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Research Article

Development Of Novel TLC Method for Separation of Glycyrrhizic Acid from Aqueous Extract of Glycyrrhiza *Glabra*

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ABSTRACT

This study aimed at exploring the use of non-hazardous, sustainable solvents adhering to the principles of Green Chemistry for performing Thin Layer Chromatographic elution of Glycyrrhizic acid from aqueous extract of Liquorice. Despite its broad therapeutic potential, the pharmaceutical industry still faces challenges in accurate separation and quantification of Glycyrrhizic acid. Current methods rely on the use of hazardous, toxic chemicals such as methanol, formic acid and chlorinated solvents like chloroform raising concerns about environmental safety. Recent advancements have emphasized on developing analytical techniques on the principles of green chemistry while ensuring analytical accuracy. The absence of a green and reproducible method for Glycyrrhizic acid separation highlights the need for development of a green analytical method. After using a wide range of solvent mixtures, this study found that using a combination of n-Butanol: Acetic acid: Toluene in the ratio of 4.2:0.2:0.4 volume/volume, resulted in the separation of extract mixture into 3 distinct components indicating accurate separation. Resulting Rf of components was found to be Rf1 = 0.7, Rf2 = 0.59, Rf3 = 0.27. This study has both pharmaceutical and environmental implications. The use of n-Butanol: Acetic acid: Toluene as a solvent system proves that accurate separation of Glycyrrhizic acid can be achieved using green solvents. This study aligns with the principles of Green Chemistry offering a safer method to develop chromatographic techniques.

INTRODUCTION

Liquorice, derived from its biological source dried, unpeeled, stolon and root of Glycyrrhiza glabra Linn. It is a perennial herb cultivated in the states of Punjab, Maharashtra in India, and in some parts of Afghanistan and Europe. It is also popularly known as Mulethi and Yashtimadhu. Glycyrrhiza glabra has been extensively used in Indian Ayurveda for its hepatoprotective, anti-viral, and

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anti-inflammatory properties [2-6]. Recent studies showcase focused research on liquorice for the treatment of respiratory infections such as SARS COVID-19 [2] [11-12] due to the presence of glycyrrhizin present in the root of the plant. These findings indicate that glycyrrhiza glabra remains a vital component in the pharmaceutical industry due to its vast medicinal benefits, justifying its use for the preparation of asavas, arishtas, churnas [1]. This study focuses on separation of components from an aqueous extract of liquorice. Glycyrrhizic acid [GA], an oleanane class of pentacyclic triterpenoid saponin with 2 glucuronic acid units linked at 3-beta hydroxyl position, is the most prominent bioactive phytoconstituent in both the root and aqueous extract of Glycyrrhiza glabra [13]. GA is a natural sweetener with a sweetness 50 times more than sucrose, without the calorific load. This makes it a healthier alternative to processed sugar [2,4]. All these findings make Glycyrrhizic acid a "Component of interest". To use GA for preparation of pharmaceutical products, checking its availability on a large scale in an extract is of utmost importance. Extraction is defined as the separation of medicinally active portions of plant tissues from crude drug [14]. For extraction various polar solvents such as ethanol, methanol, water using conventional methods such as Decoction, Soxhlet extraction, Maceration, Sonication can be utilized [14-15]. This study used water as the solvent for extraction. A ratio of 1: 10 for crude drug and solvent was used. 50.72 gm of dried liquorice powder was taken along with 500 ml distilled water and heated at 80 degrees Celsius for 4 hours. The hot extract was allowed to cool to room temperature, before filtration using a clean white muslin cloth. The muslin cloth was previously wetted using warm distilled water, this removes any particulate matter in the cloth and prevents sticking of syrupy extract allowing for faster filtration. The filtered extract was further concentrated followed by lyophilization to obtain dried powder extract. This step enhances the shelf-life of the extract along with improving easy of handling.

MATERIALS

Normal phase TLC was performed using a polar Siliga gel 60 F254 as stationary phase. 60 represents pore size of silica, F254 stands for a fluorescent dye which absorbs UV light at 254nm and emits fluorescence at a higher wavelength.

Analytical grade n-Butanol, Acetic acid, Toluene were used. GA standard of 150 µg/ml was used.

