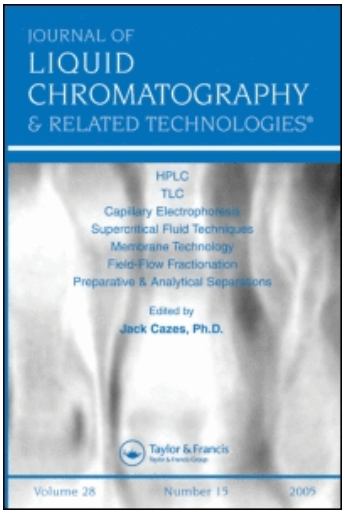

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Evaluation of HPLC Mobile Phases and Extracts for the Determination of Glycyrrhizic Acid in Licorice and Tobacco

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Evaluation of HPLC Mobile Phases and Extracts for the Determination of Glycyrrhizic Acid in Licorice and Tobacco

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Abstract: An improved high performance liquid chromatography (HPLC) analysis of glycyrrhizic acid (GA) in licorice and tobacco to which licorice was applied was developed. An acetate buffer was used in the mobile phase, which resulted in more constant retention times for GA. Nine extractants were found to remove GA from licorice, but only six were capable of removing GA from both licorice and tobacco. Precision was less than 4% relative standard deviation for licorice and tobacco and percent recoveries were at least 92. Aqueous solutions of 1,4-dioxane, ethanol, tetrahyrofuran, 2-butoxyethanol, 2-methoxyethanol, and 1,3-dioxolane can be used to extract GA from both licorice and tobacco. Glycyrrhetinic acid and compounds found in tobacco do not interfere with the analysis.

Keywords: Glycyrrhizic acid, Glycyrrhetinic acid, Licorice, Tobacco

INTRODUCTION

Mobile phases used for the determination of glycyrrhizic acid (GA) in licorice usually employ acetonitrile-water and an acid to reduce the pH of the mobile phase.[1–3] Acetic acid (HAc) is the most commonly used mobile phase modifier. Although these mobile phases containing HAc are capable of eluting GA on a reverse-phase column, it has been our experience that the retention time of GA varies with only slight variations of HAc. To better

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understand this change in retention time, a series of experiments were performed using phosphate and acetate buffers at various pH's and concentrations. Upon determining suitable (constant retention time of GA) mobile phase conditions for the elution of GA, GA was analyzed in licorice and tobacco with applied licorice.

GA is a main component of licorice root where it is found as a potassium-calcium-magnesium salt, $K_{0.6}Mg_{0.5}Ca_{0.2}$.[4] It appears that GA salts can be extracted from licorice using water or a portion of the mobile phase containing $>60\%$ water.[1] This may not be the case for GA salts in tobacco even though water and a portion of the mobile phase have been suggested for use as the extract.[1] It may not be possible to extract GA under aqueous conditions given the solubility of GA in water to be estimated at 0.053 $\mu g\ mL^{-1}$ and since GA also can be present as more insoluble calcium and magnesium salts.[4,5]

This work describes the extraction of GA from both licorice and tobacco with applied licorice using a variety of solvents.

EXPERIMENTAL

Samples

Licorice samples were obtained from common licorice suppliers. Tobacco with applied licorice was from a US commercial cigarette blend composed of flue-cured, burley and Turkish tobaccos and was ground to pass through a 40-mesh screen.

Chemicals and Reagents

Glycyrrhizic acid ammonium salt ($GANH_4$) and glycyrrhetinic acid (GR) were obtained from Fluka Chemical Corporation (Milwaukee, WI) and were used as received (without further purification). Acetonitrile (ACN) (Burdick & Jackson, Muskegon,MI) was used as a portion of the mobile phase. Tetrahydrofuran (THF) and methanol (MeOH) were obtained from Burdick & Jackson, 1,4-dioxane, 2-butoxyethanol, 2-ethoxyethanol, 2-methoxyethanol, and 1,3-dioxolane and sodium acetate (NaAc) were purchased from Sigma-Aldrich (St. Louis, MO). Ethanol (EtOH), specially denatured alcohol 3A, 200 proof was from AAPER Alcohol and Chemical, Shelbyville, KY. Glacial acetic acid (HAc) was purchased from Fisher Scientific Company L. L. C., Suwanee, GA.

Standards

Stock standards were prepared in a variety of solvents (see above). Figure 1 is a chromatogram of a $GANH_4$ standard in water.

