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ORIGINAL RESEARCH PAPER

Separation and quantification of nine bioactive compounds in traditional Unani formulations by High Performance Liquid Chromatography–Photodiode Array Detector

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ABSTRACT

A new High Performance Liquid Chromatography–Photodiode Array Detector (HPLC–PDA) method has been developed for the chromatographic separation and simultaneous quantitative determination of nine bioactive compounds, i.e. four phenolic (gallic acid, ellagic acid, chebulinic acid, and tannic acid), two flavanoids (rutin and quercetin), two anthraquinones (sennoside A and B) and one oxygenated hydrocarbon (vitamin C) in a well-known Unani polyherbal formulation namely Itrifal's. Separation was accomplished on a C18 LiChrospher 100 column (5 μ m, 250 $\times$ 4.6 mm) with a gradient elution and recorded at 254 nm. The results demonstrated that the proposed method is reproducible, accurate, economic, and suitable for the quality control of traditional polyherbal Unani formulations containing complex compounds with different structures such as Itrifals.

KEYWORDS

HPLC–PDA, quality control, polyherbal formulation, simultaneous determination

INTRODUCTION

The use of herbal drugs for the prevention and treatment of various health ailments has been in practice from time immemorial. Generally, it is believed that the risk associated with herbal drugs is very less, but reports on serious reactions are indicating to the need for development of effective marker systems for isolation and identification of the individual components. Standardisation, stability and quality control for herbal drugs are feasible, but difficult to accomplish [1]. The present study attempts to develop a multi-component High Performance Liquid Chromatography (HPLC) assay method which will help in the quality control of herbal formulations.

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Fig. 1 Chemical structure of all nine bioactive compounds selected for analysis

It is believed that the therapeutic efficacy of traditional Unani multi-herbal formulations is integrative results of multiple bioactive components. These multiple constituents in the herbs/formulations may work 'synergistically' and give the optimum therapeutic effects [2]. Therefore, simultaneous determination of the biologically active components is necessary for the quality control of these types of formulations.

According to the Unani system, Itrifal or Trifaloon is actually the name of a herbal preparation of three different types of fruits i.e. Halela (*Terminalia chebula*), Balela (*Terminalia belerica*), Amla (*Emblica officinalis*). Itrifals are proven for its anti-oxidant activities; most generally it's used as an overall body tonic, thought to be effective in cleansing and detoxifying the system [3]. Additionally, some other ingredients may be added to the base formulation, 'Itrifal' and they are named like Itrifal-e-Aftimoon (IA) and Itrifal-e-Badiyan (IB), which were selected for the present study. The IA composed of twelve crude drugs namely, *T. chebula*

(15.2% w/w), *T. belerica* (15.2% w/w), *E. officinalis* (15.2% w/w), *Operculina turpenthum* (15.2% w/w), *Cuscuta epithymum* (7.4% w/w), *Cassia angustifolia* (7.4% w/w), *Plumbago zeylanica* (4.5% w/w), *Polypodium vulgare* (4.5% w/w), *Lavandula stoechas* (4.5% w/w), *Rosa damascene* (4.5% w/w), *Pimpinella anisum* (3% w/w) and *Prunus amygdalus* (3% w/w), whereas, IB composed of eight ingredients, namely, *T. chebula* (7% w/w), *T. belerica* (7% w/w), *E. officinalis* (7% w/w), *Vitis vinifera* (7% w/w), *Rosa damascene* (7% w/w), *Zataria multiflora* (7% w/w), *Foeniculum vulgare* (50% w/w) and *P. amygdalus* (7% w/w), which are official in National Formulary of Unani Medicines (NFUMs) published by Govt. of India [4]. Five components of both the formulations namely, *T. chebula*, *T. belerica*, *E. officinalis*, *R. damascene*, and *P. amygdalus* are common whereas, all the Itrifals should essentially contains *T. chebula*, *T. belerica*, *E. officinalis* as an ingredient. Two batches (Batch I and Batch II) for IA and IB were prepared in the laboratory by following the procedure mentioned in NFUM.

