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Highlights

- Efficacy and Safety of Vitamin D_3
- Tablet Swasvin D Vyro (Virofight)
- Validation of Derivative FTIR
- Pure and Pharmaceutical Dosage

Discovering Thoughts, Inventing Future

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Contents of the Issue

i. Copyright Notice
ii. Editorial Board Members
iii. Chief Author and Dean
iv. Contents of the Issue

1. Efficacy and Safety of Vitamin D_3 (Cholecalciferol) Oral Solution Compared to Tablet and Capsule: A Randomized, Parallel-Design, Active-Controlled Study. *1-6*
2. Tablet Swasvin D Vyro (Virofight) - A Proven Solution for any Viral Infection, Immunity and Inflammation. *7-12*
3. Development and Validation of Derivative FTIR Spectroscopy for Estimation of Entecavir Monohydrate in its Pure and Pharmaceutical Dosage Forms. *13-34*
4. Proniosomal Gel: Formulation and Charecterization of an Antifungal Drug (Butenafine Hcl) Loaded Proniosomes for Topical Delivery. *35-46*

v. Fellows
vi. Auxiliary Memberships
vii. Preferred Author Guidelines
viii. Index

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Efficacy and Safety of Vitamin D_3 (Cholecalciferol) Oral Solution Compared to Tablet and Capsule: A Randomized, Parallel-Design, Active-Controlled Study

By Krishnakumar M. Nandgaye, Santoshi B. Kadam & Dr. Prashant J. Palkar

Abstract- We performed a study to compare the efficacy of Vitamin D_3 oral solution with a conventional tablets and capsules in hypovitaminosis D patients. One hundred eighty subjects were divided into three different groups and received vitamin D_3 60000 IU per week for eight weeks either in the form of an oral solution or a tablet or a capsule. A significant increase in serum 25(OH)D was observed in vitamin D_3 oral solution from baseline (P=0.0001) as compared to a tablet (P=0.0001) and capsule (P=0.0001). A significant decrease in iPTH levels was seen in the vitamin D_3 oral solution group from baseline (P=0.0001) and also as compared to a tablet (P=0.0001) and capsule (P=0.0001). Oral solution of vitamin D_3 is a nanotechnology-based formulation which was found to be effective and safe. Thus, treatment with vitamin D_3 oral solution in hypovitaminosis D patients may result in faster and higher improvement in the normalization of vitamin D levels.

Keywords: nanotechnology, hypovitaminosis D, vitamin D_3 oral solution, vitamin D_3 60000 IU.

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Efficacy and Safety of Vitamin D_3 (Cholecalciferol) Oral Solution Compared to Tablet and Capsule: A Randomized, Parallel-Design, Active-Controlled Study

Krishnakumar M. Nandgaye [α], Santoshi B. Kadam [σ] & Dr. Prashant J. Palkar [ρ]

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I. Introduction

The prevalence of hypovitaminosis D indicates that it is a common and notable problem worldwide, as identified in numerous epidemiological studies (1). Environmental factors, such as increased air pollution and reduced ultraviolet B (UVB) irradiation, as well as lifestyle factors, i.e., decreased outdoor activities and less intake of vitamin D-rich food, are likely involved in the etiology of a dramatic reduction of vitamin D circulating levels (2). The insufficiency and deficiency of vitamin D raises public health concern since it is independently associated with a higher risk of all-cause mortality (3, 4). Hypovitaminosis D has long been known to increase the risk for osteoporosis and rickets. Only in the last decades, it has been linked with various chronic pathological conditions, i.e., cancer, coronary heart disease (CHD), non-insulin dependent diabetes, neurological disorders, as well as autoimmune and inflammatory diseases (5, 6). The community-based Indian studies of the past decade done on apparently healthy controls reported a prevalence ranging from 50% to 94% (7).

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Treatment with either vitamin D_2 (ergocalciferol) or vitamin D_3 (cholecalciferol) has been recommended for vitamin D deficient patients (8). Nevertheless, as per evidence, vitamin D_3 are superior at raising serum 25(OH)D concentrations than vitamin D_2, and thus vitamin D_3 could potentially become the preferred choice for supplementation (9). Vitamin D is a fat-soluble vitamin and it has a poor bioavailability, which significantly reduces its efficacy as disease-combating agent (10). Oral dosage forms like tablet, capsule, and oral solutions have different absorption rates. The efficiency of oral absorption of conventional vitamin D_3 is approximately 50% (11). In general, the availability for the absorption of a drug is more in oral solutions comparing to the capsule and tablet, respectively (12). In accordance to this, our previous bioequivalent study conducted in healthy volunteers, in which we had first time compared three different formulations (tablet, capsule, and oral solution) of vitamin D_3 and proved that the C_{max} and AUC of an oral solution of vitamin D_3 are higher than that of the tablet and capsule (13). The aim of the present study is to compare and assess the efficacy of oral solution of vitamin D_3 with that of tablets and capsules in hypovitaminosis D patients. Furthermore, this is the first study to our knowledge to compare three different formulations of vitamin D in hypovitaminosis D subjects.

II. Methods

a) Study Design

The present study was a multi-centre, parallel-group, active-controlled study to evaluate the safety and efficacy of vitamin D_3 oral solution Hi-D™ containing vitamin D_3 60000 IU of Akumentis Healthcare Limited, India (oral solution group) comparing with two reference products, D_3 MUST™ 60K, a tablet containing vitamin D_3 60000 IU of Mankind Limited (tablet group), and Uprise-D_3 60K®, a capsule containing vitamin D_3 60000 IU of Alkem Limited, India (capsule group) in patients with hypovitaminosis D. This study was performed from 9 April 2019 to 13 September 2019.

The study was carried out in compliance with the protocol by current local legislation, International

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Council on Harmonization of requirements for registration of pharmaceuticals for human use-Good Clinical Practice (ICH-GCP).

The study protocol (version no.: 01 dated 19 December 2018) and the informed consent form in English (version no.: 01 dated 26 December 2018) & Marathi (version no.: 01 dated 31 December 2018) languages were used for obtaining written informed consent from each of the subjects were approved by the Ethics Committee on 27 February 2019, i.e., before the commencement of the study.

b) Subjects

Total 180 subjects, male and female, were enrolled in this study with the mean age of 43.9±16.63 years (minimum - 19 years and maximum - 89 years). The inclusion criteria for participants were that they should be >18 years of age at the time informed consent was obtained, have subnormal serum 25(OH)D levels on screening, female patients with negative urine or serum pregnancy test within seven days before baseline visit. Participants were excluded if they were with clinical signs or symptoms of overt metabolic bone disease such as bone pains, myopathy or fractures; with history of GI malabsorption, abnormal liver, renal or heart function, or underwent gastrointestinal surgeries in the past; suffering from hypocalcemia or hyperparathyroidism; have hypersensitivity to vitamin D.

Patients were randomized in 3 equal groups of vitamin D_3 oral solution, vitamin D_3 tablet, and vitamin D_3 capsule, respectively. All three groups received 60000 IU of vitamin D_3 per week for eight weeks in the form of respective formulation. Out of 180 patients, 164 completed the study comprising 55 patients in each vitamin D_3 oral solution Hi-D™ (Akumentis Healthcare Limited, Mumbai) and D_3 MUST™ 60K (Mankind Limited, India) group and 54 patients in Uprise-D_3 60K® group (Alkem Limited, India).

c) Outcomes

Primary outcomes included efficacy of vitamin D_3 formulations, which were evaluated by comparing and assessing all three formulations on attaining vitamin D sufficiency (serum 25 (OH) D levels) at the end of treatment (8 weeks) to find out differences between vitamin D formulations. Secondary outcomes include changes in intact parathyroid activity (serum iPTH levels) at the end of treatment (8 weeks) in all the groups. Safety was evaluated by assessing and comparing all three formulations on changes in serum calcium, serum phosphorous, serum alkaline phosphatase, serum albumin, and serum creatinine levels at the end of treatment (8 weeks) and reported adverse events during the study till the end of treatment (8 weeks) in all the groups to find out differences between vitamin D formulations. Adverse Events (AEs) were categorized by investigators according to their intensity as Grade 1-mild, Grade 2-moderate, or Grade 3-severe. Patients were encouraged to report AEs spontaneously or in response to a general non-directed questionnaire.

d) Statistical Analysis

Descriptive statistical methods were used to summarize demographic, baseline characteristics, and all other analysis variables. Data was presented in terms of mean +/- SD and range for all variables. All patients were compared at baseline for homogeneity using analysis of variance (ANOVA) as appropriate Paired t-test was used for comparison. Statistical analysis was performed on the per-protocol (PP) population which included the subjects who had completed the study without any significant protocol deviation. Two-sided tests were used with $P<0.05$ being considered significant.

III. Results

All the patients enrolled in this study were Asian; the baseline demographic data are shown in Table 1. In primary outcomes, the serum 25(OH)D levels with vitamin D_3 oral solution Hi-D™ group were elevated more than three times compared to baseline in the 8th week. This increase in 25(OH)D levels by oral solution was significant as compared to the tablet and capsule group from the baseline to the 8th week (Figure 1). The iPTH levels in vitamin D_3 oral solution were suppressed significantly by 63.53% as compared to tablet and capsule group from the baseline to the 8th week (Figure 2).

Secondary outcomes were similar in all three groups after treatment (Table 2). There was no serious adverse event reported in the overall study period. No patient developed vitamin D toxicity. Six cases of non-laboratory related AEs were reported and were mild in intensity.

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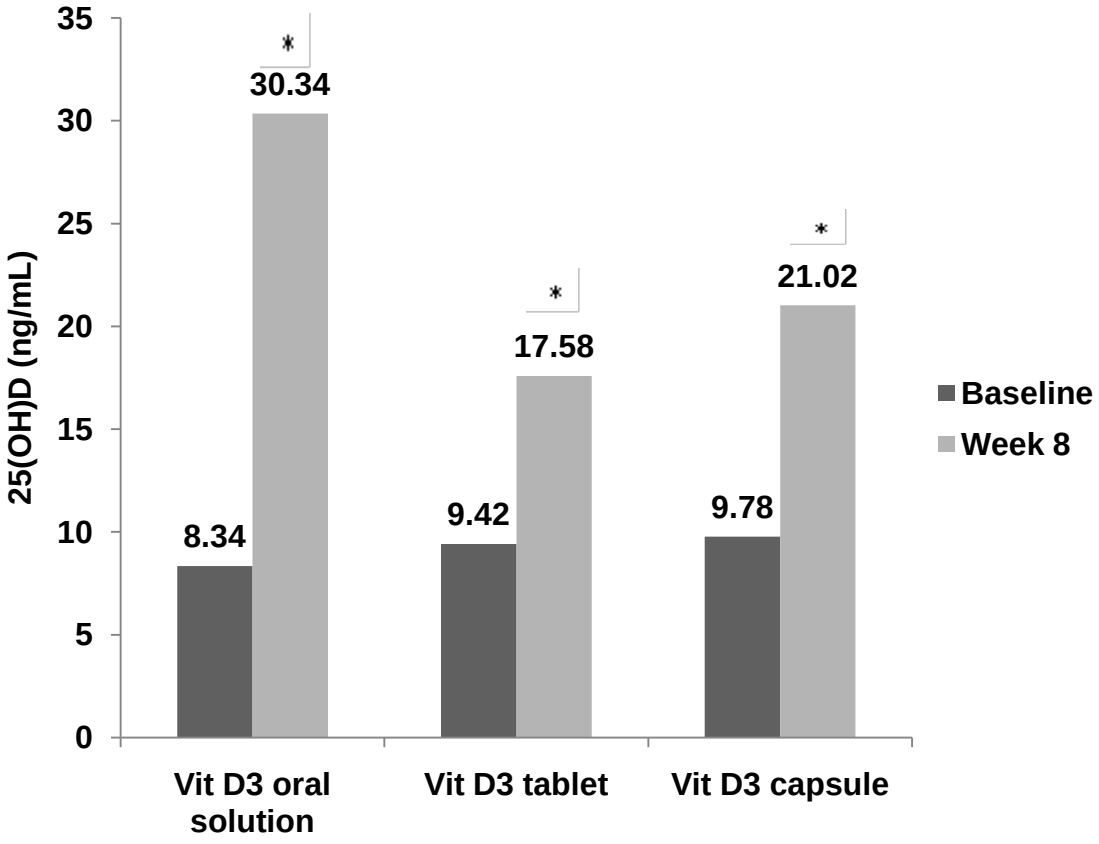


Figure 1: Change in serum 25(OH) D level at the end of eight weeks in all three treatment groups (n = 164). *P=0.0001 vs baseline; †P=0.0001 vs Tablet and Capsule.

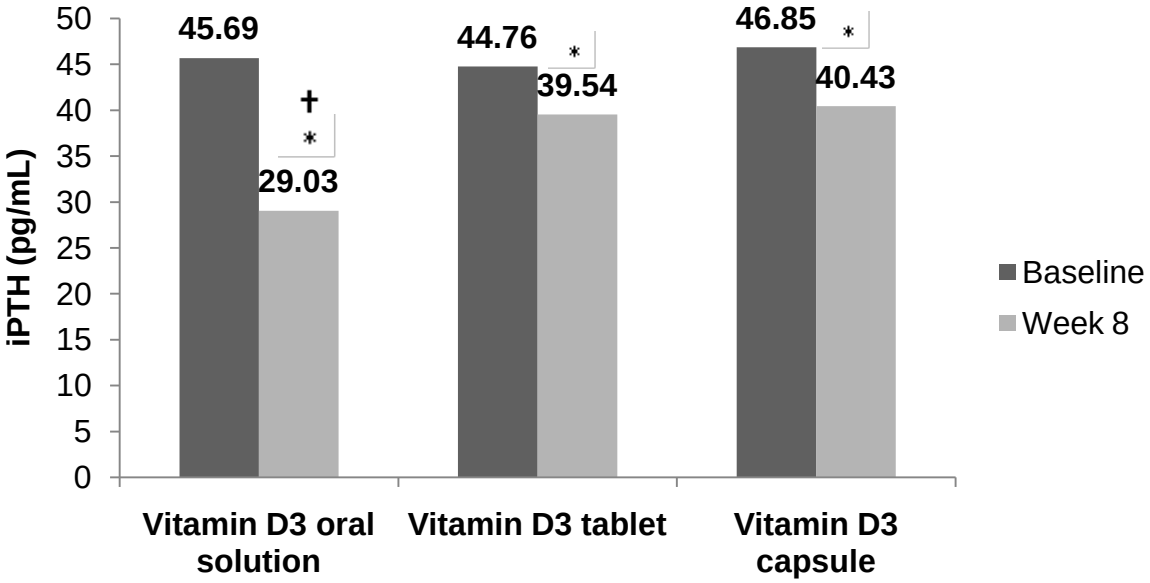


Figure 2: Change in serum iPTH levels at the end of eight weeks in all three treatment groups (n = 164). *P=0.0001 vs baseline; †P=0.0001 vs Tablet and Capsule.

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Table 1: Overall demographic profile of all subjects (N = 180)

Variable	Vit D_3 Oral Solution (N=60)	Vit D_3 Tablet (N=60)	Vit D_3 Capsule (N=60)	All Enrolled (N=180)
Age (Years) Mean (SD) Min, Max	42.7 (16.54) 20.0, 76.0	42.7 (18.52) 19.0, 89.0	46.18 (14.64) 23.0, 81.0	43.9 (16.63) 19.0, 89.0
Gender Male Female	35 25	36 24	29 31	100 80
Height (cm) Mean (SD) Min, Max	162.8 (4.17) 154, 171	162.8 (4.36) 155, 174	164.0 (3.64) 155, 171	163.2 (4.09) 154, 174
Weight (Kg) Mean (SD) Min, Max	69.1 (10.79) 47, 87	69.0 (9.61) 51, 88	71.1 (10.32) 48, 90	26.21 (4.07) 47, 90
BMI (Kg/m^2) Mean (SD) Min, Max	26.11 (4.36) 17.10, 34.90	26.04 (3.65) 19.0, 32.0	26.49 (4.24) 18.9, 35.2	26.21 (4.07) 17.10, 35.2

IV. Discussion

The status of vitamin D depends on the production of vitamin D and vitamin D intake through the diet or vitamin D supplements. Owing to its fat-soluble nature, dietary vitamin D is absorbed with other dietary fats in the small intestine. The efficient absorption of vitamin D is dependent upon the presence of fat in the lumen, which triggers the release of bile acids and pancreatic lipase. In turn, bile acids initiate the emulsification of lipids, pancreatic lipase hydrolyzes the triglycerides into monoglycerides and free fatty acids, and bile acids support the formation of lipid-containing micelles, which diffuse into enterocytes (14). In India, a current recommendation for correction of vitamin D level is by giving 60,000 IU of oral vitamin D every week for eight weeks (15).

Different dosage forms are produced to achieve the appropriate absorption through the suitable form of the drug as different drugs require different routes of administration. Absorption of each substance occurs differently by the human body. Hence different administration routes, as well as dosage forms, are provided and recommended for each substance under which the dose of the drug will be absorbed, delivered, and distributed more effectively. When it comes to oral dosage forms, solutions are one of the preferable dosage forms. Their strongest advantage is based on the fast and high absorption of soluble medicinal products. Solutions are one of the "leading" dosage forms due to their application in patients with swallowing difficulties and their easy administration (16).

The use of nanotechnology in formulation development and lifecycle management can make drug development significantly cost-effective. Also, nanotechnology can target specific drugs, which can reduce toxicity and improve efficacy. Nanotechnology-based delivery systems can also protect drugs from degradation (17). Several studies of nanotechnology-based formulations of vitamins like vitamin A and vitamin E reported significant improvement in the plasma levels of the vitamins after the administration of the formulation (18, 19).

Table 2: Changes in serum parameters (N=164)

Treatments	Vit D_3 nano oral solution (N=55)	Vit D_3 tablet (N=55)	Vit D_3 capsule (N=54)
Calcium (mg/dL) Baseline Week 8	9.28 9.40	9.32 9.31	9.42 9.40
Serum Phosphorus (mg/dL) Baseline Week 8	3.51 3.67	3.71 3.64	3.70 3.59
Alkaline Phosphatase (IU/L) Baseline Week 8	290.87 244.96	291.98 277.05	284.61 271.71

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Serum Albumin (g/dL) Baseline Week 8	4.30 3.40	4.35 4.30	4.45 4.35
Serum Creatinine (mg/dL) Baseline Week 8	0.79 0.83	0.78 0.76	0.79 0.74
Treatments	Vit D_3 nano oral solution (N=55)	Vit D_3 tablet (N=55)	Vit D_3 capsule (N=54)

The use of nanoparticle-based Vitamin D oral solution is increasing in the market. The bioavailability of nutrients that have poor water solubility can be increased by nanotechnology (11, 20). Evidence showed that nanoparticles of vitamin D_3 might also enhance important properties of vitamin D supplements, like therapeutic efficacy, photo-stability, and biodegradation (21). Moreover, in our previous study, we have compared the bioequivalence of vitamin D_3 oral solution with that of conventional vitamin D_3 tablets and capsules. We observed that the oral solution of vitamin D has higher C_{max} and AUC as compared to tablet and capsule (13).

In this study, we have evaluated the efficacy and safety of 3 different formulations of vitamin D_3 (oral solution, tablet, and capsule) in subjects with hypovitaminosis D. Results were in favour of oral solution as serum vitamin D level was increased significantly and reached up to the normal level. This result was significantly better as compared to tablets and capsules. Serum iPTH level was also improved significantly in oral solution as compared to tablets and capsules.

Manek K observed that nanoparticle-based formulation of vitamin D_3 is effective and safe in the correction of vitamin D levels in patients with documented deficiency or insufficiency of vitamin D (15). Similar results were found by Marwaha et al., documenting vitamin D_3 oral solution achieves significantly higher levels of serum 25(OH)D (18). These evidence substantiate our findings with similar observation.

To the best of our knowledge, this is the first study comparing the efficacy and safety of 3 different formulations of vitamin D_3 (oral solution, tablet and capsule) in subjects with hypovitaminosis D.

V. Conclusion

Vitamin D_3 60000 IU oral solution appears to be a better and faster treatment option for improving vitamin D levels as compared to tablets and capsules. Moreover, the nanotechnology-based formulation of an oral solution of vitamin D_3 increases plasma vitamin D levels rapidly, and it is also found to be safe. Thus, vitamin D_3 oral solution may be a better alternative than the tablet and capsule formulations in hypovitaminosis D subjects.

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Conflict of Interest

None declared

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Tablet Swasvin D Vyro (Virofight) - A Proven Solution for any Viral Infection, Immunity and Inflammation

By Dr. Smita Naram, Dr. Deepak Mahajan, Dr. Hemang Parekh & Dr. Ronak Naik

Abstract- Viral infections commonly affect both the respiratory tract, upper and lower. The first response of the immune system to the infection is Inflammation. This inflammation is produced by eicosanoids and cytokines, which are released by injured or infected cells. The immune modulation with Ayurvedic formulations as a possible therapeutic measures is need of the hour nowadays. The ancient Indian medicinal system of Ayurveda has a scope of treating many diseases by the theory of Rasayana, in other terms called preparations from plant or herbal source, including immune modulatory properties. In this article, we want to validate immunemodulatory, anti-inflammatory anti-viral role of Tablet Swasvin D vyro (Virofight) with the reference of some previous work done. In conclusion, we can say that Swasvin D vyro (Virofight) tablet is the best effective immune-modulatory, as it augments the cell-mediated as well as humeral mediated immune response, it is antiviral as it can inhibit replication of several viruses. It is anti-inflammatory by inhibiting various cytokine producing pathways, it has anti-oxidant and antiulcer properties.

Keywords: *immunomodulator, anti-inflammatory, antiviral, D vyro, virofight.*

GJMR-B Classification: *NLMC Code: QV 704*

TABLETSWASVINDVYROVIROFIGHTAPROVENSOLUTIONFORANYVIRALINFECTIONIMMUNITYANDINFLAMMATION

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Tablet Swasvin D Vyro (Virofight) - A Proven Solution for any Viral Infection, Immunity and Inflammation

Dr. Smita Naram [α], Dr. Deepak Mahajan [σ], Dr. Hemang Parekh [ρ] & Dr. Ronak Naik [ω]

Abstract- **Viral infections commonly affect both the respiratory tract, upper and lower. The first response of the immune system to the infection is Inflammation. This inflammation is produced by eicosanoids and cytokines, which are released by injured or infected cells. The immune modulation with Ayurvedic formulations as a possible therapeutic measures is need of the hour nowadays. The ancient Indian medicinal system of Ayurveda has a scope of treating many diseases by the theory of Rasayana, in other terms called preparations from plant or herbal source, including immune modulatory properties. In this article, we want to validate immune-modulatory, anti-inflammatory anti-viral role of Tablet Swasvin D vyro (Virofight) with the reference of some previous work done. In conclusion, we can say that Swasvin D vyro (Virofight) tablet is the best effective immune-modulatory, as it augments the cell-mediated as well as humeral mediated immune response, it is antiviral as it can inhibit replication of several viruses. It is anti-inflammatory by inhibiting various cytokine producing pathways, it has anti-oxidant and antiulcer properties.**

Keywords: immunomodulator, anti-inflammatory, anti-viral, D vyro, virofight.

I. Background

'Survival of the fittest' is the phrase what Darwinism theory of evolution said, indicating the natural selection. In the world of microorganisms that attacks the human body in various ways, if we are fit, our immunity is good, and we can easily tackle them. The Immune system protects from infection; in short it acts as physical barrier and prevents from external pathogens like bacteria and viruses. The first response of the immune system to the infection is Inflammation. This inflammation is produced by eicosanoids and cytokines, which are released by injured or infected cells. Common cytokines include interleukins that are responsible for communication between white cells; chemokine promotes chemo taxis and interferon that have anti-viral effects[1].

