.010 M dioctylammonium phosphate buffer (pH 6.5) (25:20:55, v/v/v) at a flow-rate of 1 ml min^{-1}.

Calibration curves

18 β-Glycyrrhetinic acid. Standard solutions of β-GA (10–150 μg ml^{-1}) in methanol, containing 10 μg ml^{-1} testosterone as internal standard, were injected in triplicate. The ratios of analyte peak area to internal standard peak area were plotted against the corresponding analyte concentration to obtain the calibration graph.

18 α-Glycyrrhetinic acid. Standard solution of α GA (0.15–1 μg ml^{-1}) in methanol, containing 1 μg ml^{-1} testosterone as internal standard, were injected in triplicate. The ratios of analyte peak area to internal standard peak area were plotted against the corresponding analyte concentration for calibration.

Analysis of commercial formulations

Sample preparation by solid phase extraction (SPE). Diol sorbent. An amount of sample equivalent to about 1 mg of β-GA was dissolved in a mixture of methylene chloride–n–hexane (20:80, v/v) in a 50-ml volumetric flask. A 6-ml aliquot of the analytical sample solution was applied to a previously conditioned SPE (2OH) cartridge. The wash step comprised 2 ml of methylene chloride–n-hexane (20:80, v/v); α- and β-GA were then eluted with 2 ml of methanol. When the creams were analysed, 100 μl of a testosterone solution (0.1 mg ml^{-1}) was added to the eluates; 100 μl of a methylhydroxyprogesterone acetate solution (0.2 mg ml^{-1}) was added to the aerosol eluates. A 20-μl volume of the resulting solutions was then injected into the chromatograph and analysed under the chromatographic conditions described. The active principle content in each sample was evaluated by comparison with an appropriate standard solution (60 μg ml^{-1}).

C18 sorbent. An amount of toothpaste sample equivalent to about 0.5 mg of β-GA was estracted with three 15-ml portions of ammonium hydroxide (5% w/w)–methanol (80:20, v/v), with magnetic stirring at 40–45°C. The resulting suspension was then centrifuged for 15 min at 3500 rpm. The clear extract was transferred and collected into a 50-ml volumetric flask and the volume was adjusted with ammonium hydroxide (5% w/w); the solid residue was discarded. An amount of cream sample, equivalent to about 0.5 mg of β-GA, was dissolved in ammonium hydroxide–methanol (80:20, v/v), with magnetic stirring at 40–45°C. An aliquot of 3 ml of the sample solutions was acidified with 150 μl of glacial acetic acid and applied to the previously conditioned SPE (C18) cartridge. The wash step comprised 2 ml of methanol–water (50:50, v/v). The elution of the two analytes (α and β-GA) was performed with 1 ml of methanol; 100 μl of a testosterone solution (0.1 mg ml^{-1}) was added to the eluate which was then injected into the chromatograph and analysed under the chromatographic conditions described. The content of active principle in each sample was evaluated by comparison with an appropriate standard solution (30 μg ml^{-1}).

Sample preparation by direct dissolution. An amount of cream sample equivalent to about 3 mg of β-GA was dissolved in a 50-ml volumetric flask with methanol containing testosterone (10 μg ml^{-1}). The sample solutions were then injected into the chromatograph and analysed under the chromatographic conditions described. The active principle content in each sample was evaluated by comparison with an appropriate standard solution.

Recovery study

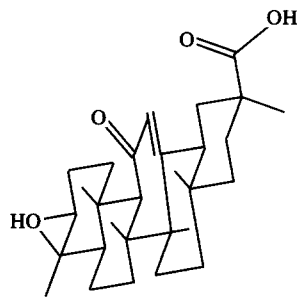
To check the accuracy of the proposed method the pharmaceutical samples were spiked with approximately 20% of the analyte declared content and the fortified sample solutions were analysed by the proposed method. The absolute recovery was calculated by comparing the peak areas from pharmaceutical samples with those obtained by a direct injection of a standard solution.

Results and Discussion

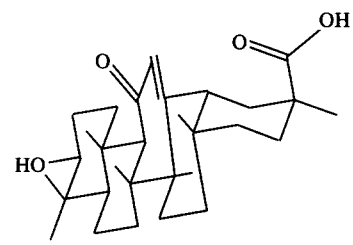
Chromatographic separation of the liquorice saponins

To optimize the chromatographic conditions for the resolution of the five triterpene saponins reported in Fig. 1, the effects of five parameters were investigated.