Fig. 2 Representative HPLC-PDA chromatogram at 254 nm of nine compounds eluting (a) standard (b) Itrifal-e-Aftmoon sample (c) Itrifal-e-Badiyan sample

Concentrated sugar syrup was first prepared by adding 500 g of sugar in 300 mL of water and boiling for 15 min with continuous stirring. The powdered and sieved drugs were added one by one to the sugar syrup with continuous constant stirring.

The major antioxidants are free phenolic acids like ascorbic acid, gallic acid, ellagic acid, chebulinic acid, and tannic acid as well as free flavonoids like rutin and quercetin forms the basis of bioactivity of these Itrifal formulations [5]. The anthraquinone glycosides like sennoside A and B were

Table 1. Linear relationships between peak area and sample concentration

Compound	Calibration curve	Linear range (µg/mL)	Slope ± SD	Intercept ± SD	Regression coefficient	LOD (µg/mL)	LOQ (µg/mL)
Ascorbic acid	$y = 201.27x - 146.07$	6.4–320	201.27 ± 4.8	144.79 ± 1.20	0.9994	1.2	5.3
Gallic acid	$y = 1154.9x + 1520.8$	1.1–474	1154.9 ± 31.5	1538.05 ± 24.40	0.9983	0.27	0.92
Rutin	$y = 458.81x + 6.0875$	1.8–257	461.14 ± 6.9	6.08 ± 0.4	0.9993	0.44	1.2
Sennoside B	$y = 36.818x + 78.931$	10–350	36.406 ± 0.97	78.9 ± 1.60	0.9989	4.1	10
Chebulinic acid	$y = 172.08x + 163.97$	10.6–355.8	172.0 ± 5.1	167.79 ± 4.74	0.9996	3.2	10.1
Sennoside A	$y = 109.88x - 2850$	10–220	109.56 ± 2.12	2875.7 ± 31.63	0.9929	3.1	10.3
Quercetin	$y = 112.31x - 86.993$	5–346.8	112.3 ± 2.0	87.5 ± 0.74	0.9967	1.4	5
Ellagic acid	$y = 473.7x - 838.12$	2.3–456	477.1 ± 3.31	841.64 ± 10.45	0.9995	0.85	2.2
Tannic acid	$y = 332.54x + 837.85$	2.8–2000	335.68 ± 3.29	842.45 ± 0.5	0.9997	0.70	2.1

selected as a specific marker for *C. angustifolia*, which is present in a significant amount in IA.

Literature survey reveals that up to now no methods have been reported on the quantitative analysis for multiple compounds in Itrifal formulations. In most of the previous studies, phenolic, and flavonoid constituents in various herbal samples had been analysed by HPTLC [6], HPLC–CAD [7], HPLC–DAD [8], HPLC–DAD–ESIMS [9], and HPLC–PDA [10] methods with the emphasis on individual components. In addition, most of the methods reported on the quality control of polyherbal formulations deals only with the quantification of one or two components [11]. The HPLC methods reported on the simultaneous quantification of multi component traditional formulation fails to suggest a proper extraction procedure in order to attain the maximum recovery of all the markers. The direct injection of such samples in HPLC will lead to interference of targeted marker components with other moieties present in the sample and the compounds in lower concentrations cannot be detected.

The aim of the study is to develop a simple, reliable and reproducible method on the simultaneous determination of multiple active components present in these commonly used traditional medicines for their quality control as well as to propose an optimised extraction procedure in order to improve the recovery of all nine marker constituents from polyherbal Unani formulations.

