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ORIGINAL ARTICLE

Quantitative HPLC Analysis of Vitamin D3 and Gallic Acid in Vivabon Syrup for Children Growth

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Keywords: *Polyherbal, Vivabon-Vitamin D3, RP-HPLC, Validation, D3, gallic acid.*

Author`s Contribution
All the authors contributed significantly to the research that resulted in the submitted manuscript.

Article info.
Received: January 17, 2017
Accepted: July 01, 2017

Funding Source: *Nil*
Conflict of Interest: *Nil*

Cite this article: *Zahoor A, Munir H, Alam MO, Ali R, Mahmood H, Hussain S, Usmanghani K. Quantitative HPLC Analysis of Vitamin D3 and Gallic Acid in Vivabon Syrup for Children Growth. RADS J. Pharm. Pharm. Sci. 2017;5(3):43-50.*

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ABSTRACT

Objective: The current study was conducted to evaluate quantitative estimation of the biomarkers gallic acid and Vitamin D_3 present in Vivabon-D_3 syrup.

Background: Vivabon-Vitamin D3 is a poly herbal syrup formulation for the promotion and growth of children. Vivabon comprises of Phoenix sylvestris, Phyllanthus emblica (synonym Emblica officinalis), Centella asiatica, Withania somnifera, Salvia haematodes, Centaurea behen, Zingiber officinalis, Trigonella foenum-greacum, Piper longum, Ammomum subulatum.

Methods: The present work has been carried out on polyherbal formulation for the quantitative determination of Vitamin D3 and gallic acid as markers.

Results: Quantitative analysis of gallic acid was conducted; using silica gel 60 F_{254} coated plates as a stationary phase to augment the identification and determination of gallic acid components.

Conclusion: A rapid method for the quantitation of Vitamin D_3 present in the Vivabon Vitamin D_3 syrup has been developed and validated in terms of linearity, precision, repeatability, limit of detection, limit of quantification and accuracy**.** The study result revealed that vivabon-D3 syrup formulation was well standardized for routine quality control procedure.

INTRODUCTION

Vivabon–Vitamin D_3 syrup is a polyherbal formulation consisting of dietary essentials of herbal origins, to provide the necessary nutrients for the growth of children. Vitamin D_3 fat soluble, helps to absorb calcium, phosphorus and procures nutritional vitamin insufficiency. The ingredients of Vivabon-D_3 syrup are rich in sugars, vitamins, glycosides, flavonoid and trace elements. Due to its nutritional and health promotive composition Vivabon-D_3 strengthens the muscles, bones, hair, teeth and generalizes body strength. Vivabon-D_3 syrup energizes the whole-body systems and increases the physical and mental capacity of children. The major chemical constituents of the fruit, leaves, roots and rhizomes pulp and bark of the poly herbal composition as given in Table **1** contains sugars, vitamin C, protein, flavonoids, saponins, tritepnes, trace elements [1-17] and many other types of chemical constituents. These chemical substances synergistically help for the growth and developments of children in their physique as well as mental performance, strength and stamina.

Vitamin D_3 can be beneficial in the treatment of rickets, hyperparathyroidism and certain bone disorders. This vitamin is also important for the absorption of calcium and phosphorus, which are essential nutrients needed for bone health.

Table 1. Active ingredients of Vivabon syrup and their medicinal uses.

S. No.	Plant Name	Common Name	Medicinal Uses	Reference
1.	*Phoenix sylvestris*	Khajoor	Oral dental care	12,15
2.	*Emblica officinalis* [3, 4]	Amla	Antioxidant, Treatment of fatigue, mental disorders, vertigo, palpitations	1,2,16,17
3.	*Withania somnifera* [5]	Asgandh	Anti-inflammatory, Antitumor, Antistress, Antioxidant, Immunomodulatory, Hemopoietic, and Rejuvenating	14
4.	*Salvia haematoides*	Behman Surkh	Anti-inflammatory, Antioxidant.	7
4.	*Centella asiatica* [6]	Brahmi booti	Wound healing, Anti-leprosy, Anti-lupus, Anti-varicose ulcers, Anti-eczema and Anti-diarrhoea	3,4,8
5.	*Ammomum subulatum* [7]	Ilaichi kalan	Anti-inflammatory, Antioxidant, Antiulcer activity, Treatment of indigestion, vomiting, biliousness, abdominal pains and rectal diseases	13
6.	*Zingiber officinale* [8]	Soanth	Antiemetic, stomachic, expectorant, anti-inflammatory, aphrodisiac	6,11
7.	*Trogonella foenum graecum* [9-11]	Methi dana	Anti-fatigue, Antioxidant properties	5,9
8.	*Centaurea behen* [12, 13]	Behman safaid	Anti-oxidant, Geriatric care	10,13
9.	*Piper longum* [14]	Filfil daraz	Anti-diarrhea, anti-cholera, anti-viral hepatitis, anti-tumors	10,13
Vitamin D3 100 IU; Water soluble vitamin D3 100IU=1mg; For 10ml dose is=400IU.				