Stationary phase. For the separation of α and β GA, a HPLC chiral stationary phase had been used [7]; however, the two isomers had



18-α-Glycyrrhetic acid (α GA)



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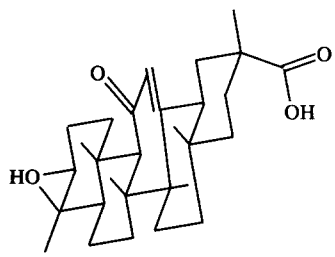
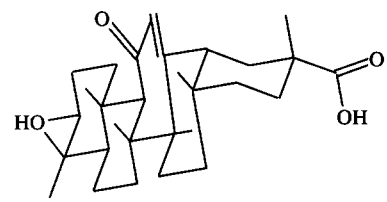
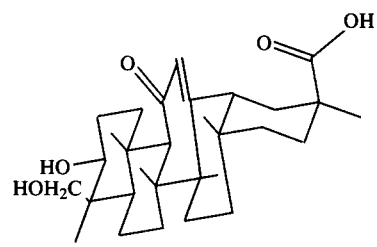


18-α-Liquiritic acid (α GA)



18-β-Liquiritic acid (β GA)



24-Hydroxy-18-β-glycyrrhetic acid
(24 OH-18 β GA)

Figure 1
Chemical structures of the analysed liquorice triterpenoids.

been well resolved also on a C18 column [8, 9]. Therefore, for the separation of the five saponins it was decided to use the HPLC reversed-phase mode and three C18 columns with a different carbon loading were tested. The best results in terms of analysis time and resolution were obtained on the Phenosphere column (18–20% carbon loading) (Fig. 2); on the Hypersil (9% carbon loading) column complete stereoisomer separation was not achieved and retention was even increased. Using the Ultracarb (30% carbon loading and exhaustive end-capping of silanols) column complete resolution was attained with increased analyte retention times owing to the highest lipophilicity of the stationary phase.

This chromatographic behaviour shows how resolution increases with the increase in lipo-

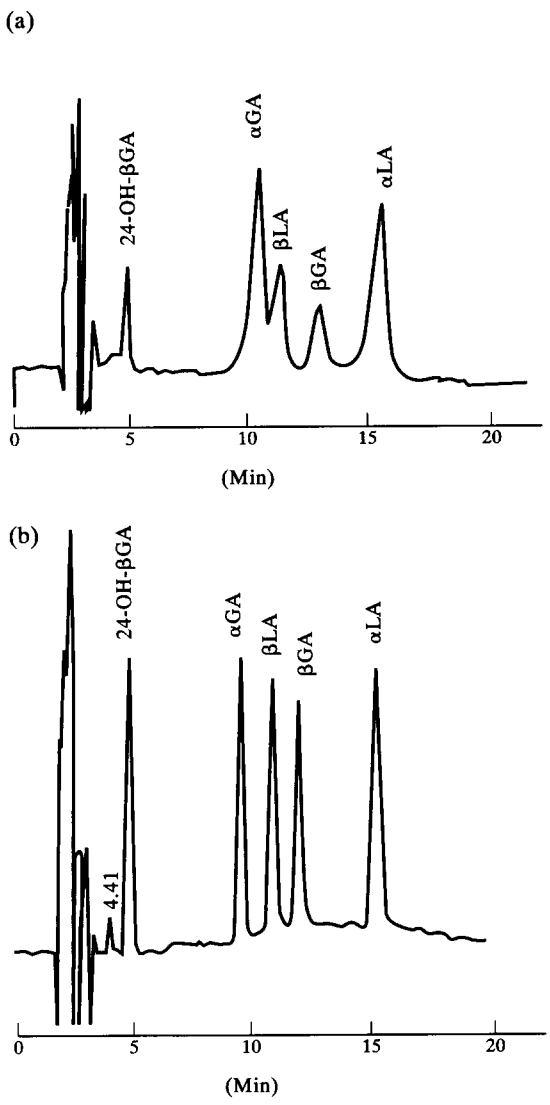


Figure 2
HPLC separations of the liquorice triterpenoids. Chromatographic conditions: (a) 5-μm Hypersil C18 column (250 × 4.6 mm i.d.) with acetonitrile–THF–0.010 M potassium phosphate buffer (pH 6.3) (27:13:60, v/v/v) at a flow-rate of 0.8 ml min^{-1}; (b) C18 Phenosphere 5 BDS 90 Å column (250 × 4.6 mm i.d.) with acetonitrile–THF–0.010 M potassium phosphate buffer (pH 6.3) (25:20:55, v/v/v) at a flow-rate of 1 ml min^{-1}. UV detection at 248 nm.

