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Review Article

A review on analytical method development, optimization and validation of combination of Azithromycin and benzoyl peroxide by RP-HPLC using design of experiment as per ICH guideline

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Abstract

Pharmaceutical analysis is one of the most challenging fields of analytical chemistry. Pharmaceutical analysts carry out the qualitative and quantitative control of APIs and drug products and also develop and validate appropriate methods. One of my main goals was to develop modern, rapid, precise and reproducible, but also cost-effective HPLC assay methods which are generally available and applicable for most users. The aim of this work was to develop LC methods for both compounds. The assay of erythromycin by LC offers several advantages, such as high specificity, the possibility of determining and quantifying impurities and degradation products, and improved accuracy. The developed methods were validated. My whole work containing following plan of work as Selection of drug, Review Literature, FITR of both drugs and Mixture, Preparation of standard solutions, Preparation of sample of pure drug in Standard solution, Method development by HPLC (as Selection of solvents to be used as diluents and mobile phase, Selection of wavelength, Selection of mobile phase and Selection of chromatographic conditions) Preparation of Mobile phase, Preparation of standard calibration curve combination of drug, Optimization of HPLC condition using box behnken design. Validation of analytical method following parameters as per ICH guidelines. (i). System suitability (ii). Linearity and range (iii). Specificity (iv).Accuracy and precision (v). Limits of detection (LOD) and Quantitation (LOQ). (vi). Selectivity and (vii).Robustness.

Introduction

This review article was prepared with an aim to review different methods developed for Azithromycin and Benzoyl peroxide drug by RP-HPLC analytical method and strategies developed and optimized for efficient method development [1]. There is a continuous research in this field and different researchers are exploiting different mobile phase combinations for their research work. Azithromycin which are used to treat various bacterial infections and Benzoyl peroxide is generally used to treat mild to moderate acne (Acne Vulgaris) [2-6].

The use of the Azithromycin and Benzoyl peroxide as a drug essential in pharmaceutical formulations highlights the requirement for its determination and quantification with appropriate analytical methods. This article also discusses the parameters that must be considered in the validation of analytical methods. A simple, fast, accurate and precise RP-HPLC method were developed and validated for the estimation of Azithromycin & Benzoyl peroxide as per ICH guidelines. Potassium dihydrogen phosphate and Acetonitrile (50:50) are commonly used as solvents. The method was developed in Eclipse C_{18} column (Waters XTerra®, 4.6X250 mm, particle 5µ) with Potassium dihydrogen phosphate and Acetonitrile are commonly used solvents in RP -HPLC having low UV cut- off of 200-400 nm respectively.

Thus this shows that the method is capable to give a good detector response, the recovery calculated was within the range of 98% to 101% of the specification limits. Hence the method was a rapid tool for routine analysis of Azithromycin/Benzoyl peroxide in the bulk and in the pharmaceutical dosage form.

Analytical method development, optimization and validation of combination of Azithromycin and Benzoyl peroxide drug formulations by RP-HPLC

Literature survey revealed that method development by reverse phase high performance liquid chromatographic has been taken for Azithromycin and Benzoyl peroxide drug

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formulations for estimation of antibacterial & ant acne activity. Till now few methods have been developed for estimation of antibacterial & ant acne activity of drugs Azithromycin and Benzoyl peroxide [7-9]. Thus the aim of this article is to be to discuss various high performance liquid chromatographic methods developed by various researchers for determination of these antibacterial & ant acne activity drugs with different mobile phase combinations. This article also discusses the parameters that must be considered in the validation of analytical methods.

Benzoyl peroxide

Kamra *et al* study

Kamra *et al* (2018) developed and validated Reversed-phase High-performance Liquid Chromatography Method for the Simultaneous Estimation of Benzoyl Peroxide and Resveratrol.

An isocratic separation of BPO and resveratrol was achieved on C18, 250 mm × 4.6 mm I.d., 5 µm particle size columns with a flow rate of 1.2 ml/min and using a UV detector to monitor the elute at 245 nm. The mobile phase consisted of an ammonium acetate (pH 4) and ethanol. Response was a linear function of drug concentration in the range of 10–100 mg/mL range with an R2 of 0.993 for BPO and 10–100 µg/mL range with an R2 of 0.995 for resveratrol, accuracy with percent relative standard deviation of 100.65 ± 0.23 (benzoic peroxide) and 100.48 ± 0.45 (resveratrol) and with a limit of detection and quantification for BPO and resveratrol, respectively. The result of analysis has been validated statistically and by recovery study. The accuracy ranged between 99.65 and 101.91%. The method was found to be precise, reproducible, and rapid [10].

Jain *et al* study

Jain *et al* (2018) developed liposomal gel of codelivery of benzoyl peroxide &adapalene for acne treatment. BPO-AD-mLipo illustrated size 256.4 ± 9.3 nm with polydispersity index ~ 0.2. Significantly enhanced dermal bioavailability (AD-2.1, 5.4; BPO-3.0, 7.83-fold) and reduction in skin irritation and papule density in animal model were observed with BPO-AD-mLipo-gel as compared with free drugs and Epiduo, respectively. Hence it has been concluded that BPO-AD-mLipo gel provides effective and safer alternative approach for codelivery of anti-acne drugs [11].

Kawashima *et al* study

Kawashima *et al* (2017) demonstrated Clinical efficacy and safety of benzoyl peroxide for acne vulgaris. Benzoyl peroxide (BPO) has been well established as a common medication for acne vulgaris in many countries (e.g. in Europe and the USA), where clinical data have been accumulated over a long time. In Japan, the use of BPO for acne treatment was approved in 2014, and the results of clinical trials in Japanese patients have recently been reported. This review compares clinical study results between Japanese and Western patients. Clinical studies that had been performed in Western countries were searched on the basis of the criteria, double-blind studies of BPO monotherapy and comparison with a vehicle group. Two reports of Japanese studies were also selected by using the same criteria. Efficacy was assessed by comparing the mean difference between the BPO and the vehicle groups for reduction rate in the number of lesions from baseline, and there were no differences between Japanese and Western patients. Safety assessment also showed that the incidence of adverse events was higher in Japanese patients than in Western patients, but the characteristics of the adverse events were not different. Therefore, we conclude that there are no significant differences in the efficacy and safety of BPO between these patient populations. The efficacy and safety of long-term use in Japanese patients are also expected to be applicable to those in Western patients [12].

Sharma *et al* study

Sharma *et al* (2016) developed Stability Indicating HPLC Method For Simultaneous Estimation of Clindamycin Phosphate and Benzoyl Peroxide In Gel Formulation. reversed phase High Performance Liquid Chromatographic method was developed for the simultaneous determination of Clindamycin Phosphate and Benzoyl Peroxide, using a C18 column and a mobile phase composed of 20 mM Ammonium acetate buffer pH 4.0: Methanol (45: 55 %v/v) as mobile phase at flow rate of 1.2 ml/min with detection wavelength of 210nm. Retention times in RP-HPLC method were found to be 4.49 min, 8.78 min for Clindamycin Phosphate and Benzoyl Peroxide, respectively. Linearity of Clindamycin Phosphate and Benzoyl Peroxide were found in the range of 10.0-30.0 µg/ml and 25.0-75.1 µg/ml. The % recovery of Clindamycin Phosphate was found to be 98.45- 101.0 and 99.8- 99.38 for Benzoyl Peroxide. Both the drugs were subjected to acid, alkali, oxidation, thermal and sunlight degradation. The degradation products of Clindamycin Phosphate and Benzoyl Peroxide were well resolved from the pure drugs with significant differences in the retention time values [13].

