Formulation of health drinks using natural sweetener, its HPTLC method development and validation

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Ashwagandha, Tulsi, Mulethi, Awala, Shatavari, Gokharu, Arjun, Giloy, Safed musli, Kalimirchi, Haldi, Jaiphal was used as an active ingredients and aqueous extract of Stevia rebaudiana as natural sweetener with nutraceutical in health dinks. The product was developed by treating concentrates of each crude drug with purified water. TLC profile, HPTLC method development and validation were carried out using Gallic acid as a standard. A new simple, sensitive, selective, precise and robust HPTLC method for analysis of Gallic acid in health drink was developed. Precoated silica health drink aluminium plate 60F-254 (20 × 10 cm) with 200 µm thickness was used as stationary phase while toluene: ethyl acetate: formic acid: ethanol (6: 4: 0.3: 0.4) system was developed as a mobile phase. Spectrum analysis showed the same Rf values and spectrum pattern of standard and sample. The method was validated by using accuracy, precision, linearity, robustness, ruggedness and recovery as applicable parameters. The developed method was quite good and most sensitive for the present products. The unpleasant and bitter taste of the product was masked by different concentrations of aqueous extract of Stevia. Sweetness potency was determined by taste evaluation method. 1% Stevia extract is sufficient to produce most palatable and acceptable sweet preparation.

Key words: Formulation, TLC profile, HPTLC method development, validation, gallic acid, health drinks, Stevia rebaudiana.

INTRODUCTION

The herbal drug preparation in it’s entirely is regarded as the active substance and the constituents are either of known therapeutic activity or are chemically defined substance generally accepted to contribute substantially to the therapeutic activity of the drug. Photochemical screening involves botanical identification, extraction with suitable solvents, purification and characterization of the active constituents of pharmaceutical importance (Wickramasimamge M). Quality control for the officially and safely of herbal product is essential .the quality control of pharmaceutical may be defined as the status of a drug which is determined either by identity, purity, constant and other chemical physical biological properties or by manufacturing process .compound with synthetic drug. The critical and approach for herbal drug are much more complex. Phytopharmaceutical are always mixtures of many constituents and are therefore vary variable and difficult to characterize. The active principles in Phytopharmaceutical are not always known.

The quality criteria for herbal drugs are based on a clear scientific definition of the raw material. Depending on the type of preparation, sensory properties, physical constants, moisture, ash content, solvent residues and adulterations have to be checked to prove identity and purity. Microbiological contamination, foreign materials, heavy metals, pesticide residues, all toxins and radio activity also need to be tested. To prove the constant composition of herbal preparations, appropriate analytical methods have to be applied and different concepts have to be used in order to establish relevant criteria for uniformity (Farmsworth NR, 1982).

Health drinks contain Ashwagandha, Tulsi, Mulethi, Awala, Shatavari, Gokharu, Arjuna, Giloy, Safed musli, Ka-limirchi, Haldi, Jaiphal have been reported as nervine tonic, immunomodulatory agents, antioxidants, tonics for heart and liver, blood purifier.

Withania somnifera (Ashwagandha) (Withania Som-nifera, 2004) is a tonic, abortifacient, astringent, deobstr-uent, nervine, aphrodisiac and sedative. It has been used in diseases such as rheumatism, leprosy and arthritis. It