Viral infections commonly affect both the respiratory tract, upper and lower respiratory tract. The respiratory infections are commonly classified clinically according to syndrome common cold, bronchitis, croup, pneumonia[2]. The viruses mostly act through a direct invasion of epithelial cells of the respiratory mucosa. There is an increase in both leucocytes infiltration and nasal secretions, includes proteins and immunoglobulin, suggesting cytokines and immune mechanisms may be responsible[3].

The immune modulation with Ayurvedic formulations as a possible therapeutic measures is need of the hour nowadays. The ancient Indian medicinal system of Ayurveda has a scope of treating many diseases by the theory of Rasayana, in other terms called preparations from plant or herbal source, including immune-modulatory properties[4].The basic concept of immune modulation practiced by Ayurvedic practitioners for centuries, as it was mentioned in Ayurvedic ancient literature and Samhitas. The goal of immune enhancement achieved by Ayurveda charyas by the use of the Rasayana concept. The toxic by-products of impaired digestion is called Aama, which clog the micro channels (Strotas) are considered as pathogenesis of Inflammation. The herbs, which improve the process of digestion, digest the Aama and purifies the micro channels is considered as an anti-inflammatory. There are many such ayurvedic herbs, and herbal combinations are available in ayurvedic literature, which is being used since ancient times to treat many acute as well as chronic inflammatory diseases. When all consumed elements of the food not digested properly, it forms Aama, this forms Abnormal Digestive juice (Sama Aahar rasa), which in term produces cells that are abnormal, and these abnormal cells are virus and other pathogens[23].

In this article, we want to validate the immunomodulatory, anti-inflammatory anti-viral role of Tablet Swasvin D vyro (Virofight) manufactured by Ayushakti Ayurveda Pvt Ltd with the reference of some previous work done.

II. Name of Herbal Combination

Tablet Swasvin D vyro (Virofight).

Author α: Founder, Chairman and Managing Director Ayushakti Ayurveda Pvt Ltd, Bhadran nagar cross road, Malad, Mumbai-64. e-mail: drdeepakm@ayushakti.com
Author σ ρ ω: Consulting Ayurveda Physician Ayushakti Ayurveda Pvt Ltd, Bhadran nagar cross road, Malad, Mumbai-64.

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III. Manufacturer

Ayushakti Ayurveda Pvt Ltd pharmacy, Plot number 78, Stice, Musalgaon, Sinnar, Nashik- 422112.

IV. Herbal Formula

Ingredients	Latin name	Quantity
Guduchi	Tinospora cardifolia	240 mgs
Dadim	Punica granatum	100 mgs
Madhuyashti	Glycyrrhiza glabra	100 mgs
Kalmegh	Andrographis paniculata	50 mgs
Kutaj	Holerrhena antidysenterica	50 mgs
Sunthi	Zinziber officinale	30 mgs
Vidarikand	Pueraria tuberosa	25 mgs
Shatavari	Asparagus racemosus	25 mgs
Godanti Bhasma		25 mgs
Bhavana Dravya	Kantakari, Tulsi patra, Jati patra	

V. Tinospora Cardifolia

The ethanol extract of Tinospora studied on delayed-type hypersensitivity, humoral response to sheep red sheep cells, skin allograft rejection, and phagocytic activity of the reticuloendothelial system in mice and found that Tinospora cordifolia improved the phagocytic function without affecting the humeral or cell-mediated immune system[5]. T. cardifolia growing on Azadiracta indiaca possesses immunomodulatory potential[6].T. cardifolia stimulates macrophages through TLR6 signaling and NF kappa B translocation, leading to cytokine production[7].Immunomodulatory protein in the stem of T .cardifolia shows lymphoproliferative and macrophage activating properties[8].

VI. Punica Granatum

Active compounds in P. granatum are punicalagin and ellagic acid, the first one attenuates the inflammatory cytokine secretion, and cell adhesion of monocytes cells stimulated with airborne dust, hence can be used against oxidative stress and inflammatory response by harmful airborne dust[9].P. granatum peel polyphenols inhibits LPS induced intracellular ROS production in RAW264.7 macrophages, Receptors of LPS, the mRNA and protein expression of TLR4 also the anti-inflammatory mechanism is associated with the NF-Kb pathway[10]. P. granatum peel's polyphenol compounds like punicalagi, ellagic acid, and hydroxyl-benzoic acid from n-butanol and ethyl acetate fractions are associated with antiviral activity against influenza virus[11].When tannins like punicalagin, punicalin, strictinin, and granatin were isolated from P.granatum, granatin was an effective anti-inflammatory by decreasing the production of PGE_2 in early-stage and decreasing NO production in late stage[12]. Polyphenols in P. granatum may prevent virus binding to the host cell receptors by blocking the cell surface receptors of the virus surface ligands[13].Punicalagin component of P. granatum has the virucidal capability; it inhibits influenza virus RNA proliferation, inhibits the replication of influenza RNA virus independent of the virucidal activity along with antioxidant effect[14].

VII. Glycyrrhiza Glabra

A phytocomponent glycerrhizin of G. glabra affects the cellular signaling pathways like protein kinase C, casein kinase II, and transcription factor-like activator protein one and nuclear factor B. it's aglycone metabolite 18 glycyrrhetinic acid up-regulate expression of inducible nitrous oxide synthase and production of nitrous oxide in macrophages, which inhibits replication of several viruses. In addition, Also glycerrhizin inhibits the absorption, both during and after the absorption period, inhibits replication and penetration of SARC type coronavirus[15]. Glycerrhiza uralensis ethanol extract inhibits the production of RANTES, potent chemotactic cytokine for monocytes, basophils, and T cells, typically detected in nasal secretions of patients with upper respiratory tract infections, involved in epithelial cell-mediated inflammation related to viral infection like influenza virus H1N1[16]. Glycyrrhetinic acid has proved inhibitory to the replication of some RNA and DNA viruses in vitro. Glycyrrhizin is reported to be effective against varicella-zoster virus and human immunodeficiency virus in vitro[17].Glabridin and isoliquiritigenin the components of G.glabra exhibits anti-inflammatory property through inhibition of PGE_2, TXB_2 and, LTB_4 in mammalian cell assay system[18].

VIII. Andrographis Paniculata

A derivative derived from A paniculata, 14-α-lipoyl and rographolide is effective in avian influenza A, ie.H9N2, H5N1 and human influenza A.ie. H1N1 in vitro [(19)].A. paniculata shows property to inhibit secretion of RANTES by H1N1 infected A549 bronchial epithelial cells[20]. Ethanol extract of A. paniculata and and rographolide inhibit expression of Epstein Barr virus lytic proteins, And rographolide inhibits the production of the mature viral particle. It also shows a significant effect on cellular immunological indicators. It was able to modulate the innate immune response by regulating activation of macrophages and regulate specific antibody production as well as antigen-specific IL-4 producing splenocytes[21]. A. paniculata enhances the WBC count, bone marrow cellularity and, β-esterase positive cells, myelosuppression found to be reversed through immunomodulatory activity, the weight of lymphoid organs, spleen and thymus were also increased[22].

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IX. Holerrhena Antidysenterica

The alkaloids from H.antidysenterica have antidiarrheal effect as similar to the standard drug diphenoxylate, by inhibiting the production of watery fluid. Also the astringent property of alkaloids reduces denaturing production of protein tannate, which reduces the secretion from intestinal mucosa[24].Hongoquercin A and Hongoquercin B alkaloid derived exhibit moderate activity against Gram-positive bacteria like E.coli by passing through outer cell membrane[25].The decoction of H. antidysenterica prevents the attaching and effecting histopathology and avert the bacteria from the opportunity to establish intimate contact with host cells and, thus, it prevents from initiating the disease process[26].

X. Zinziber Officinale

Gingerols from Fresh ginger decreases more than 70% HRSV infection and rhinoviral infection in both A549 and HEp2 epithelial cell upper and lower respiratory tract, besides fresh ginger stimulates epithelial cells to secrete IFN-β that contribute to the inhibition of virus replication also it has an anti-inflammatory effect through inhibition of production of prostaglandins and inflammatory cytokines[27]. Several sesquiterpenes like beta-sesquiphellandrene were most active as an anti-viral agent against rhinovirus in vitro[28].The rhizome aqueous extract of Z. officinale significantly reduces the PBMC (Peripheral Blood Mononuclear Cells) proliferation assay, it also inhibits the CD 14 monocyte surface marker in human PBMC showing anti-inflammatory and anti-viral activity[29].

XI. Pueraria Tuberosa

Isoorientin was isolated from tubers of P.tuberosa was identified as a COX 2 inhibiter, which showed potent anti-inflammatory properties in vitro on mouse macrophage cell line, RAW264.7, also it is effective in reducing the inflammation in vivo on pow edema and air pouch mouse models[30]. Due to the effect of some isoflavones like puerarin, daidzein and genistein, P. tuberosa holds a promising therapeutic potential as an immunomodulator. Also P. tuberosa extracts augmented some innate as well as humeral immune responses in rats[31]. Anti-inflammatory mechanism of Mangiferin extracted from P.tuberosa was confirmed via inhibiting the NF-Kb signaling, COX-1, COX-2, and inactivation of NLRP3 inflammoasomes[32]. Tuberosin is one of the active compounds in P.tuberosa, which have anti-inflammatory effect by inhibiting the free radical scavengers, it also has metal chelation property, and also it shows anti-oxidant property[33]. The ethanoic extract of P.tuberosa increases the phagocytic capacity of macrophages, inhibits both cell-mediate immunity and humeral immunity suggesting a suppressive effect on adaptive immunity without affecting the innate immune system and bone marrow proliferation[34].

XII. Asparagus Racemosus

Extract of A. recemosus is recommended for the use of positive immunomodulator I normal and immune-compromised broiler chicks as it augments the humoral and cell-mediated immune response providing better protection against infection by a rise in HI antibody[35]. Steroidal saponins like Shatavarin IV, Immunoside significantly increases CD_3+ and CD_4/ CD_{8+} suggesting T cell activation, also the regulation of Th_1 ($IL_{-2,}$ IFN-g) and Th_2 like IL_{-4} cytokines suggesting activated lymphocytes ultimately showing an immunomodulatory.[36] The aqueous extract of A.racemosus significantly inhibits suppression of chemotactic activity and production of $IL_{-1,}$ and TNF-α by murine macrophages[37].

XIII. Ocimum Sanctum

O. sanctum leaves when steam distilled shows modification in humoral immune response in albino rats may be due to antibody production, the release of mediators of hypersensitivity reaction and tissue response to mediators, also fixed oils and lonolenic acid indicates significant anti-inflammatory activity against PGE_{-2}[38]. It inhibits inflammation in rats by affecting the cyclo-oxygenase and lipo-oxygenase pathways, seed oils shows maximum percentage inhabitation of leukotriene induced paw edema[39].Ocimum sanctum seed oil appears to modulate both humoral and cell mediated immune response and this immunomodulatory response is mediated by GABAergic pathways[40]. Crude extract derived from O.sanctum leaves may inhibit the viral intracellular multiplication and masking/blocking of HA glycoprotein, terpenoid effective in virucidal and therapeutic activity, and polyphenol for prophylactic activity against influenza virus H9H2 virus in ovo model, hence crude extract from the leaves of Ocimum sanctum leads to a reduction in H9N2 influenza virus in assessing the all three; virucidal, therapeutic and prophylactic activity[41].

XIV. Solanum Xanthocarpum

The methanolic extract of Solanum nigrum has anti-inflammatory activity. Solanine showed the most potent inhibitory activity against the LPS-induced NO production in murine RAW264.7[43].

XV. Jasminum Grandiflorum

The extract of leaves of J.grandiflorum possesses the anti-ulcer potential as well as antioxidant activity. It reduces gastric fluid volume, acidity and increases the pH of the gastric fluids; which proves anti-secretory[44]. Leaves extract to decrease the ulcer index, increase pH, reduces free and total acidity, gastric

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volume proving it's an anti-secretory and hence anti-ulcer[45]. Hydro alcoholic extract of J.grandiflorum shows Anti-inflammatory and anti-conversant acivity[46].

XVI. Discussion

Nowadays, various medicinal plants and herbs are attracting interest in the development of new, more effective, and specific agents, as they may be useful in the production of phytochemicals that have activity against microbes. These plants in the form of decoctions, preparations, essential oils, and extracts widely used in ancient Indian medicine. People are preferring the use of Ayurvedic medicines as an alternate therapy for many chronic diseases as well as acute diseases nowadays. Though always there is a question, how exactly ayurvedic medicines works, by which pathway, or by which mechanism it attack on the microorganism. This manuscript was conducted just to justify the mechanism of our medicine by using some modern tools.

In conclusion, we can say Tinospora cardifolia improved the phagocytic function of the reticuloendothelial system without affecting the humeral or cell-mediated immune system (Atal CK et al. 1986, 5)T. cardifolia possesses immunomodulatory potential (Narkhede AN et al. 2014,6). It stimulates macrophages through TLR6 signaling and NF kappa B translocation, leading to cytokine production (Shyma K et al. 7). An active compounds in P. granatum, punicalagin, and ellagic acid, the first one attenuates the inflammatory cytokine secretion hence can be used against oxidative stress and inflammatory response by harmful airborne dust (Soojin Parket al; 2016, 9). Peel polyphenols inhibit LPS induced intracellular ROS production in RAW264.7 macrophages, Receptors of LPS, the mRNA and protein expression of TLR4 (Du, Lin,et al; 2019, 10). punicalagi, ellagic acid and hydroxyl-benzoic acid from n-butanol and ethyl acetate fractions are associated with antiviral activity against influenza virus (Mohammad-Taghi et al. 2019,11). Tannin, like granatin, is an effective anti-inflammatory by decreasing the production of PGE_2 in the early-stage and decreasing NO production in late-stage (Lee, C.J; 2016, 12). Polyphenols in P. granatum may prevent virus binding to the host cell receptors by blocking the cell surface receptors of the virus surface ligands (Howell ABet al; 2013, 13). Punicalagin component has the virucidal capability; it inhibits influenza virus RNA proliferation, inhibits the replication of influenza RNA virus independent of the virucidal activity (Haidari, M, et al.2009, 14). Glycerrhizin Up regulates expression of inducible nitrous oxide synthase and production of nitrous oxide in macrophages, which inhibits replication of several viruses, inhibits replication and penetration of SARC type coronavirus (J Cinatl et al; 2003, 15) Glycyrrhiza uralensis ethanol involved in epithelial cell-mediated inflammation related to viral infection like influenza virus H1N1 (Cristina Fiore et al. 2007, 16), Glabridin, and isoliquiritigen exhibits anti-inflammatory property through inhibition of PGE_2, TXB_2 and LTB_4 in mammalian cell assay system (Nirmala. P et al. 2011, 18).14-α-lipoyl and rographolide is effective in avian influenza A, ie.H9N2, H5N1 and human influenza A,i.e. H1N1 in vitro (Wen-Wan Chao et al; 2010, 19). Andrographolide inhibit the production of mature viral particle. It also shows significant effect on cellular immunological indicators and innate immune response by regulating activation IL-4 producing splenocytes (Churiyahet al. 2015, 21). Hongoquercin A and Hongoquercin B alkaloid derived exhibits moderate activity against Gram-positive bacteria like E.coli by passing through the outer cell membrane (Abbanat el al; 1998, 25). Gingerols from Fresh ginger decreases more than 70% HRSV infection and rhinoviral infection in both A549 and HEp2 epithelial cell upper and lower respiratory tract, secrete IFN-β that contribute to the inhibition of virus replication also it has anti-inflammatory (J.S. Chang et al.2013, 27). Isoorientin was isolated from tubers of P.tuberosa was identified as a COX 2 inhibiter, which showed potent anti-inflammatory properties in vitro on mouse macrophage cell line, RAW264.7 (Kotha Anilkumar et al. 2017,30). Isoflavones like puerarin, daidzein, and genistein, P. tuberosa are immunomodulator. Also P. tuberosa extracts augmented some innate as well as humeral immune responses in rats (A. K. Majiet al, 31)Extract of A.recemosus is recommended for the use as positive immunomodulator I as it augments the humoral and cell mediated immune response (Kumari R et al.2012,35). Steroidal saponins like Shatavarin IV, Immunoside significantly increases CD_3+ and CD_4/CD_{8+} suggesting T cell activation, also regulation of Th_1 (IL_{-2}, IFN-g) and Th_2 like IL_{-4} cytokines suggesting activated lymphocytes ultimately suggesting immunomodulatory effect of A.recemosus (Manish Gautam et al. 2009,36). Sanctum leaves when steam distilled shows modification in humoral immune response in albino rats due to antibody production, release of mediators of hypersensitivity reaction and tissue response to mediators, also fixed oils and lonolenic acid indicates significant anti-inflammatory activity against PGE_{-2} (S Mondal et al;2009). It inhibits inflammation in rats may be it affects the cyclo-oxygenase and lipo-oxygenase pathways (P.K Mediratta et al. 2002).

XVII. Result

We can say that Swasvin D vyro (Virofight) tablet is the best effective immunomodulator, as it augments the cell mediated as well as humeral mediated immune response, it is antiviral as it can inhibit replication of several viruses, and it is anti-inflammatory by inhibiting various cytokine producing pathways, it has anti-oxidant and antiulcer properties.

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Development and Validation of Derivative FTIR Spectroscopy for Estimation of Entecavir Monohydrate in its Pure and Pharmaceutical Dosage Forms

By Ashraf A Khanam, Y Padmavathi & Raghavendra Babu

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Keywords: entecavir monohydrate, FTIR, second derivative FTIR, sandell's sensitivity, statistical analysis.

GJMR-B Classification: NLMC Code: QV 701



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Abstract- We developed a unique analytical technique for the evaluation of Entecavir monohydrate (ETV) in its pharmaceutical dosage form using derivative spectroscopy assisted FTIR. This approach requires the formation of solid pellets of Entecavir using potassium bromide (KBr) with the aid of geometrical mixing. The spectra were calculated by direct measurement technique using reduced path length in the absorbance mode, and the equipment was configured to secure it at 8cm^{-1} resolution. We scanned the spectra between the ranges of 4000 to 400 cm^{-1}. FTIR spectra drug exhibited overlapped functional group peaks with baseline correction at 1631 cm^{-1} corresponding to C=O stretching. From these FTIR spectra, we detected intense, clear, and proportional second derivative peaks between 1639.38 and 1620.09 cm^{-1}. These peaks, in the range of concentration 12.5-200 µg/mg, obeyed Beer-Lambert's law. Therefore, we elected C=O stretching for the quantitative evaluation of Entecavir employing second-order derivative spectroscopy. The developed technique was validated conforming to the International Conference on Harmonisation (ICH) guidelines. The validation criteria linearity, precision, accuracy, the limit of quantitation, the limit of detection, and Sandell's sensitivity was established and turned up to be within limits. We successfully implemented this technique for the analysis of the marketed formulation of Entecavir monohydrate. We also compared the second derivative FTIR technique for evaluation of Entecavir monohydrate with HPLC methods published in various journals and Indian Pharmacopoeia, statistically.

Keywords: entecavir monohydrate, FTIR, second derivative FTIR, sandell's sensitivity, statistical analysis.

I. Introduction

Hepatitis B is a viral infection worldwide that invades the liver and can provoke both severe and persistent diseases. HBV: Hepatitis B virus transmits sexually, parenterally, or perinatally. HBV chronically infects over 248 million people worldwide [1-2].

Antivirals are drugs that kill a virus or suppress their capability to reproduce. The focus of antiviral medicine is to reduce symptoms, infectivity, and to minimise the span of illness. Antiviral drugs act at various stages by arresting the cycle of viral replication [3].

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Entecavir Monohydrate: The hydrated form of Entecavir is Entecavir Monohydrate: a synthesized analog of 2'-deoxyguanosine and a nucleoside reverse transcriptase inhibitor with selective antiviral action against the hepatitis B virus (Fig. 1). It phosphorylates intracellularly with the dynamic triphosphate form, which contests with deoxyguanosine triphosphate (a natural substrate of the virus hepatitis B reverse transcriptase), suppressing every phase of the enzyme's action; at the same time, it bears no activity against HIV. USFDA authorized it in March 2005.

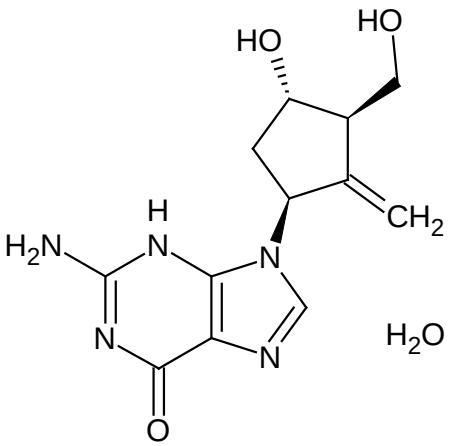


Figure 1: Structure of Entecavir Monohydrate

The IUPAC name of Entecavir monohydrate is a 2-amino-9- [(1S, 3R, 4S)-4-hydroxy-3 (hydroxymethyl)-2-methylidenecyclopentyl]-1H-purin-6-one; hydrate. Its molecular formula and molecular weight is $C_{12}H_{17}N_5O_4$ and 295.29 g/mole, respectively.

It's a non-hygroscopic, off white to white powder, practically insoluble in acetonitrile, sparingly soluble in N, N-dimethylformamide, slightly soluble in methanol, ethanol (99.5%) and water (2.4 mg/ml at pH 7.9, 25°C) [4]. Store Entecavir tablets in a tightly closed container at 25° C (77° F); excursions permitted between 15-30° C (59-86° F) [5].

Technique: Spectroscopy is the measurement of the interaction of light with various materials. To determine a chemical substance, analyze the amount of light

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absorbed or emitted by a sample. *Infrared spectroscopy (IR spectroscopy*) is a technique based on the vibrations of the atoms of a molecule. An infrared spectrum is obtained by passing infrared radiation through a sample and determining what fraction of the incident radiation absorbs at a particular energy. The energy at which any peak in an absorption spectrum appears corresponds to the frequency of vibration of a part of a sample molecule [6].

Fourier-transform infrared (FTIR) spectroscopy is based on the idea of the interference of radiation between two beams to yield an interferogram. The latter is a signal produced as a function of the change of path length between the two beams. The two domains of distance and frequency are interconvertible by the mathematical method of Fourier-transformation [7].

Derivative spectroscopy (DS) has been brought in for resolving overlapping peaks. DS approach is extensively adopted to intensify the signal and work out the overlapped peak-signals for its improvements in separating closely adjacent peaks and finding weak peaks covered by sharp peaks. When derivatized, the crests and troughs of the original peak function take hold of zero values, and the inflections are modified into maxima or minima, correspondingly. The curves of derivatization are better structured than the authentic spectra, therefore facilitating very slight distinctions to be singled out.

Advantages of DS are it clears up opportunities for enhancing selectivity and sensitivity; is employed to detect elements with significant accuracy with no preceding step; is incredibly practical when overlap or interference occurs; it extends a dynamic medium for qualitative and quantitative analyses of mixtures; and it is easy to eliminate specimen turbidity matrix background, to improve spectral details and to get rid of the effect of baseline shifts and baseline tilts [8].

After reviewing ample of available literature, we planned this work to develop and validate a sensitive second derivative technique based on FTIR, for estimation of Entecavir Monohydrate in its pure and pharmaceutical dosage form [9-31].

