Sudan University of Science and Technology

College of Graduate Studies

Development and validation of HPLC method for simultaneous determination of Chlorhexidine and Para-Chloroaniline

التطوير والتحقق من طريقة كروماتوغرافيا السائل عالية الأداء للتعيين الآني لكلورهيكسيدين وبارا كلوروانيلين

A Thesis Submitted in Fulfillment For The Requirements For The Degree of M.Sc in Chemistry

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Dedication

I dedicate this work to my family, my wifeand

my friends. A special feeling of gratitude to my

loving parents.

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Acknowledgements

I would like to thank Allah, for giving me strength to complete this work.

I would like to thank my supervisor Professor MohamedEl Mukhtar Abdel Aziz who guided and advised me in kindly fatherly manner.

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Abstract

A simple, precise and rapid isocratic HPLC-UV method for simultaneous determination of chlorhexidine (CHD) and its degradation product, para chloroaniline (pCA) in their pharmaceutical formulations was developed. Simple isocratic elution was selected, the optimized mobile phase was composed of methanol and acetate buffer solution at 55: 45 ratio, with flow rate of 1.0 ml/min, injection volume was 20µl, and the separation was performed using C18 column (200 × 4.6 mm, 5 µm particle size) at ambient temperature. Both components were determined at 254nm. Linearity of this method was checked using concentration range of 20 –160µg/ml for chlorhexidine and 0.3 –1.2µg/ml for p-chloroaniline, the linearity correlation was (R^2 =1), for both components.

The limit of detection was (1.07 and 0.012µg/ml) for chlorhexidine and p-chloroaniline respectively. The limit of quantitation was (3.25 and 0.038µg/ml) for chlorhexidine and p-chloroaniline respectively. The specificity tests were checked to find that there was no interference between the excipients used and the active ingredient and its impurity. The average percentage of accuracy for chlorhexidine and p-chloroaniline was 99.82 (0.34 RSD) and 100.37 (0.38 RSD), respectively (Not more than 2.0, USP and ICH acceptable limit). For intraday precision for 80%, 100% and 120%, the RSD for recovery percentage for chlorhexidine and p-chloroaniline was 0.08, 0.09 and 0.18, and 0.04, 0.21 and 0.21, respectively. For interday precision, was 0.81, 0.24 and 0.95, and 0.24, 0.35 and 0.28, respectively. (Not more than 2.0 acceptable limit). System suitability parameters at all different conditions were also found to be within the accepted limit.

مستخلص البحث

تم تطوير طريقه سهلة، دقيقة وسريعة وأحادية الطور المتحرك لتحليل عقار كلورهكسيدين والمادة الناتجة من تكسره بارا كلوروانيلين في وقت واحد في محاليلهما الصيدلانية باستخدام كروماتوغرافيا السائل عالية الاداء مع مكشاف الاشعة فوق البنفسجية. وقد تم استخدام تقنية الازاحة احادية الطور المتحرك للفصل. تم اختيار طور متحرك مناسب وهو يتكون من الميثانول ومحلول الخل المنظم بنسبة 45:55 وقد كان معدل سريان الطور المتحرك 1.0 مل/ دقيقه، تم حقن العينة بحجم 20 ميكروليتر. تمت عملية الفصل في درجة الحرارة المحيطة باستخدام عمود كاربون 18 ذو الابعاد (200 ملم *4.6 ملم* 5 مايكروميتر). وتم تقدير كلا المادتين عند طول موجي 254 نانوميتر. تمت دراسة العلاقة الخطية لعقار كلورهكسيدين فى مدى التراكيز 20-160 ميكروجم/مل، ولعقار بارا كلوروانيلين في مدى التراكيز 0.3 -1.2 ميكروجم/مل، فكان معامل الخطية يساوي 1.000 للمادتين. تم حساب الحد الأدنى للكشف (1.07 و0.012 ميكروجم/مل) للكلورهيكسيدين وبارا كلوروانيلين على التوالي، والحد الأدنى لتحديد الكمية (3.25 و0.038 ميكروجم/مل) للكلورهيكسيدين وبارا كلوروانيلين على التوالي في هذه الطريقة، وجد وأنها في حدود المدي المسموح به. تم اجراء اختبارات النوعية للطريقة، ووجد انه لا يحدث أي تداخل بين المواد المضافة المستخدمة والمادة الفعالة وشوائبها. كان متوسط النسب المئوية لصحة الكلورهيكسيدين وبارا كلوروانيلين 99.82 (0.34 الانحراف المعياري النسبي) و100.37 (0.38 الانحراف المعياري النسبي)، على التوالي (الحد المقبول ليس أكثر من 2.0، حسب دستور الادوية الأمريكي والمؤتمر الدولي للتنسيق) .وبالنسبة للدقة اللحظية ل 80٪ ،100٪ و120٪، كان الانحراف المعياري النسبي لنسب الاسترداد للكلورهيكسيدين وبارا كلوروانيلين 0.08 ،0.09 و0.18 و 0.04 ، 0.21 و 0.21 على التوالي .أما بالنسبة إلى الدقة اليومية، فقد كان 0.81 ،0.24 و0.95 و0.24 ،0.35 و0.28 على التوالي(لا يزيد عن 2.0 كحد مقبول). كما وجد أن معلمات ملاءمة النظام في جميع الظروف المختلفة تقع ضمن الحدود المقبولة. وجد ان عوامل نظام الملائمة للطريقة في ظروف مختلفة، جميعها أيضا في حدود المسموح حسب دستور الادوية الأمريكي والمؤتمر الدولي للتنسيق