Vitamin D_3 is also called Cholecalciferol and is a form of vitamin D. This is a fat-soluble vitamin that is produced by the body through the skin's exposure to sunlight, as noted by Everyday Health. Deficiencies in this vitamin can cause health problems, such as rickets in children. A characteristic of this disease is bowed legs. Bone disorders, including osteomalacia and osteoporosis, can also develop when there are low levels of vitamin D in the body. Osteomalacia occurs when bones become soft. For these deficiencies, supplementation is necessary. Taking vitamin D in combination with calcium can help with osteoporosis, which is when there is bone loss that leads to weakened bones. Maintaining healthy levels of vitamin D has been shown to be an important part of overall health [18-21].

MATERIALS AND METHODS

Manufacturing Process

All herbs are purchased from the local market then cleaned and examined for their impurities and adulteration. All the herbs were powdered through grinding machine and passed through the sieve no 50. The powder is further checked for its homogenous consistency. The material

was soaked in water for a night and then boiled along with continuous stirring. After boiling and cooling the solution is filtered and the impurities are separated. This cooled filter solution was then processed to make syrup. The quality of syrup was then check by below methods for the qualitative and quantitative determination of gallic acid and vitamin D_3.

Chemical and Regents

All the chemical and reagent used for the experiments analysis were of analytical grade. All solvents and sample were filtered through Millipore 13mm, 0.2µm, non-sterile membrane sample filter paper, before injecting into system.

Quantitative Estimation of Gallic acid in Vivabon Syrup by HPTLC-Densitometry

Equipment

CAMAG Scanner III, CAMAG Linomat 5 or Equivalent; TLC Plates: HPTLC silica gel $G60F_{254;}$ Solvent System: Toluene: Ethyl acetate: Formic acid: Methanol (12 :9: 4: 0.5); Wave length: 273 nm.

Standard Preparation

Prepare standard solution containing known concentration (0.4 mg/ml) by dissolving 4mg standard of Gallic acid monohydrate in 10 ml of methanol.

Sample Preparation

Weigh about 12.0g of Syrup (Note exact Weight) in to 100ml of conical flask. Add 30ml of water and mix thoroughly. Carefully transfer the solution in 250ml separating funnel and add 50ml of ethyl acetate in the funnel. Shake carefully for 3 minutes. After complete separation of layers, filter upper ethyl acetate layer through the paper filter with anhydrous sodium sulphate (about 10g) in 500ml round bottom flask. Repeat extraction of the lower water layer four times more using 50X4ml portions (5 times in total) as mention above. Collect ethyl acetate fraction into the same round-bottomed flask. evaporate the organic fraction under vacuum. Dissolve the dry residue in 5ml of methanol and transfer quantitatively into a 10ml volumetric flask. Bring the solution's volume to the mark with methanol.

Procedure

TLC Preparation Perform analysis on 10x10 cm HPTLC silica gel $G60F_{254}$ plates with fluorescent indicator. Before start the analysis, HPTLC plate cleans by predevelopment with methanol by ascending method. (Note: Immerse HPTLC Plate in a CAMAG glass chamber (20x20 cm), contains 30ml methanol as solvent system cover the chamber with glass lid and wait to develop the plate to the top with methanol. After complete development, remove the plate from TLC glass chamber and dry it in an oven at 105° C for 5 min).

Application Procedure

Apply three spots of 10µl (in the form of band) of standard preparation along with three spots of 10µl of sample preparation as the bands on the same plate by means of a CAMAG Linomat 5 (automated spray-on applicator equipped with a 100µl syringe and operated with the settings band length 6mm, distance between bands 14mm, distance from the plate side edge 15mm, and distance from the bottom of the plate 15mm).