II. Method

Materials and Reagents (Table 1, 2 & 3)

Table 1: List of chemicals and their grades

S. No.	Chemicals	Category
1.	Potassium Bromide Anhydrous	IR Grade
2.	Dimethyl Sulfoxide	AR Grade
3.	Chloroform	HPLC Grade
4.	Water	HPLC Grade
5.	Methanol	HPLC Grade

Table 2: List of standard and sample utilized

S.No.	Name	Manufacturer/ Supplier
1.	Entecavir Monohydrate (Pure form)	Gift sample from Dr.Reddy's Laboratories, Hyderabad.
2.	X-VIR* Tablets (Marketed Formulation)	Bought from a local pharmacy store

Table 3: List of instruments employed

S.No.	Instruments	Make and model	Software
1.	FTIR Spectrophotometer	Shimadzu –8400S	IR Solutions (Ver. 1.21)
2.	UV-VIS Spectrophotometer	Shimadzu –1800	UV Probe (Ver. 2.43)
3.	HPLC	Shimadzu –LC-20AT	LC Solution (Ver. 1.25)
4.	Electronic Balance	Shimadzu –BL220H	– NA –
5.	Ultra-Sonic Bath Sonicator	PCI Analytics –6.5 li200H	– NA –
6.	Hot Air Oven	BTI Mumbai –105	– NA –

a) Method Development

Liquid cell and KBr press were utilized for sampling liquids and solids, respectively. We developed FTIR spectroscopic method using an FTIR instrument with the parameters in Table 4.

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Table 4: Method Development Parameters

S.No.	Parameter	Selected Condition
1.	Selection of Measurement Mode	Absorbance Mode
2.	Selection of Beam	Internal
3.	Selection of Detector	Standard DLATGS detector
4.	Selection of Mirror Speed	2.8 mm/sec
5.	Selection of Sampling Technique	Pressed Pellet technique
6.	Selection of Apodization	Happ-Genzel
7.	Selection of solvent (based on IR transparency window)	For Liquid: Chloroform, Dimethyl sulfoxide and methanol For Solid: Potassium Bromide
8.	Analysis of IR Spectra for Functional Group Assessment	ETV IR Spectrum: Peak at 1631 cm^{-1}, C–O stretch Clear, intense peak, increased linearly with concentration.

b) Method Optimization

i. *Preparation of standard stock of Entecavir monohydrate*

Accurately weighed 40 mg of the Entecavir monohydrate was geometrically mixed with 200 mg of dried KBr to form the stock of 200µg/mg. Mix the triturate well, such that each pellet formed contained the uniformly distributed drug.

ii. *Preparation of the working standard mixture*

From the stock (200 µg/mg), accurately weighed 6.250, 12.500, 25.000, 50.000 mg was taken and diluted to 100 mg with dried KBr to create the eventual concentrations of 12.5, 25, 50, and 100 µg/mg, respectively. We ensured uniform mixing.

iii. *Extraction Procedure*

Triturate twenty tablets (X-VIR* manufactured by NATCO Pharma Ltd., containing 1 mg of ETV) after taking their average weight. Then the tablet powder equivalent to 1 tablet was transferred to an Eppendorf tube and dissolved in methanol. It was vortexed for 2 minutes, followed by centrifugation at 5000 rpm for 10 minutes. Then the resulting supernatant was collected and evaporated overnight. The residue was collected (approximately 1 mg when weighed).

iv. *Sample Preparation for Pressed Pellet Technique*

The complete residue obtained was triturated with 50 mg of KBr to make a pellet of 20 µg/mg, which we scanned in the absorbance mode, and the peak so recovered was derivatized to second order. We then calculated peak area of the derivatized peak.

v. *Sample Preparation for Liquid Sampling Technique*

Using the above extraction procedure, Entecavir monohydrate was extracted from its marketed formulation. Accurately weighed 1 mg of extract was transferred in a 10 ml volumetric flask, and suitable solvents were added individually in each flask, i.e., methanol, DMSO, methanol in chloroform.

c) Method Validation

The FTIR method was developed and validated for quantitative evaluation of ETV in tablets using the KBr pressed pellet technique corresponding to the ICH guidelines Q2 (R1): Validation of Analytical Procedures: Text and Methodology [32].

i. *Linearity and Range*

The working standard solutions of ETV were prepared and analyzed in the FTIR instrument. We recorded absorbance of the peaks at 1631cm^{-1} for standard solutions, and plotted the standard calibration curve between concentration and absorbance. Regression analysis established linearity; It reports the regression equation and the coefficient of determination.

ii. *Limit of Detection (LOD) and Limit of Quantification (LOQ)*

We estimated the responsiveness of suggested technique for measurement of ETV in terms of LOD & LOQ; and determined it using the standard deviation method. Then calculated, the standard deviation and slope from the calibration curve established for linearity parameter using the below-mentioned formulae:

$$\text{LOD} = \frac{3.3*\sigma}{s} \qquad \text{LOQ} = \frac{10*\sigma}{s}$$

Where,

σ = standard deviation of the response (intercept);
s = slope of the calibration curve

iii. *Sandell's Sensitivity*

Sandell's sensitivity, defined as the lightest weight of a material that can be encountered in a column of a unit cross-section. The lowest concentration of ETV (12.5µg/mg) was prepared from the working standard solution (200µg/mg) and scanned several times. We noted the absorbance and calculated the Sandell's sensitivity using the formula given below:

$$Sandell's\ Sensitivity\ (\pi) = \frac{Concentration\left(\frac{\mu g}{100 mg}\right)}{Absorbance\ value} \times 0.001$$

iv. *Precision*

To establish precision of the method, we reported its repeatability. They usually use the standard deviation (SD) or percentage relative standard deviation (% RSD) of a course of evaluations to assess the rigor of a scientific technique. Precision was determined using

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repeatability, and calculated for only one stage of precision.

Repeatability

We determined repeatability by analyzing six replicates of 100μg/mg, and calculating their percent relative standard deviation (% RSD).

v. *Accuracy*

The accuracy of the method was reported as the percentage recovery of a known added measure of the analyte to a specimen or as the difference between the average value obtained and the accepted true value of a specimen, jointly with an associated confidence interval.

For the drug product

We determined the accuracy study of drug product by calculating the percentage recovery of the ETV using the standard addition method. By adding known amounts of the standard mixture of ETV (40, 50, and 60 μg/mg), respectively, to a pre-quantified test mixture of ETV (50 μg/mg). The calculation of percentage recovery was performed by measuring absorbance and qualifying these amounts into the regression equation of the calibration curve and by calculating the percent relative standard deviation (% RSD) at each stage.

vi. *Assay of Entecavir Monohydrate tablets*

Triturate twenty tablets (X-VIR* manufactured by NATCO Pharma Ltd., containing 1 mg of ETV) after taking their average weight. Then the tablet powder equivalent to 1 tablet was transferred to an Eppendorf tube and dissolved in methanol. It was vortexed for 2 minutes, followed by centrifugation at 5000 rpm for 10 minutes. Then the resulting supernatant was collected and evaporated overnight. The residue was collected (approximately 1 mg when weighed). Later, the complete residue was triturated with 50 mg of KBr to make a pellet of 20 μg/mg, which we scanned in the absorbance mode, and the peak so recovered was derivatized to second order. We then calculated peak area of the derivatized peak.

$$\text{Assay} = \frac{\text{Concentration } \left(\frac{\mu g}{mg}\right) \times \text{ Dilution Factor } \times \text{ Average Weight of the Tablet (mg)}}{\text{Weight of Tablet Powder Taken (mg)} \times \text{ Label Claim of the Drug}} * 100$$

III. Results and Discussion

a) Development and Optimization of FTIR Method

i. *Solubility Studies*

During developmental studies, we checked the drug solubility in methanol and chloroform and its combination. We found ETV solution of methanol in chloroform [50 μg/ml] to be the most reliable solution for solubility that can be studied on a UV-VIS spectrophotometer, giving λ_{max} at 257 nm.

Solution Preparation

We took 10 mg of ETV along with a few ml of methanol in a volumetric flask, which was sonicated for 2 minutes, and made up to 10 ml with methanol to make methanol stock solution of concentration 1000 μg/ml. Then, 0.1, 0.5, and 1.0 ml of this methanol stock solution were made up to volume in other 10 ml volumetric flasks with chloroform to prepare solutions of 10, 50, and 100 μg/ml concentrations, respectively. An overlay of their spectra in Fig.2.

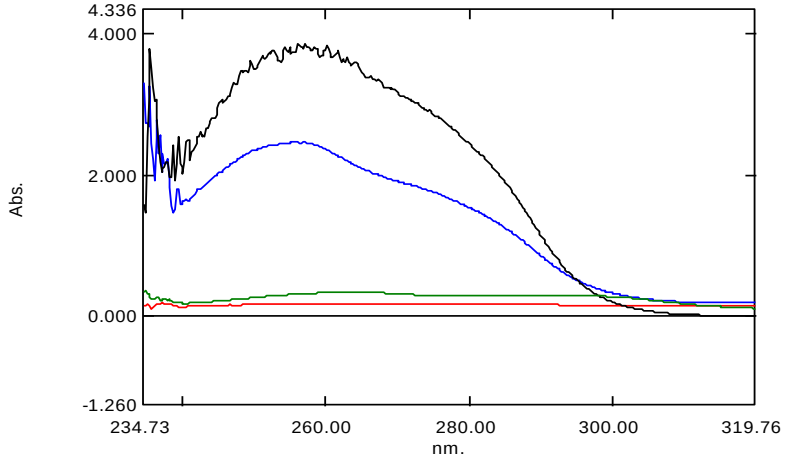


Figure 2: Overlay of drug solubility profile

Red –ETV in CH_3OH [100 μg/ml]; Green –ETV in CH_3OH in $CHCl_3$[10 μg/ml];
Blue –ETV in CH_3OH in $CHCl_3$[50 μg/ml]; Black –ETV in CH_3OH in $CHCl_3$[100 μg/ml]

ii. *Analyte Solution Stability Studies*

We found ETV solution to be stable for 1 hour after preparation, and carried out solution stability studies on UV-VIS Spectrophotometer, giving a λ_{max} at 257nm for a concentration of 50 μg/ml. So observed a slight, yet gradual decrease in absorbance in Fig. 3, Table 5.

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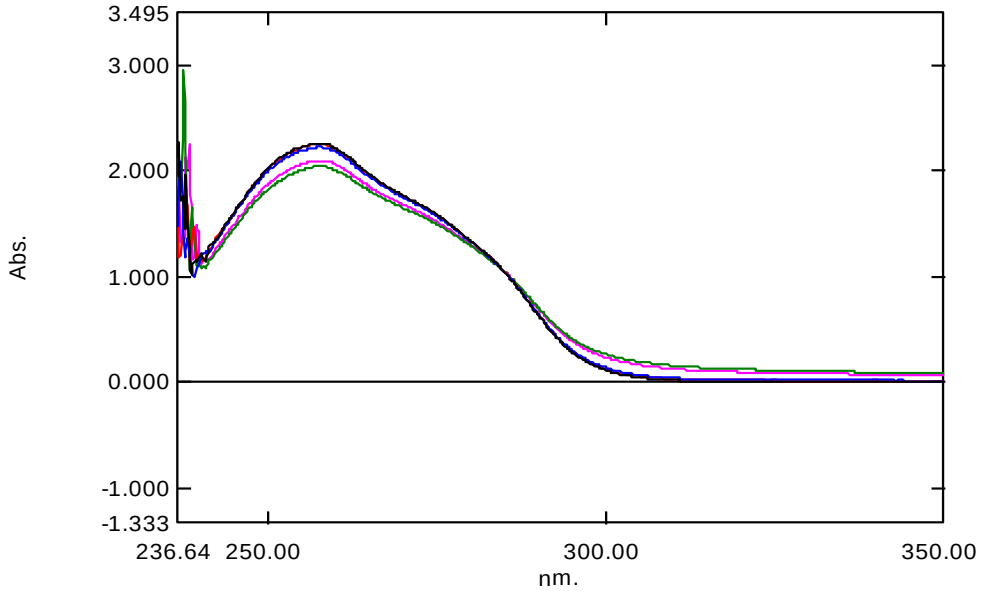


Figure 3: Overlay of analyte stability spectra for ETV [50 μg/ml]

Table 5: Analyte solution stability data for ETV [50 μg/ml]

S.No.	Time Point (hours)	Absorbance (A) at 257nm
1.	0 –Black	2.265
2.	0.5 –Red	2.258
3.	1 –Blue	2.238
4.	3 –Pink	2.102
5.	4 –Green	2.050

We carried out IR analyses using a Shimadzu 8400S FTIR instrument by pressed pellet technique and liquid sample techniques. FTIR method was developed using two sampling techniques: Liquid sampling and the Pressed Pellet Technique.

iii. *Liquid Sampling Technique: (Drug Substance)*

Characteristic functional group peaks were seen in the IR spectra of ETV solution of methanol in chloroform but not in those of methanol or DMSO alone, as shown in Fig. 4, 5 & 6. Also the required increase in functional group absorbance value with an increase in concentration, for quantitation of ETV, wasn't seen. We did not observe any sharp, functional group peaks in the IR spectra taken in DMSO.

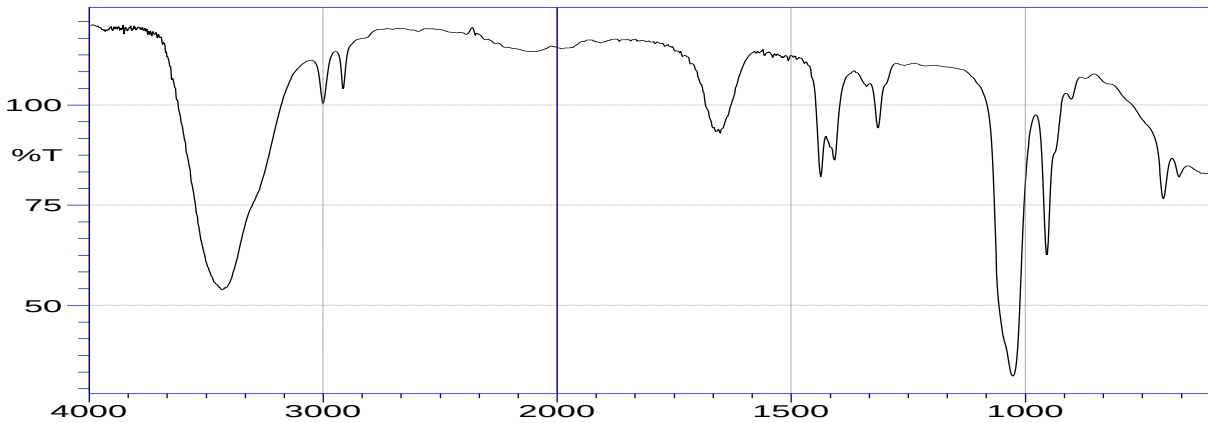


Figure 4: IR spectrum of Entecavir Monohydrate pure in methanol by liquid sampling technique (Transmittance mode)

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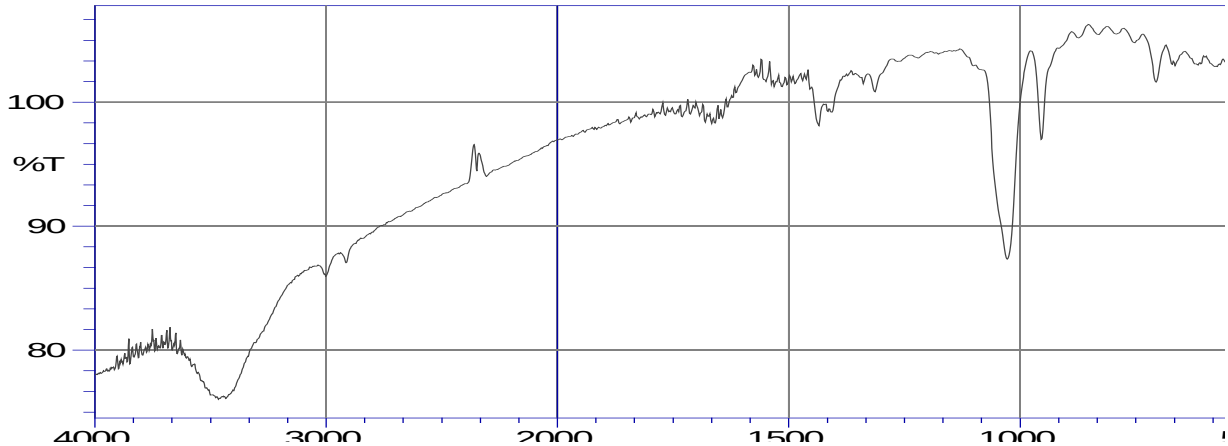


Figure 5: IR spectrum of Entecavir Monohydrate pure in DMSO by liquid sampling technique (Transmittance mode)

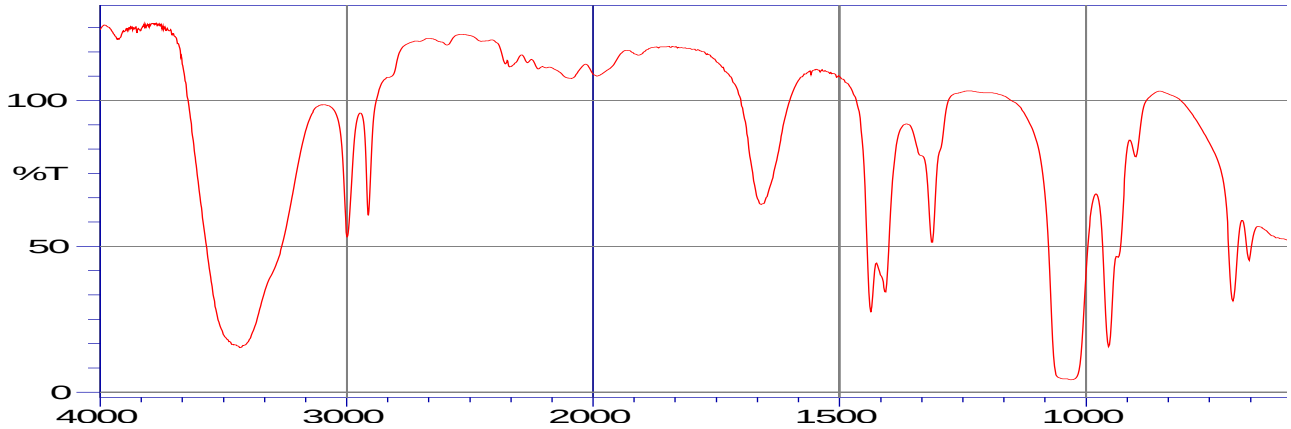


Figure 6: IR spectrum of Entecavir Monohydrate pure in methanol in chloroform by liquid sampling technique (Transmittance mode)

iv. *Pressed Pellet / Solid Pelleting Technique: (Drug Substance)*

We did pelleting by geometric mixing of KBr with ETV. They use KBr press for sampling of solids. The FTIR spectrum of ETV standard exhibited well-defined bands and peak absorbance, which increased proportionally with increasing concentration, as shown in Fig. 7.

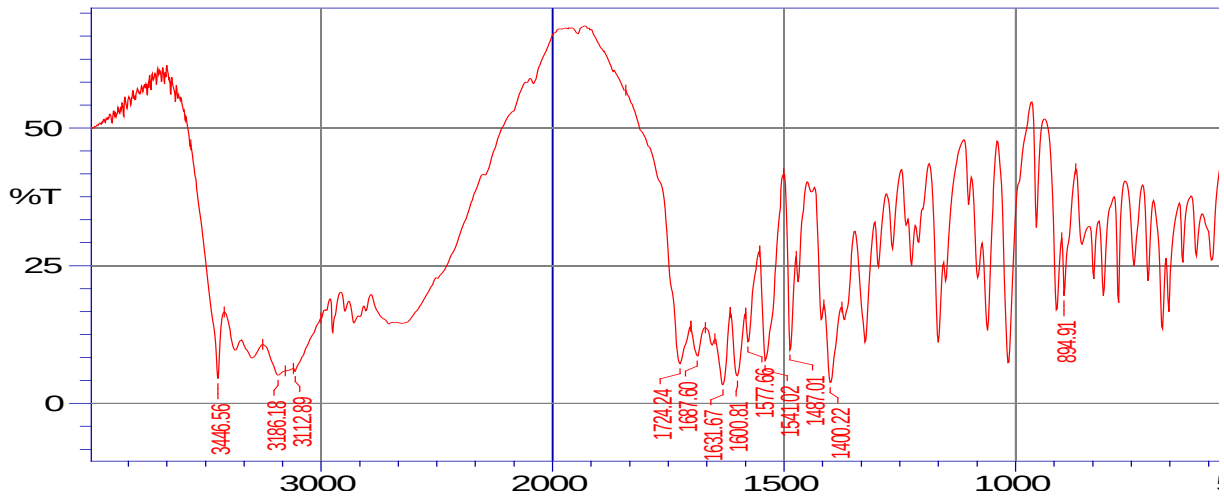


Figure 7: IR spectrum of Entecavir Monohydrate pure in KBr by pressed pellet technique (Transmittance mode)

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Thus, we developed the Derivative FTIR spectroscopic method using a solid pelleting technique on the FTIR spectrophotometer.

v. *Sample Preparation*

Performed various techniques and extraction procedures to achieve a better drug recovery from the tablet powder.

Solid Pelleting Technique (Formulation)

Trial I: Scooping Method

One X-VIR* tablet accurately weighed and finely powdered, was transferred into a vial. We randomly scooped out 10 mg of this powder into an FTIR mortar pestle, and added 100 mg of KBr to make a pellet of concentration 100 µg/mg. Then scanned this pellet, and the IR spectrum obtained for tablet by the scooping method is as in Fig. 8.

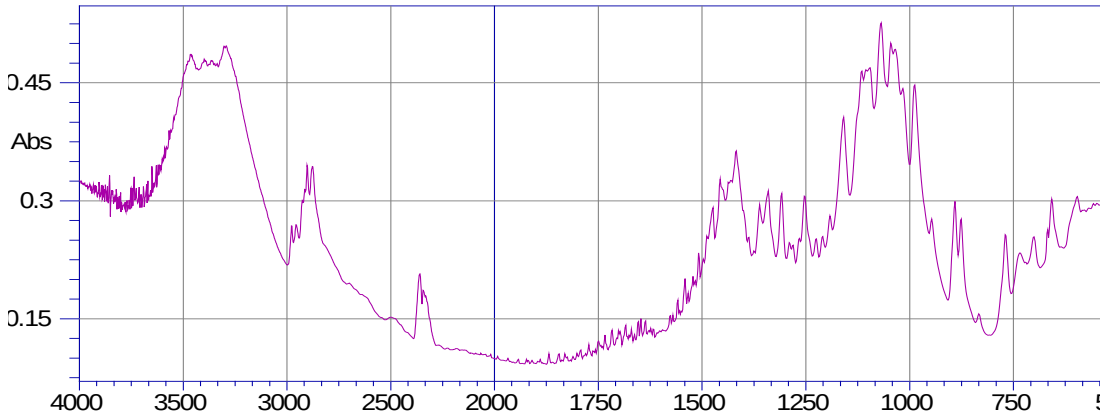


Figure 8: FTIR spectrum of X-VIR* tablet using the scooping method by solid pellet technique (Absorbance mode)

Observation: We did not observe any peaks in the region of 1600-1750cm^{-1}, which indicated the absence of the drug in the scoop taken. Thus, scooping is not a reliable technique for sample preparation from the tablet.

Trail II: Extraction Method [X-VIR Tablet in Methanol]*

One X-VIR* tablet, accurately weighed, powdered finely was transferred into an Eppendorf tube. 1 ml of Methanol was added and centrifuged at 5000 rpm for 10 mins. We obtained a clear, supernatant liquid with a pink layer on top of white precipitate, which was collected in a new Eppendorf tube; kept open overnight for evaporation. The next day, we collected the precipitate in the FTIR mortar, and added 50mg of KBr to make a pellet of concentration 20 µg/mg. This pellet was scanned to obtain IR spectrum as in Fig. 9.