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CONTENTS

Chapter One
Introduction and literature review

Chapter Two

Materials, Methods and Results

Chapter Three

Discussion

List of Tables

List of Figures

List of Abbreviations

AOAC	**Association of official analytical chemists international**
APIs	**Active pharmaceutical ingredients**
ISO	**International Organization for Standardization**
Avg	**Average**
CHD	**Chlorhexidine gluconate**
EPA	**Environmental protection agency**
FDA	**United states food and drug administration**
GMP	**Good manufacturing practices**
ICH	**International conference on harmonization**
IUPAC	**International union of pure and applied chemistry**
LOD	**Limit of detection**
LOQ	**Limit of quantitation**
NLT	**Not less than**
NMT	**Not more than**
NSAID	**Non-steroidal steroidal anti-inflammatory drug**

pCA	**Para-chloroaniline**
RSD	**Relative stander deviation**
S	**Slop of the calibration curve**
RMSE	**Root Mean Squire Error**
SPE	**Solid-phase extraction**
STD	**Standard**
STDEV	**Standard deviation**
USP	**United states pharmacopeia**

Chapter One

Introduction

And Literature review

1. Introduction and Litreture Review

1.1 Introduction

1.1.1 Analytical chemistry

Analytical Chemistry is an important part in monitoring the quality of pharmaceutical products for safety and efficacy. With the advancement in synthetic organic chemistry and other branches of chemistry including bioanalytical sciences and biotechnology, the scope of analytical chemistry has been increased to much higher levels. The emphasis in current use of analytical methods, particularly involving advance analytical installation technology has made it possible not only to evaluate the potency of active ingredients in dosage forms and Active Pharmaceutical Ingredients but also to characterize, elucidate, identify and quantify impotent constituents like active moiety, impurities, metabolites, isomers, polymers and chiral components of some of the most potent medicines. Not only it is important in today's field of pharmaceutical analytical chemistry to quantify the active ingredients in dosage form, but also have a prediction of the degradations, likely impurities being generated and understanding the impact of the impurities and degradation on the safety of a patient who has to use this medicine throughout his life. The current trends in pharmacopeias rely more on instrumental techniques