Kim *et al* study

Kim *et al* (2016) displayed role of combination of benzoyl peroxide-adeplane. The fixed-dose combination adapalene 0.1%/benzoylperoxide 2.5% (A/BPO) was introduced as an acne vulgaris therapeutic in 2007. It combines anti-inflammatory, keratolytic, comedolytic, and antibacterial properties. Thus, it addresses several pathophysiological factors involved in the Pathophysiology of acne. This review highlights the rationale for the use of this fixed-dose combination product, its therapeutic efficacy including effects on adherence and quality of life, its use for different forms of acne, and the side-effect profile. In summary, the fixed-dose combination of A/BPO gel can be regarded as a highly effective and safe formulation. It is not associated with antibiotic resistance. It reduces factors that cause nonadherence and has positive effects on the quality of life of affected patients. The tolerance is good. The initial mild irritation potential can be addressed by adequate counseling [14].

Roy *et al* study

Roy *et al* (2015) developed Validated Stability-Indicating RP-HPLC Method for the Estimation of Degradation Behaviour of Organic Peroxide and Third-Generation Synthetic Retinoids in Topical Pharmaceutical Dosage Formulation. The desired chromatographic separation was achieved on the Kinetex™

C18 (250 × 4.6 mm, 5 µm) column using gradient elution at 272 nm detection wavelength. The optimized mobile phase consisted of solvent A (mixture of 0.1% v/v glacial acetic acid in water and acetonitrile in the ratio of 80:20 v/v, respectively) and solvent B (mixture of acetonitrile: tetrahydrofuran: methanol in the ratio of 50:30:20 v/v/v, respectively). The stability-indicating capability of the developed method was established by analyzing forced degradation samples in which the spectral purity of BPO and ADP along with separation of all degradation products from the analyte peaks was achieved. The developed method was validated as per ICH guidelines with respect to specificity, linearity, limit of detection, limit of quantification, accuracy, precision, and robustness [15].

Ponhong *et al* study

Ponhong *et al* (2015) developed A rapid and sensitive Spectrophotometric method for the determination of benzoyl peroxide in wheat flour samples. The detection principle is based on BPO reacted with 2, 20-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to obtain a blue-green colored product that was detected at 415 nm by spectrophotometry. The effect of factors influencing the color reaction was investigated. Under the selected conditions, the linear range for quantification of BPO was observed between 0.2-10 mg/lit with r^2= 0.998. The limit of detection (LOD) was 0.025 mg/lit. The developed method obtained superior precision (relative standard deviation < 2%) using 11 repeatability at 0.2 mg/lit, 0.6 mg/lit, and 0.8 mg/lit. The proposed methodology was successfully applied to determine BPO in wheat flour samples [16].

Hamdu *et al* study

Hamdu *et al* (2014) developed an isocratic normal-phase high-performance liquid chromatographic method for the simultaneous determination of benzoyl peroxide and benzoic acid in one pharmaceutical preparation and their stability in different solvents. The compounds are separated on a normal phase column Eurospher-100, c18.4.6mmID. The mobile phase is methanol - water (65 + 35, v/v). Solutions are injected into the chromatographic system under isocratic conditions at a constant flow rate of 1 mL /min with UV detection at 240 nm. Analysis of stability samples in deferent solvents showed formation of BPO from BA when it dissolved in ether or methanol and very low degradation of BPO to BA when it dissolved in ether compare with when it dissolved in methanol [17].

Brandstetteret *et al* study

Brandstetteret *et al* (2011) demonstrated dose of benzoyl peroxide in treatment of acne. Benzoyl peroxide (BPO) is widely utilized in acne treatment as an alternative to antibiotics against which Propionibacterium acnes becomes more resistant. This overview examines BPO dose justification. Methods: Pub Med, Embase® and Science Citation Index searches were conducted using the keywords "benzoyl peroxide" and "acne vulgaris". Limited experimental data are available. However, there appears no significant difference in the efficacy of concentrations from 2.5% to 10%. The extent of free fatty acids and the percutaneous penetration of BPO may not play a critical role in acne vulgaris [18].

Caldwell *et al* study

Caldwell *et al* (1987) performed Analysis by HPLC of Benzoyl Peroxide Residues in Nylon Carpet Fibers. For this research, ANSO-IV nylon 6 staple carpet yams from Allied Fibers and Plastics were used. The carpet yarns were constructed into single bar, circular filling knit test sleeves which were dyed at 0.5% own with CI acid blue 25. A commercial acne med icationcreme containing 10% benzoyl peroxide was used on the fabric swatches. Two levels of benzoyl peroxide application were used (high and low levels). Both groups of samples were exposed at two levels of humidity and at constant temperature. Two procedures were used to extract BP and BA from the affected carpet tufts: a sonication method and a digestion method. The samples were analyzed by HPLC techniques developed using a gradient from 80% (v/v) A to 100% B in 15 minutes where A is an aqueous solution with pH 3 and B is acetonitrile. The results indicate two advantages of the HPLC method used in this study: a large separation of the BA peak from the void volume in terms of retention time, and the gradient elution allowing observation of both BA and BP peaks in a single chromatographic analysis of reasonable duration. For the extraction procedures, differences were noted in the results obtained with each of the methods [19].

Yong *et al* study

Yong *et al* (1979) demonstrated use of benzoyl peroxide gel in treatment of acne. The results of an open clini (al trial of200 patients in Singapore show that benzoyl peroxide, in a stablvgvl formulation, both in 2.5% and 5% strengths, was highly efficacious in the topical treatment of acne vulgaris without concomitant systemic therapy. The series included a small number of Caucasians (13 patients) who were also treated effectively. Side effects noted were generally mild and transient [20].

Azithromycin

Mokhtari *et al* study

Mokhtari *et al* (2016) made a comparison between efficacies of azithromycin gel 2% with clindamycin gel 1% in patients with acne. Average number of papules, pustules and comedones was similarly reduced in both groups and, no significant difference was observed between the two groups (P > 0.05, repeated measures ANOVA). The mean indexes of ASI and TLC also significantly decreased during treatment in both groups, no significant difference was observed between the two groups. (P > 0.05, repeated measures ANOVA). Also, impact of both drugs on papules and pustules was 2-3 times greater than the effect on comedones. Average satisfaction score was not significant between the two groups (P = 0.6, repeated measures ANOVA). Finally, frequency distribution of complications was not significant between the two groups (P > 0.05, Fisher Exact test). Azithromycin gel has medical impact at least similar to Clindamycin Gel in treatment of mild to moderate acne vulgaris, and it may be consider as suitable drug for resistant acne to conventional topical therapy [21].

Sahu *et al* study

Sahu *et al* (2015) developed and validated reversed-phase HPLC method for simultaneous estimation of azithromycin in tablet dosage form. An isocratic, precise and accurate reversed-phase liquid chromatographic method was developed for the quantitative determination of Azithromycin in tablet dosage form. The separation was carried out using a mobile phase consisting of buffer Potassium dihydrogen Phosphate: Acetonitrile (HPLC grade) (pH 7.5 adjusted with ortho phosphoric acid). The column used was Hypersil- keystone C18 (250 X 4.60 mm), 5µm column with flow rate of 1.2 ml/min using U.V. Visible detector. The detection was monitored at 215nm and the run time was 25min. The volume of injection loop was 10µl prior to injection of the drug solution the column was equilibrated for at least 15 min. The retention times of Azithromycin were found to be 9.761. Results of analysis were validated statistically and by recovery studies. Forced degradation method is used for detection of degraded impurities in body fluids shows better results than reported. The results of the study conclude that the proposed RP-HPLC method is a simple, specific, definite, precise, and less time consuming method which is useful for the routine determination of Azithromycin in its pharmaceutical dosage form [22].