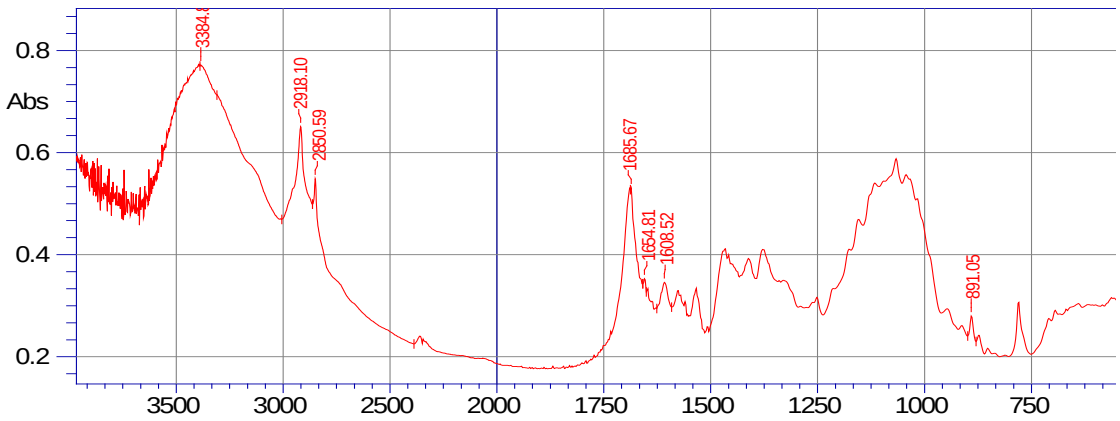


Figure 9: FTIR spectrum of X-VIR* tablet in methanol by extraction method by solid pellet technique (Absorbance mode)

Observation: We observed significant peaks as that of pure ETV. However, all the peaks shifted towards higher wavenumber. We noticed C-O peak at 1685.67 cm^{-1} instead of 1633.59 cm^{-1}. The intensity of the peak in X-VIR* Tablet (0.531) was relative to standard ETV (0.403).

Trail III: Extraction Method [X-VIR Tablet in Methanol in Chloroform]*

The Extraction procedure was the same for all solvents, except for a change in:

1. Solvent and its volume – 0.5 ml of Methanol, 0.5 ml of Chloroform
2. Precipitate observed – distinct pink layer on top of white precipitate

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However, total volume is constant for the extraction procedure. IR spectrum so obtained is as in Fig. 10.

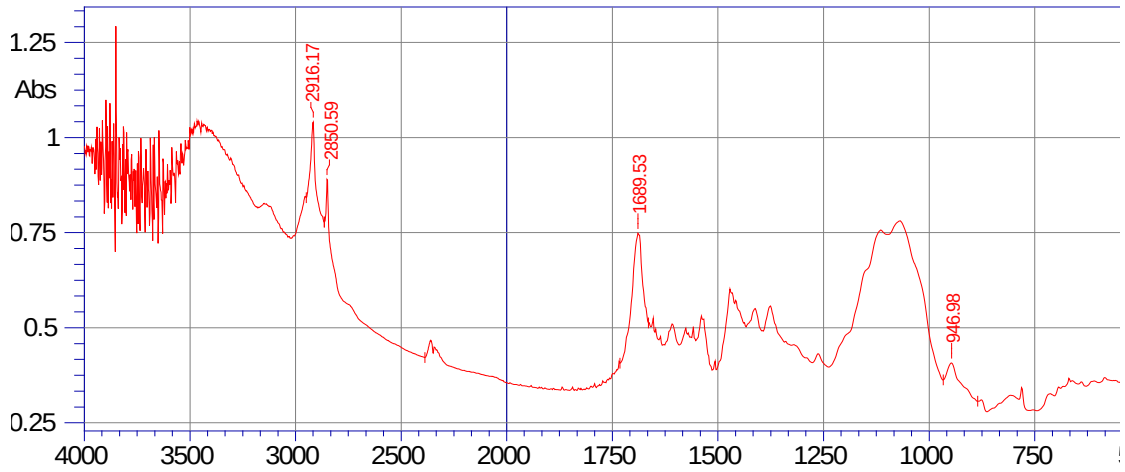


Figure 10: FTIR spectrum of X-VIR* tablet in methanol in chloroform by extraction method by solid pellet technique (Absorbance mode)

Observation: We observed significant peaks as that of pure ETV. However, all the peaks shifted towards higher wavenumber. We noticed C-O peak at 1689.53 cm^{-1} instead of 1633.59 cm^{-1}. The intensity of the peak in X-VIR* Tablet (0.749) was relative to that of standard ETV (0.403).

Trail IV: Extraction Method [X-VIR Tablet in Ethanol]*

The extraction procedure was the same for all solvents, except for a change in:

1. Solvent and its volume – 1.0 ml of Ethanol
2. Precipitate observed – white precipitate

However, total volume is constant for the extraction procedure. IR spectrum so obtained is as in Fig. 11.

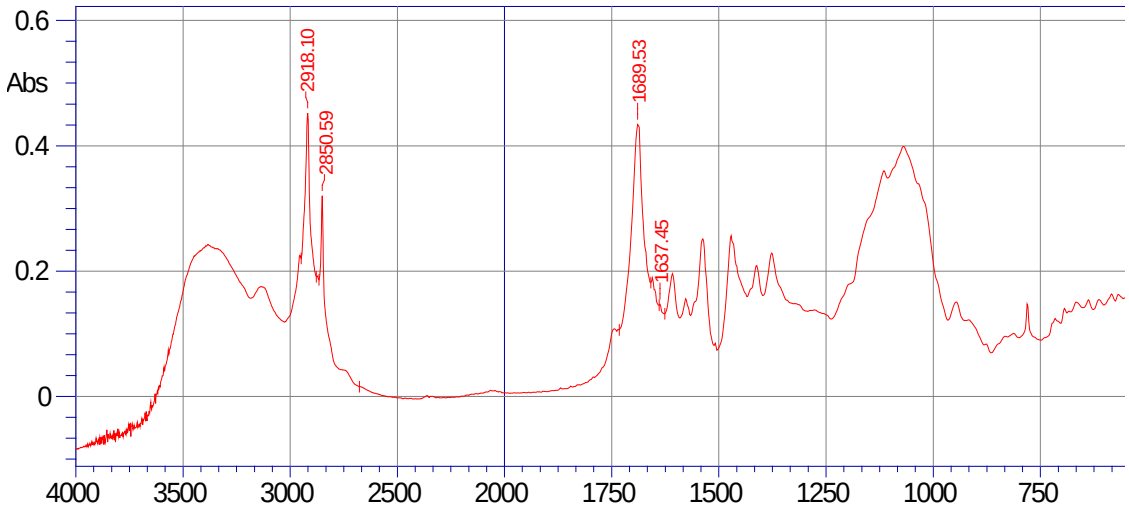


Figure 11: FTIR spectrum of X-VIR* tablet in ethanol by extraction method by solid pellet technique (Absorbance mode)

Observation: We observed significant peaks as that of pure ETV. All the peaks shifted towards higher wave number. We noticed C-O at 1689.53 cm^{-1} instead of 1633.59 cm^{-1}. The intensity of the peak in X-VIR* Tablet (0.434) was relative to standard ETV (0.403).

vi. *Liquid Sampling Technique: (Formulation)*

One tablet was weighed accurately, finely powdered, and extracted using 1 ml of Methanol. We took 1.0 ml of supernatant liquid in a 10 ml volumetric flask, and made up the volume with methanol to make a stock solution of 100µg/ml. It gave high-intensity peaks. The peak at 1708.81cm^{-1} may be due to C=O stretch, as shown in Fig. 12.

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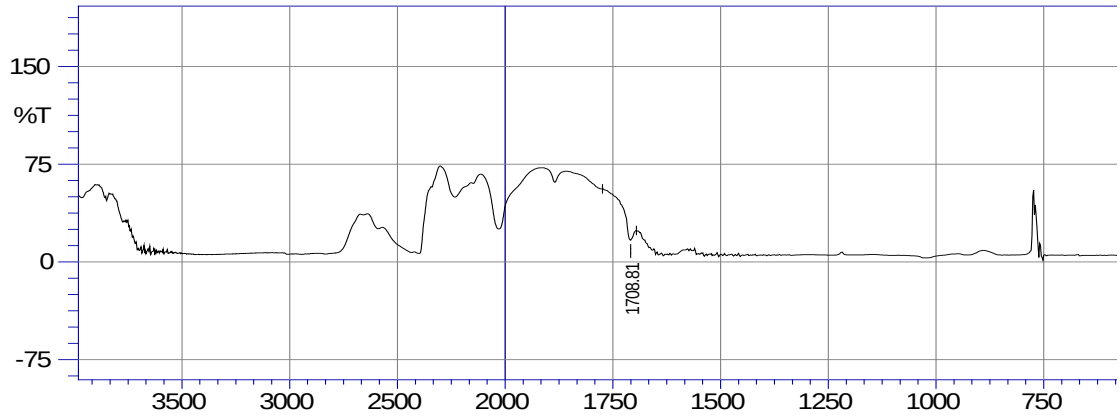


Figure 12: FTIR spectrum of X-VIR* tablet [100 µg/ml] methanol in chloroform by liquid sampling technique (Transmittance mode)

From the stock solution, 0.1, 1.0 and 5.0 ml was taken into different 10 ml volumetric flasks, and the volume was made up with chloroform to make the solutions of concentration 1, 10 and 50µg/ml respectively. Their spectra so obtained are shown in Fig. 13, 14 & 15 correspondingly. Peaks at 1600.81cm^{-1} and 1710.74 cm^{-1} may be due to C=O stretch.

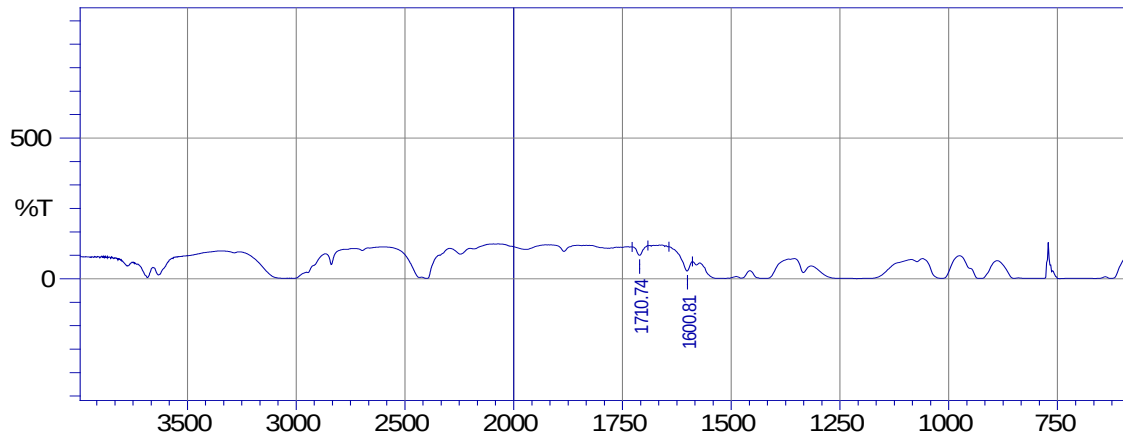


Figure 13: FTIR spectrum of X-VIR* tablet [1 µg/ml] methanol in chloroform by liquid sampling technique (Transmittance mode)

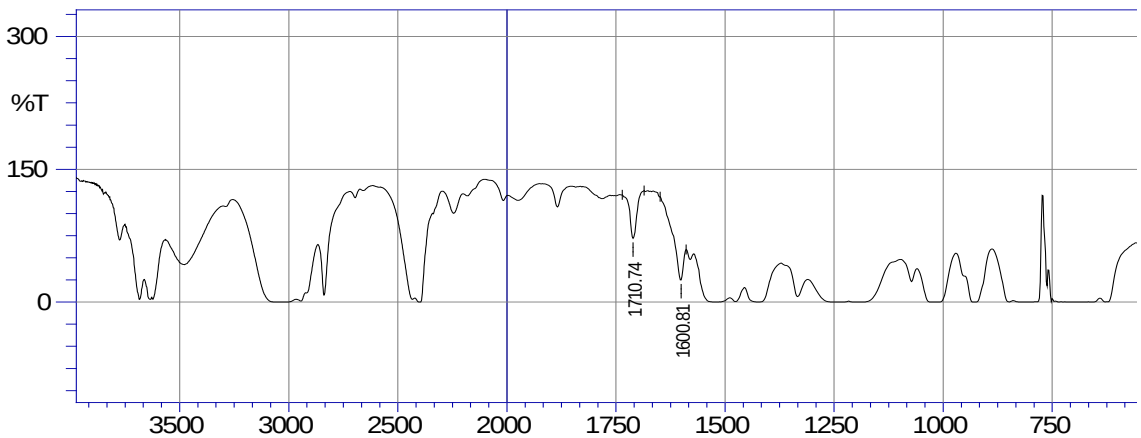


Figure 14: FTIR spectrum of X-VIR* tablet [10 µg/ml] methanol in chloroform by liquid sampling technique (Transmittance mode)

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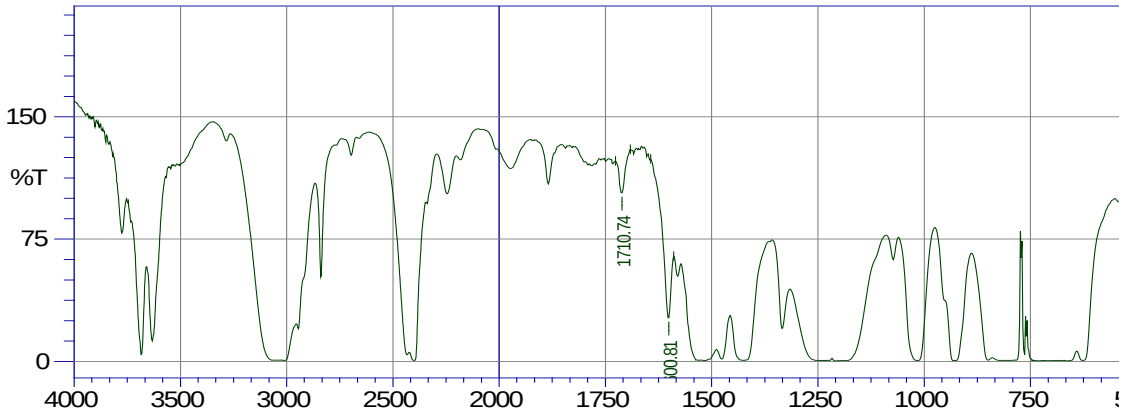


Figure 15: FTIR spectrum of X-VIR* tablet [50 μg/ml] methanol in chloroform by liquid sampling technique (Transmittance mode)

These graphs were studied as obtained for the above solutions in various concentrations. Scans for liquid sampling cell were measured in transmittance mode, to get better results. The graphs were not clear. They exhibited very high transmittance values at most concentrations. Also, functional group shifts were observed, most likely due to the interface from excipients.

vii. *Comparative Study of Sample Preparation (Table 6)*

Table 6: Comparative study between solid pelleting and liquid sampling techniques

S.No.	Parameters	Solid Pelleting Technique	Liquid Sampling Technique
1.	Sample Preparation	Tricky and requires good skill as the quantity is too small	Requires skill, however is comparatively easy
2.	Mode of Measurement	Absorbance Mode	Transmittance Mode
3.	Derivatization	Gives single, almost symmetrical peak	Gives Bifurcated, unsymmetrical peak
4.	Intensity	Within normal range, when compared to standard ETV	Very high intensities, when compared to standard ETV
5.	Sensitivity	Very High	Fairly Acceptable
6.	Selectivity	High, improved peak shape	Low, distorted peaks
7.	Stability	Partial decomposition of pellets	Complete decomposition of solution

The band chosen for quantization should be in a region of the spectrum free from absorption by other possible components of the sample. So we selected the following parameters to get better peaks that can be derivatized to estimate the amount of Entecavir Monohydrate present in the sample taken (Table 7).

Table 7: Optimized conditions for the derivative FTIR method of quantitation

S.No.	Parameter	Optimized Condition
1.	Frequency Range	400-4000 cm^{-1}
2.	Maximum No. of Scans	10 (for better S/N ratio)
3.	Resolution	8 cm^{-1} (for better peak-to-peak separation)
4.	Beer-Lambert's Concentration Range	12.5-200 μg/mg

viii. *IR Spectrum Analysis for Functional Group Assessment*

Entecavir monohydrate IR spectrum showed peaks at 1631cm^{-1}, 3112cm^{-1}, 3186cm^{-1}, and 3446cm^{-1} corresponding to the C–O stretch, primary amine's two N–H stretches and free O–H stretch, respectively. Among these, the C–O group showed a clear and intense peak, which increased linearly as the concentration was increased. Hence, we selected the C–O group for the quantitative evaluation of Entecavir monohydrate.

ix. *Verification of Beer's Law*

We observed a linear and proportional correlation linking the concentration, and absorbance in

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the range of 12.5-200 µg/mg for ETV. They use such relationships for the quantitation of drugs in their pure form and formulations. We took all the absorbance values from the C-O stretch group peak for ETV from the IR spectrum of solid pellets of drug mixtures.

b) Validation of Developed FTIR Method for Quantitative Estimation of Entecavir Monohydrate

We performed the validation for this originated FTIR approach as per ICH Q2 (R1) guidelines, and found all the specifications to be within allowable limits.

i. *Linearity of ETV*

Working standard solutions of ETV were prepared and analyzed in the investigational concentration range of 12.5–200 µg/mg, as shown in Fig 17-21 and Table 9. We recorded the peak area of the second-order derivative of the C=O peak at 1631cm^{-1} for the standard solutions. The standard calibration curve was plotted between concentration and peak area to establish linearity by regression analysis, as shown in Fig. 16, Table 8.

Table 8: Linearity regression analysis data (Second derivative mode)

S.No.	Concentration (µg/mg)	*Peak Area [1639.38-1620.09 cm^{-1}]
1.	12.5	0.0554
2.	25.0	0.0751
3.	50.0	0.1134
4.	100.0	0.1859
5.	200.0	0.3306

**Average of 3 determinations*

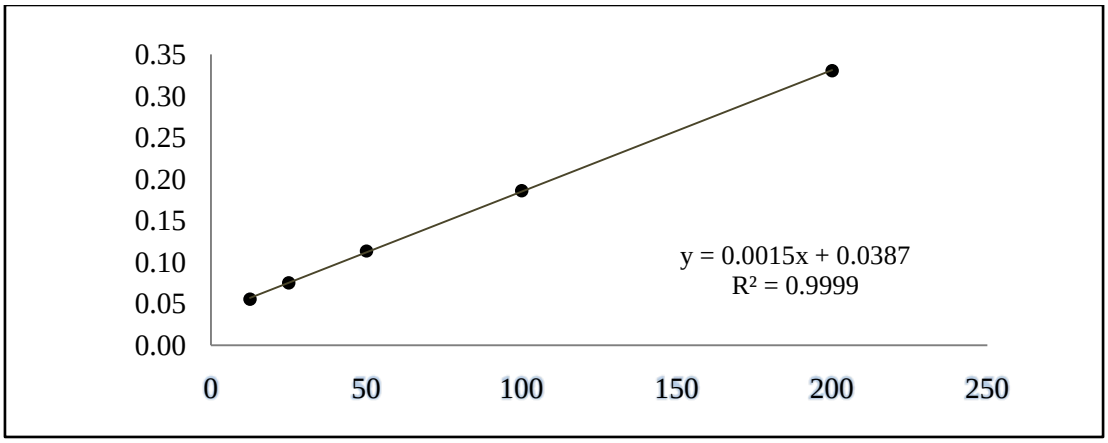


Figure 16: Standard calibration curve of ETV (Second derivative mode)

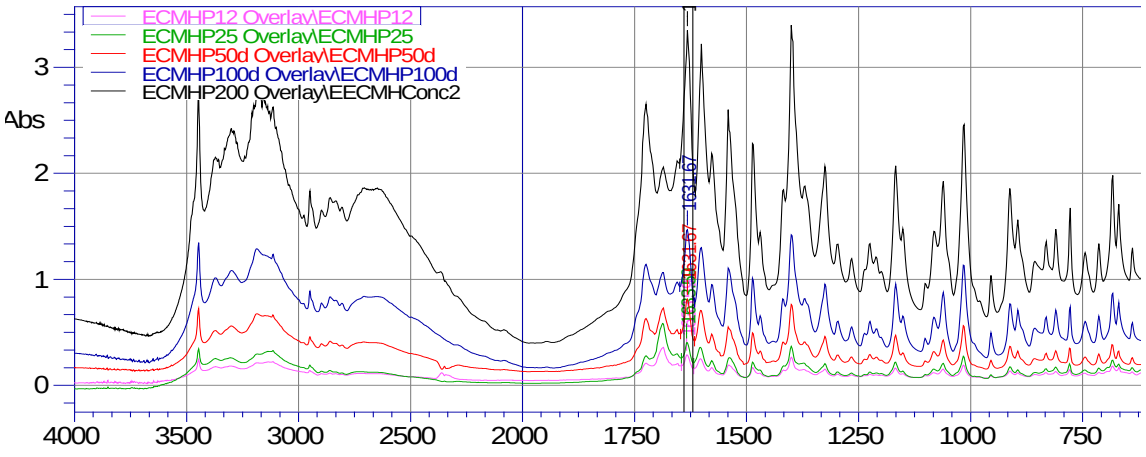


Figure 17: Overlay of linearity spectra for ETV [12.5-200 µg/mg] – Absorbance mode (Full View)

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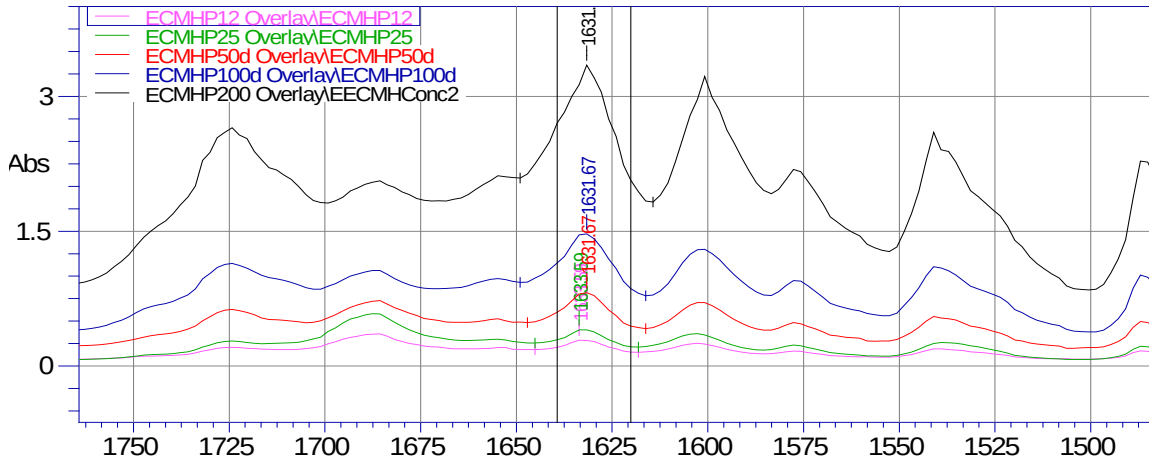


Figure 18: Overlay of linearity spectra for ETV [12.5-200 μg/mg] – Absorbance mode (Zoom View)