rather than on the classical wet chemistry methods. This has resulted in the availability of indigenous instruments like spectrophotometry, high-performance liquid chromatography (HPLC), gas chromatography (GC) and Ultra performance liquid chromatography (UPLC) etc in almost all analytical laboratories and pharmaceutical companies. Owing to the advent of automation, small sample size and high sensitivity of the instrument, very accurate and precise assay and degradation products methods can be developed on chromatographic instruments with a considerable reduction in the total analysis time. Furthermore, application of techniques like photo diode array, fourier transform infrared spectroscopy and X-ray diffraction, etc, ensure the confirmation of the identity of individual components and ensure integrity and purity of the molecule. With these advancements in analytical techniques, the ability to develop methods with short run time and relatively simple sample procedure for simultaneous estimation of individual active components in a combination drug product is central to the role of analytical chemists. Normally, individual estimation of each of the drugs would have been time consuming, with no cost effectiveness and tedious in routine analysis. (Kapil 2010, Mark 2017, Wegscheider 1996, Breaux 2003, U.S. FDA 2000)

1.1.2 Impurity

1.1.2.1 Definition

An impurity as defined by the ICH (The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) guidelines is “any component of the medicinal product which is not the chemical entity defined as the active substance or an excipient in the product**”**. Chemically a compound is impure if it contains undesirable foreign matter i.e. impurities. Thus, chemical purity is freedom from foreign matter. It is virtually impossible to have absolutely pure chemical compounds and even analytically pure

chemical compounds contain minute trace of impurities. The chemical purity may be achieved as closely as desired provided sufficient care is observed at different levels in the manufacturing of a pharmaceutical. The level of purity of the pharmaceutical substance depends partly on the cost-effectiveness of the process employed, methods of purification, and stability of the final product. Setting higher standards of purity for pharmaceutical substances than that of desirable and pharmacologically safe level will unduly result in wastage of money, material, labour and time. Purification of chemical compounds is a very expensive process hence one has to strike a balance in order to obtain a pharmaceutical substance at reasonable cost yet sufficiently pure for all pharmaceutical purposes. (ICH-Q3B 2006, Neelima 2007, FDA-Q3A 2003, Lakshmana 2010, Sanjay 2007)

1.1.2.2 Sources of impurities in pharmaceutical substances

The origin of impurities in drugs is from various sources and phases of the synthetic process and preparation of pharmaceutical dosage forms. Majority of the impurities are characteristics of the synthetic route of the manufacturing process. There are several possibilities of synthesizing a drug; it is possible that the same product of different sources may give rise to different impurities. According to the ICH impurities are classified as organic impurities, inorganic impurities and residual solvents. Organic impurities may arise from starting materials, by products, synthetic intermediates and degradation products. Inorganic impurities may be derived from the manufacturing process and are normally known and identified as reagents, ligands, inorganic salts, heavy metals, catalysts, filter aids and charcoal, etc. Residual solvents are the impurities introduced with solvents. Of the above three types, the number of inorganic impurities and residual solvents are limited. These are easily identified and their physiological effects and toxicity are well known. For this reason the limits set by the pharmacopoeias and the ICH guidelines can guarantee that the harmful effects of these impurities do not

contribute to the toxicity or the side effects of the drug substances. The situation is different with the organic impurities. Drugs prepared by multi-step synthesis results in various impurities; their number and the variety of their structures are almost unlimited, highly dependent on the route, reaction conditions of the synthesis and several other factors, such as, the purity of the starting material, method of isolation, purification, conditions of storage, etc. In addition, toxicity is unknown or not easily predictable. For this reason the ICH guidelines set threshold limit above which the identification of the impurity is obligatory. (Usatinsky 2013, Grekas 2005, Qiu 2007)

1.1.3 Antiseptics

An antiseptic is a substance, which inhibits the growth and development of microorganisms. It may be either bacteriocidal or bacteriostatic. Their uses include cleansing of skin and wound surfaces after injury, preparation of skin surfaces prior to injections or surgical procedures, and routine disinfection of the oral cavity as part of a program of oral hygiene. Some commonly used antiseptics for skin cleaning includes chlorhexidine, iodine compounds, and alcohol.

Some antiseptics are true germicides, capable of destroying microbes (bacteriocidal), while others are bacteriostatic and only prevent or inhibit their growth.