1. After sample application, dry the plate in hot air oven at 105 °C for 5 min.

TLC Development

Develop the plate by immersing sample HPTLC Plate in a CAMAG glass chamber (20x20 cm) contained the solvent system (Toluene: Ethyl acetate: Formic acid: Methanol (12: 9: 4: 0.5), wait to develop the plate to the distance of 8 to 9cm. After complete development, allow the plate to dry by keeping in fume cupboard for 10

minutes and then keep in hot air oven for 5 min at 105 °C.

TLC Scanning

Scan the plate in the densitometer by linear scanning at 273nm (Adsorption) by use of a TLC Scanner III CAMAG with a deuterium source and integrate the area of the spots corresponding to Gallic acid standard.

Calculate the amount of Gallic acid in mg per 10ml in Vivabon Syrup by following formula.

Content of Gallic acid:

$$\frac{A_{SMP} \times W_{STD} \times f \times \text{Dilution of Smp} \times \text{Application vol. of Smp} \times P \times D \times 10}{A_{STD} \times \text{Dilution of Std} \times W_{SMP} \times \text{Application of vol. Std} \times 100}$$

Table 2: High Performance Liquid Chromatography Assay Conditions.

Column specification	C-18,4.6mm×250mm,5µ
Mobile phase	Methanol 100%
Flow rate	1.2ml/min
Column temperature	ambient
Wavelength	265nm
Injection volume	100µl

A_{SMP} = Avg. Area of Sample; A_{STD} = Avg. Area of Standard; W_{STD} = Weight of Standard, mg; W_{SMP} = Weight of Sample, g; Dilution of Smp = Dilution of Sample, ml; Dilution of Std = Dilution of Standard, ml; P = Percent Purity of Standard; *f* (0.904) = conversion factor of Gallic acid monohydrate in Gallic acid; D = Density of syrup, g/ml; **Note:** Quantity of Gallic acid in Vivabon syrup should be NLT **1 mg/10ml.**

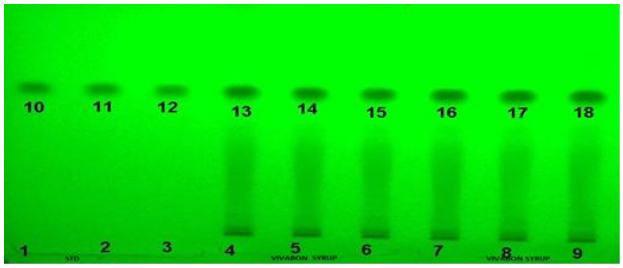


Figure 1. TLC image of Gallic acid.

The gallic acid content in vivabon syrup was found to be 10 mg/10ml.

Quantitative determination of Vitamin D3 in Vivabon syrup

The chromatographic conditions and mobile phase were present as in Table **2**.

Reference Solution

Weigh and transfer accurately about 8mg of vitamin D3 standard into 50ml amber glass volumetric flask and dissolve and make up the volume to the mark with the methanol. Filter the obtained solution with pore size 0.45 µm and use filtrate for chromatography. Use only freshly prepared solution. Inject 100µL of this solution

Test Solution

Take approximately 24gm of syrup in the 50ml amber glass volumetric flask and make up the volume to the mark with methanol, sonicate for 30 min and filter the solution through whatman 41 filter paper. Then Filter the resulting solution through a HPLC filter with pore size 0.45 µm and use filtrate for chromatography Inject 100 µL of this solution.

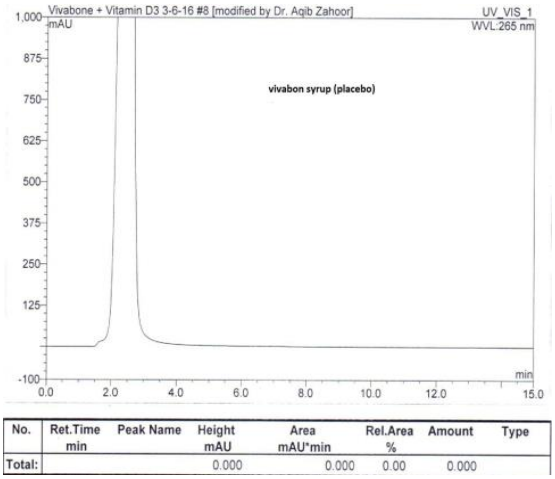
Calculation:

$$\mathbf{X=} \quad \frac{A_{SMP} \times W_{STD} \times D_{SMP} \times P \times D \times 10}{A_{STD} \times D_{STD} \times W_{SMP}}$$