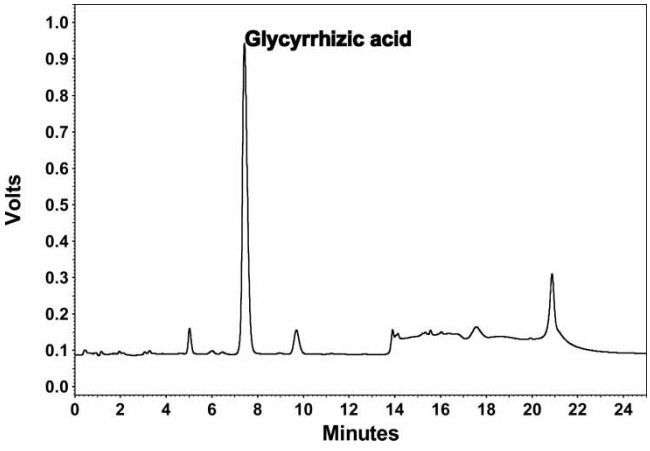

Figure 1. Chromatogram of glycyrrhizic acid ammonium salt standard (87 μg mL^{-1}) in water.

Extraction and Sample Preparation

Licorice

Weigh ~0.005 g into a 16 × 100 mm test tube. Add 5.0 mL extract, stopper and vortex 15 minutes using a VWR VX-2500 Multi-Tube Vortexer (Henery Troemner LLC, Thorofare, NJ). After vortexing, filter the extract using a 0.45 μm pore size polyvinylidene fluoride (PVDF) for aqueous extracts or polytetrafluoroethylene (PTFE) for solvent extracts (Whatman Autovial, Whatman, Clifton, NJ) into a glass autosampler vial and seal with a screw cap containing a septum.

Tobacco

Weigh ~0.2 g into a 16 × 100 mm test tube. Proceed as described above for licorice.

Preparation of 50 mM pH 4 Acetate Buffer

Weigh 0.65 g NaAc and transfer to a 1 L volumetric flask. Add 500 mL water and 2.52 g HAc, dilute to volume with water, and stir with a magnetic stirrer until dissolved. Adjust pH to 4.0, if needed, with 50% NaOH using a pH meter calibrated at a pH of 4. Filter through a 0.45 μm pore membrane filter.

HPLC System and Conditions

The HPLC system consisted of a 680 gradient controller, two 515 pumps, a 717 plus autosampler, and a 2487 absorbance detector (Waters, Milford, MA). Analysis was conducted using a 150 × 2.0 mm I.D., 5 μm particle size, 110 Å pore size, Gemini C18 analytical column preceded by a guard column containing a Gemini C18, 2 mm I.D. cartridge (Phenomenex, Torrance, CA). The injection volume was 5 μL and the autosampler temperature set at 10°C. The absorbance detector was set at 256 nm using various sensitivity settings to accommodate the concentration of the standards and samples. The run time was 25 minutes. The mobile phase flow rate was 0.5 mL min^{-1} using two mobile phases: 75:25, 50 mM pH4 acetate buffer: ACN (A) and ACN (B). Initial conditions were 93% A and 7% B, and held for 10 min. After 10 min, the mobile phase was changed to 100% B for 7 min after which time the column was equilibrated using the initial conditions for 8 min prior to the next injection.

Calculation of Results

Quantification was performed by using the peaks height of external standards of $GANH_4$, serially diluted to the concentration range of the samples from a stock solution. The stock solution and diluted working standards were refrigerated at 5°C when not in use. Data were acquired and results calculated in ppm using EZChrom Elite version 3.0.0 (Scientific Software, Pleasanton, CA).

RESULTS AND DISCUSSION

Chromatographic Conditions

It was observed early in this work while using reverse phase conditions, that $GANH_4$ did not elute within 60 minutes, even when as much as 50% of the mobile phase was composed of ACN. It was found, that only when HAc was incorporated into the mobile phase did $GANH_4$ elute from the chromatographic column. Even when 2% HAc was added, retention times of standards and samples changed on a day-to-day basis and changed even between runs in the same day. Since HAc is not a buffer and is, therefore, not capable of maintaining a constant pH, a series of phosphate and acetate buffers were evaluated with pH from 2.1 to 8.1 at concentrations from 10 to 50 mM as a portion of the mobile phase. This was an attempt to better understand the elution characteristics of GA using a true buffer under different ionic strength conditions.