EXPERIMENTAL

Samples, chemicals, solvents and standards

Three batches of Itrifal-e-Aftiomoon and Itrifal-e-Badiyan were chosen for analysis, batch I was prepared by the ingredients collected from northern part of India (Delhi),

whereas batch II was formulated using the ingredients purchased from southern part of India (Hyderabad). Both batches were prepared as per the procedure mentioned in National Formulary of Unani Medicine (NFUM) published by Govt. of India. Batch III was obtained as gift sample from Central Council for Research in Unani Medicines (CCRUM, Hyderabad). All the component drugs of formulations were identified by a botanist of University and same were authenticated by a Pharmacognosist of laboratory. The voucher specimens of all the drugs and formulations have been deposited in Bioactive Natural Product Laboratory of University. Ortho-phosphoric acid (H_3PO_4) 88% was purchased from Spectrochem Pvt. Ltd., Mumbai, acetonitrile and methanol were of HPLC grade from Merck Specialities Private Ltd., Mumbai. Milli Q water was used throughout the experiment, which was prepared using Millipore water purification system.

Gallic acid 97%, ellagic acid 97%, and tannic Acid 95% standards were obtained as gift samples from Sami Labs Ltd., Bangalore, (India). Ascorbic acid 99%, Rutin 98%, and Quercetin 96% reference standards were obtained from Sigma Aldrich (USA). Chebulinic acid 95%, Sennoside A 94%, and Sennoside B 89% standards were procured from ChromaDex, India (Bangalore). The chemical structures of all nine components were given in Fig. 1.

Chromatographic conditions and instrumentation

The analysis was carried out on a Waters Alliance e2695 separating module (Waters Co., MA, USA) using photo diode array detector (Waters 2998) with autosampler and column oven. The instrument was controlled by use of Empower software installed with equipment for data collection and acquisition. Compounds were separated on a C18 reverse phase (RP) column (250×4.6 mm, particle size 5 µm, Merck, Germany) maintained at room temperature.

Table 2. Accuracy study of phenolics, flavonoids and anthraquinones

Compound	Mean of actual amount ($\mu\text{g/mL}$)	Mean amount spiked ($\mu\text{g/mL}$)	Mean recovered amount ($\mu\text{g/mL}$)	Mean % recovery	% RSD
<i>Ascorbic acid</i>					
1	39.60	19.77	59.68	100.52	0.27
2	39.55	39.62	79.49	100.41	0.31
3	39.66	59.44	98.97	99.87	1.21
<i>Gallic acid</i>					
1	174.4	87.88	257.29	98.10	2.33
2	175.0	174.2	340.82	97.6	1.01
3	174.9	263.15	440.67	100.6	0.67
<i>Rutin</i>					
1	35.27	17.68	54.88	103.65	0.56
2	35.42	35.87	68.80	96.51	0.33
3	36.11	54.11	88.32	97.9	1.12
<i>Sennoside B</i>					
1	10.82	5.25	17.4	108.27	0.55
2	10.69	10.98	21.65	99.90	0.76
3	10.89	16.19	27.18	100.36	0.74
<i>Chebulinic acid</i>					
1	12.43	6.27	18.54	99.18	2.11
2	12.46	12.56	23.70	94.36	3.56
3	12.51	18.82	32.96	105.21	0.56
<i>Sennoside A</i>					
1	17.25	8.72	26.12	100.6	0.43
2	17.38	17.30	34.53	99.56	0.89
3	17.32	24.99	42.10	99.52	1.44
<i>Quercetin</i>					
1	6.82	3.54	10.73	103.59	2.51
2	6.69	6.81	13.55	100.40	0.34
3	6.49	9.66	16.51	102.27	0.92
<i>Ellagic acid</i>					
1	56.32	28.11	82.20	97.36	1.30
2	57.11	57.77	115.44	100.49	3.93
3	56.81	85.76	142.13	99.69	4.44
<i>Tannic acid</i>					
1	1932.1	956.2	2855.0	98.85	2.4
2	1911.3	1906.6	3869.8	101.36	0.88
3	–	–	–	–	–

The mobile phase consisted of solvent A (0.05%, v/v solution of orthophosphoric acid) and solvent B (Acetonitrile containing 0.5% mobile phase A) with the elution profile as follows: 0–20% B at 0–10 min, 20–22% B at 10–15 min, 22–30% B at 15–20 min, 30–40% B at 20–25 min, 40–50% B at 25–30 min, 50–60% B at 30–35 min, 60–80% B at 35–60 min and the re-equilibration time for each gradient elution was 15 min. The flow rate was 1.0 mL/min; the column was maintained at room temperature.