Our skin is an essential barrier to warding off infection and disease. Healthy skin that may have bacteria, viruses, or fungi living on it can see rapid growth in these microorganisms when the skin is broken (e.g., scrape, burn, cut), possibly leading to serious infection or disease unless this growth is stopped. Antiseptics can be applied to the site to prevent infection until the injury can heal. (Noormah 2010, Asif 2008)

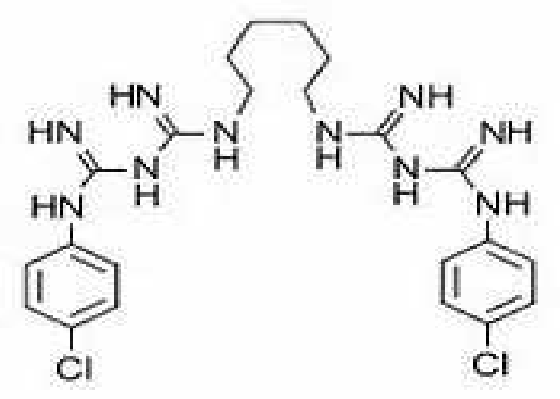
1.1.4 Disinfectants

Disinfectants are usually more caustic and concentrated than antiseptics and are therefore used on inanimate objects to kill pathogenic organisms. Not all disinfectants are antiseptics because an antiseptic additionally must not be so harsh that it damages living tissue. With this constraint imposed on antiseptics, in general, antiseptics are either not as cheap or not as effective at killing microbes as disinfectants.

Disinfectants do not necessarily kill all organisms but reduce them to a level, which does not harm health or the quality of perishable goods. Disinfectants are applied to inanimate objects and materials such as instruments and surfaces to control and prevent infection. Disinfectants are not safe for use on human skin especially substances with bleach or cleaning agent. (Noormah 2010, Asif 2008).

1.1.5 Chlorhexidine

Chlorhexidine [CHD; 1,1'-hexamethylenebis [5-(4-chlorophenyl) biguanide]] has a wide spectrum of bactericidal and antiviral activity and is a common ingredient in various formulations ranging from skin disinfectants in healthcare products to antiplaque agents in dentistry. The presence of two symmetrically positioned basic chlorophenyl guanide groups attached to a lipophilic hexamethylene chain (Figure 1.1) aids in rapid absorption through the outer bacterial cell wall, causing irreversible bacterial membrane injury, cytoplasmic leakage, and enzyme inhibition. This molecule exists as various forms of salts: diacetate, dihydrochloride, or digluconate, mainly differing by their solubilizing abilities in aqueous or oily media. CHD digluconate (or gluconate), as most soluble in water or alcohol, is the most used form in topical dermatology or cosmetic preparations.



(Figure 1.1 Chlorhexidine)

Aqueous solutions of CHD are most stable within the pH range of 5-8. Above pH 8.0 CHD base is precipitated and in more acid conditions there is gradual deterioration of activity because the compound is less stable. Chlorhexidine is a chemical antiseptic. It is effective on both gram-positive and gram-negative bacteria. It has both bactericidal and bacteriostatic mechanisms of action: the mechanism of action being membrane disruption, not ATPase inactivation as previously thought. It is also useful against fungi and enveloped viruses, though this has not been extensively investigated. Chlorhexidine is harmful in high concentrations, but is used safely in low concentrations in many products, such as mouthwash and contact lens solutions. By ionization, it produces positive ions.

Chlorhexidine is probably the most widely used biocide in antiseptic products, in particular in hand washing and oral products but also as a disinfectant and preservative. This is due in particular to its broad-spectrum efficacy, substantivity for the skin, and low irritation. Of note, irritability has been described and in many cases may be product specific. Despite the advantages of chlorhexidine, its activity is pH dependent and is greatly reduced in the presence of organic matter.