METHODOLOGY

Glycyrrhizic acid is polar in nature due to presence of carboxylic acid functional groups, the stationary phase is the silica gel which has silanol functional groups making it polar as well. The higher retention of the analyte (GA) on the stationary phase is due to their alike polarities Hence to obtain distinct and accurate separation of components the mobile phase composition must comprise of a balance between polar and non-polar components. Making the mobile phase very polar will result in the elution of analytes to the solvent front, and a non-polar solvent system will result in poor to no elution as it will not have enough strength to pull our "compound of interest", Glycyrrhizic acid, from the stationary phase. Our mobile phase system comprising of n-Butanol, Acetic acid, Toluene fulfils all the analytical parameters along with providing a safer and sustainable method adhering to the principles of Green Chemistry. Conventionally used solvents such as chloroform, ethyl acetate, methanol have recently been under scrutiny owing to their environmental impact along with their handling and disposing protocols. The study conducted by Liu, Y., & Zhang, H. (2020) underscores the importance of adopting green chemistry principles

in chromatographic practices. Birch Biotech, formerly known as Birch Biosciences, is an organization that contributes to the cause of developing sustainable analytical chromatographic technologies by offering high quality greener solvents that minimize environmental impact, ensuring higher reproducibility in experimental protocols. They emphasize on minimal consumption of solvents and promote alternatives such as using supercritical CO_2 and other bio-based solvents. Such organizations initiate a broader trend that will lead to the adoption of such techniques. Butanol has increasingly gained popularity as it is recognized as a safer and environment friendly alternative to chlorinated solvents. The major drawback for using chlorinated solvents is the rigorous handling and disposal protocols. Butanol is more sustainable and does not require special disposal protocols, reducing its ecological footprint [10]. It is also an appropriate starting point for developing a chromatographic system due to its moderate polarity index of 4.0. Methanol which is commonly used for TLC elution has several toxic effects if not handled with care, use of chemical safety goggles, hand gloves is always recommended. Toluene is less toxic compared to methanol which can cause acute toxicity if not handled with care [7-10] Our solvent system also has analytical implications apart from its positive environmental implications. It ensures a sharp separation of spots with each solvent playing a key role. Butanol (polarity index 4.0) being adequately polar in nature ensures good interaction with GA allowing for its better separation from the polar stationary phase. Acetic acid (polarity index 6.0) improves resolution by minimizing tailing. Toluene (polarity index 2.4) is non-polar in nature enhances quality of separation by improving separation from non-polar impurities.

Laboratory Protocol

Both standard and sample are spotted on a clean and dry silica gel plate. Before spotting the plate is viewed under UV light to check for any existing spots/contamination that may interfere with separation of desired compound. This is followed by placing the plate in a pre-heated oven maintained at 110 degrees Celsius for 10 minutes. This step is called “activation” heating activates the silica layer which is coated on the aluminium plate. Activation helps in complete drying (moisture interferes with adsorption properties of silica), this ensures optimal interaction with the sample by increasing adsorption capacity of silica. Spotting on silica plate should be a precise (spot should be concise and should not spread over a large area). To ensure a concise spot, an applicator was made by dividing a capillary tube into 2 parts over a burner, this creates an aperture of a very small diameter. Spotting was performed twice to ensure adequate concentration of sample and standard are adsorbed on silica. Time is taken between subsequent spotting’s to ensure one spot is dry before applying the second one. This further ensures that a concise spot is developed. Final check is performed under UV light to ensure adequate concentration of sample and test is applied in a concise manner.

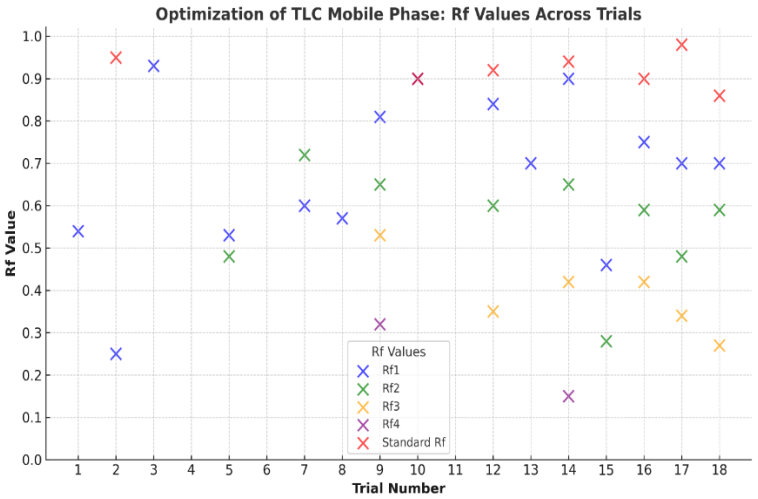
Method Development

Trial No	Mobile phase	No. of spots	RF	Remark
1	n-Butanol: Acetic acid: Water (3.5:0.5:0.5)	1 spot, standard at solvent front	Rf= 0.54	The standard spot was observed at the solvent front.

