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PHYSICOCHEMICAL ANALYSIS AND STANDARDIZATION OF ANDROGRAPHOLIDE AND GALLIC ACID BY RP-HPLC IN VILOCYM PREMIX: A HERBAL VETERINARY FORMULATION

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ABSTRACT

Background: Vilocym Premix, a polyherbal veterinary formulation of Ayurvet Limited, Baddi, is a multiple mycotoxin binder, immunomodulator and growth promoter for use across species, containing herbs like *Andrographis paniculata*, *Terminalia chebula*, *Phyllanthus emblica* and *Solanum nigrum* as the major ingredients. **Objective**: The present study was undertaken to develop standardization parameters for Vilocym Premix. Evaluation of various standardization parameters like organoleptic characters, physicochemical evaluation and estimation of bioactive markers Andrographolide and Gallic acid by reverse phase high performance liquid chromatography (RP-HPLC). **Material and method:** Vilocym Premix was obtained from the QA/QC department of Ayurvet Limited Baddi. Andrographolide and gallic acid were used as standards. Standards and sample dilutions were prepared as per Standard Test Procedure. Vilocym Premix was evaluated for its organoleptic and physicochemical properties, RP-HPLC analysis was done using mobile phases acetonitrile: water (0.1% orthophosphoric acid):: 40:60 for andrographolide and acetonitrile: water :: 20:80 (pH were adjusted to 3 with orthophosphoric acid) for gallic acid. **Results:** The RP-HPLC chromatograms of Vilocym Premix, showed standard andrographolide and gallic acid at retention time of 4.83 min and 3.60 min. The percentage of andrographolide was 0.097 % w/w and the percentage of gallic acid was 0.071 % w/w in Vilocym Premix formulation. Further, data from the physicochemical and organoleptic study of the formulation added the quality optimization of the product. **Conclusion:** The characterization parameters presented

in this communication shall serve as a standard reference for quality control analysis of Vilocym Premix for its batch to batch consistency.

KEYWORDS: RP-HPLC, phytoconstituents, andrographolide, gallic acid, standardization, Vilocym Premix.

INTRODUCTION

Vilocym Premix is a multiple mycotoxin binder, immunomodulator and growth promoter for use across species, containing different herbs.[1] It is a solid powder formulation, prepared by grinding various herbs like Kalmegh (*Andrographis paniculata)*, Haritaki chhilka (*Terminalia chebula*), Amla (*Phyllanthus emblica),* Haldi (*Curcuma longa)*, Neem (*Azadirachta indica*), Makoy (*Solanum nigrum*) and other additives.

Kalmegh (*Andrographis Paniculata*) is one of the ingredients of the formulation and the whole plant is used for the preparation of Vilocym Premix formulation. *Andrographis paniculata* contains a group of biologically active constituents such as andrographolide [Figure 1], andrographine, neoandrographolide and panicoline. Andrographolide is an important bioactive constituent present in *Andrographis paniculata*.[2] The formulation also comprises herbs like amla dry and haritaki chhilka, which are a rich source of tannins like gallic acid [Figure 1] chiefly responsible for its activity.[3-4]

In recent years, there has been a great demand for plant-derived products in developed countries. These products are increasingly being sought as medicinal products, nutraceutical and cosmetics for human and animals. To have good coordination between the quality of raw materials, in-process materials and the final products, it has become essential to develop reliable, specific and sensitive quality control methods using a combination of the classical and modern instrumental method of analysis. It is not easy to establish the quality control parameters for herbal formulations due to the complex nature and inherent variability of the bioactive chemical constituents of the polyherbal formulations. Standardization is an essential measurement for ensuring the quality control of herbal drugs.[5-7]

The present study aimed to develop a standardization protocol for Vilocym Premix by analyzing organoleptic characters, physico-chemical evaluation and estimation of the bioactive markers andrographolide and gallic acid by RP-HPLC.

