Research Article

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Simultaneous Estimation of Scopoletin, Bacopaside-II, Bacopasaponin-C, Withanolide-A, and Withanoside-IV in a Clinically Proven Polyherbal Formulation for Treatment of Insomnia

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ABSTRACT

The present work is aimed to develop and validate a reverse phase high performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of Scopoletin, Bacopaside-II, Bacopasaponin-C, Withanolide-A, and Withanoside-IV in a proprietary polyherbal formulation containing *Bacopa monnieri* (Brahmi), *Convolvulus pluricaulis* (Shankhapushpi), *Withania somnifera* (Ashwagandha), *Nardostachys jatamansi* (Jatamansi), *Myristica fragrans* (Jatiphal) and *Valeriana wallichii* (Tagar) extracts intended for the treatment of insomnia. The HPLC analysis was performed on a Inertsil ODS, 3V, 250 x 4.6 mm x 5µm, C18 column using 0.1% orthophosphoric acid buffer as the mobile phase (solvent A) and acetonitrile (solvent B) with the gradient: 0-5 min, 10-20% B; 5-10 min, 20-30% B; 10-25 min, 30% B; 25-30 min, 30-40% B; 30-40 min, 40% B; 40-45 min, 40-60% B; 45-48 min, 60% B; 48-50 min, 60-30% B; 50-52 min, 30-10% B and 52-55 min, 10% B at a flow rate of 0.8 ml/min. The detection wavelength was chosen at 227 nm for Withanoside-IV and Withanolide-A, for Scopoletin, Bacopaside-II and Bacopasaponin C, it was 205 nm. The HPLC method was validated as per ICH guidelines for linearity, LOD and LOQ. The calibration curve of all the five phytomarkers showed excellent linear correlation coefficients with values (r2=0.996) for Scopoletin, (r2=0.995) for Withanoside-IV, (r2=0.996) for Withanolide-A, (r2=0.996) for Bacopaside-II and (r2=0.999) for Bacopasaponin-C. Limits of detection (LOD) were 0.04, 0.43, 0.35, 0.39 and 0.18 µg/ml and limits of quantification (LOQ) were 0.12, 1.29, 1.06, 1.18 and 0.54 µg/ml for Scopoletin, Withanoside-IV, Withanolide-A, Bacopaside-II and Bacopasaponin-C respectively. The developed HPLC method showed good separation of all the five constituents, enabling efficient analysis of Scopoletin, Withanoside-IV, Withanolide-A, Bacopaside-II, and Bacopasaponin-C in the polyherbal formulation.

Keywords: Scopoletin, Withanoside-IV, Withanolide-A, Bacopaside-II, Bacopasaponin-C, Insomnia

INTRODUCTION

Sleep is an essential part of human life and is necessary for maintaining an optimal health and has effect on hormonal levels, mood and weight. It is a naturally recurring feature of all species where mind and body undergo a state of unconsciousness with its surrounding for some time from which one can be aroused. It is the relatively inhibition of sensory activity and reduced muscle activity with rapid and non-rapid eye movement [1]. Sleep occurs in repeating periods according to the internal circadian clock that regulates cycles of sleepiness and alertness based on changes in the light in the surrounding environment [2].

Sleep helps to restore the immune [3], nervous, skeletal and muscular systems [4] and is regulated by the brain through cellular and molecular mechanism [5] and skeletal muscles through sleep regulatory signals to the brain [6].

Insomnia is dissatisfaction with sleep quality or quantity (*Levenson et al.,* 2015) [7] in addition to at least one other symptom among difficulty in initiating, maintaining sleep [8] and frequent awakening with inability to return to sleep

[9]. There are various factors responsible for insomnia like; unhealthy lifestyle, over intake of alcohol [10], central nervous system stimulants beverages like tea and coffee [11], working in shifts, peer pressures [12], overuse of mobile phones [13], age-related comorbid diseases and other medications [14]. According to various studies, 10-60% of the world population has a problem of insomnia [15].

Sleep disorders have a socio-economic impact like; consequent reduction in labor productivity and increased risk of accidents [16]. Chronic insomnia can be led to health problems such as cardiovascular disease [17,18], diabetes [19], and obesity [20]. Most of the time people with sleep problems avoid taking any step to resolve them, and the majority of people hide it from their physicians [21].