Sample preparation

The extraction procedure was optimised prior to preparation of sample for complete recovery of all desired nine components of analysis using different compositions of methanol: water varying time for extraction by sonication, which was monitored using proposed HPLC method. One g each

of IA and IB samples (in triplicate) were taken separately from each batch in a 50 mL conical flask and 25 mL of solvent (70% methanol) was added and mixed well. It was sonicated for 40 min at room temperature and filtered using Whatman filter paper no. 4 (the extraction solvent and time of sonication was optimised and selected after several trials for maximum recovery of all nine components). The filtrate obtained was transferred to a separating funnel (100 mL volume) and extracted with 25 mL of hexane to remove undesired nonpolar compounds. Aqueous methanol extract was dried using rotavapor below 40 °C and the residues obtained was reconstituted in 10 mL of solvent i.e. methanol: water (1:1, v/v), further transferred to 25 mL of volumetric flask and make up the volume to obtain a concentration of 40 mg/mL. All the sample solutions were filtered through 0.22 μm syringe filter before injecting.

Table 3. Intra and inter-day variations of the method

Compound	Actual retention time	Intra-day precision (% RSD)				Inter-day precision (% RSD)			
		Standard		Sample		Standard		Sample	
		RT	Area	RT	Area	RT	Area	RT	Area
Ascorbic acid	2.278	1.72	0.22	1.11	2.44	2.23	0.84	2.22	1.02
Gallic acid	3.518	1.30	0.43	0.66	1.12	0.88	0.70	1.51	0.69
Rutin	16.002	0.83	1.22	0.44	0.44	0.97	1.77	1.37	1.07
Sennoside B	16.591	0.66	0.87	0.45	0.78	0.74	1.64	0.89	0.59
Chebulinic acid	18.639	0.23	1.55	1.86	0.89	1.96	0.67	1.69	0.93
Sennoside A	20.286	0.76	3.1	0.50	0.66	1.07	0.54	2.49	0.87
Quercetin	20.214	2.5	2.6	0.58	1.52	1.69	0.71	1.95	0.14
Ellagic acid	33.327	0.97	0.77	0.63	1.99	0.73	0.93	2.50	1.22
Tannic acid	44.915	1.66	0.99	2.22	2.96	0.39	0.76	1.75	0.66

RESULTS AND DISCUSSION

Optimisation of chromatographic conditions

Since, the polarity range of the components is very narrow, gradient elution was carried out to separate maximum components of formulation. The mobile phase was selected using different compositions of methanol–water (50:50 and 80:20) and acetonitrile–water (20:80 and 60:40) with some modifiers including orthophosphoric acid, formic acid, acetic acid, phosphate buffer, acetate buffer with different pH values adjusted using triethyl amine and ammonia, which were investigated under different gradient elution modes. After many trials, excellent separation of all components were achieved on solvent A (0.05%, v/v solution of orthophosphoric acid) and solvent B (acetonitrile containing 0.5% mobile phase A), in gradient elution upto 60 min, the representative HPLC–PDA chromatograms of all the nine reference compounds and samples are shown in Fig. 2.

The UV spectra of each analyte was determined independently to get the $\lambda_{\max}$ of all nine components viz.; vitamin C, gallic acid, rutin, sennoside A, chebulinic acid, sennoside B, quercetin, ellagic acid, and tannic acid at 243, 271, 255, 267, 278, 269, 255, 256, and 273 nm, respectively. In order to detect all nine components with good sensitivity, 254 nm wavelength was selected as the detecting wavelength for the analysis.