A 150 × 3.0 mm I.D., 5 μm particle size, 110 Å pore size, Gemini C18 analytical column preceded by a guard column containing a Gemini C18, 2 mm I.D. cartridge (Phenomenex, Torrance, CA) was used at a flow rate of 1.0 mL^{-1} with a mobile phase of 6:4, buffer:ACN during this pH/buffer concentration work.

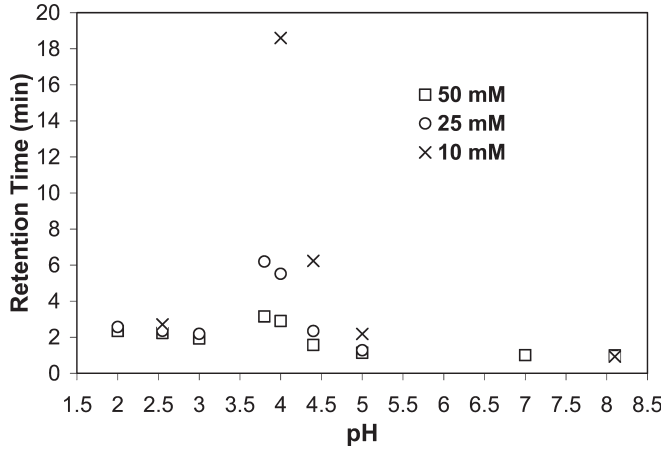

Figure 2. Plots of retention time change of glycyrrhizic acid ammonium salt standard versus pH at three buffer concentration levels.

Figure 2 shows the retention time change of $GANH_4$ at three buffer concentrations when pH is varied. At every buffer concentration, the retention time reaches a maximum around a pH of 4 and drops on either side of this value. The pKa of HAc is 4.6 and its buffer capacity (resistance to change in pH) will function from 3.6 to 5.6 pH units. This makes acetate buffer the choice to maintain a pH of 4. The retention mechanism appears complicated in that $GANH_4$ has changed to another more retentive species of GA around the pH of 4. To obtain a practical retention factor where chromatographic separation occurs, it appears one must use a buffer concentration between 25 and 50 mM, 10 mM buffer yielding retention times >18 minutes at pH 4.

Having established a pH and buffer concentration range, variations in the amount of ACN were evaluated. Upon decreasing ACN in the mobile phase by 5%, the retention time increased $\sim$ 80%; reducing ACN 10% resulted in a retention time increase of $\sim$450%. From these results, it is clear that not only pH, and buffer concentration impact on retention time, but the amount of ACN in the mobile phase is also critical.

Isocratic conditions were used when evaluating the modifications of the mobile phase. It was found that by continually injecting standards (40 injections) the retention time decreased from 6.85 to 5.56 minutes, a 23% change. The retention time could be stabilized by running the mobile phase overnight at a low flow rate of 0.2 mL min^{-1}. To eliminate this overnight equilibration in order to minimize retention time change, an ACN mobile phase wash was incorporated into the mobile phase program. This was an attempt to make the initial conditions (point of injection) constant for each analysis and to rid the column of retained components that may be affecting retention. Only when the buffer concentration was increased to 50 mM acetate buffer and used at around 70% did the retention time become

constant, but GA was eluting near or in the mobile phase ACN wash. A smaller dimension column, 50 × 2.0 mm I.D., 5 μm particle size, 110 Å pore size, Gemini C18 analytical column preceded by a guard column containing a Gemini C18, 2 mm I.D. cartridge (Phenomenex, Torrance, CA) 50 × 2 mm, containing less stationary phase, and 10% more ACN added to the mobile phase was used to elute GA before the ACN wash and worked well for licorice monitor samples. However, this 50 × 2 mm column could not resolve the other components found in the extract of licorice fortified tobacco, a longer column was needed. A 150 × 2 mm column and conditions described in the Experimental section was able to better resolve the compounds in the licorice fortified tobacco extract and was chosen to analyze both licorice and the licorice fortified tobacco.

Extractants, Extraction Conditions, and Precision

There was no problem extracting GA quantitatively from licorice with water (Table 1). This was accomplished by sonification or wrist action shaking for 1/2 h or microwave extraction at 100°C for 10 minutes of a 0.02 g sample in 25 mL water. The same result was obtained using any of these three conditions. To reduce sample weight and volume of extract, the sample weight and extract volume were proportionately reduced to a 0.005 g sample weight of licorice extracted in 5 mL water. Vortexing for 15 minutes was found to be sufficient to extract GA from licorice when results were compared to other extraction conditions. MeOH was found to be a poor extractant for licorice.