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is used to treat general debility, arthritis, depression, chronic fatigue, insomnia, anxiety, depressed immunity, infertility and memory loss. It increases the iron content. *Myristica fragrans* (Varro E, 1981) (Jaiphal) is aromatic, carminative, digestive, anti-inflammatory, diuretic, lactagogue, aphrodisiac, hypnotic, hallucinogenic, antispasmodic and stimulant agent. *Piper nigrum* (Trease and Evans’s, 1997) (Kaliimirchi) stimulates appetite, encourages peristalsis, tones the colon muscles and is a general digestive tonic. Sometimes it is used in gonorrhoea. On account of its stimulant action it aids digestion and is especially useful in a tonic dyspepsia and turbid condition of the stomach. *Tinospora cardiofolia* (Singh SS, 2003) (Giloy) is antiperiodic, antipyretic, alternative, diuretic, anti-inflammatory. It is a constituent of several compound preparations. It clears out brain toxin that hinders mental activity. *Curcuma longa* (Chattopadhyay I, 2004) (Haldi) is also used as an anti-inflammatory agent, and remedy for gastrointestinal discomfort associated with irritable bowel syndrome, and other digestive disorders. It is currently being investigated for possible benefits in Alzheimer's disease, cancer and liver disorders. *Terminalia arjuna* (Kokate CK, 2008) (Arjuna) is mainly used in heart disease, contusions, and fractures. It is prescribed for all sorts of conditions of cardiac failure and dropsy. The tonic made from bark is believed to have a stimulant effect on the heart. It helps strengthen the body's natural rejuvenate processes, hastening the replacement of dead or weak cells with fresh, vital ones. *Chlorophytum borivilianum* (Kokate CK, 2003) (Safed musli) is a rare divine-graced herb to offer all the effects required for achievement of health par excellence or for attaining the ultimate positive health. It treats male sexual inadequacies like oligospermia, lack of libido, impotency, etc, general debility. *Asparagus officinalis* (Kokate CK, 2008) (Awala) fruit is the richest known source of vitamin 'C' and used as a diuretic, appetizer, laxative, hair dye, and shampoo etc. It cures insomnia and is healthy for hair. It is also used as a Cardio protective, useful in hemorrhage, menorrhagia, leucorrhoea, and discharge of blood from uterus. *Stevia rebaudiana* (Savita SM, 2004) is a natural sweetener with nutraceutical. *S. rebaudiana* contains stevioside and rubarubioside as diterpene glycosides. These are 300 times sweeter than sugar. It also contains sequiterpene lactones that are responsible for bitter after taste. *S. rebaudiana* acts as anti-diabetic, anti-hypertensive, anti-hyperlipidemic, anti-yeast, antibacterial, antineoplastic and antifungal. It is considered as GRAS by USFDA.

Health drinks are the liquid preparation that contains vitamins, amino acids, minerals and other dietary supplements. Health drinks are useful (Naram KU, 2000) for body maintenance, to prevent and treat disease. As per the guidelines given in different references, different samples of health drink were prepared. Five different samples were developed using 0.25, 0.5, 0.75, 1 and 1.25% aqueous extract of *S. rebaudiana*. Sweetness potency of different samples was determined by taste evaluation method. Stevioside is a marker compound that could not absorb in UV region. *Stevia* contains 10 diterpene glycosides and gallic acid. TLC is possible for evaluation of health drink using stevioside as a standard. Therefore it was our intention to develop HPTLC method using gallic acid as a standard.

Thin layer chromatography (TLC), (Scinto S, 1988) also known as planar chromatography or flat bed chromatography is like all other chromatographic techniques, a multi-stage distribution process.

HPTLC is a powerful analytical technique (Agarawal H, 2004) due to its merits of reliability, simplicity, reproducibility and speed. A new, simple, sensitive, selective, precise and robust high-performance thin-layer chromatographic (HPTLC) method for analysis of Gallic acid was developed and validated for the determination of Gallic acid in health drinks. Gallic acid is mainly present in *Emblica officinalis* but also present in every crude materials used in the formulation. The aim of this work was to develop an accurate, specific, repeatable and robust method for the determination of Gallic acid.

The proposed method was validated in compliance with ICH guidelines (Q2A, ICH, 2005).

**EXPERIMENTAL**

**Materials**

*Ashwagandha, Tulsi, Mulethi, Awala, Shatavari, Gokharu, Arjun, Giloy, Safed musli, Kaliimirchi, Haldi, Jaiphal* were procured from authentic sources (Satara Arkshala Satara) and also authenticated by botanist, Prof. B. D. Patil, Botany department, SGM College, Karad, (M.S.). Standard Gallic acid was purchased from Loba Chemie.
Aqueous extract of each crude drug was obtained separately by cold maceration method (WHO Quality control). The health drink was prepared by aqueous extraction method. Each concentrate of different crude drugs was mixed together and was treated with purified water for 7 days with occasional shaking at room temperature and homogeneous product was developed.

Recovery studies

Repeatability of the sample application and measurement of peak area were carried out using six replicates of the same spot (600 ng spot−1 of Gallic acid) and the %R.S.D.

Limit of detection and limit of quantification

LOD and LOQ were experimentally verified by diluting the known concentrations of Gallic acid until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations.