For the treatment of sleep disorders, commonly used FDA approved allopathic drugs include benzodiazepine (triazolam, estazolam, temazepam (quazepam and flurazepam), non-benzodiazepine drugs such as (eszopiclone, and zaleplon) and z- drugs (zolpidem, zopiclone, and zaleplon) [22,23]. There are few other drugs which are not targeting GABAergic system are melatonergic agonist drugs (Agomelatine, Ramelteon, Tasimelteon,) Orexin Receptor Antagonist Drugs (suvorexant), antidepressant drugs (Amitriptyline, Mirtazapine, Trazodone doxepin) [24], anticonvulsant drugs (Gabapentin, Pregabalin) and Antipsychotic Drugs (Olanzapine, Quetiapine). These drugs have negative side effects such as daytime drowsiness, dependency, depression, decreased concentration, vertigo, cognitive impairment, tolerance, memory impairment, loss of coordination, dizziness, disinhibition, gastrointestinal upset, rebound insomnia upon discontinuation and car accidents and dependence liability [23,25,26, 27], hence drugs with no or minimal side effects are being explored.

The alternative medicinal herbal therapy has more advances over the conventional allopathic treatment for the sleep disorders [28]. Herbal medicines have been in use since ancient times for the treatment of various diseases [29] since they are easily available and within the reach of common people. Almost 80% of the world's population is relying on herbal medicines for primary health issues [30,31,32]. Certain plants are known as sleep inducers and give a calming and relaxing effect which in turn results in sound and good sleep effect. These include *Bacopa monnieri*, *Rauvolfia serpentina* and *Withania somnifera* [23,33] highlight the potential use of herbal remedy to address insomnia. Most of the herbal medicines used for insomnia treatment target the GABAergic system [34]. A proprietary polyherbal formulation of SAVA Healthcare Ltd., have been developed using the extracts of Brahmi (*Bacopa monnieri*), Shankhapushpi (*Convolvulus pluricaulis*), Ashwagandha (*Withania somnifera*), Jatamansi (*Nardostachys jatamansi*), Jatiphal (*Myristica fragrans*), Tagar (*Valeriana wallichii*) and has been shown to be safe and effective in the treatment of primary insomnia by human trials [35].

The secondary metabolites like; polyphenols, flavonoids, terpenoids, glycosides terpenoids, saponins from these plants have sleep-inducing properties. Numerous chemical compounds from these plants are synergistically responsible for targeted activities [36]. Phytoconstituents present in the herbal extracts and formulations are raw material dependent and vary with changes in climatic conditions, season and region of the collection, and other raw materials adulteration. Standardization of herbal medicine gained utmost importance concerning quality and safety regulations [37]. Quality control of herbal medicines covers the aspects; phytochemical screening, in vivo, in vitro and marker compound analysis using modern analytical techniques [38]. High performance liquid chromatography (HPLC) is the simplest, reliable, and efficient technique for simultaneous estimation of two or more components from herbal extracts and formulations [39].

A polyherbal formulation for Insomnia has been developed at SAVA using well-known 6-ayurvedic ingredients for the management of primary insomnia. The proprietary polyherbal formulation contains extracts of 6 well-known Ayurvedic herbs, presented in the form of tablets. Each of the herbs incorporated in this polyherbal formulation, is well known for their action on sleep and CNS. While *Nardostachys jatamansi* is well-known to reduce stress and for its soothing and sedative action on the central nervous system [40], *Valeriana wallichii* is established to relieve anxiety and curing insomnia and found to be better than [41]. *Withania somnifera* has been recommended to be used in insomnia and is known for its adaptogenic properties and calms mind and bringing about sleep naturally [42]. *Myristica fragrans*, also known as 'nutmeg tree' is an aromatic plant and shows anti-depressant and calming effect [43]. *Bacopa* is classified as a brain tonic and removes anxiety and also is an antioxidant [44] while *Convolvulus pluricaulis Choisy* is classified as sleep promoting and this plant induces feeling of calmness, gives good sleep and is known for anxiolytic action [45]. From the literature reports, we observed the dose of each extract per day quite high for the intended effects. Hence, we reasoned that if we combine these extracts and reduce the dose, can we have the same effect without compromising the therapeutic efficacy of the herbal formulation.