Optimisation of extraction procedure for sample preparation

The optimisation of extraction methodology needs to be investigated in order to obtain satisfactory extraction efficiency, which must enables complete extraction of the compounds of interest avoiding chemical modification [12]. In attempt to find the optimum solvent composition and extraction time, different solvent ratios were tried for extraction and the recoveries all nine components at different time intervals were also monitored by the HPLC

method. Aqueous methanol was reported to be a suitable solvent for the extraction of phenolic and flavonoid compounds [13–15]. Solubility studies conducted on phenolic and flavonoid compounds showed that the most suitable solvent was methanol–water (70:30). Optimisation of extraction procedure was aimed to maximise the recovery of all nine components. In this study, 70% methanol in water as a solvent and extraction time of 40 min by sonication was found to be optimum for the extraction of all the components. So, the optimum extraction time for the samples was set as 40 min in 70% methanol in water to extract the poly herbal formulations.

Method validation

The proposed method was validated as per ICH guidelines [16] for different parameters like linearity, accuracy, precision, limits of detection (LOD) and limits of quantification (LOQ) and robustness similar to the methods reported by laboratory [17–20].

Calibration curves, limits of detection and quantification

Standard stock solutions of nine reference standards, each of 500 $\mu\text{g/mL}$ concentrations (vitamin C, gallic acid, rutin, sennoside B, chebulinic acid, sennoside A, quercetin, ellagic acid, and tannic acid) were prepared by dissolving them in water:methanol (1:1). The stock solutions were then diluted to different concentrations and working standard solutions were stored at 4 °C till it was applied on HPLC after filtration using 0.22 μm syringe filter before injecting for construction of calibration plots.

The calibration curves were plotted with at least six appropriate concentrations in triplicate in the ranges of 6.4–320, 1.1–474, 1.8–257, 10–350, 10.6–355.8, 10–220, 5–346.8, 2.3–456, and 2.8–2000.0 $\mu\text{g/mL}$ for ascorbic acid, gallic acid, rutin, sennoside B, chebulinic acid, sennoside A, quercetin, ellagic acid, and tannic acid, respectively.

The lowest diluted solutions of the nine reference compounds in the calibration curves were further diluted to a

series of concentrations with 50% methanol for the determinations of LOD and LOQ. The LOD and LOQ under the present chromatographic conditions were determined at a signal-to-noise (S/N) ratio of 3 and 10, respectively. Calibration curves, regression equation along with LOD and LOQ for each compound have been shown in Table 1.

Specificity

The specificity was confirmed by the absence of any endogenous interference at retention times of peaks of interest, which is self-indicative by matching retention times, spectrums and peak purity through PDA.

Precision and accuracy

Precision of the method was determined by carrying out the intra-day and inter-day variation tests. Inter-day and intra-day precisions were done by preparing and applying three different concentrations of standard in triplicate six times a day and similarly on six different days, respectively. The Relative Standard Deviation (RSD) was taken as a measure of precision (Table 2).

To evaluate the accuracy, the pre-analysed samples were spiked with standard at three different known concentration levels i.e. 50, 100 and 150% and the mixtures were re-analysed by the proposed method. Average recoveries of investigated targets ranged from 94.36% to 108.27%, and RSD values were all <3% ($n = 3$). From acquired data it was found that the developed method was reliable and accurate for the measurement (Table 3).

Sample analysis

The HPLC–PDA method was developed and successfully employed for the identification and quantification of nine marker components in three different batches of two traditional poly herbal formulations; Itrifal-e-Aftimoon and Itrifal-e-Badiyan, which are commonly prescribed in Unani System of Medicine (USM). All the samples were analysed according to the optimised extraction procedure described earlier in the section. Identification of the peaks in the sample chromatograms were carried out by comparing retention and PDA spectra of each component. The content of each analyte was calculated from the corresponding calibration curve. The amount of markers ($n = 3$) present in each formulation were quantified in three different batches were listed in Table 4. The Itrifal-e-Aftimoon samples were identified for the presence of all nine marker components whereas in Itrifal-e-Badiyan, rutin and sennosides were absent. There were no remarkable differences in the quantities of all these components in the three batches analysed in case of both samples.