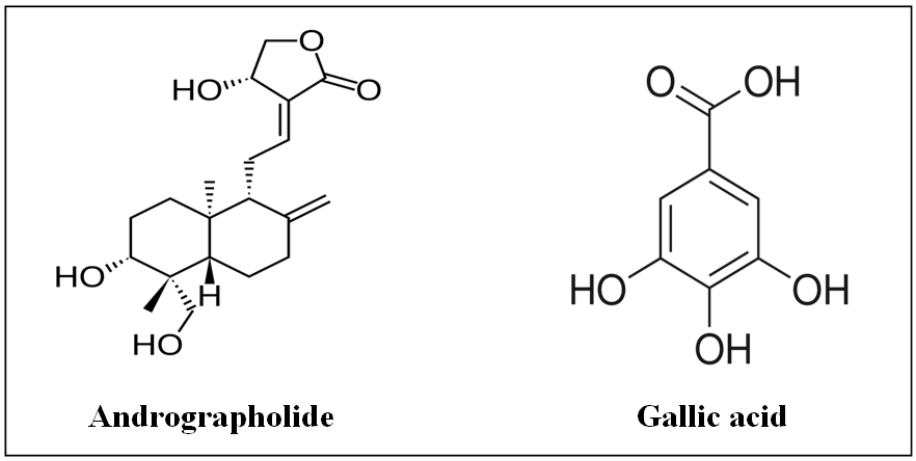

Figure 1: Chemical structure of andrographolide and gallic acid.

MATERIALS AND METHODS

Sample collection

Samples of Vilocym Premix were obtained from the QA/QC department of Ayurvet Limited Baddi.

Reagents and standards

All chemicals and solvents used were of analytical or HPLC grade and obtained from E-Merck, Rankem and SD Fine. Bioactive Markers i.e. gallic acid was purchased from HIMEDIA and andrographolide was isolated in our phytochemistry Lab and the structure was confirmed by 1H, 13C & 2D NMR spectra with reference literature.

Organoleptic characteristic

The characteristics like colour, odour, taste and texture were ascertained using sensory organ.[8]

Evaluation of physicochemical parameters

Evaluation of physicochemical parameters such as pH, total ash, loss on drying at 105º C, methanol soluble extractive value, water-soluble extractive value were carried out as per the standard pharmacopoeia methods and WHO guidelines for Ayurvedic formulations.[9-13]

RP-HPLC analysis

Standard preparation

Preparation of standard solution of gallic acid: Stock solution of gallic acid was prepared by dissolving 3 mg accurately weighed standard in a small amount of methanol and made up

the volume up to 25 ml. From the stock solution, different dilutions of 5.0 – 45.0 ppm were prepared for further analysis.

Preparation of standard solution of andrographolide: Stock solution of andrographolide was prepared by dissolving 3 mg accurately weighed standard in a small amount of methanol and made up the volume to 25 ml. From the stock solution, different dilutions of (20 – 100 ppm were prepared for further analysis.

Test sample preparation: 5 gm of the formulation was taken in 250 ml round bottom flask, 60 ml of methanol (60 ml ×3) was added and reflux on a water bath for one hour. Combine all the solutions and concentrated up to 100ml, finally filtered the solution through a 0.45µ membrane filter. This filtrate was used for HPLC analysis.

High-performance liquid chromatography apparatus and conditions:
Gallic acid and andrographolide content were analyzed by reverse phase-high performance liquid chromatography (WATERS, binary pump 515 with PDA 2996 detector, USA). The data was acquired on the Empower 2.0 controlling software. The separation was obtained on Phenomenex luna C18 column (250 mm x 4.6 mm, 5µm).

Good separations and suitable retention time of andrographolide and gallic acid were obtained in isocratic elution using optimized chromatographic conditions described in **[Table 1]**.

Table 1: Chromatographic conditions.