Our proprietary in-house polyherbal formulation contains a mixture of 6- herbal extracts viz. Brahmi, Ashwagandha, Shankhapushpi, Jatamansi, Jatiphal and Tagar was used for the simultaneous estimation of phytoconstituents present in it by reverse phase-high performance liquid chromatography (RP-HPLC). Ashwagandha (*Withania somnifera*) is also known as Indian Ginseng. Plant preparation shows anti-inflammatory, anticancer and immunomodulatory activities [46] and has sleep-inducing properties [47]. Ashwagandha boosts brain functions, lower stress, improve immunity and balance sexual health [42]. Ashwagandha contains different chemical constituents are alkaloids, steroidal lactones and saponins. Prominent constituents of *Withania somnifera* roots are Withanolides and Withaferins [48]. Brahmi (*Bacopa Monnieri*) has been used since ancient times and is known as a memory enhancer and anxiety reducer [49]. Bacosides, Bacopasaponin, Cucurbitacins and flavonoids are the phytoconstituents present in the Bacopa [50]. Major Bacosides are Bacopaside-II, Bacopasaponin-C, Bacoside A3 and Bacopaside-X [51]. Shankhapushpi (*Convolvulus pluricaulis*) herb is well known for its effects against chronic cough, anxiety and sleeplessness [52]. The major phytoconstituents from Shankhapushpi are alkaloids Convolvine, Convolamine, Phyllabine, Convolidine, Convoline, Convosine and β-sitosterol and flavonoid Scopoletin [53]. Jatiphal (*Myristica fragrans*) is commonly used as a spice in Indian kitchens. It is also used as an anti-diarrheal, stomachic, aphrodisiac, stimulant and has sedative, sleep-inducing properties [54]. Stigmasterol, Beta-stigmasterol, Licarin, Neolignan, Viroline [55], Eugenol, Elemicin and Myristicin are the major chemical constituents of Jatiphal [56]. Tagar (*Valeriana wallichii*) is also known as Indian Valerian and has been used as a sleep aid in the treatment of insomnia [57]. Tagar (*Valeriana wallichii*) and Jatamansi (*Nardostachys jatamansi*) are both plants that belong to the Valerianeaceae family. Prominent phytoconstituents of the Tagar roots are Valerinic acid, Valepotriates whereas; roots of Jatamansi contain Valeranomne or Jatamansone, β-sitosterol and Jatamansin [58].

Since the sole aim of our effort was to develop HPLC methods for detection of presence of all the plant extracts in the current polyherbal formulation, we chose Withanoside IV and A from *Withania somnifera* and Scopotelin from *Convolvulus pluricaulis* which constitute 29% and 15% of total weight of the final formulation. Also, since valerenic acid is a common marker for both *Valeriana wallichii* and *Nardostachys jatamansi,* we decided not to estimate this marker for identification of these two extracts and opted for estimation of marker Bacopaside II and Bacopasaponin C from the third herb- *Bacopa* in this polyherbal formulation. Since the Myrsitin content in *Myristica fragrans* is 13 mg/g which is lower than the detection limit of 0.75 ppm of Myrsitin, we did not include this marker for the analysis by this HPLC method.

There are reported methods available in the literature for the determination of Bacopaside-II and Bacopasaponin-C from *Bacopa monnieri* [59,60,61] and Withanolide-A and Withanoside-IV from *Withania somnifera* [62,63] individual plants and their extracts. As well HPLC method was reported by [64] for simultaneous estimation of Scopoletin, Withaferin-A, Bacoside A3, Bacopaside-II, Jujubogenin and Bacosaponin-C in the extract from plant's *Convolvulus pluricaulis*, *Withania somnifera*, and *Bacopa monnieri* respectively. But, we report the simultaneous estimation of prominent markers Scopoletin, Bacopaside-II and Bacopasaponin-C, Withanolide-A and Withanoside-IV by HPLC in the polyherbal formulation containing extracts from six different plants for the first time. This method has been validated for linearity and LOD and LOQ as per ICH guidelines. The present work was aimed at simultaneous estimation of 5- active constituents in the in-house proprietary polyherbal formulation of 6-herbal extracts and will be useful for the quantification of these marker compounds given the quality and safety of the product.

MATERIALS AND METHODS

Standards and reagents

Analytical reference standards, Scopoletin (HPLC Purity 98%), Withanoside-IV (HPLC purity 97.7%), Withanolide A (HPLC purity 97.5%), Bacopaside-II (HPLC purity 99.0%) and Bacopasaponin C (HPLC purity 95%) were purchased from Natural Remedies Ltd., Bangalore, India. HPLC grade methanol and acetonitrile were obtained from Rankem India. The Milli-Q water was used for mobile phase preparation.

Before use, the solvents were filtered through a 0.45µ nylon filter (MDI Membrane Technologies, USA) and degassed in an ultrasonic bath (PCI Analytics, Mumbai). Orthophosphoric acid was purchased from Finar India Ltd.

Plant materials

Myristica fragrans extract was purchased from Kisalaya Herbals Ltd, Madhya Pradesh, India. The raw materials of *Bacopa monnieri* and *Withania somnifera* were purchased from Noor Nihal Herbal Trading Company, Bangalore and *Valerian wallichii*, *Nardostachys jatamansi* and *Convolvulus pluricaulis* raw materials from Gurukripa Agrotech, Bangalore.