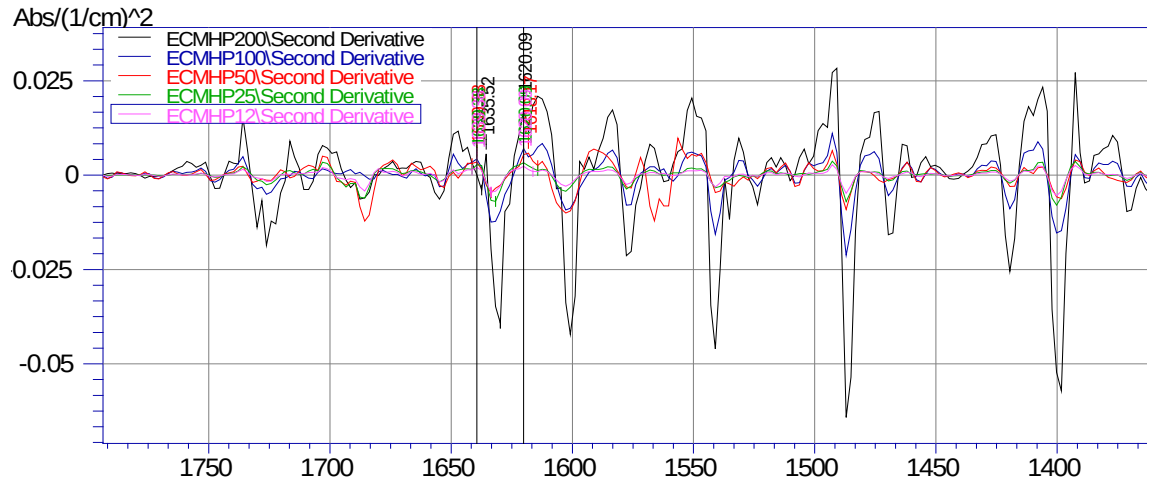


Figure 19: Overlay of linearity spectra for ETV [12.5-200 μg/mg] – Second derivative mode (Zoom Out View)

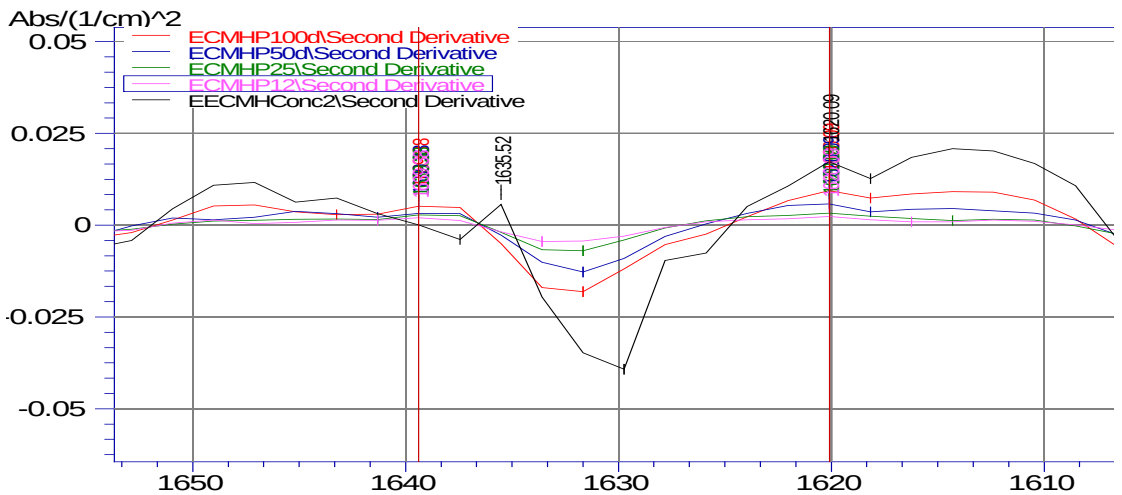


Figure 20: Overlay of linearity spectra for ETV [12.5-200 μg/mg] – Second derivative mode (Zoom In View)

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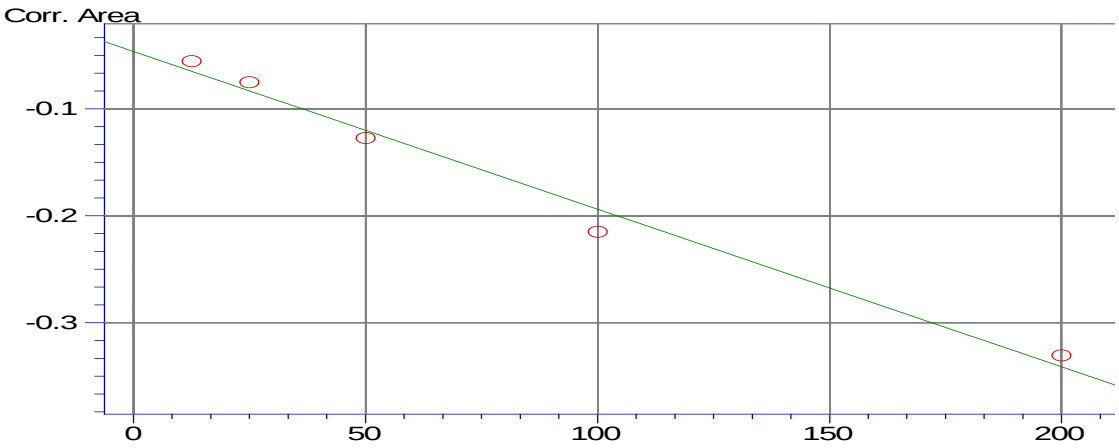


Figure 21: Five-point calibration curve for linearity of ETV from IR Solution software [12.5-200 µg/mg] –Second derivative mode

Table 9: Report of multi-point calibration of ETV for linearity from IR Solution software [12.5-200 µg/mg] –Second derivative mode

Report of Multi Point Calibration	
Calibration of:	E:\Ashraf M.Ph 2017-19\quant.tmp
Evaluation mode:	Peak area
Order:	Linear
Origin:	Ignore
Peak:	from 1639.380 to 1620.090 cm^{-1}
Corrected value:	Yes
Equation:	Corr. Area = -4.642E-2 - 1.474E-3 * c ^ 1, r = 0.992494

We found the response of the drug to be linear in the investigational concentration range 12.5-200 µg/mg by acquiring the regression equation, y = 0.0015x + 0.0387, and coefficient of determination, R^2 = 0.9999 for the second derivative of obtained spectra in absorbance mode. ETV obeyed Beer –Lambert's law in the investigational concentration range.

ii. *Limit of Detection (LOD) and Limit of quantitation (LOQ) of ETV*

We estimated the sensitivity of the proposed method for measurement of ETV for both UV and Derivative FTIR values in terms of LOD & LOQ, which were determined using the standard deviation method. Standard deviation (σ) and slope (s) were calculated from the calibration curve for linearity of each method, respectively, as shown in Table 10.

Table 10: LOD and LOQ Values of ETV

Name of the drug	LOD (µg/mg)	LOQ (µg/mg)
Entecavir Monohydrate	3.29	9.96

We found the LOD and the LOQ values to be 3.29 and 9.96 µg/mg, respectively, which indicates the sensitivity of the method.

iii. *Sandell's Sensitivity*

The Sandell's sensitivity was calculated based on the absorbance value of the lowest concentration, 12.5 µg/mg when scanned several times and derivatized to second order. We noted the absorbance(s) and found the Sandell's sensitivity to be 0.0437 µg/cm^2/0.001 Abs unit.

iv. *Precision*

We reported the precision of the originated analytical technique in terms of repeatability, which was determined by analyzing 6 replicates at 100% concentration [100µg/mg] of ETV to obtain spectra from IR Solution software in second derivative mode. Later,

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we calculated the mean, standard deviation, and %RSD in MS-Excel (Method Precision).

Finally, we calculated the percentage relative standard deviation (%RSD) and found it to be within limits (NLT 2.0% and NMT 10.0%) [32], as shown in Table 11 and Fig. 22. Hence the method is repeatable and precise.

Table 11: Repeatability data of ETV (Method Precision)

S.No.	Concentration (g/mg)	Peak Area	Mean*±Standard Deviation	%RSD
1.	100	0.2296	0.2370 ± 0.0124	5.23
2.	100	0.2242		
3.	100	0.2527		
4.	100	0.2556		
5.	100	0.2323		
6.	100	0.2275		

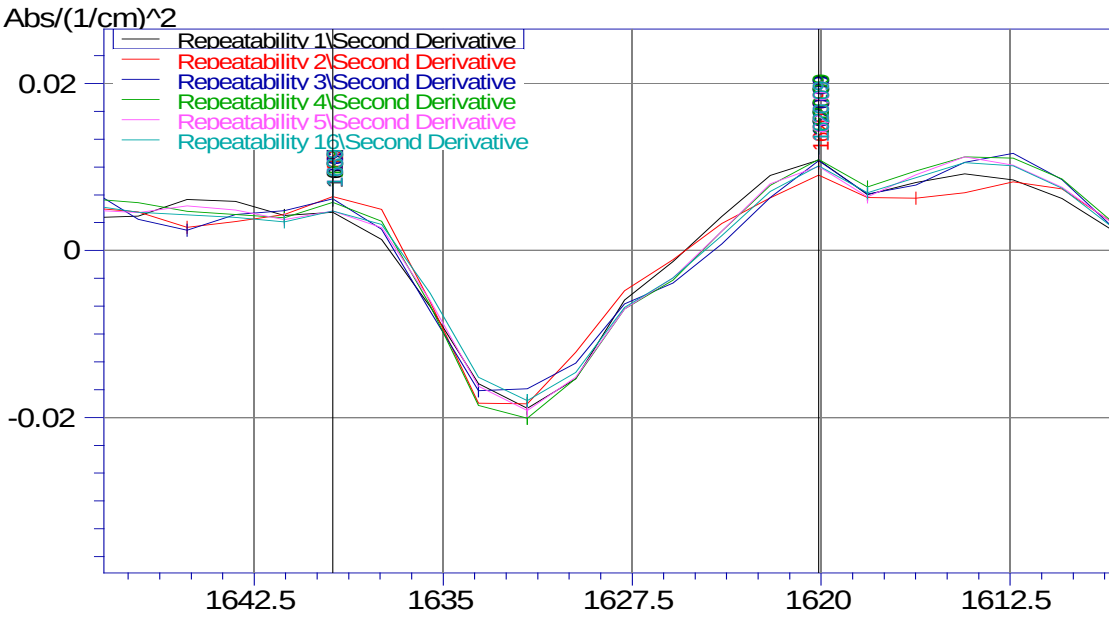


Figure 22: Repeatability curves of six different sample preparations of ETV (Method Precision)

To check system precision, we scanned one sample of ETV at 100% concentration [100 µg/mg] six times, and found the %RSD to be within limits (NMT 2.0%) [32], as shown in Table 12 and Fig. 23. Hence the system is capable of giving precise results.

Table 12: Repeatability data of ETV (System Precision)

S.No.	Concentration (g/mg)	Peak Area	Mean*±Standard Deviation	%RSD
1	100	0.2381	0.2365 ± 0.027	1.16
2	100	0.2389		
3	100	0.2365		
4	100	0.2317		
5	100	0.2342		
6	100	0.2394		

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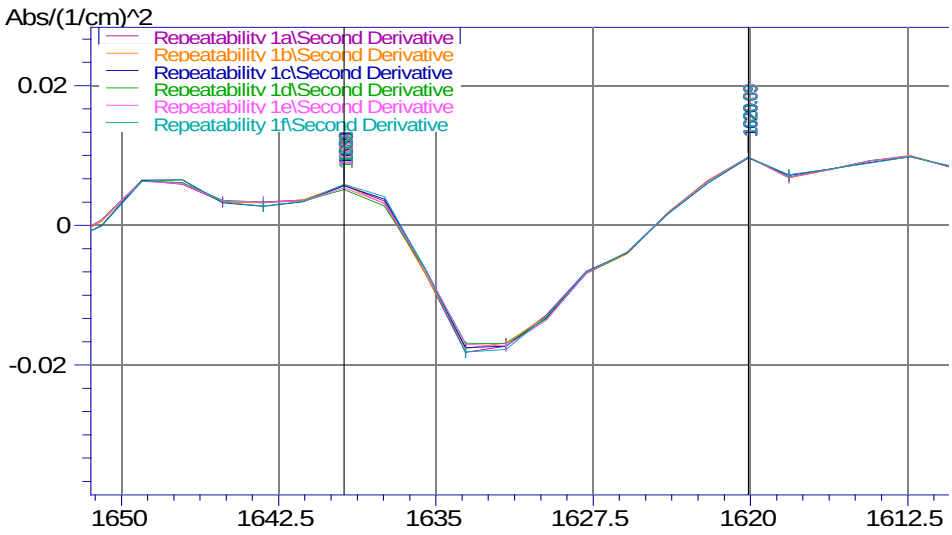


Figure 23: Repeatability curves of single sample preparation of ETV (System Precision)

v. *Accuracy*

We carried out an accuracy study by calculating the percent recovery of ETV by the standard addition method. Known amounts of standard ETV (40, 50, and 60µg/mg) were added to a pre-quantified test mixture of X-VIR* tablet extract (50 µg/mg). The percent recovery was calculated by measuring the peak area, and fitting these values into the regression equation of the calibration curve. Concentrations recovered are tabulated in Table 13.

Table 13: Recovery data for Entecavir Monohydrate drug product (X-VIR* tablets)

S.No.	Spike Level (%)	Concentration of pure ETV added (g/mg)	Concentration of X-VIR tablet extract added (g/mg)	Total Concentration (g/mg)	Peak Area*	Concentration Recovered (%)
1.	80	40	50	90	0.0975	100.89
2.	100	50	50	100	0.1334	101.40
3.	120	60	50	110	0.1261	99.88

**Average of 3 determinations*

Overlay spectra of the three recovery curves of Entecavir Monohydrate recovered from the marketed formulation of X-VIR* tablets at the spike levels of 80-120% in absorbance and second derivative modes are as in Fig. 24 & 25 respectively.

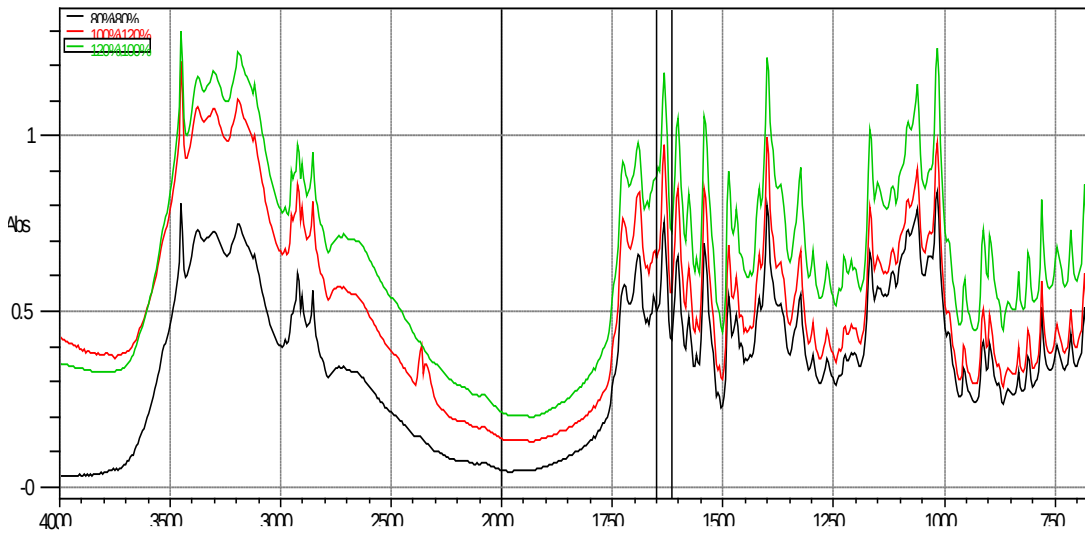


Figure 24: Recovery curves for Entecavir Monohydrate from X-VIR* tablets in Absorbance mode (80-120%)

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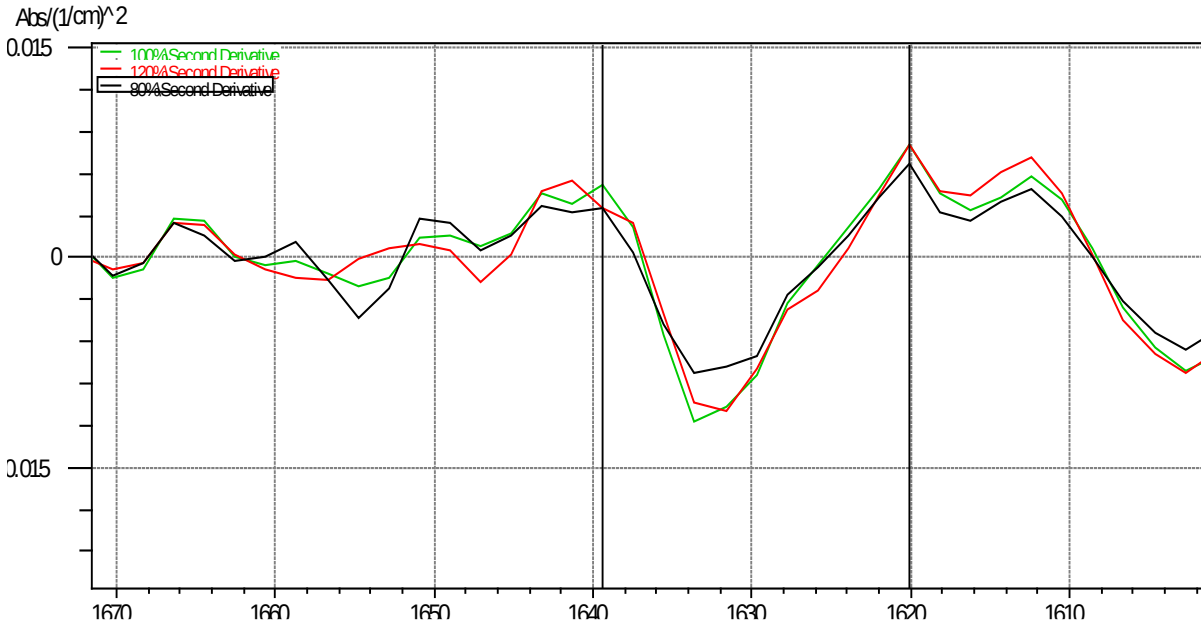


Figure 25: Recovery curves for Entecavir Monohydrate drug product (X-VIR* tablets) in Second derivative mode

We found the method to be accurate for the determination of Entecavir monohydrate in tablets as the percentage recovery values calculated were found to be within the acceptable limits (100±2%) [32].

vi. *Assay*

Assay means to provide an exact result that allows an accurate statement on the content or potency of the analyte in a sample. –ICH Q2(R1). The peak area value of the specimen scanned in absorbance mode (Fig. 26) and derivatized to second-order (Fig. 27) was substituted into the regression equation of the calibration curve to obtain its concentration, which we used ultimately to calculate its purity as shown in Table 14.

Table 14: Assay results of marketed tablets

S.No.	Brand Name	Chemical Name	% Purity*
1.	X-VIR Tablets	Entecavir Monohydrate	99.75

*Average of 3 determinations

USP drug content limits for commercially available tablets is 98-102% [33].

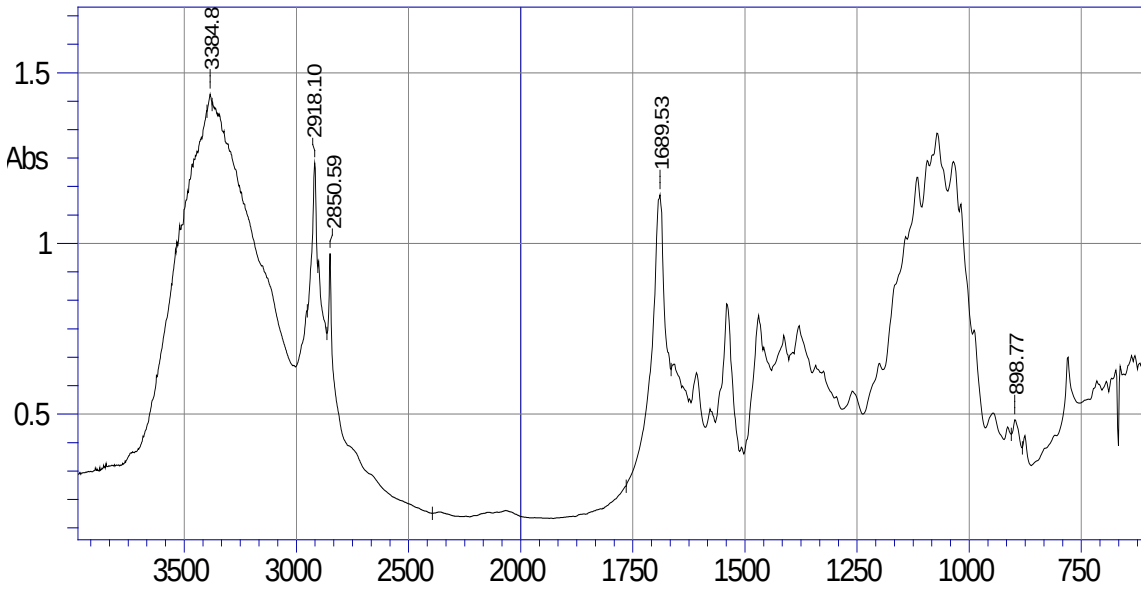


Figure 26: FTIR spectrum for assay of marketed tablets (Absorbance mode)

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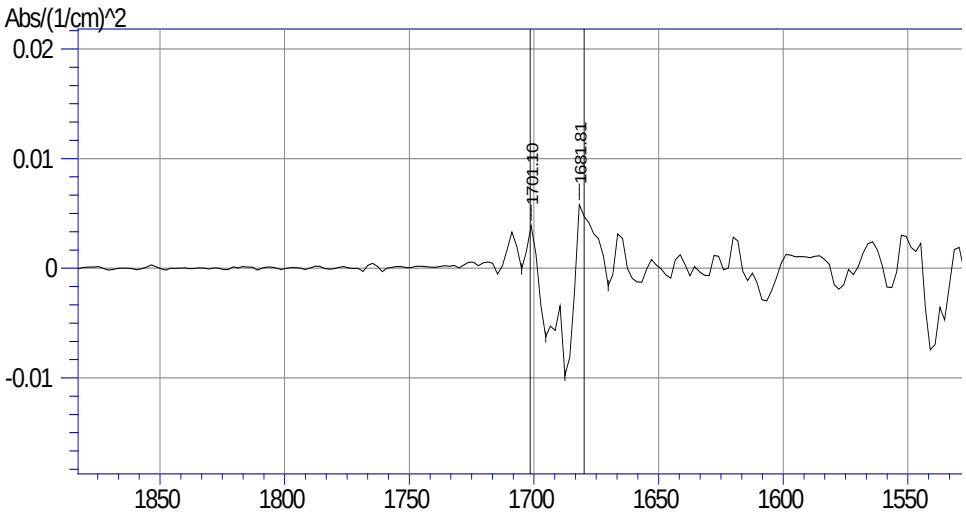


Figure 27: FTIR spectrum for assay of marketed tablets (Second derivative mode)

The shift in the absorbance value of the C=O peak from 1631.67 cm^{-1} to 1689.53 cm^{-1} is due to the interference of excipients in the marketed formulation [34].

IV. Comparitive Analysis

To ensure this developed technique is appropriate and superior to existing analytical methods, we performed a few validation parameters on previously developed and published UV and HPLC methods from various journals [9-31] and Indian pharmacopeia [35,36]. The results so obtained were compared with the current derivative FTIR method to prove this new technique is equally good.

a) Linearity of ETV on UV-VIS Spectrophotometer

The linearity was established on UV-VIS Spectrophotometer by performing linear regression analysis for the calibration curve constructed between concentration and absorbance.