Ghari *et al* study

Ghari *et al (*2014) developed azithromycin encapsulated nanoparticles by using response surface methodology. AZI-loaded poly (DL-lactide-co-glycolide) (PLGA) nanoparticles were prepared using the nanoprecipitation method and characterized. The Box-Behnken design (BBD) was employed to optimize the effects of different variables on particle size and drug encapsulation efficiency of nanoparticles. The results of optimized formulations showed a polydispersity index of 0.06 %, an average diameter of 183 ± 1.8 nm, and the encapsulation efficiency of 57.83 ± 4.8 %. The prepared particles had the spherical shape, and the in vitro drug release profile showed a biphasic pattern, showing an initial burst release of 23 % in the first 2 h followed by a sustained release for up to 24 h. [23].

Ghari *et al* study

Ghari *et al* (2013) developed a Simple RP-HPLC-UV Method for Determination of Azithromycin in Bulk and Pharmaceutical Dosage forms as an Alternative to the USP Method. The best stationary phase was determined as C18 column, 5 µm; 250 mm × 4.6 mm. Mobile phase was optimized to obtain a fast and selective separation of the drug. Flow rate was 1.5 mL/min, Wavelength was set at 210 nm and the volume of each injection was 500 µL. An isocratic methanol/buffer mobile phase at the ratio of 90:10 v/v gave the best separation and resolution. The proposed method was accurate, precise, sensitive, and linear over a wide range of concentration of azithromycin. The developed method has the advantage of using UV detector compared to the USP method in which electrochemical detector has been used. The validated method was successfully applied to the determination of azithromycin in bulk and pharmaceutical dosage forms [24].

Sudheer *et al* study

Sudheer *et al* (2012) a sensitive and specific isocratic RP-HPLC was developed for quantitative estimation of Azithromycin and AmbroxolHCl tablet formulation. The developed method consisting the mobile phase K2HPO4 – pH 6.5 : (68 : 32) with isocratic programming, Hypersil, BDS, C 8, column (150 mm x 4.6 mm i.d., 5µm particle size) as stationary phase with a flow rate of 1.5 mL/minute by using max 215nm and PDA detector. Proposed method was found to be linear in the concentration range of 100.0 to 360.0 µg/mL for Azithromycin and 15.0 to 54.0µg/mL for AmbroxolHClrespectively, the correlation coefficient was found to be 0.999. Precision study showed that the percentage relative standard deviation was within the range of acceptable limits, and the mean recovery was found to be 100.36 % for Azithromycin and 100.24% for AmbroxolHCl. The developed method was validated for specificity by stress studies. AmbroxolHCl and Azithromycin were subjected to stress condition and products were analyzed by using photo diode array detector. It was found to be stable in milder condition of stress (0.1 M HCl, 0.1 M NaOH, 3% H2O2, at 60°C/10 minutes). The analyte peaks were well resolved from the degraded impurities [25].

Al-Rimawi *et al* study

Al-Rimawi *et al* (2010) developed a method of HPLC analysis of azithromycin and its related compounds. Liquid chromatography with a UV detector at a wavelength of 210 nm using a reversed-phase C(18) stationary phase has been employed in this study. Isocratic elution is employed using a mixture of phosphate buffer-methanol (20:80). This new method is validated in accordance with USP requirements for new methods for assay determination, which include accuracy, precision, specificity, linearity, and range. This method shows enough selectivity, sensitivity, accuracy, precision, and linearity range to satisfy Federal Drug Administration and International Conference of Harmonization regulatory requirements. The current method demonstrates good linearity over the range of 0.3-2.0 mg/mL of azithromycin. The accuracy of the method is 100.5% with a relative standard deviation of 0.2%. The precision of this method reflected by relative standard deviation of replicates is 0.2%. The method is sensitive with a detection limit of 0.0005 mg/mL for azithromycin. Impurities and degradation products of azithromycin can be selectively determined with a good resolution in both raw material and pharmaceutical forms [26].

Senthil Raja *et al* study

SenthilRaja *et al* (2010) A simple reverse phase liquid chromatographic method has been developed and subsequently validated for simultaneous determination of Azithromycin and Ambroxol Hydrochloride in combined dosage form. The separation was carried out using a mobile phase consisting of acetonitrile and mono basic potassium phosphate buffer of pH 8.5 in the ratio of 65:35 v/v. The column used was C18 phenomenex Gemini 5m, 250cm x 4.6mm id with flow rate of 2ml/min using PDA detection at 220nm. The described method was linear over a concentration range of 96-145mg/ml and 80- 125mg/ml for the assay of

Azithromycin and Ambroxol Hydrochloride respectively. The retention times of Ambroxol and Azithromycin were found to be 3.7min and 6.1min respectively. Results of analysis were validated statistically and by recovery studies. The limit of quantification (LOQ) for Azithromycin and Ambroxol Hydrochloride were found to be 96.7mg/ml and 8.35mg/ml respectively. Then the limit of detection (LOD) for Azithromycin and Ambroxol Hydrochloride were found to be 31.91 mg/ml and 2.75 mg/ml respectively [27].

Nájlaet *et al* study

Nájlaet *et al* (2010) the objective of this research was to develop and validate an alternative analytical method for quantitative determination of Levofloxacin in tablets and injection preparations. The calibration curves were linear over a concentration range from 3.0 to 8.0 µg mL-1. The relative standard deviation was below 1.0% for both formulations and average recovery was 101.42 ± 0.45% and 100.34 ± 0.85% for tablets and injection formulations, respectively. The limit of detection and limit of quantitation were 0.08 and 0.25 µg mL-1, respectively. It was concluded that the developed method is suitable for the quality control of Levofloxacin in pharmaceuticals formulations [28].

Singh *et al* study

Singh *et al* (2010) A RP-HPLC method was developed and validated for quantitative determination of azithromycin in pharmaceutical suspension dosage forms. The chromatography was carried out on a Phenomenex C 18 (150 x 4.6 mm i.d., 5µ) column with Acetonitrile: 0.5 % Formic acid as mobile phase (Isocratic A: B = 40: 60 % v/v), at 215 nm detector wave length with a flow rate of 1 ml/min. Clarithromycin was used as an internal standard. The linearity was established in the range of 20 - 600 ng/ml for HPLC. The HPLC method was accurate and precised for azithromycin suspension with a recovery of 98.75 to 99.44%. The spiked sample solutions were stable upto 1 month. This validated method can be used for estimation of azithromycin in pharmaceutical suspension [29].

Shizarpour *et al* study

Shizarpour *et al* (2008) deiplayed comparison between Topical Azithromycin and Clindamycin in the Treatment of Mild to Moderate Acne Vulgaris. Dermatologists are in agreement about topical treatment in the mild to moderate acne vulgaris, but extensive using of tropical antibiotics and drug resistance have decreased their therapeutic benefits. In this study, we tried to compare the therapeutic effects of tropical azithromycin and clindamycin. This study was designed and performed as a double blind, randomized clinical trial. Thirty two patients with mild to moderate acne were treated with azithromycin and 30 patients, who were matched with the former group based on age, sex and severity of the disease, were treated with clindamycin for 12 weeks and results of their treatment were compared with each other. Results of this study showed that ratio of response to treatment and decreasing the grade of the disease and number of nodules, papules and pustules were not significantly different in the first month of the treatment in both groups while just the number of nodules in the group on azithromycin showed more decrease in the last months of treatment in comparison with clindamycin (0.88±0.75 vs. 0.25±0.75, p=0.015). Also, clindamycin had more side effects, but the rate of satisfaction with both drugs were high and showed no difference. In this study, no significant association was found between sex and response to treatment and evaluation of association between age and response to treatment showed a significant reverse association between age and decreasing the number of pustules [30].