Sr. No.	Chromatographic conditions	Andrographolide analysis	Gallic acid analysis
1	Column	Phenomenex luna C18 column (250 mm x 4.6 mm, 5µm)	Phenomenex luna C18 column (250 mm x 4.6 mm, 5µm)
2	Mobile Phase	Acetonitrile : Water (0.1% orthophosphoric acid) :: 40:60	Acetonitrile : Water :: 20:80 pH=3 with orthophosphoric acid
3	Wavelength	228 nm	272 nm
4	Flow rate	1.0 ml/min	1.0 ml/min
5	Run time	15 minutes	10 minutes
6	Injection volume	20.0 µl	20.0 µl

Analytical method validation

The analytical method was validated for linearity, the limit of detection (LOD), the limit of quantification (LOQ), accuracy, intraday and interday precision, and repeatability according to the International Conference on Harmonization (ICH) guidance for Q2B validation of analytical procedures. Namely, linearity was evaluated by the coefficient of determination (r^2) of the calibration curve in the tested linear range of each compound.

LOD and LOQ values were calculated as LOD = 3.3 signal/noise and LOQ = 10 × signal/noise, based on the standard deviation (r) of the y-intercept in each calibration curve and the slope of the calibration curve (s).[14-15]

Accuracy, tested as percentage recovery, was determined by using the standard addition method and calculated as

Recovery (%) = (recorded concentration – original concentration) / spiked concentration × 100.

Intraday precision was determined by analyzing a single sample five times within a day and interday precision was determined by measuring the sample on three consecutive days. Repeatability was measured six times using a standard solution. Repeatability was evaluated by calculating the relative standard deviation (RSD) and calculated by the following equation. RSD (%) = standard deviation (SD) / mean×100.

RESULTS AND DISCUSSION

As part of standardization, Vilocym Premix polyherbal formulation was tested for relevant physical and chemical parameters and quantification of marker compound by HPLC. Physicochemical analysis can be employed for routine evaluation at the site of manufacturing and established physicochemical standards can furnish information for further investigation and facilitate identification of formulations. Hence the pH, moisture content, water-soluble extractive, methanol soluble extractive, ash value, acid insoluble ash, sieve analysis and tapped density were determined, which will serve as a reference for future analysis **[Table 2].** As herbs mentioned under experimental investigation are among the main active ingredients in the polyherbal formulation, quantifying them with their respective bioactive markers and setting the limits will help us in ensuring authenticity and efficacy of the product.

Table 2: Physico-chemical parameters of Vilocym Premix.

S. No.	Parameters	Results
1	Colour, Odour	Creamish to green coloured fine powder with characteristic odour
2	pH	4.9
3	Moisture content	5.8 %w/w
4	Water-soluble extractive	15.5 %w/w
5	Methanol soluble extractive	9.1 %w/w
6	Ash value	35.0 w/w
7	Acid insoluble ash	25.3 %w/w
8	Sieve analysis (retained on #40)	0.25 %w/w
9	Tapped Density	0.61 g/cc

Quantitative estimation of andrographolide and gallic acid in Vilocym Premix by RP-HPLC

Calibration curve of standard andrographolide and gallic acid: The linearity of an analytical method is its ability to elicit a test result. The linearity of the method was observed within the expected concentration range demonstrating its suitability for analysis.

Different concentrations of andrographolide and gallic acid standards were prepared and injected in HPLC. A calibration curve was established for peak area vs. the concentration of standard applied. The calibration peak summary was tabulated in **[Table 3]** and the Calibration curve was depicted in [**Figure 2]**.

The marker compound andrographolide is exhibited at retention time 4.83 minutes for standard and 4.830 minutes for formulation in the chromatogram [Figure 3]. The retention time of gallic acid was 3.501 minutes for standard and 3.589 minutes for sample chromatogram. From the calibration curve, the correlation coefficient was obtained [Figure 4]. The correlation coefficient 'r^2' values were found to be 0.999 each for both andrographolide and gallic acid. The value of intercept was less than 2% of the response of 100% of the test concentration in all the cases indicating a functional linear relationship between the concentration of analyte and area under the peak area. Quantification was done based on peak area. The proposed method was validated as per ICH guidelines.[13]