An increase in demand in the usage of traditional medicine is found worldwide in recent years.

There are many reports available on the standardisation of poly herbal formulations, where bioactive compounds were estimated by HPLC method [21]. By considering this fact in mind, a RP–HPLC method reported here represents

Table 4. Determination of bioactive compounds in Itrifal-e-Aftimoon and Itrifal-e-Badiyan formulations by HPLC–PDA method

	Ascorbic acid	Gallic acid	Rutin	Sennoside B	Chebulinic acid	Sennoside A	Quercetin	Ellagic acid	Tannic acid
	<i>Itrifal-e-Aftimoon Mean contents (% w/w) ± SD (n = 3)</i>								
Batch 1	0.099 ± 0.002	0.436 ± 0.013	0.0886 ± 0.003	0.026 ± 0.009	0.033 ± 0.0019	0.044 ± 0.0044	0.016 ± 0.0003	0.140 ± 0.0039	4.79 ± 0.106
Batch 2	0.101 ± 0.002	0.433 ± 0.011	0.0879 ± 0.001	0.024 ± 0.0085	0.035 ± 0.002	0.043 ± 0.0051	0.017 ± 0.0006	0.139 ± 0.0033	4.83 ± 0.089
Batch 3	0.098 ± 0.002	0.434 ± 0.014	0.0881 ± 0.0015	0.023 ± 0.0088	0.032 ± 0.0015	0.041 ± 0.0033	0.017 ± 0.0007	0.138 ± 0.0031	4.95 ± 0.085
	<i>Itrifal-e-Badiyan Mean contents (% w/w) ± SD (n = 3)</i>								
Batch 1	0.119 ± 0.0012	0.058 ± 0.0051	Not detected	Not detected	0.027 ± 0.0019	Not detected	0.064 ± 0.0051	0.099 ± 0.0101	4.36 ± 0.101
Batch 2	0.101 ± 0.0011	0.055 ± 0.0047	Not detected	Not detected	0.023 ± 0.0051	Not detected	0.066 ± 0.0071	0.103 ± 0.0098	4.33 ± 0.110
Batch 3	0.111 ± 0.0181	0.044 ± 0.0031	Not detected	Not detected	0.022 ± 0.0055	Not detected	0.064 ± 0.0061	0.86 ± 0.0131	4.19 ± 0.088

a simple, accurate and rapid technique for simultaneous determination of four phenolics (gallic acid, ellagic acid, chebulinic acid, and tannic acid), two flavonoids (rutin and quercetin), two anthraquinones (sennoside A and B) and one oxygenated hydrocarbon (vitamin C) in Itrifals. This is the first report on the simultaneous determination these nine markers in a traditional Unani polyherbal formulations. The main advantage of the method includes the simplicity of extraction procedure, simultaneous detection and quantification of all these nine bioactive components in a single chromatographic run. The quantification limits were found to be low enough for the successful employment of the method in different polyherbal formulations which contains these components in very minute quantities. The efficiency of the method was evaluated from conducting recovery experiments and the results found to be promising, which indicates that the method can be executed successively in various multi component herbal formulations with high accuracy and precision. The linearity experiments conducted proved that the method can be applied for the samples which contain these components in a wide range.

CONCLUSION

In conclusion, the proposed method is useful as a reliable, fast and effective tool for the quality control as well as standardisation of different formulations, especially traditional polyherbal formulations like Itrifals and Triphala of Unani and Ayurvedic Systems of medicines. This simple multi-component assay method will be helpful in quality control of a large no of traditional formulations and can also be extended for the pharmacological, biopharmaceutical and pharmacokinetic studies.

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