Bardazzi *et al* study

Bardazzi *et al* (2007) demonstrated role of azithromycin in acne treatment on adolescents. A majority of patients (47/52) showed remarkable improvement in the first 4 weeks with a more than 20 percent reduction of their inflammatory papulo-pustular lesions. Maximum clearance was observed in 32 patients at 8 weeks. Slow improvement with eruptions of new lesions was seen in 6 patients. Adverse events, such as heartburn and nausea, were reported by 3 patients. All patients completed the 8-week study period. The beneficial effect was maintained at 4 months after the conclusion of treatment. Therefore it has been concluded that Azithromycin, 500 mg thrice weekly for 8 weeks, appears to be a safe and effective treatment for acne vulgaris in adolescents, with excellent patient compliance [31].

Ghoshal *et al* study

Ghoshal *et al* (2007) performed Comparative evaluation of effectiveness of adapalene and azithromycin, alone or in combination, in acne vulgaris. Acne vulgaris, a disorder of the pilosebaceous structure, is a common disorder in adolescents and young adults that is associated with significant morbidity. The aim of this study was to compare the effects of the drugs adapalene and azithromycin, given separately and in combination in acne vulgaris. Methodology: A total of 61 newly attending cases of inflammatory acne vulgaris were considered the study. They were randomly allocated into three groups. Group 1 received topical adapalene (0.01%) gel, group 2 received oral azithromycin, whereas group 3 was given a combination of these two. The patients were treated for a period of 12 weeks, being reviewed every fortnightly. The results obtained were analyzed in detail using statistical methods. The combination of adapalene and azithromycin caused the highest reduction in the inflamed lesion count followed by azithromycin given singly. Further, monotherapy with adapalene was used. However, this difference in efficacy was small and not statistically significant (P = 0.717). Azithromycin lead to a rapid reduction in the inflammatory lesion count, but it had negligible action on noninflamed lesions. At the end of 12 weeks of treatment, the three treatment groups showed no statistically significant difference in the efficacy in inflammatory acne [32].

Suhagia *et al* study

Suhagia *et al* (2006) a simple and sensitive Spectrophotometric method has been developed for determination of azithromycin in its pharmaceutical dosage forms. In the proposed method, azithromycin is oxidized with potassium permanganate to liberate formaldehyde, which is determined in situ using acetyl acetone, in the presence of

ammonium acetate. A yellow colouredchromogen was obtained, having an absorption maximum at 412 nm. The method is found to be linear in the concentration range of 10-75µg/ml, with regression coefficient of 0.9978. Various reaction parameters such as concentration of potassium permanganate and reagent, time required for oxidation, and maximum colour intensity were optimized. The method was validated, and can be used successfully to assay azithromycin in its pharmaceutical dosage forms viz. tablets, capsules, and injections [33].

Kulikov *et al* study

Kulikov *et al* (2004) developed and validated Micellar Liquid Chromatographic Method with UV Detection for Determination of Azithromycin in Tablets and Capsules. A rapid and simple micellar liquid chromatographic method that does not require use of specific chromatographic columns has been developed and validated for azithromycin determination. The method uses a Hypersil C18 column at 60 °C, 1-butanol–pH 6.86 phosphate buffer solution–water, 15:25:60 (v/v), containing 0.10 M sodium dodecyl sulfate, as mobile phase, and UV-detection at 215 nm. Different characteristics of the method were validated satisfactorily. The specificity, accuracy, linearity, precision (repeatability), and robustness of the method were demonstrated. The method proved suitable for determination of the azithromycin content of capsules and uncoated tablets [34].

Kamau *et al* study

Kamau *et al* (2002), developed Isocratic Liquid Chromatographic Method for the Analysis of Azithromycin and Its Structurally Related Substances in Bulk Samples. AZT is separated from its synthesis intermediates and a degradation product as well as from six unknown impurities on an XTerra RP18 column at 70°C using a mobile phase consisting of acetonitrile–pH 6.5 0.2M K2HPO4–water (35:10:55, v/v/v) at 1.0 mL/min. The XTerrastationary phase contains methyl groups that are incorporated in the bulk structure of the material. This allows for special selectivities. Robustness is evaluated by a full factorial design experiment. The method shows good selectivity, repeatability, linearity, and sensitivity [35].

Zubata *et al* study

Zubata *et al* (2001) developed a new HPLC method for azithromycin quantitation. A simple liquid chromatographic method was developed for the estimation of azithromycin raw material and in pharmaceutical forms. The sample was chromatographed on a reverse phase C18 column and eluants monitored at a wavelength of 215 nm. The method was accurate, precise and sufficiently selective. It is applicable for its quantitation, stability and dissolution test [36].

Combination in treatment of Acne

Akter *et al* study

Akter *et al* (2018) performed a clinical trial study of Oral Azithromycin Pulse Therapy and Daily Topical Benzoyl Peroxide in the Treatment of Acne Vulgaris. It was an open, controlled, clinical trial, conducted on 37 out patients with acne vulgaris. Patients were clinically assessed at baseline &at week 0,4, 8 and 12. Evaluation included success rate (subjects clear or excellent improvement, good response), lesion count & percentage change in lesion count from baseline, cutaneous tolerability & adverse events. The combination of oral azithromycin pulse therapy & daily topical benzoyl peroxide was very safe & effective with significant differences in percentage of lesion count change observed as early as 1-4 weeks. Adverse events were more frequent with the combination therapy that occurred early in the study &were transient. This study revealed that combination regimen of azithromycin &benzoyl peroxide (4%) is indeed very much efficacious & safe in the management of acne vulgaris [37].

Sardana *et al* study

Sardana *et al* (2016) performed a cross-sectional pilot study of antibiotic resistance in Propionibacterium acnes strains in Indian acne patients using 16s-RNA polymerase chain reaction: A comparison among treatment modalities including antibiotics, benzoyl peroxide, and isotretinoin. The follicular content was sampled and the culture was verified with 16S rRNA polymerase chain reaction, genomic sequencing, and pulsed-field gel electrophoresis. Minimum inhibitory concentration (MIC) assessment was done for erythromycin (ERY), azithromycin (AZI), clindamycin (CL), tetracycline (TET), doxycycline (DOX), minocycline (MINO), and Levofloxacin (LEVO). The four groups of patients were compared for any difference in the resistant strains. Results: Of the 52 P. acnes strains isolated (80 patients), high resistance was observed to AZI (100%), ERY (98%), CL (90.4%), DOX (44.2%), and TETs (30.8%). Low resistance was observed to MINO (1.9%) and LEVO (9.6%). Statistical difference was seen in the resistance between CL and TETs; DOX/LEVO and DOX/MINO ($P < 0.001$). High MIC90 (≥256 µg/ml) was seen with CL, macrolides, and TETs; moreover, low MIC90 was observed to DOX (16 µg/ml), MINO (8 µg/ml), and LEVO (4 µg/ml). Though the treatment group with isotretinoin/BPO had the least number of resistant strains there was no statistical difference in the antibiotic resistance among the various groups of patients. Conclusions: High resistance was seen among the P. acnes strains to macrolides-lincosamides (AZI and CL) while MINO and LEVO resistance was low [38-39].