CHD is incompatible with inorganic anions in all but extremely dilute solutions. CHD is also incompatible with organic anions, such as soaps, sodium lauryl sulphate, sodium carboxymethyl cellulose, alginates, and many pharmaceutical

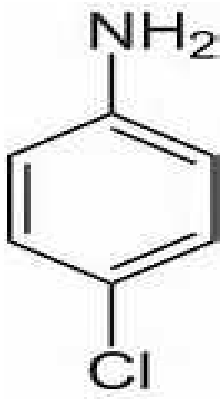
dyes. In certain instances, there will be no visible signs of incompatibility, but the antimicrobial activity may be significantly reduced because of the CHD being incorporated into micelles (ionic clusters). (Jenkins 1988, Decker 2008, ICH 2006)

1.1.6 p-Chloroaniline

Hydrolysis of chlorhexidine yields p-chloroaniline (pCA); the amount is insignificant at room temperature, but is increased by heating above 100°C, especially at alkaline pH. This cationic molecule (positively charged species) is thus generally compatible with other cationic materials, although compatibility will depend on the nature and relative concentration of the second cationic species. It is, however, possible for a reaction to occur between CHD and the counter-ion (anion) of a cationic molecule which is negatively charged, resulting in the formation of a less soluble CHD salt, which then may precipitate. pCA is very toxic if inhaled, swallowed or absorbed through the skin. It may act as a human carcinogen. It is readily absorbed through the skin and it may act as a sensitizer. However, as pCA is the principal product of degradation, and because of his toxicity and to be in line with actual recommendation for genotoxic impurities, it is important to quantify pCA in CHD solution. CHD and pCA can be determined using several methodologies such as high-performance liquid chromatography, gas chromatography-mass (GC-MS), fluorometry, UV spectroscopy and time-of-flight secondary ion mass spectrometry.

p-Chloroaniline is a colourless to slightly amber-coloured crystalline solid aniline derivative with a mild aromatic odour. It has the chemical formula C_6H_6ClN, and its relative molecular mass is 127.57. Its molecular structure is shown in Figure 1.2. Its IUPAC name is 1-amino-4-chlorobenzene; other names include pCA, p-chloroaniline, 1-chloro-4-aminobenzene, 4-chloro-1-aminobenzene, 4-chlorobenzenamine, 4-chloroaminobenzene, and 4-chlorophenylamine. Depending on the purity of the product, the substance melts between 69 and 73 °C. Its boiling

point is given as 232 °C. (Denton 2001, European Medicines Agency 2006, Hebert 2003, Tsuchiya 1999, Pesonen 1995, Cheung 1991, Zhu 2003, Zhang 1995, Lam 1993, Haand 1995, Middleton 2003, Gavlick 1992, Havlikova 2007, Below 2017, Antonio 2016, Alain 2011, Marco 2011, Barbin 2008, Matsushima 1982, Alder 1980, Read 1978, Gavlick 1994, Ono 1982, Barbin 2013, Bettina 2010, Vries 1991, Jensen 1971, Kamil 2014)



(Figure 1.2 p-Chloroaniline)

pCA is used as an intermediate in the production of several urea herbicides and insecticides (e.g., monuron, diflubenzuron, monolinuron), azo dyes and pigments (e.g., Acid Red 119:1, Pigment Red 184, Pigment Orange 44), and pharmaceutical and cosmetic products (e.g., chlorohexidine, triclocarban [3,4,4'-trichlorocarbanilid], 4-chlorophenol). In 1988, about 65% of the global annual production was processed to pesticides. In Germany, in 1990, about 7.5% was used as dye precursors, 20% as intermediates in the cosmetics industry, and 60% as pesticide intermediates. The use for the remaining 12.5% of the production quantity was not specified.

The pCA-based azo dyes and pigments are especially used for the dyeing and printing of textiles. Triclocarban is a bactericide in deodorant soaps, sticks, sprays,

and roll-ons, and chlorohexidine is used in mouthwashes and spray antiseptics. 4-Chlorophenol is also listed as an antimicrobial agent for cosmetic products in the European inventory of cosmetic ingredients. However, no information is available on the products in which it is used. All of these products may contain residual pCA, or pCA may emerge during their degradation.