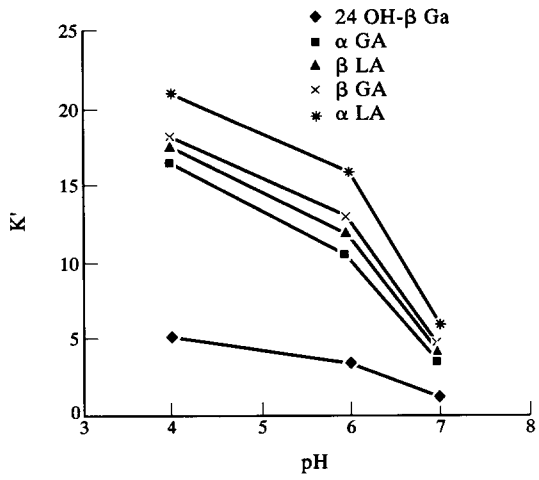


Figure 3
Capacities factors of the liquorice saponins at various pH values.

philicity of the stationary phase, even if the intermediate lipophilicity column was successful in a shorter analysis time. Accordingly, the Hypersil C8 column showed lower selectivity and all five saponins were unresolved. However, α and β GA could be separated using a higher percentage of buffer; the mobile phase was: acetonitrile–THF–DOA buffer (pH 6.5) (28:9:63, v/v/v) at a flow-rate of 1.2 ml min^{-1}.

pH of the mobile phase. With all the stationary phases under investigation, resolution of the five saponins was strongly influenced by the mobile phase pH (Fig. 3). At lower pH (4.0) the isomers gave overlapping peaks at undesirably long retention times. Increasing the pH up to a value of 7.0 (measured on the mixed global mobile phase) or 6.5 (on the buffer) a decrease of retention time was observed and well resolved chromatographic peaks were produced with relatively short retention times (within 20 min). Thus separation was improved and the retention times were shortened as the pH increased, as a result of ionisation of the compound.

Organic modifiers. Separation was achieved simply by using aqueous mixtures of either acetonitrile (more efficaceous) or methanol. Substitution of THF for part of the acetonitrile or methanol (up to 30% with Ultracarb, the most lipophilic stationary phase) resulted in a decrease in retention time and an improvement of resolution. THF was demonstrated to be a very selective organic modifier.

Amine modifers. The addition of amine modifiers did not produce any appreciable improvement in the separation obtained on the Phenosphere column. When an Hypersil column was used (9% and uncapped), the amine modifiers were useful to improve peak symmetry and to decrease slightly the retention times, but did not enhance significantly the resolution. This effect, observed using 5 mM

DOA, 10 mM quinuclidine and 10 mM TEA, shows the ability of the amine modifier in suppressing the adverse sylanol interactions.

On the Ultracarb column, the effect of amine modifiers depended on the nature of the amine. For separations with 60% (v/v) of aqueous phase, the addition of 10 and 30 mM quinuclidine and 10 mM TEA produced an increase in retention times in comparison to the use of 10 mM potassium phosphate buffer. In contrast, 15 mM DOA caused retention times to decrease with an improvement in chromatographic efficiency (Fig. 4). This behaviour might be explained on the basis of the different lipophilicity of the amine modifiers which appear to act as ion-pairing agents for the analytes [10].

Nature of the counterions. Phosphate, perchlorate and camphorsulphonate anions used in the buffers did not seem to produce any significant effect on the separation under all the conditions examined.

These results obtained with different stationary phases and amine modifiers suggest that the separation of the various isomers can be achieved on the basis of their different lipophilicity (solvation effects might be involved) rather than interaction with free silanols as previously supposed [7].