Where, A_{SMP} – Mean value of peak area of tested solution samples; A_{STD} – Mean value of peak area of standard solution samples; W_{SMP} – Preparation weight, g; W_{STD} – Standard weight, mg

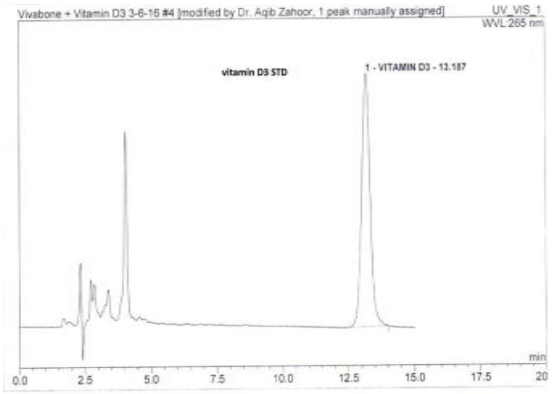
$I.V_{SMP}$ – Injection volume of sample; $I.V_{STD}$ injection volume of standard.; D – Density of syrup (gm/ml), P – Percent Purity of standard sample, %

The chromatograms were obtained as mentioned below and the amount of Vitamin D_3 where found to be 2mg/10ml syrup.



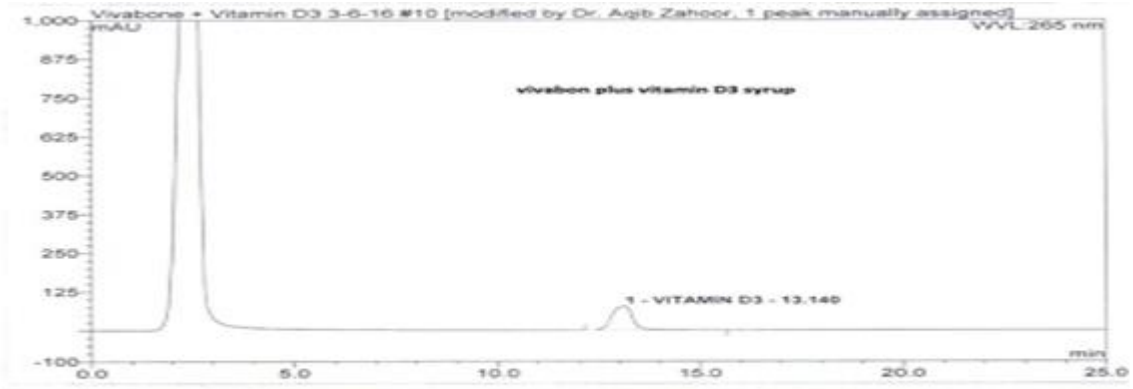
No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
Total:			0.000	0.000	0.00	0.000	

A: Placebo



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	13.19	STD	2.920	1.090	100.00	n.a	BMB^
Total:			2.920	1.090	100.00	0.000	

B: Standard



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	13.14		77.068	45.223	100.00	n.a	BMB^
Total:			77.068	45.223	100.00	0.000	

C: Syrup (sample)

Figure 2. Chromatograms of Vitamin D_3.

RESULT

The current study was conducted to evaluate quantitative estimation of the biomarkers gallic acid and Vitamin D_3present in Vivabon-D_3 syrup. Quantitative analysis of gallic acid was conducted; using silica gel 60 F_{254} coated plates as a stationary phase to augment the identification and determination of gallic acid components as shown in Figure **1**. Thin layer chromatographic analysis of gallic acid was conducted by using toluene - ethyl acetate - formic acid - methanol - 12:9:4:0.5 (v/v/v/v) as a solvent system. After developing and drying, the plates were observed under UV light for the presence of gallic acid, which was detected by prominent dark brown color spot (Figure **2**). The R*f* value (0.58) of gallic acid in both sample (Figure **3**) and reference standard (Figure **4**) was found comparable under UV light at 273 nm.

Vitamin D_3 was determine by HPLC, using 100% methanol as mobile phase with flow of 1.2ml/minute by using 100µL of injection volume and by detecting vitamin D_3 at 265 nm on ambient temperature.

Standardization of specific biologically active gallic acid and vitamin D_3 components were

identified in the poly herbal formulation thereby establishing the standard of those particular compounds for validation.