Extraction of GA from tobacco, where licorice was applied, was not as straight forward as extraction of GA from licorice. Vortexing a 0.2 g tobacco sample using water or MeOH resulted in a lower result than that obtained using other extracts (Table 1).

Extracting licorice applied to tobacco using solvents similar to the mobile phase (1:1 50 mM Ac^-pH4:ACN and 75:25 50 mM Ac^-pH4:ACN) increased the amount of GA extracted, but still may not be as effective as other extractants (Table 1). 1,4-Dioxane was evaluated since it has been used as the solvent during the hydrolysis of GA to glycyrrhetinic acid (GR).[6,7] Indeed, the author states that licorice is more readily soluble in this medium.[7] However, 1,4-dioxane is a known carcinogen and should be used with caution.

Noting the two ether units found in 1,4-dioxane, THF, 2- butoxyethanol, 2-methoxyethanol and 1,3-dioxolane were evaluated as extracts for licorice cased tobacco. These solvents were found to extract GA from both licorice and tobacco with applied licorice (Table 1). Figures 3 and 4 are chromatograms of the 1:1 ethanol: water extract of licorice and tobacco with applied licorice.

The minimum detectable quantity (MDQ) for GA in licorice was 1060 ppm and 26 ppm for GA in tobacco with applied licorice. The MDQ is based on a 5 mL extraction of 0.005 g licorice and 0.2 g tobacco with applied licorice using a low standard of 1.06 μg mL^{-1} $GANH_4$.

Table 1. Precision for glycyrrhizic acid extracted from licorice and licorice fortified tobacco tobacco[a] (n = 6, ppm ± standard deviation)

Extract	Licorice	Tobacco with applied licorice
Water	65509 ± 1399	119 ± 4.5
MeOH	41494 ± 2599	107 ± 6.4
1:1 50 mM Ac^-pH4:ACN	63234 ± 1082	245 ± 2.2
75:25 50 mM Ac^-pH4:ACN	n. a.	206 ± 6.7
1:1 1,4-Dioxane:water	67025 ± 1742	252 ± 2.0
1:1 EtOH:water	66444 ± 1758	285 ± 8.3
1:1 THF:water	64357 ± 2323	288 ± 4.7
1:1 2-Butoxyethanol:water	63903 ± 1756	322 ± 8.0
1:1 2-Methoxyethanol:water	64172 ± 2271	246 ± 10.0
1:1 1,3-Dioxolane:water	62951 ± 1979	282 ± 9.0

n. a. = not analyzed.
[a]Standards prepared in and samples extracted with the extract.

The detection limits can be improved by increasing sample weight, injection volume, sensitivity, and decreasing extraction volume.

Recovery/Standard Addition

Table 2 shows the percent recoveries of GA from licorice using various extracts to be >98%. The y-intercepts for licorice are in the range with

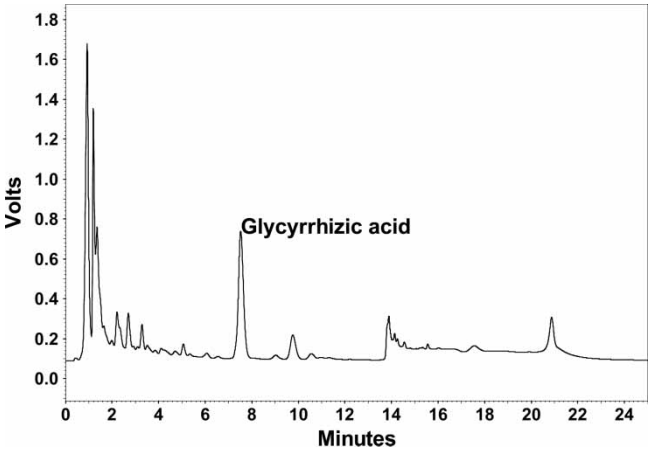

Figure 3. Chromatogram of the 1:1 ethanol:water extract of licorice. Conditions: see experimental section.