Table 15: Linearity regression analysis data for ETV [15-50 μg/ml]

S.No.	Concentration (μg/ml)	Absorbance *(A) at 257 nm
1.	15	1.904
2.	20	2.050
3.	25	2.102
4.	30	2.238
5.	35	2.338
6.	40	2.471
7.	45	2.605
8.	50	2.730

**Average of 3 determinations*

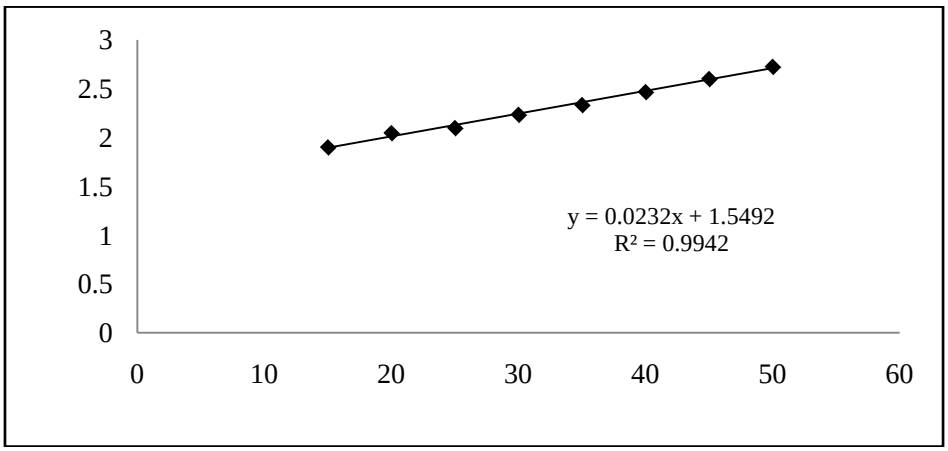


Figure 28: Standard calibration curve of ETV [15-50 μg/ml]

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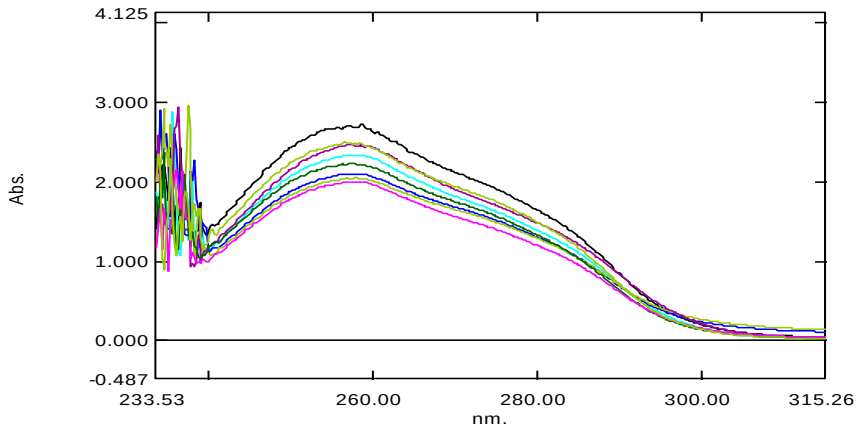


Figure 29: Overlay of linearity spectra for ETV [15-50 μg/ml]

The investigational concentration ranges of 15-50 μg/ml (Fig. 29) were found to be linear and obeying Beer –Lambert's Law, as shown in Table 15 and Fig. 28. We found the regression equation to be y = 0.0232x + 1.5492 with correlation coefficient, R^2 = 0.9942.

b) UV–VIS Spectroscopy v/s Second Derivative FTIR Spectroscopy (Table 16)

Table 16: Comparison between UV and FTIR spectroscopies

S.No.	Parameters	UV –VIS Spectroscopy	Second Derivate FTIR Spectroscopy
1.	Concentration Range	15-50 μg/ml	12.5-200 μg/mg
2.	Regression Equation (y = mx + c)	y = 0.0232x + 1.5492	y = 0.0015x + 0.0387
3.	Coefficient of Determination (R^2)	0.9942	0.9999
4.	Standard Deviation (STDEV)	0.285555	0.111541
5.	Standard Error between Y and X (STEYX)	0.023520	0.001457
6.	Slope (*s*)	0.023248	0.001463
7.	Limit of Detection (LOD)	3.39 μg/ml	3.29 μg/mg
8.	Limit of Quantitation (LOQ)	10.12 μg/ml	9.96 μg/mg

c) Assay of ETV on RP-HPLC

We further performed the assay of Entecavir Monohydrate on RP-HPLC using water: methanol as mobile phase. The optimized conditions are as in Table 17.

Table 17: Optimized conditions of RP-HPLC

S.No.	Parameters	Conditions
1.	Column	Enable-18H C-18 column
2.	Column Dimensions	250mm × 4.6mm, 5μm
3.	Mobile Phase	Water:Methanol (80:20)
4.	Flow Rate	1.2 ml/min
5.	Injection Volume	20 μL
6.	Wavelength	254 nm
7.	Runtime	15 minutes

We dissolved the pure drug of ETV and the residue obtained from extracted X-VIR* tablet in methanol (1000 μg/ml) and spiked it in 10 ml chloroform to obtain the standard stock solutions of 100 μg/ml each, respectively.

Then we injected these solutions into the RP-HPLC, and the overlay chromatogram so obtained is shown in Fig. 30, where Data 1 represents chromatogram of Standard pure ETV solution, and Data 2 represents the chromatogram of X-VIR* tablet solution.

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Table 18: Assay results of marketed X-VIR* tablets on HPLC

S.No.	Chemical Name	Label Claim (mg)	Amount found (mg)	% Purity
1.	Entecavir Monohydrate	1 mg	0.9006	90.06
2.	Entecavir Monohydrate	1 mg	0.8543	85.43
3.	Entecavir Monohydrate	1 mg	0.9462	94.62
Mean			0.9004	90.04

**Average of 3 determinations*

We found the mean value of % purity for the second derivative FTIR method to be 99.75% and that of RP-HPLC to be 90.04% from Table 18.

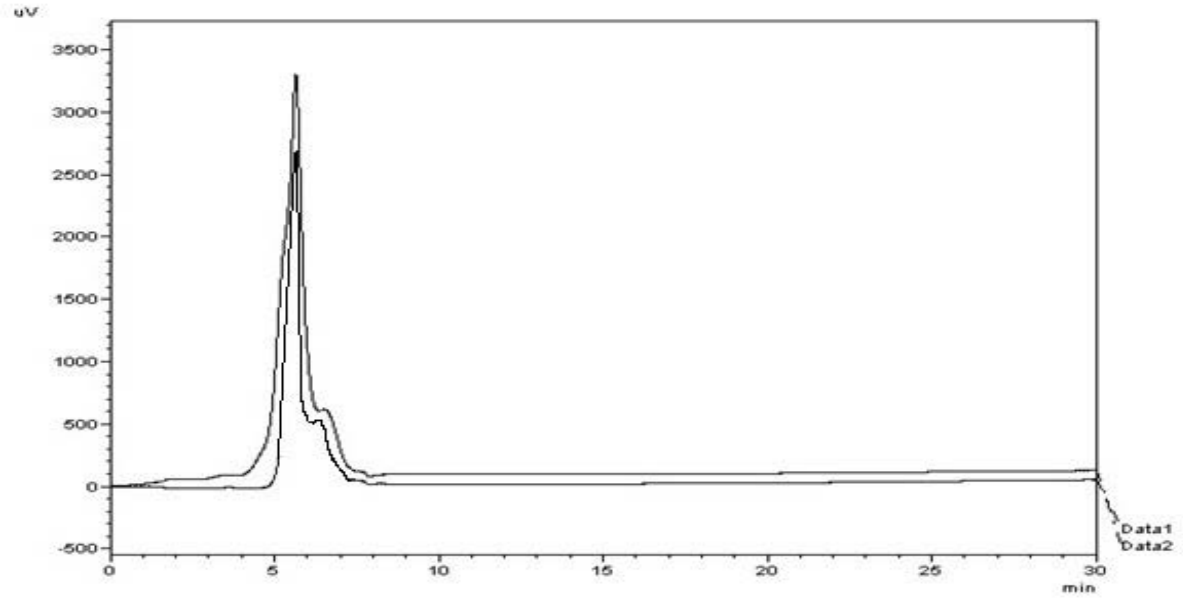


Figure 30: Overlay of chromatograms of standard ETV (Data 2) and X-VIR* tablet formulation (Data1)

d) Statistical Analysis for Second Derivative FTIR v/s RP-HPLC

Student t-test

We calculated the assay result of Entecavir monohydrate by both methods. Statistical analysis of the outcomes of the two techniques showed a significant difference between the techniques at a significance level (α) of 5% ($t_{calculated} > t_{critical}$). Furthermore, the amount of Entecavir monohydrate calculated by both procedures was within the range between 90 – 110%.

Table 19: Statistical data for *t*-test of percentage purity of ETV

Method	Mean of percentage purity	Standard deviation of individual data	Size of sample
Second Derivative FTIR	$\bar{x}_1 = 99.75$	$s_1^2 = 0.808$	$n_1 = 3$
RP-HPLC	$\bar{x}_2 = 90.04$	$s_2^2 = 4.595$	$n_2 = 3$

Hypothesis: The two analytical methods, to determine the percentage purity of Entecavir monohydrate, are not significantly different.

$$H_0 : \mu = \mu_0$$

Against $$H_1 : \mu \neq \mu_0$$

Since variances of the population were not known and size of the samples was small, *t*-test for difference in means was adopted assuming the populations to be normal and we worked out the test statistic *t* under the given formula:

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$$t = \frac{\overline{x_1} - \bar{x}_2}{\sqrt{\frac{(n_1-1)s_1^2 + (n_2-1)s_2^2}{(n_1+n_2-2)}} \times \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

P-value (Probability of rejection) = 0.05 (two-tailed)

$$t_{calculated} = 3.453$$

$$t_{critical}\,(0.05) = 2.776$$

Degrees of freedom (df) = $n_1 + n_2 - 2$; (3+3−2) = 4

As our hypothesis was two-sided, we applied a two-tailed test for determining the rejection regions at 5 percent level which came to as under, using the table of *t*-distribution for 4 degrees of freedom:

$$R: \mid t \mid > 2.776$$

The observed value of *t* was 3.453 ($t_{calculated} > t_{critical}$), which falls in the region of rejection of our hypothesis. So we reject our hypothesis of both methods not being significantly different and conclude that the two ways to determine the percentage purity of Entecavir monohydrate differ significantly.

V. Conclusion

The developed method for estimation of Entecavir monohydrate is based on the application of FTIR with derivative assistance by using the solid pellet technique, which was compared statistically with the pharmacopoeial method (HPLC), and the results revealed that the developed new technique was significantly different. Hence it proves good applicability. It fulfilled all validation requirements in a range of concentrations, and they can use this technique as an alternative to the official methods.

It is suitable for quality control of both pure and marketed solid dosage form, and similar methods can be developed for other categories of drugs for their estimation in the formulations.

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We thank Prof. Dr. B. Madhava Reddy, Principal, for his encouragement to involve in practical approaches and to allow us to carry out this research work. His endless support and constructive suggestions have been precious during the entire course of work.

We are also immensely grateful to the Almighty God for giving us the intellect, strength, determination and power to succeed no matter the challenges we had to face to make this research a success.

Any errors, if encountered in the future, are our own and should not tarnish the reputations of any of the esteemed persons whose work we took as reference for this research.

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Proniosomal Gel: Formulation and Charecterization of an Antifungal Drug (Butenafine Hcl) Loaded Proniosomes for Topical Delivery

By Thimmaraju D. R & Dr. Abdul Nasir Kurnool

Adichunchanagiri University

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Keywords: *butenafine hydrochloride, fungal infection, proniosomes, slurry method, in-vitro diffusion studies, antifungal activity.*

GJMR-B Classification: *NLMC Code: QV 252*



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Keywords: butenafine hydrochloride, fungal infection, proniosomes, slurry method, in-vitro diffusion studies, antifungal activity.

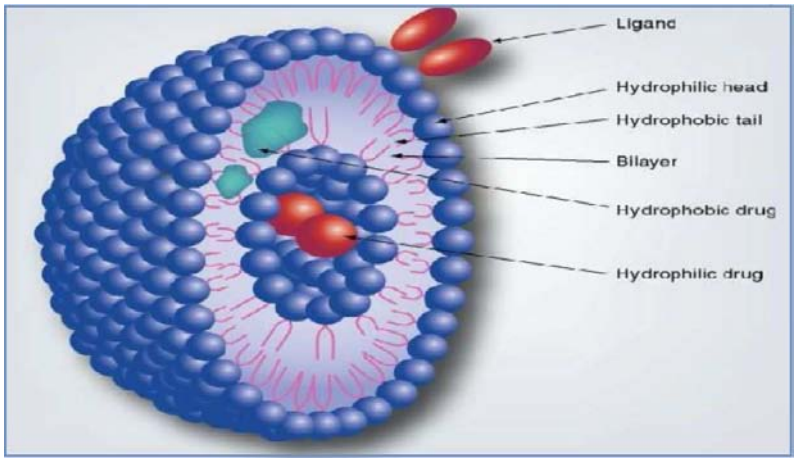
I. Introduction

The main target of drug therapy is to provide therapeutic amount of drug concentration to proper site in the body to produce desired therapeutic efficacy. Now a days in the field of pharmaceutical industries are put great efforts towards the prefabrications of existing drugs and their delivery systems, to break the problem related to poor solubility, stability, toxicity, bioavailability, dosage problem etc.[1]Topical administration is a preferable route for direct, local therapy, in that it is Non-invasive and is directly applied to the invading site and reduces systemic adverse effects. However, it is not possible to use all types of drugs (antifungal drugs) through transdermalroute.[2] The drawbacks of topical antifungal formulation such as cream, lotions, spray etc. may include difficult to apply in deep dermatophytic infections, inadequate amount leads poor response, inability to apply difficult to reach area such as natal cleft, low effectiveness, redness of skin, stinging and burning sensations as side effects.[3] Various attempts have been made to improve the skin permeation of drugs like use of permeation enhancers, electroporation, microneedles, needleless injection, thermophoresis, etc. But these methods damaging the protective barrier function of the skin and may cause irritation or other skin problems.[4] Hence, to overcome the problems associated with topical antifungals formulations, this study is intended to formulate novel drug delivery system such as Proniosomal gel for topical administration with model anti-fungal drug, in order to enhance skin permeation as well as to sustain the drug release for prolonged period of time.[5] There are many novel drug delivery systemshave been investigated by pharmaceutical scientists to fulfil these criteria and considerations for topical delivery of drugs. In that nanocarrierssuch asProniosomescan make their way easily to hair follicles and they may show accumulation between corneocytes, skin having high lipid content so it can easily combining with lipidic layer, Proniosomes also have the capability to control/sustain the drug release, which reduces the side effects and dosing frequency of drugs.[6]

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a) *Structure of Proniosomes*



Proniosomes are dry, free-flowing solid colloidal carrier particles that are coated with surfactants and can be converted to niosomal dispersion by hydrating instantly before use on agitation in hot aqueous media within few minutes. Proniosomes can entrap both hydrophilic and lipophilic drugs (figure-1). Proniosomal gels are usually present in white semisolid gel texture or transparent and translucent. Proniosomal gels are physically stable throughout storage and transport.[7]

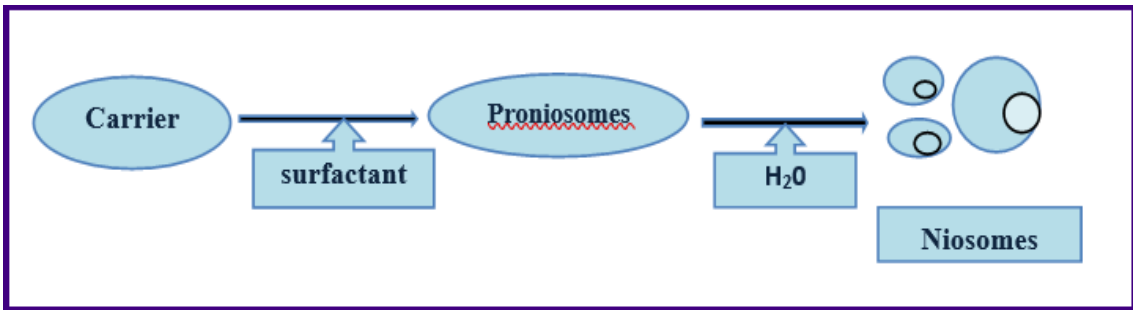


Figure 1: Action of proniosomes

b) *Advantages of Proniosomes over the other vesicular systems:*

- ✓ Proniosomes reduces the physical stability problems of niosomes such as fusion, aggregation, leaking on storage.
- ✓ It also controls hydrolysis of encapsulated drugs which limiting the shelf life of the dispersion.
- ✓ The entrapment of both hydrophilic and hydrophobic drugs can be done easily by this vesicular system.[8]
- ✓ Sustained and controlled release of drugs can be done due to depot formation.
- ✓ More stable and Ease of use.
- ✓ It leads to ease the transportation, better size distribution and storage uniformity of dose.
- ✓ These formulations are biodegradable, biocompatible and non-immunogenic to the body.[9]

Butenafine hydrochloride is a synthetic benzylamine antifungal agent. It is indicated for the topical treatment of the following dermatologic infections: Interdigital Tinea pedis (athlete's foot), Tinea corporis (ringworm) and Tinea cruris (jock itch) due to E. floccosum, Tinea mentagrophytes, T. rubrum, and T. tonsurans, tinea (pityriasis) versicolor due to M. furfur.[10] Butenafine Hcl is a synthetic antifungal agent that is structurally and pharmacologically related to allylamine antifungals. The exact mechanism of action has not been established, but it is suggested that butenafine's antifungal activity is exerted through the alteration of cellular membranes, which results in increased membrane permeability, and growth inhibition. Butenafine is mainly active against dermatophytes and has superior fungicidal activity against this group of fungi when compared to that of terbinafine, naftifine, tolnaftate, clotrimazole, and bifonazole. It is also active against Candida albicans and this activity is superior to that of terbinafine and naftifine. Butenafine also generates low MICs for Cryptococcus neoformans and Aspergillus fumigatus as well.[11]

The aim of the current study was to develop a topical formulation which would be effective against transdermal fungal infection as well as overcome the drawbacks of current topical/ oral therapy. The butenafine hcl loaded proniosomal gel formulation is

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prepared by slurry method with some modifications. The formulations are evaluated for its vesicle size, entrapment efficiency (EE), viscosity, spreadability, skin permeation, and stability.

II. Materials and Methods

Materials and sources: Butenafine hydrochloride were supplied as a gift sample by Glenmark Pharmaceuticals Ltd. Mumbai. Cholesterol and soya lecithin were purchased Yarrow chem products, Mumbai, India. Span-60, Tween-60 were purchased from SD fine Chem. Ltd. Mumbai, India, Chloroform and Ethanol Merck Specialties PVT. LTD. All other chemicals and reagents used were of analytical grade and were used without further purification.

Methods:

Characterization of drug and other excipients: Characterisation was done by Fourier transfer infrared spectroscopy (FT-IR) (Make: Brukers alpha t- series, Software: Opus) of butenafine hydrochloride and other was performed.The drug and excipients were kept in 1:1 ratio at 5°C in refrigerator to observe any reaction which may take place between drug and excipients. The mixtures of samples were analysed by FT-IR after one month along with standard drug and excipients as the reference.

Procedure for preparation of proniosomes

The current proniosomes were prepared by adopting slurry method using maltodextrin as carrier. The composition of different proniosome formulation were prepared by using various non-ionic surfactants and cholesterol in different molar ratios and drug is represented in Table-01. The solvent such as chloroform and ethanol (2:1) were used to dissolve ingredients. The physical mixtures and solvent were mixed thoroughly in the beaker using sonicator/ glass rod. Then, an accurately weighed amount of maltodextrin was added slowly to above resultant solution with continuous stirring to obtain slurry, otherwise it forms clump mass because maltodextrin was water soluble. Additional quantity of solvent was added to form slurry, in case of lower surfactant loading. The obtained solution was immediately transferred to round bottomed flask and attached to rotary flask evaporator to evaporate solvents at temperature 45± 2°C, 60-70 RPM and reduced pressure 600-700mm of Hg respectively. After complete removal of solvent from the flask, thin layer of proniosomes was obtained. Then, further dried overnight in a desiccator (containing $cacl_2$/ silica) under vacuum at room temperature to get dry, free-flowing proniosomal powder. The obtained proniosomes was stored in a tightly closed container at 4°C until further evaluation. The composition of different proniosomal formulations were represented in Table-01.

Proniosomes were transformed into niosomes by hydrating using phosphate buffer (pH 6.8), the niosomes were sonicated twice for 2 min. usinga sonicator. These niosomes were used in the formulations of gels.[12]

Preparation of butenafine hydrochloride proniosomal gel

Butenafine hydrochloride proniosomal gel was prepared by using 1% w/w of Carbopol-940 as a gel base. Carbopol-940 was soaked overnight in 100ml distilled water then mixed with niosomal suspension and required quantity of preservatives (methyl/ propyl paraben) were added.Triethanolamine was added drop wise to the formulation for an adjustment of required skin (pH5.8-6.0) and also to obtain gel at required consistency. The prepared butenafine hcl. proniosomal gel was stored in the refrigerator until further evaluation studies.[13]

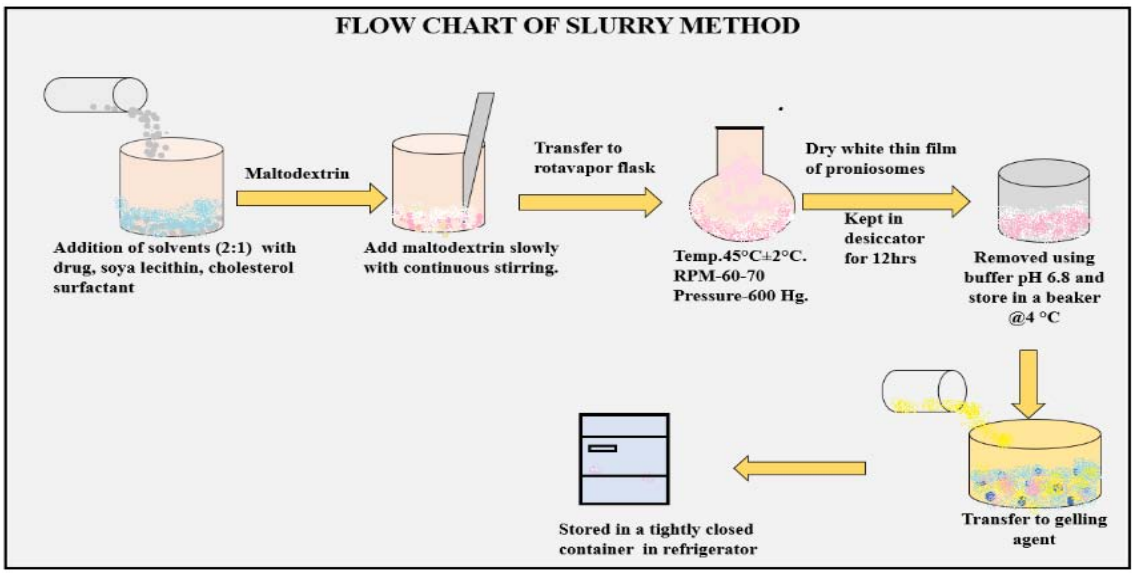


Figure 2: Schematic representation of preparation of proniosomes

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Table 1: Different proniosomal formulation chart

Excipients → / Formulation code ↓	Drug Butenafine Hcl (mg)	Soya lecithin (mg)	Maltodextrin (mg)	*Surfactants (mg)			*Cholesterol (mg)
				Span 40	Span 60	tween60	
PF1	20	100	500	100	-	-	100
PF2	20	100	500	200	-	-	100
PF3	20	100	500	100	-	-	200
PF4	20	100	500	-	100	-	100
PF5	20	100	500	-	200	-	100
PF6	20	100	500	-	100	-	200
PF7	20	100	500	-	-	100	100
PF8	20	100	500	-	-	200	100
PF9	20	100	500	-	-	100	200

*Surfactant: cholesterol-(1:1), (2:1), (1:2) ratios respectively.
Solvents: - Chloroform: Ethanol (2:1)- PF1-PF9

III. Evaluation Studies of Proniosomal Gel

a) Characterization of Proniosomes

i. *Photomicrography*

The photomicrography was carried out using optical microscope. A dry Proniosomes were hydrated using saline buffer pH 6.8, then drop of suspension were mounted on glass slide and observed under the microscope with magnification of 40X for morphological observation. The photomicrograph of the preparation obtained from the optical microscope by using a digital SLR camera.

ii. *Morphology study by Scanning electronic microscopy (SEM)*

The sample of proniosomes is placed in an evacuated chamber and scanned in a controlled manner by an electronic beam. The dried proniosomes were mounted on to stubs by using double-sided adhesive carbon tape. Then proniosomes were analyzed after gold sputtering to yield a gold film of 30 nm thickness. In the SEM (Joel, JSM-5600LV, japan), interaction between electronic beam and vesicles produces a variation in the physical phenomenon that can be obtained in the form of images. These obtained images usedfor surface characteristics.[14]

iii. *Particle size analysis*

Particle size analysis was carried out using optical microscope. The optical microscope was fitted with a stage micrometer to calibrate the eyepiece micrometer. A dry Proniosomes were hydrated usings a line buffer pH6.8 or NaCl then drop of suspension were transferred onto a clean glass slide and observed under the microscope. Before placing the cover slip sample was dispersed uniformly with the help of a brush, size of 100 niosomes from the batch were measured in terms of eyepiece division.[15]

b) % Entrapment efficiency

To determine the % EE, 20mg of Proniosomes were taken in beaker and was dissolved in 20ml of co-solvents of Ethanol and buffer pH 5.8. The free butenafine hydrochloride was separated from proniosomes by ultra-centrifugation (Eppendorf centrifuge 5430 R) at a speed of 14000 RPM for 30-45 min. at 4^0C. after centrifugation 1ml of supernatant was taken and observed at 223nm using UV spectrophotometer (shimadzu-1800), to determine the amount of free drug in the formulations.[16]

c) Viscosity and pH studies

Viscosity of the prepared proniosomal gel is evaluated by using Brookfield viscometer (model-DV2TRVTJ0) with spindle No. RV-07(7) at 100 rpm. 100 g of the proniosomal gel was taken in a beaker and the spindle was dipped in it. The viscosity of gel was measured at temperature of 25°C. The readings of 03 were taken for average ofsamples then calculate the viscosity.