Recovery studies

The HPTLC method was validated for the determination of Gallic acid in the health drink. A stock solution of Gallic acid (100 µg/mL) was prepared in methanol. All the mixtures were spiked with Gallic acid until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations.

Ruggenedness

A solution of concentration 1000 ng spot−1 was prepared and analyzed on day 0 and after 6, 12, 24, 48 and 72 h. Data were treated
for %R.S.D. to assess ruggedness of the method.

Specificity

The specificity of the method was ascertained by analyzing the Standard drug and Health Drink. The spot for Gallic acid in the sample was confirmed by comparing the \( R_f \) values of the spot with that of the standard. The peak purity of the Gallic acid was assessed by comparing the spectra at three different levels, viz. peak start (S), peak apex (M) and peak end (E) positions of the spot.

Detection of related impurities

The related unknown impurities were determined by spotting higher concentrations of the Gallic acid. Gallic acid solution was prepared at a concentration of 2000 \( \mu \text{g mL}^{-1} \) in methanol, and this solution was termed as sample solution. One milliliter of the sample solution was diluted to 40 mL with methanol and this solution was termed as standard solution (50 \( \mu \text{g mL}^{-1} \)). Two microliters of both the standard (100 ng spot\(^{-1}\)) and the sample solution (4000 ng spot\(^{-1}\)) were applied on HPTLC plate and the chromatograms were run as described.

Analysis of gallic acid in health drink

An accurately weighed quantity of Health Drink equivalent to about 100 ng of Gallic acid, that is, 8.5 g of Health Drink was extracted with 25 ml methanol by sonication for 30 min. This extract was centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was filtered and the filtrate was dried to constant weight at room temperature. The residue was redissolved in 5 ml of methanol. Six micro liters of the filtered solution was applied on the TLC plate followed by development and scanning as described in Section 2.2. The analysis was repeated in triplicate.

RESULT AND DISCUSSION

Development of the optimum mobile phase

The TLC procedure was optimized with a view to quantify the Health Drink. Initially toluene: ethyl acetate: formic acid: methanol in varying ratios was tried. The mobile phase toluene: ethyl acetate: formic acid: methanol (3:3: 0.8:0.2 v/v) gave good resolution with \( R_f \) = 0.59 for Gallic acid but typical peak shape was missing. Finally, the mobile phase consisting of (3:3:0.8:0.2 v/v) gave a sharp and well-defined peak at \( R_f \) = 0.59. Well-defined spots were obtained when the chamber was saturated with mobile phase for 30 min at room temperature.

Calibration curves

The developed HPTLC method for estimation of Gallic acid showed a good correlation coefficient \( (r^2 = 0.9991 \pm 0.0002) \) in concentration range of 100 - 1200 ng spot\(^{-1}\) with respect to the peak area. Fig. 3 displays three-dimensional image of the calibration samples at 254 nm. The mean value (±S.D.) of slope and intercept were 4.1312 ± 0.0491 and 208.2135 ± 4.5092, respectively. No significant difference was observed in the slopes of standard curves (ANOVA, \( P > 0.05 \)).

Method validation

The %R.S.D. for repeatability of sample application (600 ng spot\(^{-1}\)) and measurement of peak areas were found to be 0.09 and 0.15% respectively. The measurement of the peak area at three different concentration levels showed low values of S.E. and % R.S.D. (<1%) for inter- and intra-day variation, which suggested an excellent precision of the method (Table 1). The low values of S.D., %R.S.D. and S.E. obtained after introducing small deliberate changes in the developed HPTLC method indicated the robustness of the method. The proposed method when used for extraction and subsequent estimation of Gallic acid from the formulation afforded recovery of 98.91 - 101.34% as listed in (Table 3). Low %R.S.D. value of 0.0551 between the peak area values proved the ruggedness of the method indicating that Gallic acid is stable during the extraction procedure as well as during analysis. The peak purity of Gallic acid was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot as shown in Graph 1 and 2. Good correlation (\( r = 0.9992 \)) was obtained between the standard and the sample overlain spectra of Gallic acid.

Analysis of the prepared formulation health drink

A single spot at \( R_f \) = 0.59 was observed in the chromatogram of the Gallic acid extracted from health drink (Graph 2). There was no interference from the excipients and the other active components present in the herbal health drink formulation. The % recovery of the Gallic acid from the health drink formulation was found to be 98% and was well within the limits (label claim ±5%).