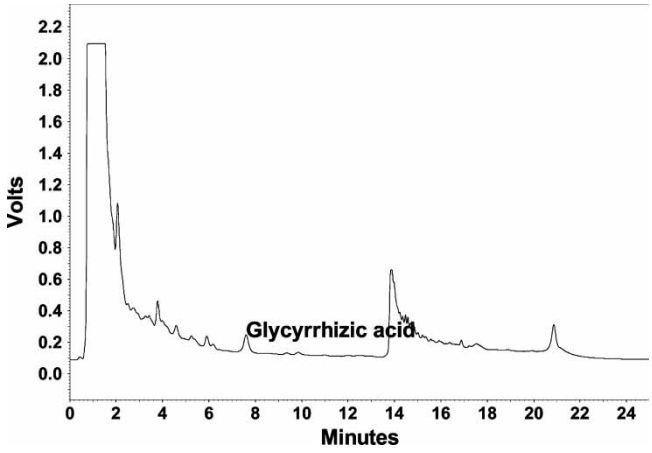

Figure 4. Chromatogram of the 1:1 ethanol:water extract of tobacco with applied licorice. Conditions: see experimental section.

those obtained by the external standard (Table 1). The percent recoveries of GA from tobacco with applied licorice are >92% and the y-intercepts, in most cases, close to those obtained by the external standard (Table 1).

Investigation of Potential Interferences with Glycyrrhizic Acid

GR, the aglycon of GA, could be a potential interference with GA. To resolve whether GR interfered with GA, standards of each compound were analyzed

Table 2. Recovery of glycyrrhizic acid (three levels, n = 2)

	Monitor licorice		Tobacco with applied licorice	
Extract	Recovery (%)	y-Intercept	Recovery (%)	y-Intercept
Water	99.1 ± 0.5	66341	n.a.	n.a.
1:1 Dioxane:water	100.3 ± 0.9	67058	92.9 ± 1.5	278
1:1 50 mM Ac^-pH4:ACN	n.a.	n.a.	93.3 ± 0.2	290
1:1 EtOH:water	98.2 ± 0.5	66290	106.3 ± 1.7	253
1:1 THF:water	100.8 ± 1.0	69853	99.4 ± 0.5	308
1:1 2-Butoxyethanol:water	101.2 ± 1.3	69802	105.6 ± 1.6	324
1:1 2-Methoxyethanol:water	100.1 ± 1.2	75430	98.0 ± 2.6	251
1:1 1,3-Dioxolane:water	98.8 ± 1.1	66520	103.5 ± 4.1	266

n. a. = not analyzed.

using the conditions described in the Experimental section. GR eluted in the ACN wash of the mobile phase and should not interfere with GA.

Tobacco with no licorice applied was extracted in 1:1, THF: water; 1:1, EtOH: water; 1:1, 1,4-dioxane:water; 1:1, 50 mM pH4 Ac^-:ACN; and water where no interferences with GA (peaks having the same retention time) were observed. In addition, the extractants listed in Table 1 were also evaluated for potential peaks that may co-elute with GA.

CONCLUSIONS

After optimizing the pH of the mobile phase using an acetate buffer, a precise and accurate HPLC procedure was used for the determination of GA in licorice and tobacco with applied licorice. Several extractants were found which extract GA from the more complicated tobacco matrix. No interferences from GR, tobacco without licorice and the extractants were observed, making the technique selective for the determination of GA.

REFERENCES

1. Vora, P.S. High pressure liquid chromatographic determination of glycyrrhizic acid or glycyrrhizic acid salts in various licorice products: collaborative study. JOAC **1982**, *65*, 572–574.
2. Shen, S.; Chang, Z.; Liu, J.; Sun, X.; Hu, X.; Liu, H. Simultaneous determination of glycyrrhizic acid and liquiritin in Glycyrrhiza uralensis extract by HPLC with ELSD detection. J. Liq. Chromatogr. & Rel. Technol. **2006**, *29*, 2387–2397.
3. Sabbioni, C.A.; Ferranti, A.; Bugamelli, F.; Forti, G.C.; Raggi, M.A. Simultaneous HPLC analysis, with isocratic elution, of glycyrrhizin and glycyrrhetic acid acid in licorice roots and confectionary products. Phytochem. Anal. **2006**, *17*, 25–31.
4. Baltina, L.A. Chemical modification of glycyrrhizic acid as a route to new bioactive compounds for medicine. http://www.uea.ac.uk/che/carbohydrate/CCCPeople/Baltina/review.htm. [12 December 2006].
5. Meylan, W.M.; Howard, P.H. SRC PhysProp Database. http://esc.syrres.com/interkow/webprop.exe?CAS = 1405-86-3 [10 August 2005].
6. Killacky, J.; Ross, M.S.F.; Turner, T.D. The determination of β-glycyrrhetinic acid in liquorice by high performance liquid chromatography. Planta. Med. **1976**, *30*, 310–316.
7. Cunndiff, R.H. Spectrophotometric determination of glycyrrhizic acid in licorice extract. Anal. Chem. **1964**, *36*, 1871–1873.

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