Central composite design for RP HPLC

Siregar *et al* study

Siregar *et al* (2017), central composite design (CCD) was used for optimization of high performance liquid chromatographic (HPLC) method for simultaneous analysis of curcumin (CUR) and demethoxycurcumin (DMC) in tablets containing Curcuma extract. Separation of CUR and DMC was performed using X-Bridge C18 column (250 x 4.6 mm i.d; 5 µm). Four factors that were investigated include the concentration of acetic acid (X1), ratio of acetic acid (X2), flow rate of mobile phase (X3) and column temperature (X4). Based on responses obtained (retention time, peak area, resolution and tailing factor), the optimum condition selected was X1 = 3.00%, X2 = 51%, X3 = 1.05 mL/min and X4 = 45oC. This HPLC condition was validated by assessing several validation parameters including system suitability test, selectivity, linearity, precision, accuracy and robustness

according to International Conference Harmonization (ICH). All validation parameters meet the acceptance criteria set by ICH. The validated method was successfully used for analysis of CUR and DMC in tablets containing Curcuma extract. CCD was effective means in optimization of HPLC for analysis of CUR and DMC in pharmaceutical formulation [40].

Fatma *et al* study

Fatma *et al* (2017) performed Central composite design and response surface methodology for the optimization of Ag+-HPLC/ELSD method for triglyceride profiling. The study presents the application of central composite design (CCD) and response surface methodology (RSM) for the optimization of silver-ion normal phase HPLC/ELSD (Ag+-HPLC/ELSD) method parameters to profile the isomers of triglycerides in vegetable oils. The significance of a second-order polynomial model for predicting the optimal values of Ag+-HPLC/ELSD method parameters was evaluated by the analysis of variance, ANOVA, and 3D response surface plots for the interactions between three variables were constructed. Three experimental parameters were chosen as independent variables which are the flow rate of mobile phase, temperature of column compartment and concentration of sample. A multivariate five-level CCD and RSM were used to confirm a quadratic model as a functional relationship between the response values (Rs, N, α and k′) and variables. The optimum values of parameters were found to be a flow rate of 1.25 mL min−1, temperature of column compartment of 20 °C, and sample concentration of $5 \times 10-2$ mg μL−1. Regression analysis with an R2 values indicated as an adequate correlation between the experimental and predicted response values. ANOVA test results were also confirm that the models can be successfully used to predict the optimum parameters of Ag+-HPLC/ELSD method. Therefore, the proposed model provides an efficient, automated, and robust Ag+-HPLC/ELSD method for triglyceride profiling and is also suitable for a number of applications and analytical method developments for vegetable oils [41].

Kalariya *et al* study

Kalariya *et al* (2017) applied experimental design and response surface technique for selecting the optimum RP-HPLC conditions for the determination of moxifloxacinHCl and ketorolac tromethaminein eye drops. A method has been developed for the separation of moxifloxacin HCl and ketorolac tromethamineusing reverse phase high-performance liquid chromatography (RP-HPLC) on C18 column (250 · 4.6 mm, 5 lm) with UV detection at 308 nm. Experimental designs were applied for multivariate optimization of the experimental conditions of RP-HPLC method. Three independent factors: methanol content in the mobile phase composition, buffer pH and flow rate were used to design mathematical models. Here faced central composite (FCC) experimental design was used to study the response surface technique and to study in depth the effects of these independent factors. Derringer's desirability function was applied to simultaneously optimize the retention time of last eluting peak (ketorolac tromethamine) and tailing factor of moxifloxacin. The predicted optimum assay condition consisted of methanol and potassium dihydrogen phosphate buffer (pH 3.2; 25 mM, 0.5% Triethylamine) in a proportion of 60:40% v/v, respectively, as the mobile phase at a flow rate of 1.2 mL min1. Using this optimum condition, baseline separation of both drugs with good resolution and a run time of less than 7 min were achieved. The optimized assay condition was validated according to ICH guidelines to confirm specificity, linearity, accuracy and precision [42].

Ayoub *et al* study

Ayoub *et al* (2016) utilized experimental design in chromatographic method development. A guide experimental design in chromatographic method development was described and applied successfully to the analysis of different recently approved anti-diabetic pharmaceutical combinations. Enhancement of UHPLC analysis of alogliptin benzoate either with pioglitazone hydrochloride or with metformin hydrochloride was achieved. The optimal chromatographic conditions were not attained by trial and error that requires a large number of experiments. Alternatively, a computer program was used as a systematic optimization strategy for the design of the experiment which accurately predicts the combined effect of different factors simultaneously. Resolution between peaks was studied by the proposed fractional factorial design approach performed by the Minitab® Program using screening and optimization steps. Application of the central composite design was implemented. A Pareto chart was used to exclude the insignificant variables. Linearity ranges were found to be 0.5-40 μg ml-1, 1-20 μg ml-1 and 1-32 μg ml-1 for alogliptin benzoate, pioglitazone hydrochloride and metformin hydrochloride, respectively. The proposed method is applicable for the analysis of six pharmaceutical dosage forms namely, Nesina®, Actos®, Glucophage®, Oseni®, Kazano® and Actoplus MET® tablets [43].

Bapatu *et al* study

Bapatu *et al* (2016) applied QbD Approach Method Development for Estimation of Dabigatran Etexilate along with Its Impurities and Identification of Degradants in Capsule Dosage Form. Method design, method evaluation, method control and life cycle management were explained by systematic flow chart. Analytical Target Product profile was defined. The method was developed using the Inertsil ODS-3V, 150 mm × 4.6 mm, 5 μm column using the gradient program with ammonium formate buffer as mobile phase A and acetonitrile as mobile phase B. Risk assessment was performed as part of method evaluation. Design of experiment tools was used to optimize the chromatographic conditions. A two-level Full Factorial Design along with Face Centered Central Composite design augmentation was employed and statistical analysis of the experimental data uncovered the significant influential of chromatographic factors. The design space and the contour plot suggest that the current center point parameters can be further modified, resulting in better acceptability of the response parameters. The performance of the optimized method was validated according to current ICH guidelines. Dabigatran Etexilate Capsules was subjected to

various stress conditions like oxidative, acid, base, hydrolytic, thermal, humidity, and photolytic degradations and evaluated chromatograms at 220 nm. The degradation products were well separated from each other and main peak, demonstrating the stability-indicating power of the method. One of the major degradants impurities, which are forming in neutral hydrolysis stress condition, is isolated and characterized by using analytical techniques like IR, LC-MS and NMR. Degradation pathway for Dabigatran Etexilate was proposed based on forced degradation data along with reaction mechanism [44].

Shakyaya *et al* study

Shakyaya *et al* (2016) developed and validated Stability-Indicating Liquid Chromatographic Method for Determination of Valsartan and Hydrochlorothiazide Using Quality by Design. Optimized mobile phase (v/v/v) was water (containing 0.25 ml/L triethylamine), methanol and acetonitrile (50:38:37, pH adjusted to 3.0±0.1). Chromatographic separation was achieved on Hypersil®-Gold C18 (150 x 4.6 mm, 5 µm, Thermo Fisher Scientific, USA), column at 25 ± 2 °C. The method was validated as per the "International Conference on Harmonisation" (ICH) guidelines. Chromatographic run time was 10.0 minutes. The linearity range for valsartan and hydrochlorothiazide were 1.25-64.00 µg/ml and 0.195-10.00 µg/ml respectively. The limits of detection (LOD) for valsartan and hydrochlorothiazide were 0.253 and 0.0226 µg/ml while the limits of quantitations (LOQ) were 0.767 and 0.068 µg/ml respectively, using 10µl sample. Stability studies indicate that the degradation of valsartan was higher during oxidative stress than other stress conditions [45].