Preparation of Bacopa monnieri plant extract

500 Kg *Bacopa monnieri* whole plant dried raw material was processed for extraction with six volumes of ethanol three times (each extraction for three hours) at 75-80°C. After extractions, all ethanol extractions were pooled and filtered through Polypropylene (PP) cloth using Sparkler filter. Then this filtered extract was concentrated in reactor and dried in vacuum tray dryer at 55-60°C.

Preparation of *Valeriana wallichii, Withania somnifera, Convolvulus pluricaulis* and *Nardostachys jatamansi* plant extracts

500 Kg Shankhapushpi whole plant dried raw material was processed for extraction with six volumes of 70% ethanol three times (each extraction for three hours) at 75-80°C. After extractions, all extractions were pooled and filtered through Polypropylene (PP) cloth using Sparkler filter. Then this filtered extract was concentrated in reactor and dried in vacuum tray dryer at 55-60°C. The same extraction process was applied for the *Withania* roots and valerian roots extracts preparation using 70% ethanol and *Nardostachys jatamansi* extract preparation using 50% ethanol as the extraction solvent.

Polyherbal tablet preparation

Each tablet contained 75 mg extract of *Valeriana wallichii,* 150 mg of *Withania somnifera,* 120 mg of *Convolvulus pluricaulis,* 70 mg of *Bacopa monnieri,* 70 mg of *Nardostachys jatamansi* and 15 mg of *Myristica fragrans* [35]. This is a proprietary formulation for treatment of Insomnia manufactured by SAVA Healthcare Ltd., was taken for the study. The tablets were prepared by using by non-aqueous wet granulation method containing the above herbal extracts in the ratio as per the [21] and the tablets were prepared by mixing all the herbal extract with crospovidone, sodium starch glycolate and microcrystalline cellulose in a Rapid mixer granulator. Granules were then prepared by adding sufficient quantity of granulating liquid i.e. isopropyl alcohol to make a damp mass. This wet mass was dried and passed through sieve no. 16 to get uniform granules. The prepared granules were later lubricated with magnesium stearate and compressed into tablet shaped punches using the Rotary Tablet Compression machine (Cadmach Machinery Company Private Limited, Ahmedabad, India) with an average weight of 750 mg. Further tablet were coating with a non-aqueous solvent system. The tablets were evaluated for the average weight, hardness, thickness, disintegration test and polyphenol content. Identical placebo in terms of color, size, shape, weight was prepared and also primary packing of the drug and placebo was kept absolutely similar in order to keep both investigator as well as the subject blind of which drug he/she was receiving. The placebo tablet (microcrystalline cellulose powder) contained the same excipients as the active tablets as described by [65].

Chromatographic system and conditions

HPLC analysis was performed using Waters Alliance e2695 HPLC system consisting of an intelligent pump with a high pressure mixer, auto sampling injection valve equipped with 1mL loop, and a PDA detector 2998. The compounds were separated on the Inertsil ODS, 3V, 250 x 4.6 mm and 5µm column (make-LCGC) with the mobile phase consisting of solvent A (Orthophosphoric buffer pH 2.0) and solvent B (acetonitrile). Orthophosphoric acid was prepared by adding 1 ml of Orthophosphoric acid in 1000 ml of Milli Q water. A constant flow gradient composition of 0-5 min, 10-20% B; 5-10 min, 20-30% B; 10-25 min, 30% B; 25-30 min, 30-40% B; 30-40 min, 40% B; 40-45 min, 40-60% B; 45-48 min, 60% B; 48-50 min, 60-30% B; 50-52 min, 30-10% B and 52-55 min, 10% B at a flow rate of 0.8 ml/min. The injection volume was 20 µl and the detection wavelength was set to 205 nm for Bacopaside-II, Bacopasaponin-C, Scopoletin, and 227 nm for Withanoside-IV and Withanolide-A by wavelength scanning from 190-400 nm using PDA detector. HPLC functioned at column oven temperature 35°C and sample temperature 15°C. Data were analyzed on a computer equipped with Chromeleon software. Before analysis, both the mobile phase and sample solutions were degassed by the use of a sonicator and filtered through 0.45 µl filter paper. The identities of four compounds were established by comparing the retention time of the sample solution with those of standard solutions.