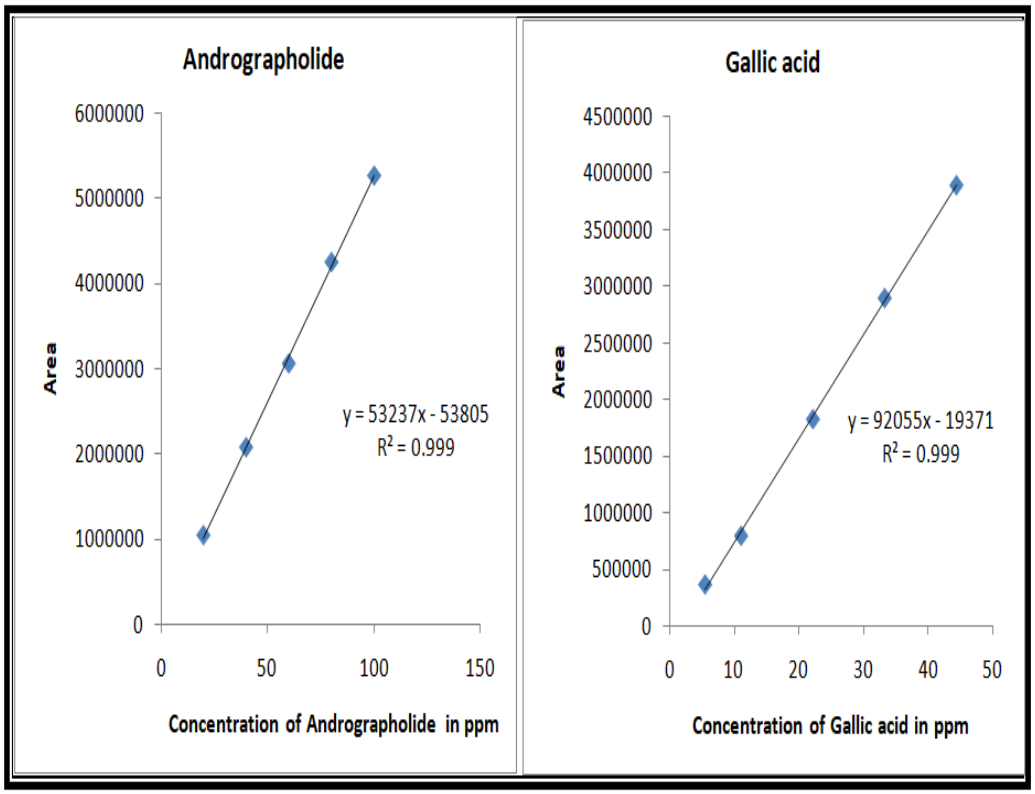

Figure 2: Calibration curves of andrographolide and gallic acid.

Table 3: Precision, LOD, LOQ, linear regression analysis and their correlation coefficient for quantitative analysis of marker compounds.

Sr.No.	Parameters	Andrographolide	Gallic Acid
1	Concentration range (ppm)	20.0 - 100.0	5.0 - 45.0
2	Regression equation	y = 53237x - 53805	y = 92055x - 19371
3	Correlation Coefficient (r2)	0.999	0.999
4	Amount of marker compound in Vilocym Premix (% w/w)	0.097	0.071
5	Method precision (Repeatability n=7) – RSD %	0.92	0.98
6	Intermediate precision (Reproducibility) - RSD [%] Intraday 1 Interday 3	 0.88 0.84	 0.87 0.83
7	LOD	0.015 ppm	0.292 ppm
8	LOQ	0.045 ppm	0.972 ppm