DISCUSSION

Child growth and physique is one of the most prevalent ailments in children for their mental faculty and physical build up. Herbal medicines are usually obtainable as a mixture of more than one plant constituent and its therapeutic activity depends on its phytochemical constituents. Accurate identification and quality reassurance is an essential prerequisite to make sure reproducible quality which contributes to safety and efficacy of herbal medicine. The results of a double-blind clinical trial on *Centella asiatica* indicated that there was a significant increase in the general mental ability of mentally retarded children after three months and six months of drug administration. Significant improvement was found in the overall general adjustment, attention and concentration after six months. In an experimental study it was concluded that *Centella asiatica* leaf extract had a neuronal dendritic growth stimulating property; hence, the extract could be used for enhancing neuronal dendrites in stress, neurodegenerative and memory disorders [38]. These studies indicate that *Centella asiatica* improves memory and learning ability. Multiple micronutrients such as vitamins and minerals when delivered through either supplements or fortified foods were found to have a positive effect on reasoning ability and academic performance in school children [22-24].

Immunomodulatory activity

An experimental study showed that the presence of *Amla (Emblica officinalis)* was effective against the cytotoxic effects of chromium induced oxidative damage of murine macrophages and resulted in an enhanced cell survival, decreased free radical production and higher antioxidant levels, similar to that of control cells. Further, chromium (VI) treatment resulted in decreased phagocytosis and gamma-interferon (gamma-IFN) production while *Amla (Emblica officinalis)* inhibited chromium induced immune suppression and restored both phagocytosis and gamma-IFN production by macrophages significantly [33]. These findings suggest the cytoprotective and immunomodulatory potential of *Emblica officinalis* fruit. In another in-vitro study, *Amla (Emblica officinalis)* relieved the immunosuppressive effects of Chromium on lymphocyte proliferation even restored the IL-2 and gamma-IFN production considerably [34]. An experimental study conducted on mice demonstrated that the aqueous extract of *Emblica officinalis* was very effective in reducing cyclophosphamide induced suppression of humoral immunity. All the above studies indicate the immunomodulatory potential of *Emblica officinalis*. In a clinical study it was proved that Bovine colostrum had the ability to increase IgA which indicated that it had the potential to enhance human special immune response [25,26].

Standardization is an imperative aspect for evaluating the quality and safety of the polyherbal formulation as these are combinations of more than one herb to accomplish the desire therapeutic effect. Phytochemical assessment is a mean for the quality measurement, including preliminary phytochemical screening, chemo profiling, and marker compound analysis employing innovative analytical techniques.

High-performance thin-layer chromatography has been emerged as a significant tool for the qualitative, semiquantitative, and quantitative phytochemical analysis of the naturally occurring drugs. It consists of developing TLC fingerprinting profiles and estimation of biomarkers. In the current study quantitative estimation of specific biologically active gallic acid and Vitamin D3 were conducted in the

poly herbal formulation. HPTLC densitometry analysis of gallic acid was conducted by using toluene - ethyl acetate - formic acid - methanol 12:9:4:0.5 (v/v/v/v) as a mobile phase. Sample preparation and development of appropriate mobile phase are two imperative stages in analytical procedures, which becomes more considerable for plant-based medicines owing to their complexity of the chemical compounds and their affinity towards different solvent systems. The Rf value (0.58) of gallic acid in both sample and reference standard was found comparable under UV light at 273nm. Standardization promise constant composition of all herbals including analytical operations for identification, markers and assay of active principles. Thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC) are routinely used as valuable tools for qualitative determination of small amounts of impurities [15].

CONCLUSION

An accurate, rapid and simple HPTLC method for quantitative estimation of gallic acid. While a rapid method for the quantitation of Vitamin D_3 present in the Vivabon Vitamin D_3 syrup has been developed and validated in terms of linearity, precision, repeatability, limit of detection, limit of quantification and accuracy. The methods employed in current study resulted in good peak shape and enabled high-quality resolution of gallic acid and Vitamin D_3. It does not suffer any positive or negative interference due to common other component present in the formulation and would also serve as a tool for authentication of herbal products containing gallic acid and vitamin D_3. The present standardization provides a specific and rapid tool in the herbal research, permitting to set quality specifications for identity, transparency and reproducibility of biomarkers in Vivabon-Vitamin D_3 syrup.

Acknowledgement

The authors are grateful to honorable Mr. Nadeem Khalid, CEO, Herbion Pakistan (Pvt.) Limited Karachi, Pakistan for providing necessary facilities to carry out the research work.

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