For the analysis of pharmaceutical formulations, chromatographic conditions were chosen to enable α and β-GA to be separated from the other formulation components. This was accomplished using a reversed-phase

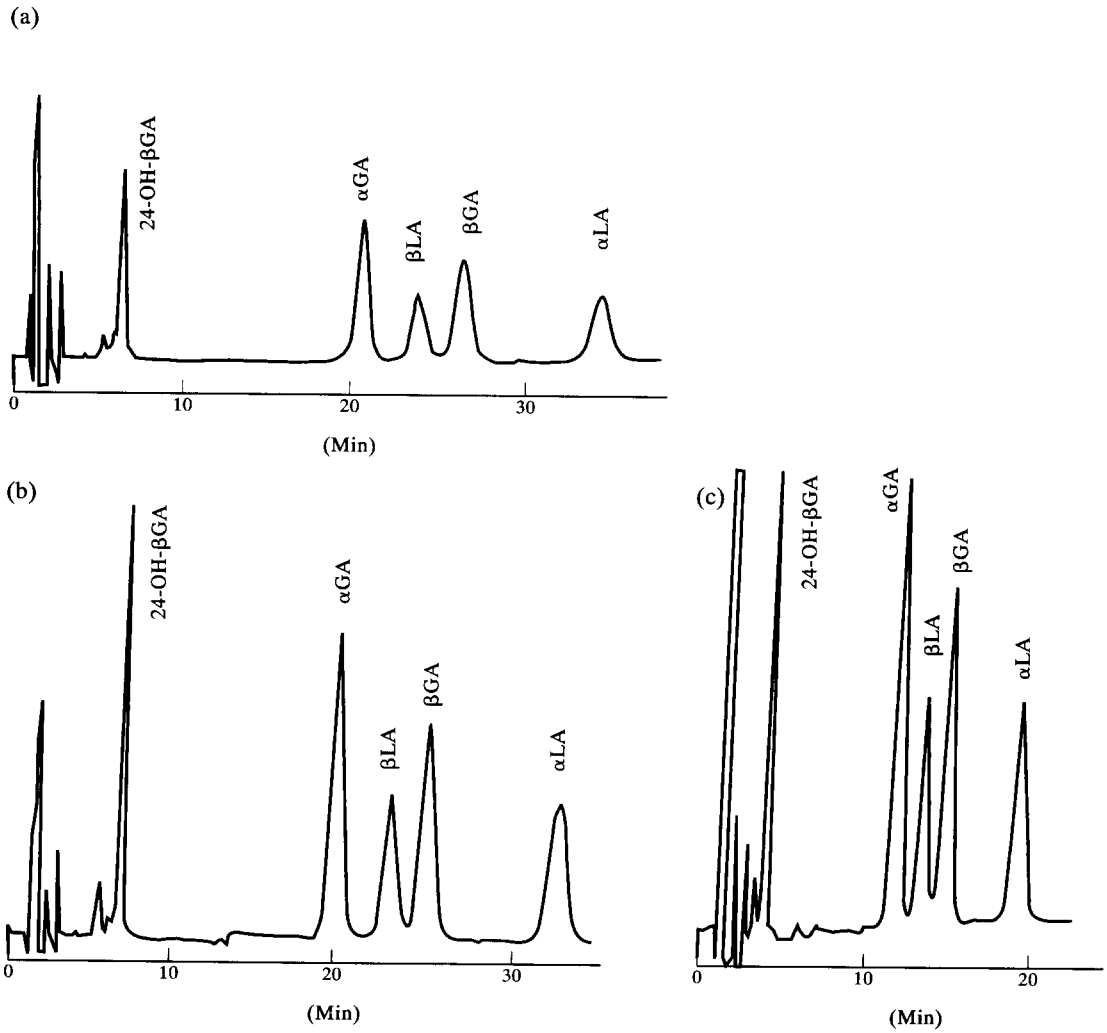


Figure 4
Chromatographic separation of the liquorice saponins. Phenomenex Ultracarb 5 ODS (30) column (150 × 4.6 mm i.d.) with acetonitrile–THF–pH 6.5 buffer (27:13:60, v/v/v) at a flow-rate of 0.8 ml min^{-1}; buffers were (a) 30 mM triethylammonium phosphate buffer, (b) 30 mM quinuclidinium phosphate buffer and (c) 10 mM dioctylammonium phosphate buffer. UV detection at 248 nm.