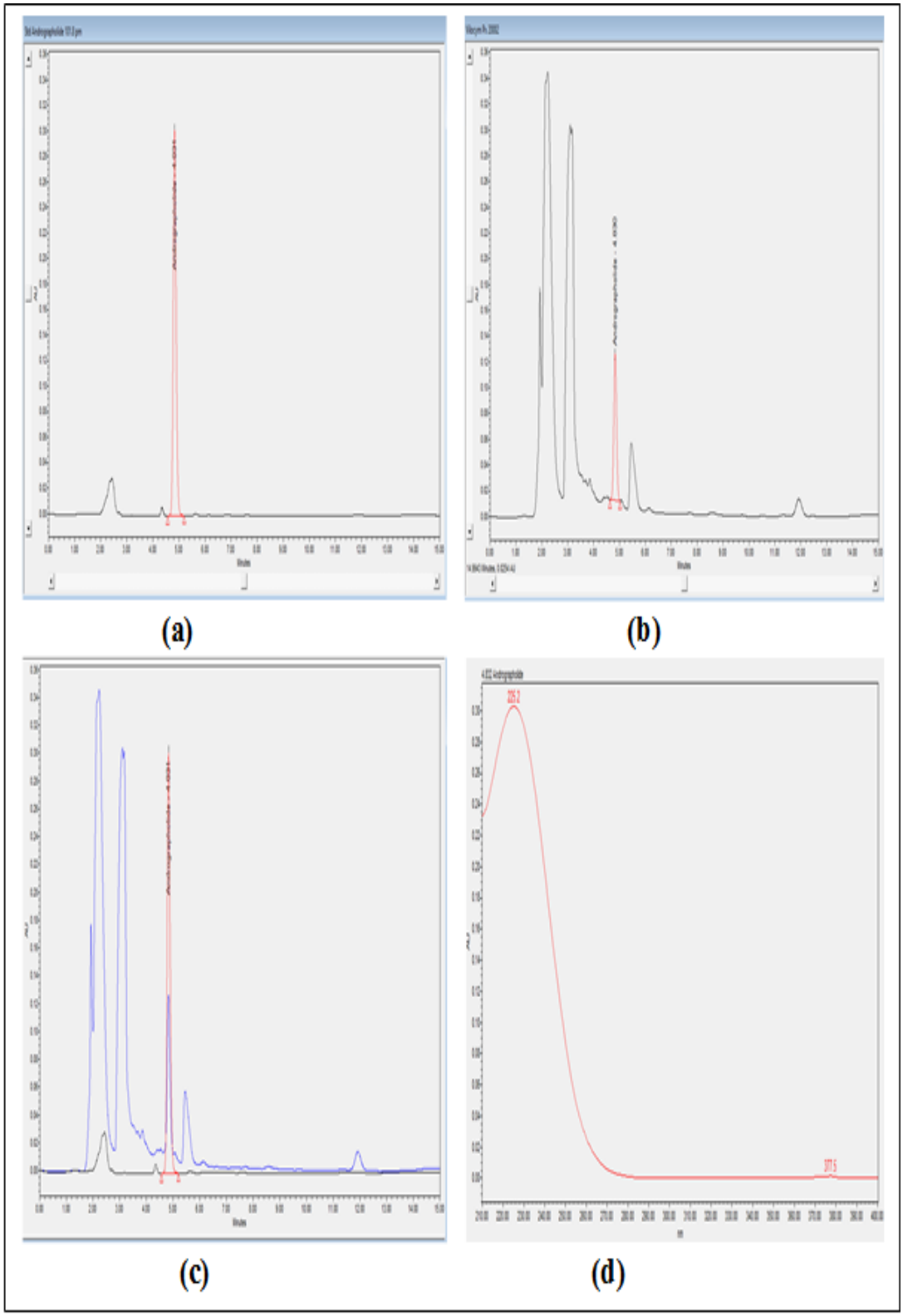

Figure 3: Chromatograms showing the resolution of marker compound in the formulation Vilocym Premix. (a) Chromatogram of standard andrographolide, (b) Chromatogram of test Vilocym Premix, (c) Overlay of the andrographolide chromatograms i.e. sample against the standard, (d) Spectral scan of standard andrographolide and spectral scan of andrographolide in Vilocym Premix.