2	n-Butanol: Acetic acid: Water (4:1:0.5)	1spot, standard just below solvent front	Rf (std) = 0.95 Rf (sample)= 0.25	After slightly decreasing the polarity, the standard spot continued to remain with the solvent front.
3	n-Butanol: Acetic acid (4.5:0.5)	1 spot of sample and standard with solvent front.	Rf (sample)=0.93	Elimination of the most polar component in the mobile phase the issues with migration of standard remained.
4	n-Butanol: water (4.5:0.5)	Spots with solvent front	N/A	Variation of trail 3 was attempted by eliminating acetic acid.
5	CHCl3: Methanol: water (3:1.6:0.4)	2 spots seen, and standard with solvent front	Rf spot 1=0.53 Rf spot 2= 0.48	While optimum separation was observed for sample, the standard spot continued to migrate excessively
6	n-Butanol: hexane: acetic acid (4.5:0.5:0.5)	All spots at solvent front	N/A	First iteration by using a non-polar component hexane
7	n-Butanol: Toluene: acetic acid (4.5:0.5:0.5)	2 spots seen, standard at solvent front	Rf spot 1= 0.6 Rf spot 2 = 0.72	Better separation for sample was seen by use of toluene in the solvent composition.
8	n-Butanol: acetic acid (3.75:1.25)	1 spot seen, standard at solvent front	Rf = 0.57	A repeat of trial 3 was performed by adjusting concentration of components.
9	n-Butanol: acetic acid: toluene (4.2:0.4:0.4)	4 spots seen, standard at solvent front	Rf1= 0.81 Rf2= 0.65 Rf3= 0.53 Rf4= 0.32	Four spots for sample were observed, indicating excellent separation. However, standard remained an issue
10	n-Butanol: acetic acid: toluene (3.5:1:0.5)	1 spot seen, standard just below solvent front	Rf (standard)= 0.9 Rf = 0.73	The migration of standard was reduced, but resolution seen in previous trials.
11	Hexane: butanol: Chloroform (2.5:2:0.5)	All spots at solvent front	N/A	
12	Butanol: Hexane: acetic acid: toluene (3.5:0.7:0.4:0.4)	3 spots seen, and 1 of standard	Rf1= 0.84 Rf2= 0.6 Rf3= 0.35 Rf std= 0.92	As we proceed to obtain good resolution for sample, attempts were made to reduce the standard R_f
13	Butanol: Chloroform: Toluene: acetic acid (1: 3:0.5:0.5)	1 spot seen, standard just below solvent front	Rf1 = 0.7	Chloroform although not intended to use, was added to check its influence on standard R_f
14	Butanol: hexane: acetic acid: Toluene (3.5: 0.7: 0.2: 0.4)	4 spots seen and 1 of standard	Rf1 = 0.90 Rf2 = 0.65 Rf3 = 0.42 Rf4 = 0.15 Rf standard = 0.94	Similar results as seen in trial 13

15	Butanol: Hexane: Toluene: Formic acid (3.5: 0.7: 0.6: 0.2)	2 spots seen, Standard spot at the solvent front	Rf 1 = 0.46 Rf2 = 0.28	Formic acid was introduced as the polar component for the first time.
16	Butanol: Toluene: Formic acid (4.2: 0.4: 0.2)	3 spots seen, standard just below solvent front	Rf1 = 0.75 Rf2 = 0.59 Rf 3 = 0.42 Rf standard = 0.90	Hexane was eliminated and the concentration of n-butanol was increased. This was done due to moderate polarity of n-butanol to provide better control on resolution.
17	Butanol: Hexane: Formic acid: Toluene (3.5: 0.7: 0.2: 0.4)	3 spots, standard just below solvent front	Rf1 = 0.7 Rf2 = 0.48 Rf3 =0.34 Rf standard = 0.98	
18	Butanol: acetic acid: Toluene (4.2: 0.2 :0.4)	3 spots seen and 1 of standard	Rf 1 = 0.7 Rf2 = 0.59 Rf3 = 0.27 Rf standard = 0.86	The final trial saw a significant reduction in the Rf of standard concluding the experiment.