Preparation of standard solution and construction of calibration plots

The standard stock solutions (100µg/ml) of Withanolide- A, Withanoside- IV, Bacopasaponin C and Bacopaside-II were freshly prepared by using 2.0 mg of each standard in 20 ml of methanol and 2.5 mg of Scopoletin in 25 ml of methanol. Further dilutions were prepared from these stock solutions by diluting 0.2 ml of Scopoletin standard stock solution and 2 ml of each Withanoside IV, Withanolide A, Bacopasaponin C, and Bacopaside II in to a 20 ml volumetric flask from respective initial stock solutions and made up volume up to the mark with diluent methanol and mixed well. This mixed standard solution was used to make calibration standard solutions. Seven-point calibration was done by preparing the dilutions of working standards at 5, 10%, 20%, 25%, 30%, 40% and 50% achieving concentrations in the range of 0.05-0.520 µg/ml for Scopoletin, 0.48-5.0 µg/ml for Withanoside-IV, 0.49-5.05 µg/ml for Withanolide-A, 0.48-5.07 µg/ml for Bacopaside-II and 0.48- 5.02µg/ml for Bacopasaponin-C.

Preparation of sample solution

For analysis of tablets dosage form, 10 tablets were weighed and the average weight was determined. Tablets were crushed and fine powder mixture of 300 mg was then transferred into a 25 mL volumetric flask. Added about 30 ml of methanol and sonicated for 20 minutes with intermediate shaking. The final volume was made up to the mark with methanol. The solution was mixed well, filtered through a 0.45 µ nylon filter and 20 µl was injected into the HPLC system.

Validation studies

The method was validated for the parameters like linearity, the limit of detection (LOD), and the limit of quantification (LOQ) as per the guidelines of the International Conference on Harmonization (ICH).

The linearity of an analytical procedure is its ability to obtain test results within a given range, which are directly proportional to the concentration of the analyte in the sample. The linearity study was done by sequentially diluting mixed standard solution to a given concentration range as given above. Calibration plots were formed for all four compounds, after triplicate analysis of each calibration solution, by plotting peak area against concentration (µg/ml) of the corresponding standard solution.

LOD is the lowest amount of analyte in a sample which can be detected but not necessarily quantified. LOQ of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantified. LOD and LOQ were experimentally verified by diluting known concentrations of Bacopasaponin C, Bacopaside-II, Withanolide-A and Withanoside-IV until the average response was approximately 3 to 10 times the standard deviation (SD) of response (peak area) for the three replicate determinations. For the determination of LOD and LOQ, linearity of the standards was performed three times to obtain the standard deviation of the intercept (SD) and slope of the regression equation (S) value. LOD and LOQ were determined by the standard deviation method and calculated as follows:

Limits of detection (LOD) = 3.3 × SD/S and Limits of quantitation = 10 × SD/S

RESULTS

Selection of appropriate column, mobile phase ratio, use of proper buffer reagent concerning pH of compounds to eluted, detection wavelength, and flow rate are the key parameters for development of any robust HPLC method with efficient separation of desired compounds. This paper focusses on simultaneous estimation of five constituents namely Scopoletin from *Convolvulus pluricaulis*, Withanoside-IV and Withanolide-A from *Withania somnifera*, Bacopaside-II and Bacopasaponin-C from *Bacopa monneiri* from the proprietary polyherbal formulation since these extracts constituted maximum of the total weight of the final formulation that has shown efficacy in treating Insomnia through a double blind controlled human studies [35].

A series of mobile phases containing different volume fractions of water and acetonitrile with different pH's and different flow rates were tested but we could not get proper peak resolutions for these five ingredients simultaneously. The final mobile phase consisting of solvent A (0.1% orthophosphoric acid) and solvent B (acetonitrile) yielded best results.

The chromatogram of the mixed standards solutions of all the 5 standards at a concentration of 20 µg/ mL is shown in Figure 1. The gradient used was: 0-5 min, 10-20% B; 5-10 min, 20-30% B; 10-25 min, 30% B; 25-30 min, 30-40% B; 30-40 min, 40% B; 40-45 min, 40-60% B; 45-48 min, 60% B; 48-50 min, 60-30% B; 50-52 min, 30-10% B and 52-55 min, 10% B at a flow rate of 0.8 mL/min. The peaks of Scopoletin, Withanoside-IV, Bacopaside-II, Bacopasaponin-C and Withanolide-A were eluted at 15.23, 20.75, 38.47, 41.13, and 46.54 mins respectively at 205 nm with a run time of 55 min. The flow rate of 0.8 mL/min was found optimum for the separation of these compounds through the selected HPLC column.

This method was applied for the quantification of 5-phytomarkers in the in-house polyherbal formulation and the results are presented in Table 1. Chromatogram obtained from the methanol extract of the polyherbal formulation showing Scopoletin, Withanoside-IV, Withanolide-A, Bacopaside-II and Bacopasaponin-C is shown in Figure 2.

Table 1: Active constituents in the polyherbal formulation for Insomnia.