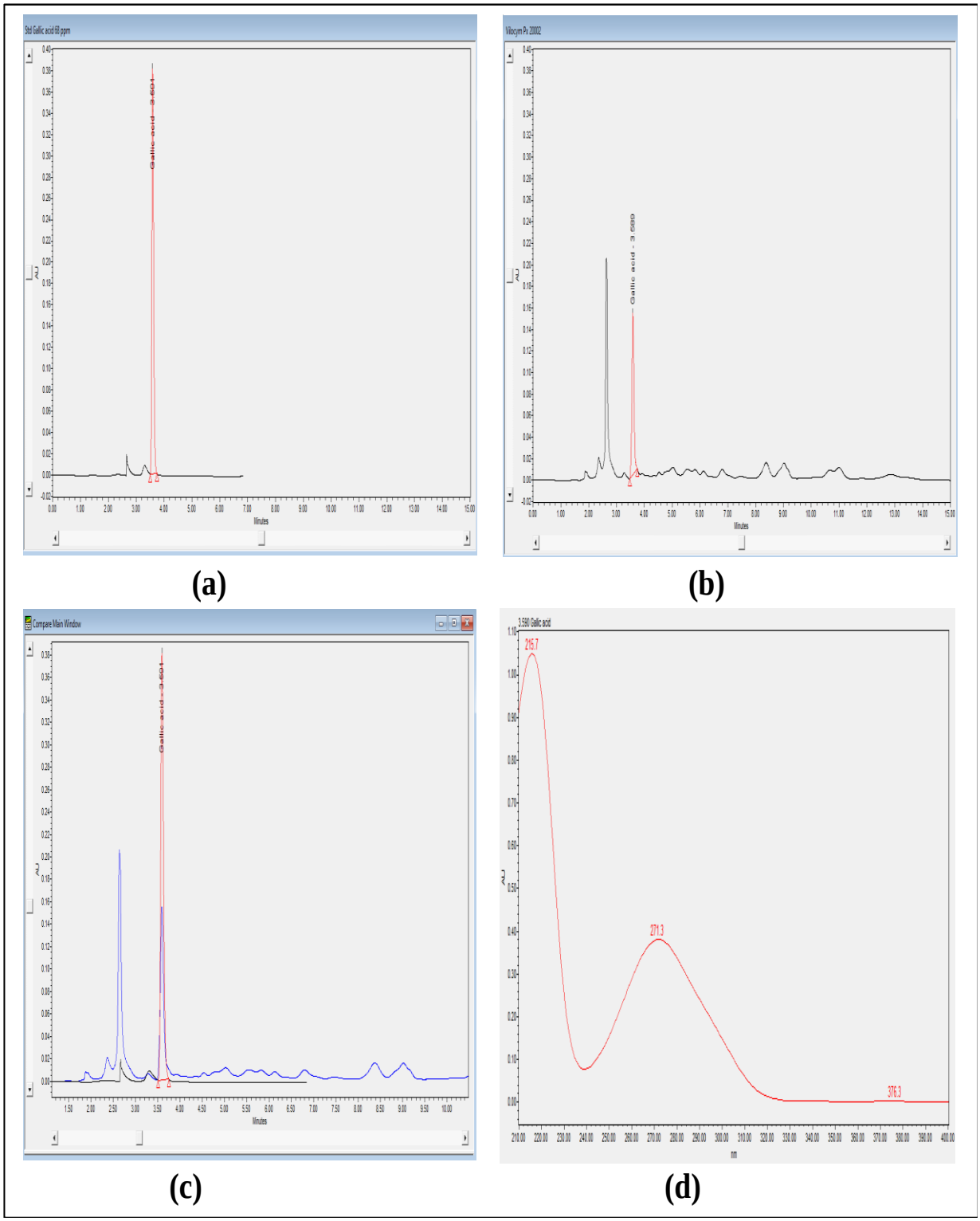

Figure 4: Chromatograms showing the resolution of marker compound in the formulation Vilocym Premix. (a) Chromatogram of standard gallic acid, (b) Chromatogram of test Vilocym Premix, (c) Overlay of the gallic acid chromatograms i.e. sample against the standard, (d) Spectral scan of standard gallic acid and spectral scan of gallic acid in Vilocym Premix.

CONCLUSION

Herbal veterinary formulation Vilocym Premix was standardized by standard analytical technique RP-HPLC. The characterization parameters presented in this communication may serve as a standard reference for quality control analysis of Vilocym Premix to ensure the batch to batch consistency of the main phytoconstituents.

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