RESULTS AND DISCUSSION



A systematic and methodical approach was employed to optimize mobile phase composition to obtain optimum separation of analyte. Across 18 iterations we attempted to adjust the polarity of the saturated mobile phase to ensure good resolution of the spot. Various solvents such as water, acetic acid, formic acid, n-butanol, hexane and toluene were mixed in different ratios to obtain a concise spot. Trial 18 employed a mobile phase composition of n-butanol, acetic acid, and toluene (4.2:0.2:0.4), yielding three distinct analyte spots with Rf values of 0.70, 0.59, and 0.27, alongside the standard at Rf = 0.86, demonstrating optimal separation. The effectiveness of this solvent system stemmed from its precise polarity balance,

achieved through the strategic inclusion of toluene as a non-polar modifier. This adjustment mitigated excessive analyte migration observed in earlier trials dominated by polar solvents. The controlled addition of acetic acid provided sufficient elution strength without compromising resolution, ensuring well-defined spot separation. This approach highlights the significance of fine-tuned polarity modulation in achieving high-resolution chromatographic outcomes. An elaborate discussion on the performed trials is outlined below-

Initial Trials (1-4)

The primary trials utilized a binary or ternary combination of polar solvents such as acetic acid, n-butanol, water to assess migration of the polar analyte against a polar stationary phase. We emphasized on gradually increasing polarity to enhance elution strength of the solvent system. These strategic adjustments allowed us to better understand the interactions between the analyte and the silica plate. In Trial 1 a single spot with R_f = 0.54 was observed, indicating limited separation. Increasing the organic solvent proportion in Trials 2 and 3 improved analyte mobility but led to insufficient resolution. For Trial 2, results obtained were R_f (std) = 0.95, R_f (sample)= 0.25. Whereas observations for Trial 3 were, R_f (sample)=0.93 and the standard was seen at solvent front. Trial 4, using n-butanol: water (4.5:0.5), resulted in analytes co-migrating to the solvent front due to excessive polarity, confirming the need for non-polar modifiers.

Polarity Modulations (5-13)

To fine-tune the issues related to excess migration and poor resolution of the analyte non-polar components such as hexane and toluene were introduced. Trial 5 (chloroform: methanol: water; 3:1:0.4) yielded two distinct spots (R_{f1} = 0.53, R_{f2} = 0.48), signifying improved resolution. However, Trial 6 (n-butanol: hexane: acetic acid) caused all analytes to migrate to the solvent front, reflecting reduced. Subsequent trials incorporating toluene (e.g., Trials 7–10) achieved intermediate retention but exhibited inconsistencies in resolution due to suboptimal polarity balancing.

Optimization And Finalization (14-18)

The final stages involved the incorporation of formic acid and toluene followed by iterative tuning of solvent compositions. Trial number 18 being the hit trial. Butanol: acetic acid: Toluene (4.2: 0.2 :0.4) resulted in 3 distinct spots of R_f =0.7, R_{f2} = 0.59, R_{f3} = 0.27 and Rf of standard being 0.86, indicating optimum resolution. The observed trends during experimentation align with the established principles associated with TLC method development, where polarity is the key driver of subsequent adjustments made to mobile phase composition. Initially the use of polar solvents promoted excess migration of analyte to the solvent front. Subsequent introduction of non-polar solvents [hexane, toluene] modulated the interaction of analyte with the stationary phase., this allowed for better control over resolution and migration.

CONCLUSION

This study conclusively proved that a green, sustainable Thin Layer Chromatographic method can be developed for separation of GA from an aqueous extract of Glycyrrhiza glabra. This study addresses a critical gap in the development of chromatographic techniques and adheres to the principles of Green Chemistry. Use of non-hazardous, green solvent system comprising of n-Butanol: Acetic acid: Toluene in a ratio of 4.2:0.2:0.4 volume/volume, the retention factors for extract were found to be Rf_1 = 0.7, Rf_2 = 0.59,

Rf_3 = 0.27, indicating high accuracy and reproducibility

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