The pH of each formulation was measured using a calibrated digital pH meter. The readings were taken for average of 3 samples. The normal range of topical gel pH is 5.5-8 respectively.[17]

d) Spreadability

For the determination of spreadability, excess of sample was applied between the two glass slides and was compressed to uniform thickness by placing 1000 gm weight for 5 min. Weight (50 gm) was added to the pan. The time required separating the two slides, i.e. the

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time in which the upper glass slide moves over the lower plate was taken as measure of spreadability (S). The standard range of gel spreadability is 8.4-15gm.cm/sec. respectively.[18]

Spreadability (g.cm/s) (S) = M×L/T

Where M = weight tied to upper slide, L = length moved on the glass slide, T= time taken.

e) In-vitro drug release studies

i. *Method for egg membrane preparation*

The contents of egg shells were removed and then it was dipped in the dilute hydrochloric acid (0.1N) for 30 min. The egg membrane was separated manually and washed thoroughly with distilled water.[19]

ii. *Drug release studies*

In vitro release studies were carried out using Franz diffusion cells with a receptor compartment volume of 20 mL and an effective diffusion area of 3.14 cm^2. Egg membrane was used as diffusion membrane. A predetermined amount of gel containing proniosomes was placed on the donor compartment and 20ml of freshly prepared phosphate buffer of pH 5.8 is placed in receptor compartment. The receptor medium was continuously stirred using magnetic stirrer at37±0.5°C. At predetermined time intervals, 0.1 mL samples were withdrawn from the receiver compartment and replaced with an equal volume of fresh buffer. The collected samples were analyzed at 223 nm by using UV spectrophotometer.[20]

f) In-vitro antifungal studies

9.75gm of Potato dextrose agar was taken in a 250 mL conical flask and dissolved in 250 mL of distilled water. The medium was sterilized in an autoclave at 15 lbs for 30 min. After sterilization, the medium was kept aside at room temperature. Then medium was poured into sterilized Petri dishes to get 3-4 mm depth uniformly in front of laminar airflow unit. After solidification, a loop of diluted suspension culture (Candida albicans) in nutrient broth was added on to the surface of solidified agar and was spread homogeneously with the help of L shape rod. After stabilization of culture, Gels of known concentration along with pure drug were fed into the petridish with the help of sterile disk. Then Petri dishes were incubated for 48 h at 37 °C. After incubation the zone of inhibition was measured.[21]

g) Stability studies of the most satisfactory formulation

Optimized formulations of butenafine loaded proniosomal gel were subjected to accelerated stability testing as per ICH guidelines for short term and placed in a screw capped glass container and stored at ambient humidity conditions and temperatures 40°C (75 ± 5RH) for a period of 30 days. The samples were analyzed for physical appearance, pH, drug content, and in vitro drug release at regular interval of 30 days.[22]

IV. Result and Discussion

a) Characterization of proniosomes

i. *Photomicrography*

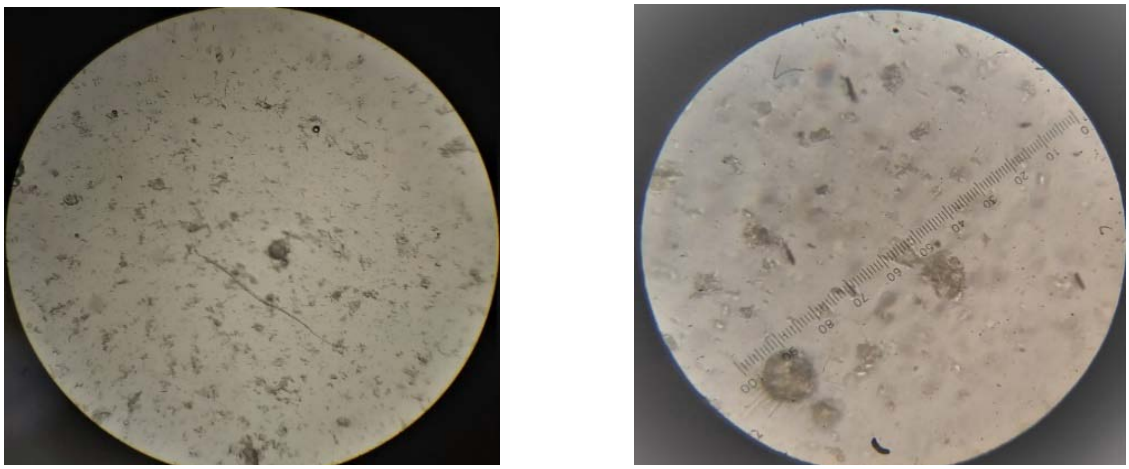


Figure 3: Optical photomicrograph of proniosomal formulation F3(10x, 40x)

ii. *Scanning electron microscopy*

Scanning electron microscopy reveals that the optimized Butenafine hcl loaded proniosome formulation (F3) have homogeneous solid spherical structure. And shows size ranging from 30-50µm respectively

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Figure 4: SEM photograph of optimized formulation (F3)

iii. *Particle size by photomicrography*

Particle size has increased with increase in concentration of polymer, with constant drug load and concentration of other agents. However, size was reduced with increase in concentration of surfactant and mean size of particles depends upon on the rotational and temperature of the rotavapor. The results of particle size of each formulations were found to be 33.85-40.03µm and tabulated in table no. 02.

b) % Entrapment efficiency

Entrapment efficiency was found to increase with increase in polymer (cholesterol) Concentration. Increase in size of proniosomes with increase in concentration of polymer and drug has resulted in increase in drug entrapment efficiency and also improves the stability of the bilayer membrane of the vesicles. Cholesterol decreases leakage of drug molecule from bilayer vesicle structure and also provides spherical smooth surface to the bilayer vesicles.

Entrapment efficiency of proniosomes formulations ranged from 64.41% to 79.87%. niosomes formed from span 60 proniosomal gel exhibits higher EE than other surfactant formulations (i.e. span 40, tween 60). Span 60 is solid at room temperature and have highest phase transition. Span 60 is having the same head group with different alkyl chains and might lower the HLB value and thus increases the EE of the drug. Formulation F3 containing span 60: cholesterol in 1:2 ratio showed highest EE (79.87%) and tabulated in table no. 02.

Table 2: Mean Particle size and Entrapment Efficiency of formulation F1-F9

SL. NO.	FORMULATION	MEAN PARTICLE SIZE (µm)	% ENTRAPMENT EFFICIENCY
1	F1	35.69 ± 1.56	69.01 ± 2.71
2	F2	34.15 ± 1.45	71.34 ± 1.21
3	F3	40.03 ± 0.14	79.87 ± 1.93
4	F4	36.60 ± 1.81	64.41 ± 3.10
5	F5	34.74 ± 2.11	70.53 ± 1.58
6	F6	38.81 ± 0.56	72.32 ± 2.17
7	F7	33.85 ± 1.54	73.87 ± 3.89
8	F8	35.15 ± 2.21	65.37 ± 0.98
9	F9	37.80 ± 2.92	77.64 ± 1.89

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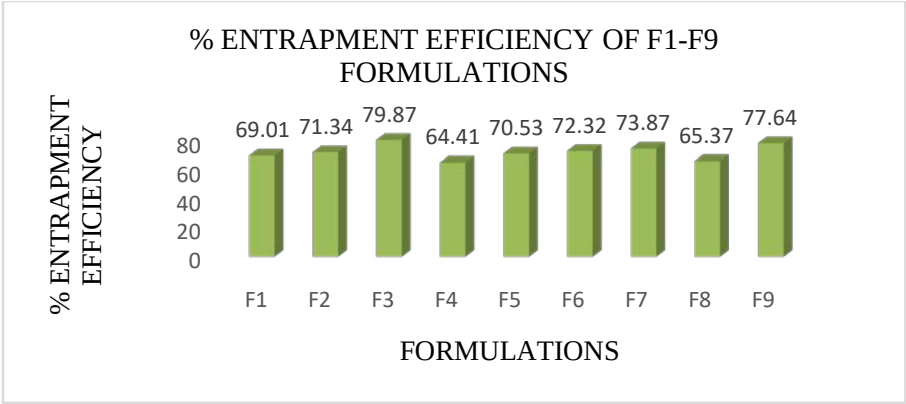


Figure 5: Comparative Entrapment efficiency of prepared proniosomes (F1-F9)

c) pH and Viscosity

The pH values exhibited by gels are tabulated in table no. 03 and found in range of 5.40 to 6.11 at 25^{0}C which is physiologically acceptable range for topical preparations.

The viscosity of the proniosomal gels were found in range 8920-14400 at 25^{0}C, as concentration of Carbopol used in all the formulations is same (1%). The results of viscosity of each formulation were tabulated in table no. 03. when using spindle no. 07 respectively.

d) Spreadability studies

Spread ability of the different proniosomal gel formulations were determined and tabulated in table no. 03.The spread ability range of gel were found to be 16.40-22.19 gm.cm/sec.

Table 3: pH, viscosity, spreadability of formulation F1-F9

FORMULATION	MEAN pH	VISCOSITY (CPS)	SPREADABILITY (gm.cm/sec)
F1	5.69±0.04	8,920±0.02	17.40
F2	5.55±0.02	9,960±0.01	21.27
F3	5.97±0.02	13,960±0.03	16.76
F4	5.84±0.04	10,920±0.01	18.80
F5	5.40±0.03	11,680±0.04	22.19
F6	5.47±0.02	12,360±0.03	17.25
F7	6.11±0.01	9,800±0.05	21.26
F8	6.00±0.02	13,360±0.02	19.03
F9	5.93±0.01	14,400±0.01	16.40

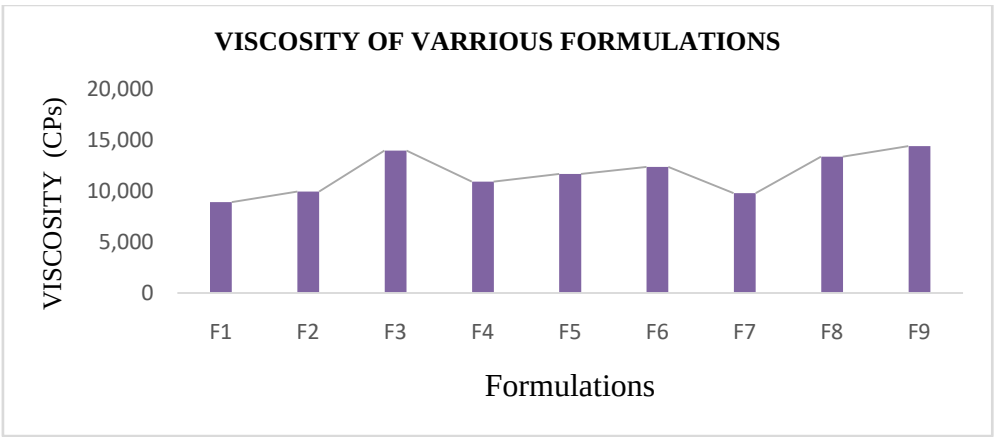


Figure 6: Comparative viscosity of prepared proniosomes (F1-F9)

e) In-vitro drug release studies of the proniosomal gel

The release studies were carried out for all the butenafine hcl loaded formulations. The formulation which shows the percentage of drug release maximum at 30 hrs. was considered as optimum. The percentage drug release of all prepared formulation is compared with optimized formulation (F3). The prepared butenafine hcl loaded proniosomes might illustrated

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initially slow release due to bursting of improper niosomes in the formulations, after bursting proniosomal vesicle observed rapid release process (table no. 04). The amount of drug released from different batches of proniosomal gel formulations was in the order of F3>F9>F6>F1>F7>F5>F4>F2>F8. The percentage drug release of all prepared formulation is in the ranging from of 55.08to 75.46%.

Table 4: In-vitro release study of butenafine Hcl formulations F1-F9

Time in Hour	F1	F2	F3	F4	F5	F6	F7	F8	F9
0	0	0	0	0	0	0	0	0	0
1	4.728	3.886	5.189	3.821	3.825	3.886	5.181	3.562	4.533
2	6.389	5.699	7.050	6.602	5.895	6.544	6.805	5.812	7.202
3	7.538	6.817	8.170	7.440	6.628	8.737	7.645	6.353	8.209
4	9.112	7.976	11.340	8.700	7.584	10.119	9.164	8.202	9.368
5	12.381	11.124	14.770	12.190	10.644	12.198	11.907	11.295	13.480
6	14.682	13.238	16.270	14.030	12.485	15.459	14.313	13.358	15.058
7	15.906	15.048	19.180	16.460	14.773	17.755	15.971	14.980	16.940
8	18.528	16.466	22.720	18.970	16.770	19.220	18.134	16.525	20.249
9	20.194	18.964	25.550	20.550	19.088	21.578	20.895	18.892	22.908
10	23.014	20.961	27.370	23.584	20.573	24.001	23.746	21.214	25.390
11	25.741	23.860	31.110	25.693	24.040	27.027	25.796	24.002	28.873
12	28.623	25.700	33.750	27.988	26.418	29.757	28.808	26.044	32.142
24	51.806	46.630	63.740	50.362	47.386	54.850	51.998	45.890	61.117
30	63.141	58.001	75.460	60.265	60.284	66.778	61.719	55.084	73.395

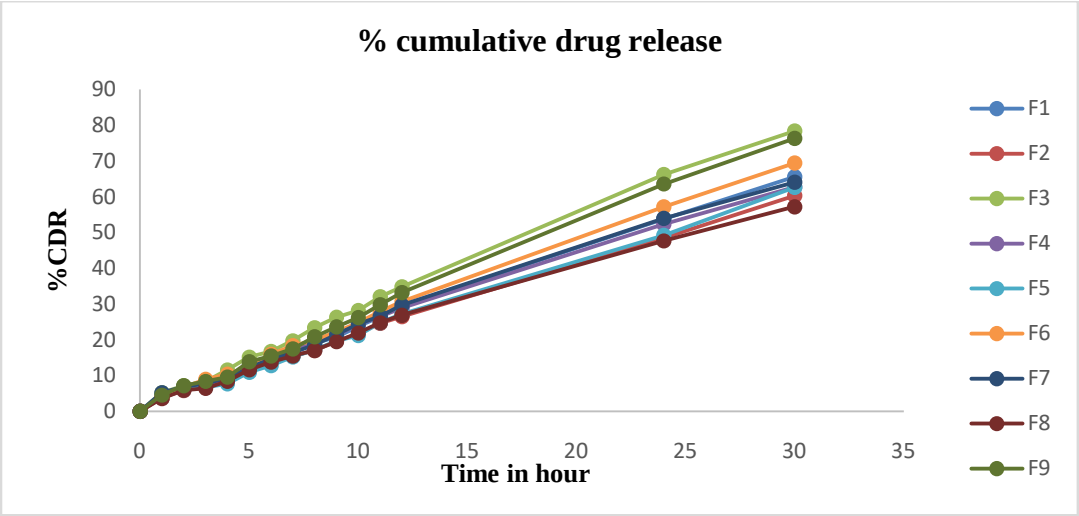


Figure 7: In vitro release profile of Butenafine Hcl formulation from F1-F9

f) Release kinetic studies

The release data was fitted to various mathematical models to evaluate the kinetics and mechanism of drug release. The kinetic data of all formulations F1-F9 could be best expressed by first order equation as the plots showed highest linearity (R^2:0.340-0.395), then zero order release kinetics (R^2:0.977-0.997).The release data of the optimal batch showed F3 value of 0.997 and 0.395 for the zero order and first order respectively. The formulations were observed to yield correlations with Higuchi model i.e. R^2=0.994 thus indicating the diffusion mechanism. The 'n' values obtained Korsmeyer-peppas model of F-3 was 0.443 which indicates that drug release swelling and mechanism of release was Anomalous (non-Fickian) diffusion.

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Table 5: Kinetics values obtained for butenafine loaded proniosomal gel

Formulation	Zero order kinetic	First order kinetics	Higuchi's model	Korr'speppas Model	n value
F1	0.991	0.373	0.989	0.916	0.416
F2	0.989	0.382	0.986	0.929	0.412
F3	0.997	0.395	0.994	0.985	0.443
F4	0.985	0.377	0.991	0.929	0.426
F5	0.983	0.371	0.987	0.979	0.404
F6	0.994	0.366	0.991	0.983	0.422
F7	0.945	0.369	0.990	0.920	0.397
F8	0.977	0.340	0.981	0.918	0.401
F9	0.995	0.393	0.993	0.984	0.441

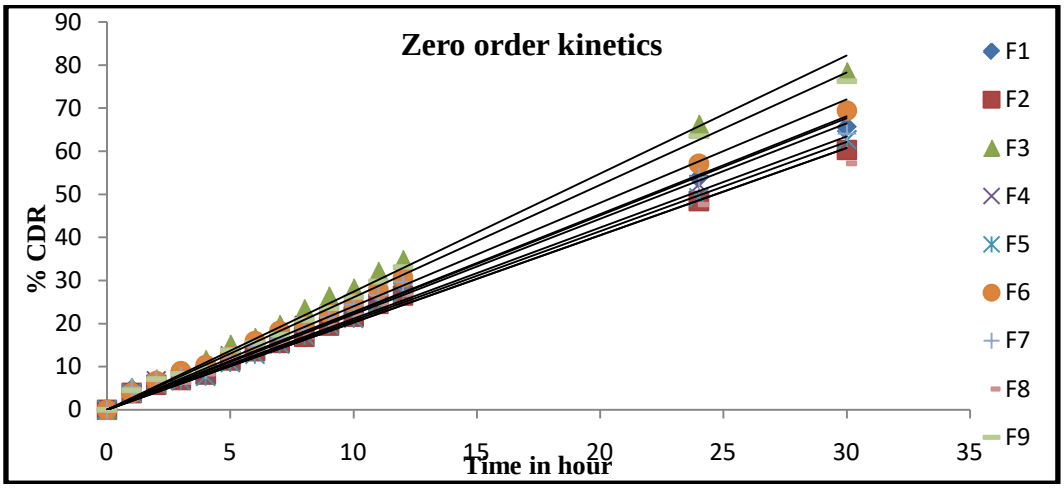


Figure 8: Comparative Zero order release profile of proniosomal gel formulations

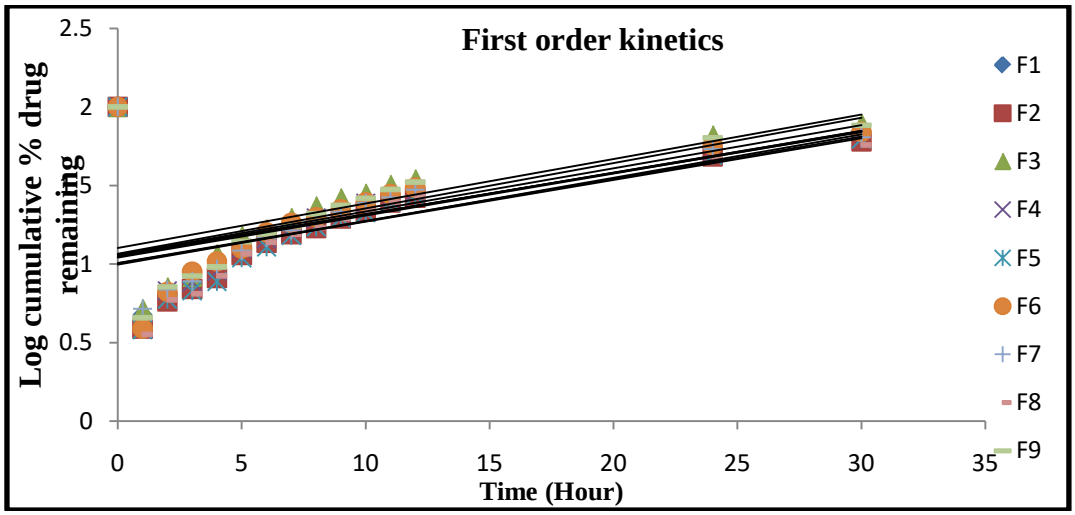


Figure 9: Comparative First order release profile of proniosomal gel formulations

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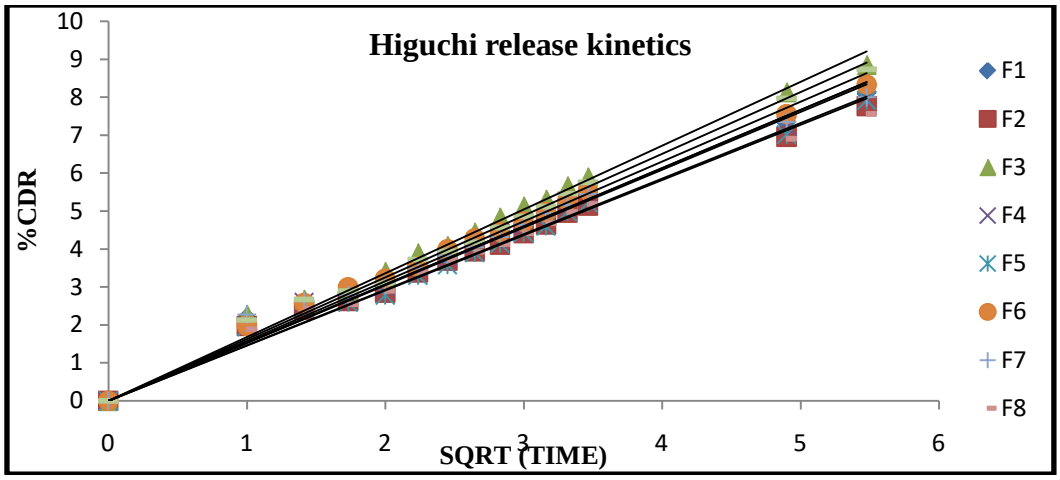


Figure 10: Comparative Higuchi release profile of proniosomal gel formulation

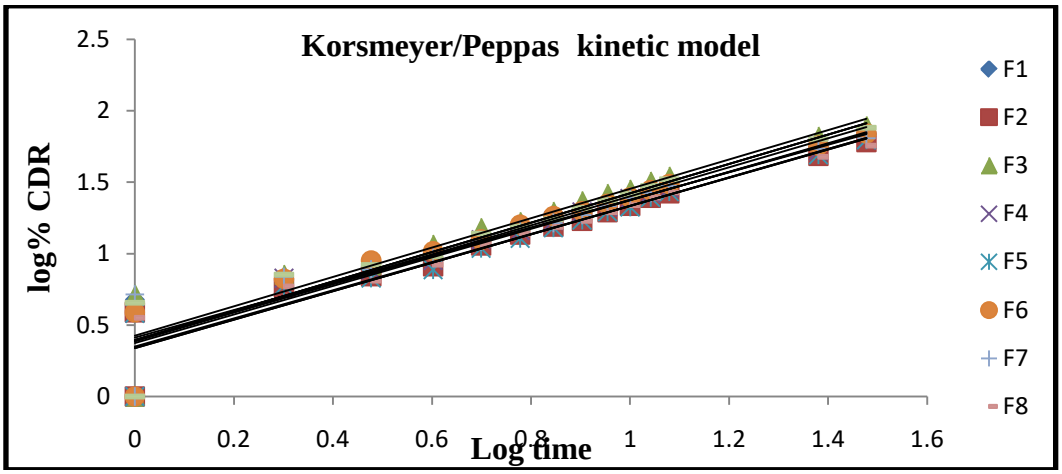


Figure 11: Comparative Korsmeyer-Peppa's release profile of proniosomal formulations

g) In-Vitro antifungal activity

The in-vitro antifungal activity of formulation was studied by cup plate method. Results of the in-vitro antifungal activity are shown in table-06. The zone of inhibition is more observed in butenafine hcl loaded proniosomal gel formulation (F3) compared to other formulation and pure drug was taken as a standard. The marketed product (Butop-1% cream) showed less zone of inhibition compared to optimized proniosomal gel formulation (F3). From the experimental outcome, it was concluded that prepared proniosomes gel formulation exhibited promising antifungal activity.