Uysal *et al* study

Uysal *et al* (2016) performed Optimization of RPLC Method for Separation of Some Acetylcholinesterase Inhibitors by using Central Composite Design. Method development, acetonitrile concentration, pH of mobile phase and column temperature were investigated using central composite design (CCD). Afterwards, the optimal conditions were found employing central composite design and Derringer's desirability function. Effect of these variables on the output responses such as retention factors, resolutions (Rs) and retention time (tR) were evaluated. The separation was applied by using X Terra C18 column (250 × 4.6 mm ID, 5 µm). The optimum assay conditions were: acetonitrile-water binary mixture (45:55, v/v) and pH 9.5 as the mobile phase and at column temperature 33°C. Total chromatographic analysis time per sample was approximately 12.5 min. The method showed good agreement between the experimental data and predictive value throughout the studied parameter space. By using equations obtained CCD, protonation constant values (pKa) of donepezil, galantamine and rivastigmine were also predicted [46].

Sathiyasundar *et al* study

Sathiyasundar *et al* (2015) optimized liquid chromatographic method for the simultaneous separation and estimation of zaltoprofen and paracetamol in human plasma sample. The new drug combination of zaltoprofen and paracetamol are widely used as analgesic and antiinfilamatery medication. There is no method reported for simultaneous estimation of this combination for plasma samples. On considering these facts a new reliable, sensitive and accurate reverse phase liquid chromatographic method has been developed for simultaneous quantitative estimation of these drugs in human plasma sample using probinicid as internal standard. In the preliminary screening steps, a factorial design was employed to identify the factors that had significant effects on the selected chromatographic responses. Based on this responses analyses time (tR3), Capacity factor (k1) and selectivity (α) was optimized using Rotatable Central Composite Design (RCCD) and Response Surface Methodology (RSM). Further, this optimized condition was used to analyze the spiked plasma samples, reconstituted by a plasma protein precipitation method. The sample was efficiently separated using Reverse Phase Liquid Chromatography method equipped with PDA detector. The chromatographic separation was carried out using supelcosil LC-8 (150mm × 4.6mm I.D and 5µm particle size) analytical column measured at 254 nm. The developed RP-HPLC method can be applied for the estimation of quantitative pharmacokinetics in preclinical and clinical studies [47].

Chen *et al* study

Chen *et al* (2015) optimized and validated High-Performance Chromatographic Condition for Simultaneous Determination of Adapalene and Benzoyl Peroxide by Response Surface Methodology. An optimized mobile phase composed of acetonitrile, tetrahydrofuran and water containing 0.1% acetic acid at a ratio of 25:50:25 by volume was successfully predicted by using RSM. An isocratic separation was achieved by using the condition. Furthermore, the analytical method was validated in terms of specificity, linearity, accuracy and precision in a range of 80% to 120% of the expected concentration. Finally, the method was successfully applied to the analysis of a commercial product [48].

Singh *et al* study

Singh *et al* (2014) optimized RP-HPLC method for Investigation of Emtricitabine Loaded Poly(lactic-co-glycolic acid) Nanoparticles. NPs were evaluated for *in vitro* release and *in vivo* absorption study. The desired chromatographic separation was achieved on a Phenomenex C18 (250mm × 4.6mm I.D., 5 μm) column, under isocratic conditions using UV detection at 280 nm. The optimized mobile phase consisted of a mixture of 40mM phosphate dihydrogen phosphate buffer (pH 6.8), methanol, and 2% acetonitrile in a ratio of (83 : 15 : 2, v/v/v) at a flow rate of 1 mL/min. The linear regression analysis for the calibration curves showed a good linear correlation over the concentration range 0.040–2.0 μg/mL, with retention time of 4.39 min. An average encapsulation efficiency of 74.34% was obtained for NPs. In vitro studies showed zero-order release and about 95% drug being released within 15 days in PBS (pH 7.4). In conclusion, the proposed optimized method was successfully applied for the determination of in vitro and in vivo release studies of emtricitabine NPs [49].

XIN *et al* study

XIN *et al* (2014) Optimized HPLC Condition of Ursolic Acid and Oleanolic Acid by Central Composite Design. Effects of

HPLC mobile phase and column on the separation and determination of ursolic acid and oleanolic acid were in vestigated. By using the resolution and the analysis time as response values, the main influencing factors of HPLC(the volumetric ratio of methanol and aqueous phase in the mobile phase, the volume fraction of phosphoric acid in the aqueous phase, as well as the flow rate of the mobile phase and the column temperature) were optimized by central composite design. An effective and fast method for the separation and determination of ursolic acid and oleanolic acid by HPLC was established. The results showed that oleanolic acid and ursolic acid were effectively separated within 23 min with the resolution of 1.739, when the chromatographic separation was performed on a Shim-pack ODS-CLC (M) column kept at 21℃, using the methanol and aqueous phase containing 0.05% phosphoric acid with the volumetric ratio of 91.7:8.3 as the mobile phase at a flow rate of 0.6 mL/min, and the detection wavelength was set at 210 nm [50].

Khodadoust *et al* study

Khodadoust *et al* (2013) optimized HPLC UV method of dispersive liquid–liquid micro extraction with central composite design for preconcentration of chlordiazepoxide drug. A simple, rapid, and sensitive method based on dispersive liquid–liquid micro extraction combined with HPLC-UV detection applied for the quantification of chlordiazepoxide in some real samples. The effect of different extraction conditions on the extraction efficiency of the chlordiazepoxide drug was investigated and optimized using central composite design as a conventional efficient tool. Optimum extraction condition values of variables were set as 210 μL chloroform, 1.8 mL methanol, 1.0 min extraction time, 5.0 min centrifugation at 5000 rpm min−1, neutral pH, 7.0% w/v NaCl. The separation was reached in less than 8.0 min using a C18 column using isocratic binary mobile phase (acetonitrile/water (60:40, v/v)) with flow rate of 1.0 mL min−1. The linear response ($r2 > 0.998$) was achieved in the range of 0.005–10 μg mL−1 with detection limit 0.0005 μg mL−1. The applicability of this method for simultaneous extraction and determination of chlordiazepoxide in four different matrices (water, urine, plasma, and chlordiazepoxide tablet) were investigated using standard addition method. Average recoveries at two spiking levels were over the range of 91.3–102.5% with RSD < 5.0% (n = 3). The obtained results show that dispersive liquid–liquid micro extraction combined with HPLC-UV is a fast and simple method for the determination of chlordiazepoxide in real samples [51].

Petkovska *et al* study

Petkovska *et al* (2008) applied experimental design approach for the development and validation of an enantiospecificrp-hplc method for simultaneous determination of clopidogreland related compounds. Experimental design was applied during the method optimization (Full factorial 23 design) and robustness testing (Central Composite Face Centered design). Laboratory mixtures of clopidogrel and its impurities in a concentration ratio of 1: 5.0×10–4 were used as an investigation matrix. The three independent variables were the acetonitrile content in the mobile phase, pH of the mobile phase, and the column temperature. A Chromatographic Response Function (CRF) was used for estimation of the system response resolution (Rs). Separation was achieved using mobile phase composition of ACN: Buffer solution pH 6.5 (40:60 v/v) at 30ºC. A CHIRAL-AGP 4.0 mm × 100 mm, 5.0 μm particle size column was used. The total time for chromatographic separation was approximately 10.0 min. The method was validated for its selectivity, linearity, precision, accuracy and robustness [52].