By considering \( R_f \) values of standard Gallic acid and spots observed of samples, fingerprint analysis, presence of this active chemical marker compound is detected. Chromatogram of sample solution showed other peaks than those of standards which might be due to the presence of other minor of major phytoconstituents present in it.

Stevia extract in various concentration potentiate sweetness index of health drink. 0.5% aqueous extract of Stevia is sufficient to produce acceptable and palatable product. Stevioside have no any chromospheres group that can absorb in UV region and hence gallic acid is the only one chiefly available marker compound which was used as a standard for HPTLC work.

Conclusion

The sweet formula for health drink was prepared which
Table 1. Intra and Inter-day precision of HPTLC method (n = 6).

<table>
<thead>
<tr>
<th>Amount (ng spot⁻¹)</th>
<th>Intraday precision</th>
<th>Inter-day Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean area</td>
<td>S.D.</td>
</tr>
<tr>
<td>400</td>
<td>5826.10</td>
<td>1.55</td>
</tr>
<tr>
<td>600</td>
<td>6720.68</td>
<td>1.51</td>
</tr>
<tr>
<td>300</td>
<td>7450.55</td>
<td>1.41</td>
</tr>
</tbody>
</table>

Table 2. Robustness of the HPTLC Method (n = 3,600 ng/spot).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>S.D. of peak area</th>
<th>% RSD</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase composition</td>
<td>1.62</td>
<td>0.3859</td>
<td>0.1575</td>
</tr>
<tr>
<td>Mobile phase Volume (18,20,22 ml)</td>
<td>1.79</td>
<td>0.2857</td>
<td>0.1167</td>
</tr>
<tr>
<td>Duration of Saturation (20,30,40 min)</td>
<td>1.42</td>
<td>0.2541</td>
<td>0.1180</td>
</tr>
<tr>
<td>Activation of prewashed TLC plates (2.5 and 7 min)</td>
<td>1.25</td>
<td>0.1874</td>
<td>0.0850</td>
</tr>
</tbody>
</table>

Table 3. Recovery Studies (n = 6).

<table>
<thead>
<tr>
<th>Excess drug added to analyte (%)</th>
<th>Theoretical content (ng)</th>
<th>Amount Found (ng)</th>
<th>Recovery (%)</th>
<th>% RSD</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>400</td>
<td>395.54</td>
<td>98.85</td>
<td>0.254</td>
<td>1.85</td>
</tr>
<tr>
<td>50</td>
<td>600</td>
<td>601.91</td>
<td>100.15</td>
<td>0.161</td>
<td>1.71</td>
</tr>
<tr>
<td>100</td>
<td>800</td>
<td>805.71</td>
<td>100.35</td>
<td>0.995</td>
<td>1.35</td>
</tr>
<tr>
<td>150</td>
<td>1000</td>
<td>981.90</td>
<td>98.19</td>
<td>0.105</td>
<td>1.81</td>
</tr>
</tbody>
</table>

Graph 1. Chromatogram of standard Gallic acid.
renders the product of appropriate sweetness potency. The developed sweet health drink constitutes major and minor chemical moieties including stevioside as a natural target molecule. The present work gives a scientific data about the formulation and development of herbal medicines using natural sweetener and a qualitative analysis or presence of antioxidant by HPTLC method. Gallic acid is a common chemical constituent of crude drugs in the formulation, used as a chemical marker compound in present study. TLC profile ensures (Soni Sapna, 2008) best resolution of a standard Gallic acid and sample. The developed HPTLC technique is a precise, specific, accurate and robust for the determination of Gallic acid. Statistical analysis proves that the method is reproducible and selective for the analysis of Gallic acid. Since the proposed mobile phase effectively resolves Gallic acid, the method can be used for qualitative as well as quantitative analysis of Gallic acid in Health Drink. Further the proposed method can be extended to study the degradation of Gallic acid under different stress conditions, as per the recommendations of ICH guidelines.

As *Stevia* acts as antidiabetic, antihypertensive, anti-hyperlipidemic, antioxidant, antimicrobial and nutraceutical, its use as a natural sweetener or versatile excipients in herbal preparations can provide a new vista to pharmaceutical industry so as to produce the product most elegant, sweet, acceptable and palatable by diabetic consumers.

REFERENCES