S.N.	Name of active constituent	Extract	Amount of extract/ tablet	Theoretical content in Tablet (mg)	Actual Content in Tablet (mg)
1	Scopoletin	*Convolvulus pluricaulis*	120 mg	0.0068	0.0043
2	Withanolide A	*Withania somnifera*	150 mg	0.39	0.22
3	Withanoside IV			2.2	1.21
4	Bacopaside II	*Bacopa monnieri*	70 mg	3.64	3.49
5	Bacopasaponin C			3.63	4.10

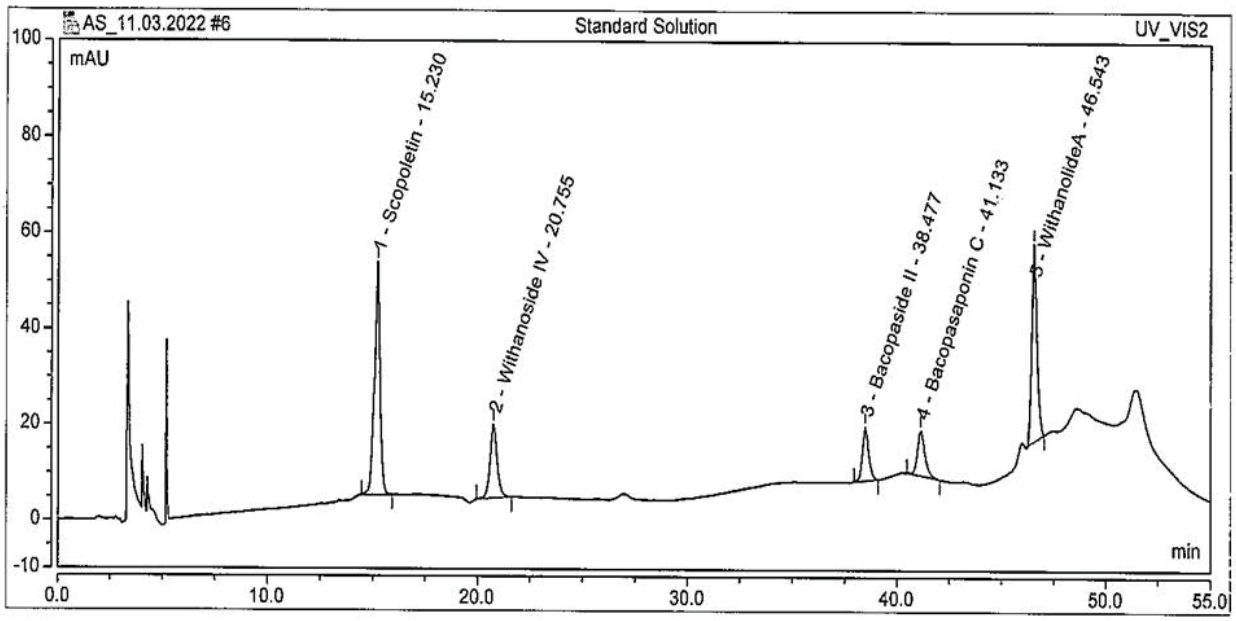

Figure 1: HPLC chromatogram of standard solutions of Scopotelin at 1 ppm concentration while Withanoside-IV, Withanolide-A, Bacopaside-II, and Bacopasaponin-C were prepared as 10 ppm solutions. The wavelength chosen was 205 nm.