Table 1
Data for the calibration graphs (n = 6)

Compounds	Slope	Intercept (±SE)	Correlation coefficient	Concentration range (μg ml^{-1})
β-GA	0.04354 (±0.00009)	0.01595 (±0.00319)	0.9999	10–150
α-GA	0.043932 (±0.00219)	0.02878 (±0.07130)	0.9994	0.15–1.5

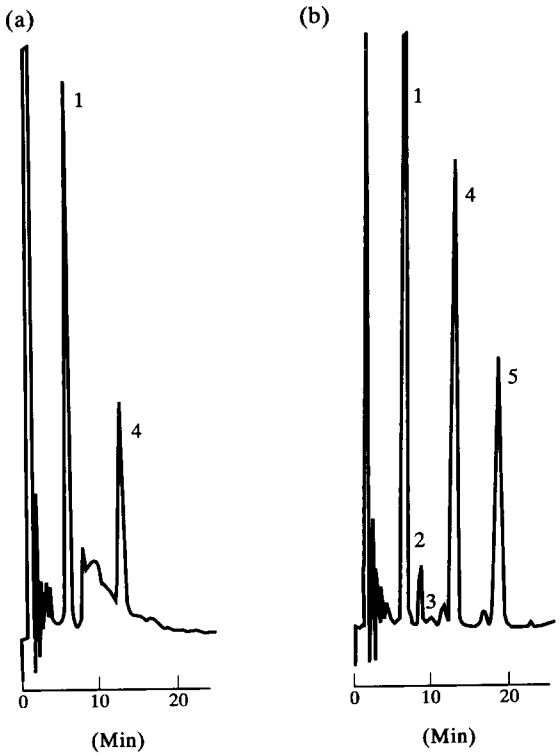


Figure 5
Chromatograms of (a) solution of aerosol before diol SPE (20 μg ml^{-1} β-GA) and (b) same solution after diol SPE. Peaks: 1 = hydrocortisone acetate, 2 = lignocaine hydrochloride, 3 = α-GA, 4 = β-GA, 5 = methylhydroxyprogesterone acetate (internal standard). Phenomenex Ultracarb 5 ODS (30) Column (150 × 4.6 mm i.d.) with acetonitrile–THF–10 mM dioctylammonium phosphate buffer (pH 6.5) (25:20:55, v/v/v) at a flow-rate of 0.8 ml min^{-1}. UV detection at 248 n.

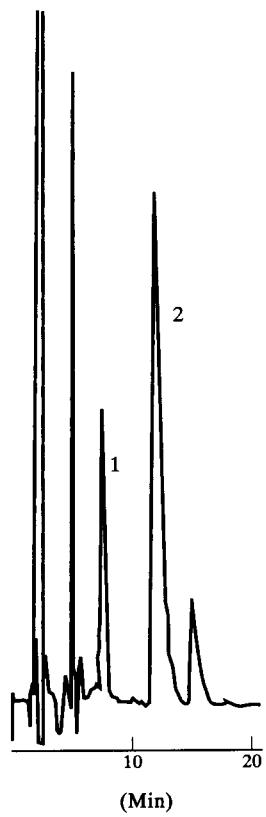


Figure 6
Chromatogram of a solution of cream A after diol SPE. Peaks: 1 = testosterone (internal standard), 2 = β GA. Chromatographic conditions as in Fig. 5.

Phenomenex Ultracarb 5 ODS (30) column (150 × 4.6 mm i.d.), using as the mobile phase acetonitrile–THF–0.010 M dioctylammonium phosphate buffer (pH 6.5) (25:20:55, v/v/v) at a flow-rate of 0.8 ml min^{-1}.

Using these chromatographic conditions for quantitative application, linear regression curves were obtained with good precision by plotting the peak-area ratio of α and β-GA to internal standard (testosterone) against the corresponding α and β-GA concentrations (Table 1).

Analysis of commercial formulations

α and β-GA were quantitatively extracted from the cream and aerosol formulations by methylene chloride–n-hexane (20:80, v/v). A further sample clean up by SPE was performed to eliminate most of the formulation excipients and to concentrate the analytes. To this end a sample solution aliquot was applied to a diol SPE sorbent and methylene chloride–n-hexane (20:80, v/v) was used in the wash step. Quantitative elution of retained α and β-GA was then performed with methanol and the resulting eluate, after the addition of the appropriate internal standard, was injected into the chromatograph (Figs 5, 6). The method was

Table 2
Results for the HPLC determination of βGA in commercial pharmaceutical preparations. The results represent the mean of five determinations and are expressed as a percentage of the content declared

Sample	Method	Found	RSD%
Cream A*	Diol SPE	98.96	2.58
	C18 SPE	99.24	2.76
Toothpaste†	C18 SPE	89.66	2.80
Cream B‡	Direct	100.65	0.50