Conclusion

It was concluded that the proposed new RP-HPLC method developed for the quantitative determination of Azithromycin and Benzoyl peroxide in bulk was simple, selective, sensitive, accurate, precise and rapid. The method was proved to be superior to most of the reported methods. The mobile phases were simple to prepare and economical. In this method, correlation coefficient was found to be 0.998 for Benzoyl peroxide and 0.997 for Azithromycin.

The method was found to be accurate, precise, repeatable and reproducible with different instruments and analysts. Limit of quantification ion for Benzoyl peroxide and for Azithromycin 0.044μg/ml and 0.02μg/ml respectively. Similarly limit of detection or Benzoyl peroxide and for Azithromycin 0.015μg/ml and 0.009μg/ml respectively.

So, simple, sensitive, accurate, precise RP- HPLC methods were developed and validated for the simultaneous estimation of Benzoyl peroxide and Azithromycin.

The method also finds use in clinical, biological and pharmacokinetic studies for the drug Azithromycin and Benzoyl peroxide. The method was validated as per ICH guidelines, and validation acceptance criteria were met in all cases. Hence, this method was specific, stability-indicating and can be successfully used for the estimation of drug in bulk and pharmaceutical dosage forms.

References

1. Xiong Y, Xiao KP, Rustum AM. Development and validation of a stability-indicating RP-HPLC method to separate low levels of dexamethasone and other related compoundsfrom betamethasone. J Pharmaceut Biomed Anal, 2009;49: 646-654.
2. Zmuida, China. "How to Give a Child Azithromycin". Retrieved 2013-01-06.
3. Adolfo C. Fernandez-Obregon, MD, Azithromycin for the treatment of acne, International Journal of Dermatology 2000;39: 45–50.
4. Tilelli, John A.; Smith, Kathleen M.; Pettignano, Robert. "Life-Threatening Bradyarrhythmia After Massive Azithromycin Overdose". Pharmacotherapy 2006;26(1): 147–50.
5. Baselt, R. (2008). Disposition of Toxic Drugs and Chemicals in Man (8th ed.). Foster City, CA: Biomedical Publications. pp. 132–133.
6. Azithromycin Side Effects". WebMD. Retrieved 28 March 2015.
7. Cartwright, Hugh (17 March 2005). "Chemical Safety Data: Benzoyl peroxide". Oxford University. Retrieved 13 August 2011.

8. "Skin tumor-promoting activity of benzoyl peroxide, a... [Science. 1981] - PubMed - NCBI".

9. Leyden, James J.; Kligman, Albert M, "Contact sensitization to benzoyl peroxide", Contact Dermatitis 3 1977;(5): 273–75.

10. [10]. Manju Kamra, Anupama Diwan, Satish Sardana, Development and Validation of Reversed-phase High-performance Liquid Chromatography Method for the Simultaneous Estimation of Benzoyl Peroxide and Resveratrol, International Journal of Pharmaceutical & Biological Archives 2018; 9(2):113-121.

11. Jain S1, Kale DP1, Swami R1, Katiyar SS, Codelivery of benzoyl peroxide & adapalene using modified liposomal gel for improved acne therapy, Nanomedicine (Lond). 2018;13(12):1481-1493

12. Makoto Kawashima, Toshitaka Nagare, and Masaharu, Clinical efficacy and safety of benzoyl peroxide for acne vulgaris: Comparison between Japanese and Western patients, J Dermatol. 2017; 44(11): 1212–1218.

13. Madhuri Sharma, Ankita Bhavsar, A.Ramkishan, Stability Indicating HPLC Method For Simultaneous Estimation of Clindamycin Phosphate and Benzoyl Peroxide In Gel Formulation, Am. J. PharmTech Res. 2016; 6(2);632-640.

14. Su Youn Kim and Falk R Ochsendorf, New developments in acne treatment: role of combination adapalene–benzoylperoxide, Ther Clin Risk Manag. 2016; 12: 1497–1506.

15. Chinmoy Roy, Lalatendu Panigrahi, and Jitamanyu Chakrabarty, Validated Stability-Indicating RP-HPLC Method for the Estimation of Degradation Behaviour of Organic Peroxide and Third-Generation Synthetic Retinoids in Topical Pharmaceutical Dosage Formulation, Sci Pharm. 2015; 83(2): 321–338.

16. Kraingkrai Ponhong , Sam-ang Supharoek, Watsaka Siriangkhawut, Kate Grudpan, A rapid and sensitive Spectrophotometric method for the determination of benzoyl peroxide in wheat flour samples, journal of food and drug analysis 2015;23:652 -659.

17. Hazim Hamid Hamdu, An isocratic normal-phase high-performance liquid chromatographic method for the simultaneous determination of benzoyl peroxide and benzoic acid in one pharmaceutical preparation and their stability in different solvents, IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS) 2014;9(1): 04-12.

18. Andreas J. Brandstetter, Howard I. Maibach, Topical dose justification: benzoyl peroxide concentrations, Journal of Dermatological Treatment 2013;24(4): 275-7.

19. J. Caldwell, T. Perenich, Recovery and Analysis by HPLC of Benzoyl Peroxide Residues in Nylon Carpet Fibers, Textile Research Journal 1987, 1.

20. Chuah Chong Yong, Benzoyl Peroxide Gel Therapy In Acne In Singapore, Intfknational Journal Of Dermatology,july-August 1979;18: 486-490.

21. Fatemeh Mokhtari, Gita Faghihi, Akram Basiri, Sadaf Farhadi, Mohammadali Nilforoushzadeh, and Shadi Behfar1, Comparison effect of azithromycin gel 2% with clindamycin gel 1% in patients with acne, Adv Biomed Res. 2016; 5: 72.

22. Adarsh Sahu, Mahendra Prasad Jatavb, Reetesh Vinodec and Kamal Yadavd, Development and validation of a reversed-phase HPLC method for simultaneous estimation of azithromycin in tablet dosage form, IJPC 2015; 05:09.

23. T.Ghari, S.A.Mortazavi M.R.Khoshay, F.Kobarfard, K.Gilani, Preparation, optimization, and *in vitro* evaluation of azithromycin encapsulated nanoparticles by using response surface methodology, Journal of Drug Delivery Science and Technology 2014;24(4): 352-360.

24. Tayebeh Ghari, Farzad Kobarfard, Seyed Alireza Mortazavia, Development of a Simple RP-HPLC-UV Method for Determination of Azithromycin in Bulk and Pharmaceutical Dosage forms as an Alternative to the USP Method, Iran J Pharm Res. 2013; 12(Suppl): 57–63.

25. M. Sudheer1, A. B. N. Nageswara Rao, D. Hari Hara Theja, M. Siva Prakash, P. Ramalingam1 and A. Madhan Mohan, Development of Stability Indicating RPHPLC Method for Simultaneous Determination of Azithromycin and AmbroxolHCl (SR) in the Tablet Formulation; Scholars Research LibraryDer Pharmacia Lettre, 2012; 4 (3):803-810.

26. Al-Rimawi F, Kharoaf M., Analysis of azithromycin and its related compounds by RP-HPLC with UV detection, J Chromatogram Sci. 2010 Feb;48(2):86-90.

27. M. Senthil Raja1, Shan. S.Ha., P. Perumala., and M.T.S. Moorthy, RP-HPLC Method development and validation for the simultaneous estimation of Azithromycin and ambroxol hydrochloride in tablets; International Journal of PharmTech Research;2010;2(1);36-39.