Table 6: In-vitro antifungal activity

SL.NO.	FORMULATION	ZONE OF INHIBITION (mm)
1	Pure drug	18±0.04
2	Marketed product (butop-1%)	12
3	F1	-
4	F2	3
5	F3	16
6	F4	8.5
7	F5	-
8	F6	9.5
9	F7	-
10	F8	7.6
11	F9	14.5

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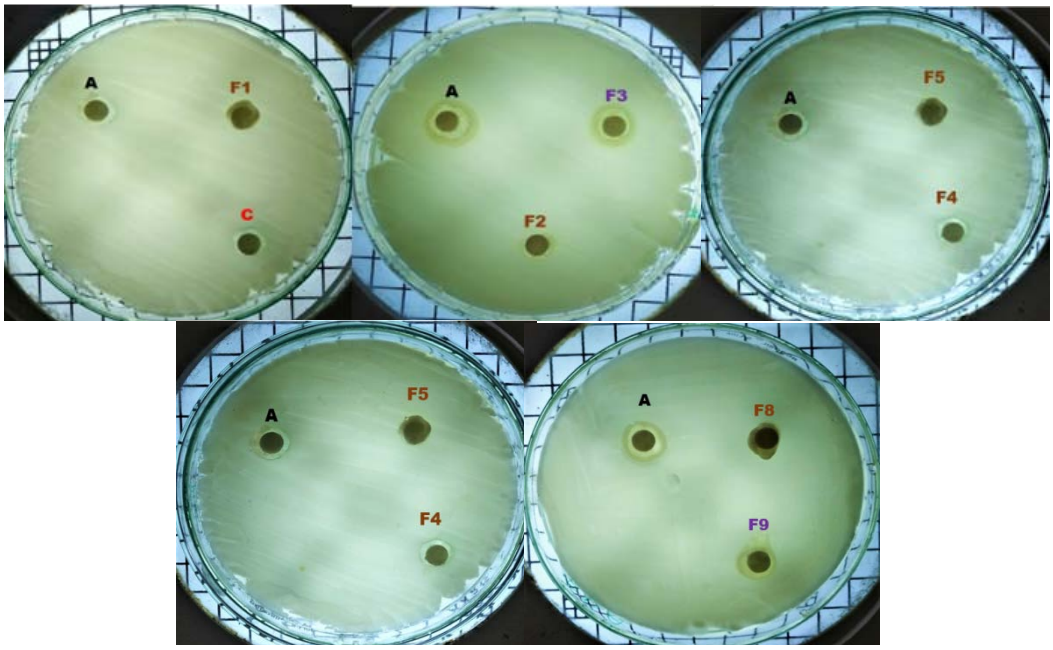


Figure 12: In vitro antifungal activity

h) Stability studies

The stability studies of the formulation were carried out according to ICH guidelines. The optimized formulations i.e. (F3) were subjected to stability studies @ 40°C (75 ± 5RH) for a period of 30 days. The physical stability was assessed by the pH, drug content, and % invitro drug release. The stability studies showed that there were no significant changes in the above-mentioned response variables. Thus, it can be concluded that the drug was found to be stable on storage.

Table 7: Stability studies of F3 formulation

Sampling Interval (days)	Storage condition		
	40^0C±20C /75% RH±5%RH		
	pH	Drug content	% in-vitro Drug release
0	5.97±0.02	99.13±2.13	75.46±0.62
30	5.93±0.01	98.91±1.12	74.78±0.91

V. Conclusion

Result of the present study indicates that prepared butenafine loaded proniosomes gel formulation is an alternative route for transdermal drug deliveryto treat fungal infection. Proniosomes were prepared by slurry method using different concentration of cholesterol, surfactants, and soya lecithin etc. The prepared proniosomes were evaluated for % entrapment efficiency, spreadability, viscosity and % drug content. The values obtained were found to be satisfactory and complies with standard range. The in-vitro drug release was found to increase with increase in cholesterol (polymer) concentration and decreased with increase in surfactant concentration. In that F3 formulation exhibited highest drug release (75.46%) compared to other formulations over a period of 30 hours. The in-vitro antifungal activity study was concluded that proniosomal gel formulation with drug (F3) shows better zone of inhibition than the other formulations and marketed product. Stability studies demonstrated there was no significant variation in pH, drug content and % in-vitro drug release and found to be stable at the end of storage period (30days).From the above experimental data, it can be concluded that the transdermal delivery of butenafine Hcl loaded proniosomal gel formulations can be used in the future for treatment of fungal infection with improved bioavailability.

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Unless specified in the notification, the Editorial Board's decision on publication of the paper is final and cannot be appealed before making the major change in the manuscript.

Acknowledgments

Contributors to the research other than authors credited should be mentioned in Acknowledgments. The source of funding for the research can be included. Suppliers of resources may be mentioned along with their addresses.

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Authors can submit papers and articles in an acceptable file format: MS Word (doc, docx), LaTeX (.tex, .zip or .rar including all of your files), Adobe PDF (.pdf), rich text format (.rtf), simple text document (.txt), Open Document Text (.odt), and Apple Pages (.pages). Our professional layout editors will format the entire paper according to our official guidelines. This is one of the highlights of publishing with Global Journals—authors should not be concerned about the formatting of their paper. Global Journals accepts articles and manuscripts in every major language, be it Spanish, Chinese, Japanese, Portuguese, Russian, French, German, Dutch, Italian, Greek, or any other national language, but the title, subtitle, and abstract should be in English. This will facilitate indexing and the pre-peer review process.

The following is the official style and template developed for publication of a research paper. Authors are not required to follow this style during the submission of the paper. It is just for reference purposes.

Manuscript Style Instruction (Optional)

- Microsoft Word Document Setting Instructions.
- Font type of all text should be Swis721 Lt BT.
- Page size: 8.27" x 11'", left margin: 0.65, right margin: 0.65, bottom margin: 0.75.
- Paper title should be in one column of font size 24.
- Author name in font size of 11 in one column.
- Abstract: font size 9 with the word "Abstract" in bold italics.
- Main text: font size 10 with two justified columns.
- Two columns with equal column width of 3.38 and spacing of 0.2.
- First character must be three lines drop-capped.
- The paragraph before spacing of 1 pt and after of 0 pt.
- Line spacing of 1 pt.
- Large images must be in one column.
- The names of first main headings (Heading 1) must be in Roman font, capital letters, and font size of 10.
- The names of second main headings (Heading 2) must not include numbers and must be in italics with a font size of 10.

Structure and Format of Manuscript

The recommended size of an original research paper is under 15,000 words and review papers under 7,000 words. Research articles should be less than 10,000 words. Research papers are usually longer than review papers. Review papers are reports of significant research (typically less than 7,000 words, including tables, figures, and references)

A research paper must include:

a) A title which should be relevant to the theme of the paper.
b) A summary, known as an abstract (less than 150 words), containing the major results and conclusions.
c) Up to 10 keywords that precisely identify the paper's subject, purpose, and focus.
d) An introduction, giving fundamental background objectives.
e) Resources and techniques with sufficient complete experimental details (wherever possible by reference) to permit repetition, sources of information must be given, and numerical methods must be specified by reference.
f) Results which should be presented concisely by well-designed tables and figures.
g) Suitable statistical data should also be given.
h) All data must have been gathered with attention to numerical detail in the planning stage.

Design has been recognized to be essential to experiments for a considerable time, and the editor has decided that any paper that appears not to have adequate numerical treatments of the data will be returned unrefereed.

i) Discussion should cover implications and consequences and not just recapitulate the results; conclusions should also be summarized.
j) There should be brief acknowledgments.
k) There ought to be references in the conventional format. Global Journals recommends APA format.

Authors should carefully consider the preparation of papers to ensure that they communicate effectively. Papers are much more likely to be accepted if they are carefully designed and laid out, contain few or no errors, are summarizing, and follow instructions. They will also be published with much fewer delays than those that require much technical and editorial correction.

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Format Structure

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All manuscripts submitted to Global Journals should include:

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The title page must carry an informative title that reflects the content, a running title (less than 45 characters together with spaces), names of the authors and co-authors, and the place(s) where the work was carried out.

Author details

The full postal address of any related author(s) must be specified.

Abstract

The abstract is the foundation of the research paper. It should be clear and concise and must contain the objective of the paper and inferences drawn. It is advised to not include big mathematical equations or complicated jargon.

Many researchers searching for information online will use search engines such as Google, Yahoo or others. By optimizing your paper for search engines, you will amplify the chance of someone finding it. In turn, this will make it more likely to be viewed and cited in further works. Global Journals has compiled these guidelines to facilitate you to maximize the web-friendliness of the most public part of your paper.

Keywords

A major lynchpin of research work for the writing of research papers is the keyword search, which one will employ to find both library and internet resources. Up to eleven keywords or very brief phrases have to be given to help data retrieval, mining, and indexing.

One must be persistent and creative in using keywords. An effective keyword search requires a strategy: planning of a list of possible keywords and phrases to try.

Choice of the main keywords is the first tool of writing a research paper. Research paper writing is an art. Keyword search should be as strategic as possible.

One should start brainstorming lists of potential keywords before even beginning searching. Think about the most important concepts related to research work. Ask, “What words would a source have to include to be truly valuable in a research paper?” Then consider synonyms for the important words.

It may take the discovery of only one important paper to steer in the right keyword direction because, in most databases, the keywords under which a research paper is abstracted are listed with the paper.

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Numerical methods used should be transparent and, where appropriate, supported by references.

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Authors must list all the abbreviations used in the paper at the end of the paper or in a separate table before using them.

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Authors are advised to submit any mathematical equation using either MathJax, KaTeX, or LaTeX, or in a very high-quality image.

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Tables: Tables should be cautiously designed, uncrowned, and include only essential data. Each must have an Arabic number, e.g., Table 4, a self-explanatory caption, and be on a separate sheet. Authors must submit tables in an editable format and not as images. References to these tables (if any) must be mentioned accurately.

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Figures are supposed to be submitted as separate files. Always include a citation in the text for each figure using Arabic numbers, e.g., Fig. 4. Artwork must be submitted online in vector electronic form or by emailing it.

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Tips for writing a good quality Medical Research Paper

1. Choosing the topic: In most cases, the topic is selected by the interests of the author, but it can also be suggested by the guides. You can have several topics, and then judge which you are most comfortable with. This may be done by asking several questions of yourself, like "Will I be able to carry out a search in this area? Will I find all necessary resources to accomplish the search? Will I be able to find all information in this field area?" If the answer to this type of question is "yes," then you ought to choose that topic. In most cases, you may have to conduct surveys and visit several places. Also, you might have to do a lot of work to find all the rises and falls of the various data on that subject. Sometimes, detailed information plays a vital role, instead of short information. Evaluators are human: The first thing to remember is that evaluators are also human beings. They are not only meant for rejecting a paper. They are here to evaluate your paper. So present your best aspect.

2. Think like evaluators: If you are in confusion or getting demotivated because your paper may not be accepted by the evaluators, then think, and try to evaluate your paper like an evaluator. Try to understand what an evaluator wants in your research paper, and you will automatically have your answer. Make blueprints of paper: The outline is the plan or framework that will help you to arrange your thoughts. It will make your paper logical. But remember that all points of your outline must be related to the topic you have chosen.

3. Ask your guides: If you are having any difficulty with your research, then do not hesitate to share your difficulty with your guide (if you have one). They will surely help you out and resolve your doubts. If you can't clarify what exactly you require for your work, then ask your supervisor to help you with an alternative. He or she might also provide you with a list of essential readings.

4. Use of computer is recommended: As you are doing research in the field of medical research then this point is quite obvious. Use right software: Always use good quality software packages. If you are not capable of judging good software, then you can lose the quality of your paper unknowingly. There are various programs available to help you which you can get through the internet.

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7. Revise what you wrote: When you write anything, always read it, summarize it, and then finalize it.

8. Make every effort: Make every effort to mention what you are going to write in your paper. That means always have a good start. Try to mention everything in the introduction—what is the need for a particular research paper. Polish your work with good writing skills and always give an evaluator what he wants. Make backups: When you are going to do any important thing like making a research paper, you should always have backup copies of it either on your computer or on paper. This protects you from losing any portion of your important data.

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11. Pick a good study spot: Always try to pick a spot for your research which is quiet. Not every spot is good for studying.

12. Know what you know: Always try to know what you know by making objectives, otherwise you will be confused and unable to achieve your target.

13. Use good grammar: Always use good grammar and words that will have a positive impact on the evaluator; use of good vocabulary does not mean using tough words which the evaluator has to find in a dictionary. Do not fragment sentences. Eliminate one-word sentences. Do not ever use a big word when a smaller one would suffice.

Verbs have to be in agreement with their subjects. In a research paper, do not start sentences with conjunctions or finish them with prepositions. When writing formally, it is advisable to never split an infinitive because someone will (wrongly) complain. Avoid clichés like a disease. Always shun irritating alliteration. Use language which is simple and straightforward. Put together a neat summary.

14. Arrangement of information: Each section of the main body should start with an opening sentence, and there should be a changeover at the end of the section. Give only valid and powerful arguments for your topic. You may also maintain your arguments with records.

15. Never start at the last minute: Always allow enough time for research work. Leaving everything to the last minute will degrade your paper and spoil your work.

16. Multitasking in research is not good: Doing several things at the same time is a bad habit in the case of research activity. Research is an area where everything has a particular time slot. Divide your research work into parts, and do a particular part in a particular time slot.

17. Never copy others' work: Never copy others' work and give it your name because if the evaluator has seen it anywhere, you will be in trouble. Take proper rest and food: No matter how many hours you spend on your research activity, if you are not taking care of your health, then all your efforts will have been in vain. For quality research, take proper rest and food.

18. Go to seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.

19. Refresh your mind after intervals: Try to give your mind a rest by listening to soft music or sleeping in intervals. This will also improve your memory. Acquire colleagues: Always try to acquire colleagues. No matter how sharp you are, if you acquire colleagues, they can give you ideas which will be helpful to your research.

20. Think technically: Always think technically. If anything happens, search for its reasons, benefits, and demerits. Think and then print: When you go to print your paper, check that tables are not split, headings are not detached from their descriptions, and page sequence is maintained.

21. Adding unnecessary information: Do not add unnecessary information like "I have used MS Excel to draw graphs." Irrelevant and inappropriate material is superfluous. Foreign terminology and phrases are not apropos. One should never take a broad view. Analogy is like feathers on a snake. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Never oversimplify: When adding material to your research paper, never go for oversimplification; this will definitely irritate the evaluator. Be specific. Never use rhythmic redundancies. Contractions shouldn't be used in a research paper. Comparisons are as terrible as clichés. Give up ampersands, abbreviations, and so on. Remove commas that are not necessary. Parenthetical words should be between brackets or commas. Understatement is always the best way to put forward earth-shaking thoughts. Give a detailed literary review.

22. Report concluded results: Use concluded results. From raw data, filter the results, and then conclude your studies based on measurements and observations taken. An appropriate number of decimal places should be used. Parenthetical remarks are prohibited here. Proofread carefully at the final stage. At the end, give an outline to your arguments. Spot perspectives of further study of the subject. Justify your conclusion at the bottom sufficiently, which will probably include examples.

23. Upon conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print for the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects of your research.

Informal Guidelines of Research Paper Writing

Key points to remember:

- Submit all work in its final form.
- Write your paper in the form which is presented in the guidelines using the template.
- Please note the criteria peer reviewers will use for grading the final paper.

Final points:

One purpose of organizing a research paper is to let people interpret your efforts selectively. The journal requires the following sections, submitted in the order listed, with each section starting on a new page:

The introduction: This will be compiled from reference matter and reflect the design processes or outline of basis that directed you to make a study. As you carry out the process of study, the method and process section will be constructed like that. The results segment will show related statistics in nearly sequential order and direct reviewers to similar intellectual paths throughout the data that you gathered to carry out your study.

The discussion section:

This will provide understanding of the data and projections as to the implications of the results. The use of good quality references throughout the paper will give the effort trustworthiness by representing an alertness to prior workings.

Writing a research paper is not an easy job, no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record-keeping are the only means to make straightforward progression.

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- Submitting a manuscript with pages out of sequence.
- In every section of your document, use standard writing style, including articles ("a" and "the").
- Keep paying attention to the topic of the paper.
- Use paragraphs to split each significant point (excluding the abstract).
- Align the primary line of each section.
- Present your points in sound order.
- Use present tense to report well-accepted matters.
- Use past tense to describe specific results.
- Do not use familiar wording; don't address the reviewer directly. Don't use slang or superlatives.
- Avoid use of extra pictures—include only those figures essential to presenting results.

Title page:

Choose a revealing title. It should be short and include the name(s) and address(es) of all authors. It should not have acronyms or abbreviations or exceed two printed lines.

Abstract: This summary should be two hundred words or less. It should clearly and briefly explain the key findings reported in the manuscript and must have precise statistics. It should not have acronyms or abbreviations. It should be logical in itself. Do not cite references at this point.

An abstract is a brief, distinct paragraph summary of finished work or work in development. In a minute or less, a reviewer can be taught the foundation behind the study, common approaches to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Use comprehensive sentences, and do not sacrifice readability for brevity; you can maintain it succinctly by phrasing sentences so that they provide more than a lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study with the subsequent elements in any summary. Try to limit the initial two items to no more than one line each.

Reason for writing the article—theory, overall issue, purpose.

- Fundamental goal.
- To-the-point depiction of the research.
- Consequences, including definite statistics—if the consequences are quantitative in nature, account for this; results of any numerical analysis should be reported. Significant conclusions or questions that emerge from the research.

Approach:

- Single section and succinct.
- An outline of the job done is always written in past tense.
- Concentrate on shortening results—limit background information to a verdict or two.
- Exact spelling, clarity of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else.

Introduction:

The introduction should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable of comprehending and calculating the purpose of your study without having to refer to other works. The basis for the study should be offered. Give the most important references, but avoid making a comprehensive appraisal of the topic. Describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will give no attention to your results. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here.

The following approach can create a valuable beginning:

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- Defend the model—why did you employ this particular system or method? What is its compensation? Remark upon its appropriateness from an abstract point of view as well as pointing out sensible reasons for using it.
- Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
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When a technique is used that has been well-described in another section, mention the specific item describing the way, but draw the basic principle while stating the situation. The purpose is to show all particular resources and broad procedures so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step-by-step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

Materials may be reported in part of a section or else they may be recognized along with your measures.

Methods:

- Report the method and not the particulars of each process that engaged the same methodology.
- Describe the method entirely.
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- Simplify—detail how procedures were completed, not how they were performed on a particular day.
- If well-known procedures were used, account for the procedure by name, possibly with a reference, and that's all.

Approach:

It is embarrassing to use vigorous voice when documenting methods without using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result, when writing up the methods, most authors use third person passive voice.

Use standard style in this and every other part of the paper—avoid familiar lists, and use full sentences.

What to keep away from:

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings—save it for the argument.
- Leave out information that is immaterial to a third party.



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Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part as entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Use statistics and tables, if suitable, to present consequences most efficiently.

You must clearly differentiate material which would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matters should not be submitted at all except if requested by the instructor.

Content:

- Sum up your conclusions in text and demonstrate them, if suitable, with figures and tables.
- In the manuscript, explain each of your consequences, and point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation of an exacting study.
- Explain results of control experiments and give remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or manuscript.

What to stay away from:

- Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- Do not include raw data or intermediate calculations in a research manuscript.
- Do not present similar data more than once.
- A manuscript should complement any figures or tables, not duplicate information.
- Never confuse figures with tables—there is a difference.

Approach:

As always, use past tense when you submit your results, and put the whole thing in a reasonable order.

Put figures and tables, appropriately numbered, in order at the end of the report.

If you desire, you may place your figures and tables properly within the text of your results section.

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Discussion:

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Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implications of the study. The purpose here is to offer an understanding of your results and support all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of results should be fully described.

Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact, you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved the prospect, and let it drop at that. Make a decision as to whether each premise is supported or discarded or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."

Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work.

- You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- Give details of all of your remarks as much as possible, focusing on mechanisms.
- Make a decision as to whether the tentative design sufficiently addressed the theory and whether or not it was correctly restricted. Try to present substitute explanations if they are sensible alternatives.
- One piece of research will not counter an overall question, so maintain the large picture in mind. Where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

When you refer to information, differentiate data generated by your own studies from other available information. Present work done by specific persons (including you) in past tense.

Describe generally acknowledged facts and main beliefs in present tense.

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Topics	Grades		
	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

INDEX

A

Akumentis · 1, 2
Anomalous · 43
Antiulcer · 7, 10
Aspergillus · 37

B

Basophils · 8
Bifonazole · 37

C

Corneocytes · 36
Cryptococcus · 37
Cytokines · 7, 9, 10

E

Eicosanoids · 7
Epidemiological · 1, 4
Epithelial · 7, 8, 9, 10, 11
Ethanol · 8, 10, 13, 20, 38

G

Glycyrrhetinic · 8
Granatum · 8, 10, 11

H

Hongoquercin · 9, 10
Hypocalcemia · 2

I

Immunomodulator · 7, 9, 10

L

Lonolenic · 9, 10
Lymphoproliferative · 8

M

Mucosa · 7
Myopathy · 2

P

Pancreatic · 3
Phagocytic · 8, 9, 10
Polyphenols · 8, 10
Punicalagin · 8, 10

R

Reticuloendothelial · 8, 10

S

Sesquiterpenes · 9, 12
Splenocytes · 10

T

Terpenoid · 9
Therapeutic · 4, 7, 9, 36
Tinospora · 8, 10, 11
Toxicity · 2, 3, 11, 36
Transdermalroute · 36
Triglycerides · 3

V

Virucidal · 8, 9, 10





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