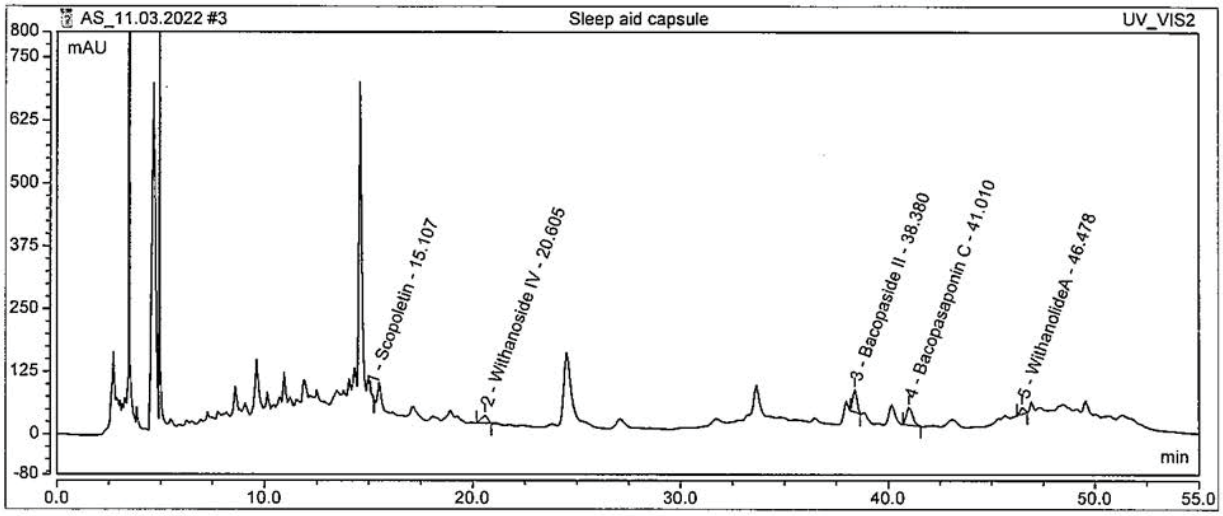

Figure 2: HPLC chromatogram of a sample of the polyherbal formulation. See materials and methods section for sample processing details. The peaks of all the five active constituents are labelled and denoted by in arrows. The wavelength of detection was 205 nm.

This method was validated for linearity, LOD and LOQ. All the validation studies were carried out by triplicates. Linearity was determined for all the five markers namely Scopoletin, Withanoside-IV, Withanolide-A, Bacopaside-II, and Bacopasaponin-C separately by plotting a calibration graph of peak area against the respective concentration (supplementary Figuress. S1, S2, S3, S4 and S5). From the calibration curve, it was clear that Scopoletin showed linearity between 0.05-0.52, Withanoside-IV showed the linearity between 0.48-5.0 µg/ mL, for Withanolide-A had linearity between 0.49-5.05 µg/ mL, Bacopaside-II showed linearity between 0.48-5.07 µg/ mL and Bacopasaponin-C had between 0.48 to 5.02 µg/ mL. The linear correlation equation for the five markers were; Scopoletin: y=154,919.6526x - 817.2862 with r2= 0.996, Withanoside-IV: y = 10,505.4200x - 467.5208 with r2 =0.995; Withanolide-A: y =53,084.6362x - 5,234.9166 with r2 =0.996;

Bacopaside-II: y = 9,992.1808x - 1,308.6186 with r2= 0.996 and Bacopasaponin-C: y = 10,440.2964x - 182.1004 with r2= 0.999, where y is peak area and x is concentration.

Determination of LOD and LOQ, linearity of the standards was performed in triplicates to obtain the standard deviation of the intercept (SD) and slope of the regression equation (S) value. LOD and LOQ studies were carried out to evaluate the detection and quantitation limits of the method to determine the presence of any impurities by using the following equations: LOD=3.3 σ/S and LOQ=10 σ/S, where σ is the standard deviation and S is the slope of the curve. Limits of detection (LOD) were 0.04, 0.43, 0.35, 0.39 and 0.18 µg/ mL and limits of quantification (LOQ) were 0.12, 1.29, 1.06, 1.18 and 0.54 µg/ mL for Scopoletin, Withanoside-IV, Withanolide-A, Bacopaside-II and Bacopasaponin-C respectively (Table 2).

DISCUSSION

The HPLC method described in this article is advantageous for the estimation of phytomarkers in the polyherbal formulation from the mixture of different extracts. It showed appreciable resolution of marker compounds in standard solution and extract mixture. This method was able to separate and quantify bulky steroidal lactones and triterpene saponins from the extract mixture.

There are reported methods available in the literature for the determination of Bacopaside-II and Bacopasaponin-C from *Bacopa monnieri* [59-61] and Withanolide-A and Withanoside-IV from *Withania somnifera* [62,63] individual extracts. Also, a HPLC method for simultaneous estimation of Scopoletin, Withaferin-A, Bacoside A3, Bacopaside-II, Jujubogenin and Bacosaponin-C in the extract from plant's *Convolvulus pluricaulis*, *Withania somnifera*, and *Bacopa monnieri* respectively is reported. However the detection limit observed by [64] for Scopoletin, Bacopaside II and Bacopasaponin C were 0.8, 4.07 1.89 µg/ mL respectively while the quantification limits was found to be 2.45, 12.34, 5.74 µg/ mL for Scopoletin, Bacopaside II and Bacopasaponin C respectively.

The HPLC method described here shows the better limit of detection and quantification as compared to earlier method [64]. The detection limit for Scopoletin, Bacopaside II and Bacopasaponin C are 0.04, 0.39 and 0.18 µg/ mL respectively, while the limit of quantification was found to be 0.12, 1.18 and 0.54 µg/ mL for Scopoletin, Bacopaside II and Bacopasaponin C respectively.