28. Nájla Mohamad Kassab, Marcos Serrou do Amaral; Anil Kumar Singh; Maria Inês Rocha Miritello SantoroIII, Development and validation of UV Spectrophotometric method for determination of levofloxacin in pharmaceutical dosage forms,Quim. Nova, 2010;33(4);4-37.

29. Sachin Kumar Singh, Dev Prakash, Tamizh Mani T, Development and Validation of Stability indicating RP-HPLC Method for the estimation of Azithromycin Suspension; International Journal of Chem Tech Research;2010;2(4);1939-44.

30. Mohammad Shizarpour, Alireza Firooz, Hamidreza Pazooki, Samad Rezai Khiabanloo, Comparison of Topical Azithromycin and Clindamycin in the Treatment of Mild to Moderate Acne Vulgaris, Iranian Journal of Dermatology, 2008; 11(2): 67-72.

31. Bardazzi F1, Savoia F, Parente G, Tabanelli M, Balestri R, Spadola G, Dika E, Azithromycin: a new therapeutical strategy for acne in adolescents, Dermatol Online J. 2007 Oct 13;13(4):4.

32. Loknath Ghoshal, Sabyasachi Banerjee, Sadhan Kumar Ghosh, Dwijendra Nath Gangopadhyay, Sukumar Jana, Comparative evaluation of effectiveness of adapalene and azithromycin, alone or in combination, in acne vulgaris, 2007;52(4): 179-183.

33. BN Suhagia, SA Shah, IS Rathod, HM Patel, KR Doshi,Determination of Azithromycin in pharmaceutical dosage forms by Spectrophotometric method,IJPS;2006; 68(2); 242-245.

34. A.U. KulikovEmail authorA. G. Verushkin, Development and Validation of a Micellar Liquid Chromatographic Method with UV Detection for Determination of Azithromycin in Tablets and Capsules, Chromatographia July 2004;60(1-2): 33–38.

35. F.N. Kamau, H.K. Chepkwony, J.K. Ngugi, D. Debremaeker, E. Roets, and J. Hoogmartens, Isocratic Liquid Chromatographic Method for the Analysis of Azithromycin

and Its Structurally Related Substances in Bulk Samples, Journal of Chromatographic Science, 2002;40: 529-533.

36. Patricia Zubata, Rita Ceresole, Maria Ana Rosasco, Maria Teresa Pizzorno, A new HPLC method for azithromycin quantitation, Journal of Pharmaceutical and Biomedical Analysis 2002;27:833–836.

37. Bilqis Akter, Oral Azithromycin Pulse Therapy and Daily Topical Benzoyl Peroxide in the Treatment of Acne Vulgaris: An Open Clinical Trial Study, Journal of Bangladesh College of Physicians and Surgeons, 2018;36 (1):11-15.

38. Kabir Sardana, Tanvi Gupta, Bipul Kumar, Hemant K Gautam, Vijay K Garg, A cross-sectional pilot study of antibiotic resistance in Propionibacterium acnes strains in Indian acne patients using 16s-RNA polymerase chain reaction: A comparison among treatment modalities including antibiotics, benzoyl peroxide, and isotretinoin, Indian journal of dermatology,2016;61(1):45-52.

39. Semwal A, Ramandeep S. Preparation, characterization and biological evaluation of chitosanmoxifloxacin prodrugs for pharmaceuticals. Marmara Pharm. J 2014; 18(1): 036-042.

40. Chairany Siregar, Niken K. Prabaningdyah, Syaiful Choiri, Sugeng Riyanto and Abdul Rohman, Optimization of HPLC Using Central Composite Design for Determination of Curcumin and Demethoxycurcumin in Tablet Dosage Form, Dhaka Univ. J. Pharm. Sci. 2017;16(2): 137-145.

41. Fatma Nur ArslanEmail authorHuseyin Kara, Central composite design and response surface methodology for the optimization of Ag+-HPLC/ELSD method for triglyceride profiling, Journal of Food Measurement and Characterization June 2017; 11(2): 902–912.

42. Pradipbhai D. Kalariya, Deepak Namdev, R. Srinivas, S. Gananadhamu a, Application of experimental design and response surface technique for selecting the optimum RP-HPLC conditions for the determination of moxifloxacin HCl and ketorolac tromethamine in eye drops. Journal of Saudi Chemical Society 2017; 21: S373–S382.

43. B. M. Ayoub, O. ABDEL-AZIZ, A guide for using experimental design in chromatographic method development: applied to the analysis of selected anti-diabetic pharmaceutical combinations. Pharmazie 2016;71:683-687.

44. Hanimi Reddy Bapatu1, Ravi Kumar Maram, Woo Hyung Cho, Venkat Bhaskar Rao Pasagadugula,, QbD Approach Method Development for Estimation of Dabigatran Etexilate along with Its Impurities and Identification of Degradants in Capsule Dosage Form, AJAC2016;7(6):494-524.

45. [45]. Asho k K. SHakyaya, Development and Validation of a Stability-Indicating Liquid Chromatographic Method for Determination of Valsartan and Hydrochlorothiazide Using Quality by Design, Orient. J. Chem., 2016; 32(2): 777-788.

46. Ramazan Uysal, Yaşar Doğan Daldal, Zehra Üstün, Ebru Çubuk Demiralay, Optimization of RPLC Method for Separation of Some Acetylcholinesterase Inhibitors by using Central Composite Design, Eurasian J Anal Chem 2016;(Sp 1):23–43.

47. R. Sathiyasundar, P. Venkatesan, M. Swamivelmanickam, R. Suresh, K. Valliappan and K. Kannan, Multicriteria optimization of an liquid chromatographic method for the simultaneous separation and estimation of zaltoprofen and paracetamol in human plasma sample: Application of chemometric protocols. Der Pharmacia Lettre, 2015; 7 (10):262-269.

48. Yi-Cheng Chen, Pi-Ju Tsai, Yaw-Bin Huang, 1 and Pao-Chu Wu , Optimization and Validation of High-Performance Chromatographic Condition for Simultaneous Determination of Adapalene and Benzoyl Peroxide by Response Surface Methodology, PLoS One. 2015; 10(3): e0120171.

49. Gurinder Singh and Roopa S. Pai, Optimization (Central Composite Design) and Validation of HPLC Method for Investigation of Emtricitabine Loaded Poly(lactic-co-glycolic acid) Nanoparticles: *In Vitro* Drug Release and *In Vivo* Pharmacokinetic Studies, The Scientific World Journal, 2014;1-12. Article ID 583090, 12 pages http://dx.doi.org/10.1155/2014/583090

50. XING Ya-li, BI Liang-wu, ZHAO Zhen-dong, HAN Lu-lu, XIA Tian-juan, Optimization of the HPLC Condition of Ursolic Acid and Oleanolic Acid by Central Composite Design, Chemistry and Industry of Forest Products 2014; 22(4): 37-41.

51. Saeid Khodadoust Mehrorang Ghaedi, Optimization of dispersive liquid–liquid micro extraction with central composite design for preconcentration of chlordiazepoxide drug and its determination by HPLC-UV, Journal of separation science, 2013;36(11): 1734-1742.

52. Rumenka Petkovska1, Claus Cornett2, Aneta Dimitrovska, experimental design approach for the development And validation of an enantiospecific rp-hplc method For simultaneous determination of clopidogrel And related compounds, Macedonian Journal of Chemistry and Chemical Engineering, 2008;27(1):53–64.

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