*Cream A: Anolan Pommade, Laboratoires Delagrange (Chilly-Mazarin, France); for topical use, indicated in haemorrhoidal attacks. Formula: β-glycyrrhetic acid 2 g, amyleine hydrochloride 2 g, excipients to 100 g.
†Toothpaste: Arthrodont, Laboratoires Veyron (Marseille, France); indicated in gingivitis, gingivorrhagia, inflammatory reactions to prostheses. Formula: β-glycyrrhetic acid 1 g, formaldehyde solution 0.1 g, excipients to 100 g.
‡Cream B: Vidermina, Ganassini (Milano, Italy); indicated as skin disinfectant. Formula: β-glycyrrhetic acid 1 g, 8-hydroxyquinoline sulphate 0.25 g, excipients to 100 g.

applied to the analysis of a cream and an aerosol; the results obtained with the cream (Table 2) showed good precision and were in good agreement with the declared content. The formulations were spiked with 1–3% α GA of the β-GA declared content. The lower limit of detection for both drugs was found to be 0.15 μg ml^{-1} (0.15% α-GA in β-GA declared content). With this sensitivity, traces of α-GA in the examined preparations were not observed. The SPE step and the chromatographic system were found to be suitable also for the aerosol formulation (Anginovag, Laboratorios Novag S.A., Barcelona, Spain), a pressurized suspension indicated for topical application in oral inflammation. This sample was submitted only to qualitative analysis; the principal formulation ingredients were all separated and identified using a photodiode-array detector (Fig. 5).

Since the toothpaste formulation is a hydrophilic mixture, the above SPE method is not suitable. Therefore, the analytes were extracted with methanol–ammonium hydroxide (5% w/w) (20:80, v/v) and a clear solution was obtained after elimination of the abrasive powder and colloids in the toothpaste by centrifugation. The clean up was performed selectively on a C18 SPE cartridge by loading the clarified ammoniacal mixture acidified with acetic acid to obtain α and β GA in the undissociated form. The wash step was performed with methanol–water (50:50, v/v). The analytes were eluted with methanol and, after the addition of testosterone as internal standard, the solution was injected into the chromatography (Fig. 7).

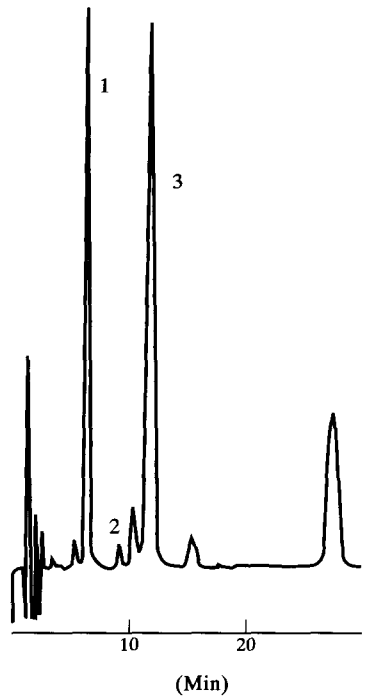


Figure 7
Chromatogram of a solution of toothpaste spiked with 3% of α-GA after C18 SPE. Peaks: 1 = testosterone (internal standard), 2 = α-GA, 3 = β-GA. Chromatographic conditions as in Fig. 5.

The cream was also submitted to this procedure by solubilizing it with methanol–ammonium hydroxide (5% w/w) (20:80, v/v). The results obtained from the analysis of commercial formulations were compared with those from appropriate standard solutions submitted to the same procedure and the β GA content in each sample was calculated (Table 2).

The accuracy of the methods (SPE plus HPLC assay) was verified by analysing β GA standard solutions and formulations spiked with known amount of analytes. The recovery values obtained were 98–99% with the diol sorbent and 90–91% with the C18 sorbent.

For sample preparation, the SPE with diol sorbent can, therefore, be considered as a first choice owing to the higher recovery obtained; however, when the characteristics of the formulation excipients do not allow this procedure, the reversed-phase (C18 sorbent) approach can be adopted, even if the recovery is lower. Direct analysis of the sample can be applied when the sample clean up and the analyte concentration are not critical to obtain a reliable analysis.

Conclusions

The methods described allow the HPLC chromatographic separation of five liquorice saponins with potentially different biological activity and the determination of α and β GA at various concentrations in different kinds of complex pharmaceutical formulations. By the SPE procedure with C18 and diolic cartridges the analytes are selectively extracted, concentrated and freed from excipient interference; the whole method (SPE and HPLC assay) showed satisfactory accuracy and precision with high sensitivity. The method is suitable for the selective determination of α and β GA in pharmaceutical formulations for quality control purposes.

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