We report the simultaneous estimation of prominent markers Scopoletin, Bacopaside-II, Bacopasaponin-C, Withanolide-A and Withanoside-IV by HPLC in the polyherbal formulation containing extracts from six different plants for the first time. This method has been validated for linearity and LOD and LOQ as per ICH guidelines. The present work disclosing an efficient method for simultaneous estimation of 5- active constituents in the in-house proprietary polyherbal formulation is novel, sensitive and can be applied to polyherbal formulations with similar extracts for quantification of the marker compounds ensuring the quality and safety of the product.

The human trials study with the polyherbal formulation on Insomnia subjects was registered on: 04/04/2018 (Clinical Trial Registry of India {CTRI}/2018/04/013035) and the treatment group subjects were given two dose comprising 2 polyherbal tablets orally after evening/night meal with water for 28 days. The treated subjects showed a sleep on-set of sleep onset was 93.00 min on visit 1 (day 7) which reduced to 31 min on day 28. The placebo treated group it increased from 113 to 137 min. The total number of awakenings was 0.4 on day 28 for treated groups while it increased to 3.23 min in placebo treated group.

Bacopa monnieri supplementation, 150 mg of a standardized *Bacopa monnieri* extract, per day did not improve sleep patterns more than the placebo in adults with self-reported sleep problems since sleep onset after 28 days supplementation was same as placebo. In Bacopa treated group, the no. of awakenings was reduced from 3 to 2.13, which is low as compared to the observed results with our polyherbal formulation. It is reported that *Withania somnifera* at a treatment dosage ≥600 mg/day, for as long as 8 weeks shows

Table 2: Limit of Detection and Limit of Quantification for active constituents from the polyherbal formulation effective against Insomnia.

S.N.	Active constituent	LOD (µg/ml)	LOQ (µg/ml)	Linearity	
				Regression equation	R^2
1	Scopoletin	0.04	0.12	y=154,919.6526x - 817.2862	0.996
2	Withanoside IV	0.43	1.29	y = 10,505.4200x - 467.5208	0.995
3	Withanolide A	0.35	1.06	y =53,084.6362x - 5,234.9166	0.996
4	Bacopaside II	0.39	1.18	y = 9,992.1808x - 1,308.6186	0.996
5	Bacopasaponin C	0.18	0.54	y = 10,440.2964x - 182.1004	0.999

improvement in alertness and anxiety level but no significant effect on quality of life. In our study, we see 87% improvement in quality of life in comparison to placebo indicating the superior properties of our polyherbal formulation with other individual extracts [66]. *Valeriana wallichii* and *Nardostachys jatamansi*, at a dose of 12 g daily each for 1 month provided significant improvement in sleep onset and improved sleep disturbances better than *Nardostachys jatamansi* in humans suffering from insomnia [41]. The dose of these plant extracts used in this study is several folds higher that what we have used in our polyherbal formulation, hence our study with least amount of these extracts per day assumes critical importance.

Wellness is not just confined to physical health but also applied to mental health [59]. However, due to unhealthy and busy lifestyles, people are suffering from different mental disorders like; depression [68] anxiety and insomnia [33]. Analyses across 53 mammalian species indicate a strong correlation between duration of sleep and metabolic rate [69], hence interventions/strategies to address insomnia appears to be the need of the hour [70].

CONCLUSION

Polyherbal formulations contain multiple active phytoconstituents that are responsible for the desired activity through a synergistic effect. Standardization of herbal polyherbal is inevitable to ensure the quality and safety of the herbal products concerning human consumption, various markets and regulatory bodies' requirements. The proposed research study discloses development and validation of a RP-HPLC method for the estimation of 5-phytomarkers in the in-house proprietary polyherbal formulation intended for the treatment of primary insomnia. Such a developed method is reproducible, sensitive and linear establishing its reliability. The standards and sample preparations in this method are very simple and a single solvent is used throughout the method. The mobile phase gradient separated the peaks of 5-markers in polyherbal formulation accurately. This method reduces the time required for estimation individual ingredients from this polyherbal formulation.

ABBREVIATIONS

µ: Micron; CNS: Central Nervous System; µm: Micrometer; mAU: Milli Absorbance unit; g: Gram; hrs: Hours; RP-HPLC: Reverse phase high-performance liquid chromatography; Kg: Kilogram; mg: Milligram; min: Minutes; mL: Millilitre; SD: Standard deviation; nm: nanometer; µl: microliter; µg: microgram; LOD: Limit of detection; LOQ: Limit of quantification (LOQ); ICH : International Conference on Harmonization; CTRI: Clinical Trial Registry of India; *r*2: Coefficient of determination

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The authors do not have any conflict of interest.

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