1.1.7 Validation

1.1.7.1 Analytical method validation

It is necessary to assure that the performance characteristics of the developed analytical procedure meet the requirements for the intended analytical application. The procedure which provides assurance for the same quality of pharmaceutical product by means of laboratory studies is defined as method validation. Method validation is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, strength and quality, for the quantification of the drug substances and drug products. Method validation has received considerable attention in the literature and from industrial committees and regulatory agencies. The U.S. FDA CGMP states for validation for the test methods employed by the firm. The U.S. FDA has also proposed industry guidance for analytical procedures and methods validation. ISO/IEC 17025 includes a chapter on the validation of methods with list of validation parameters. The ICH has developed a consensus text on the validation of analytical procedures. ICH also developed guidance with detailed methodology. The US. EPA prepared guidance for method's development and validation for the Resource Conservation and Recovery Act (RCRA). The AOAC, the EPA and other scientific organizations provide methods that are validated through multi-laboratory studies. The USP has published specific

guidelines for method validation for compound evaluation. The WHO published validation guidelines under the title, Validation of analytical procedures used in the examination of pharmaceutical materials in the 32nd report of the WHO expert committee on specifications for pharmaceutical preparations. (U.S. FDA (title 21) 2016, U.S. FDA (draft) 2000, ISO/IEC 17025 2005, ICH Q2A 1996, ICH Q2B 1996, U.S. EPA 1995, USP. 1225 2007, Hokanson 1994, Green 1996, Wegscheider 1996, Seno 1997, Winslow 1997, AOAC 1998)

1.1.8 Method validation

Analytical characteristics used in method validation were discussed in the followings:

i. System suitability

System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being evaluated. In the case of chromatographic procedures, system suitability test is performed from five or six replicate injections of standard working solution. To be sure that the system is stable. The acceptance criteria for system suitability are as follows:

- Relative standard deviation for peak area of the six injections is not more than two (NMT 2).
- Resolution between peaks is not less than two (NLT 2).
- Tailing factors of peaks is not more than two (NMT 2).
- Theoretical plate for per column is not less than two thousand (NLT 2000).

ii. Linearity and range

The linearity of an analytical procedure is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the

concentration of analyte in samples within a given range. Thus, linearity refers to the linearity of the relationship of concentration and response signal (peak area). The goal is to have a model, whether linear or nonlinear, that describes closely the concentration-response relationship. Linearity should be established across the range of the analytical procedure. It should be established initially by visual examination of a plot of signals as a function of analyte concentration. If there appears to be a linear relationship, test results should be established by appropriate statistical methods (e.g., by calculation of a regression line by the method of least squares). Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity. The correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares should be submitted. The range of an analytical procedure is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated to be determined with a suitable level of precision, accuracy, and linearity using the procedure as written. The range is normally expressed in the same units as test results (e.g., percent, parts per million) obtained by the analytical procedure. The range of the procedure is validated by verifying that the analytical procedure provides acceptable precision, accuracy, and linearity when applied to samples. It is recommended that, for the establishment of linearity, a minimum of five concentrations normally be used. It is also recommended that the following minimum specified ranges should be considered: In case of assay of a drug substance (or a finished product): from 80% to 120% of the test concentration. For content uniformity: a minimum of 70% to 130% of the test concentration, unless a wider or more appropriate range. For dissolution testing: ±20% over the specified range (e.g., if the acceptance criteria for a controlled-release product cover a region from 30%, after 1 hour, and up to 90%, after 24 hours, the validated range would be 10% to 110% of the label claim).

iii. Detection limit and quantitation limit

a) Limit of detection

The detection limit is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. The detection limit is usually expressed as the concentration of analyte (e.g., percentage, parts per billion) in the sample. The detection limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected. In the case of procedures submitted for consideration as official compendial procedures, it is almost never necessary to determine the actual detection limit. In the case of instrumental analytical procedures that exhibit background noise, the Inernational Conference of Hharmonization documents describe a common approach, which is to compare measured signals from samples with known low concentrations of analyte with those of blank samples. The minimum concentration at which the analyte can reliably be detected is established. Typically acceptable signal to- noise ratios are 2:1 or 3:1. Other approaches depend on the determination of the slope of the calibration curve and the standard deviation of responses, which is the method applied in this study.

Limit of detection = 3(SD/S)

Root-Mean-Square Error (RMSE) ≡ SD = the standard deviation of the response signal from regression line

S ≡ slope from linear regression analysis

b) Limit of quantiation

The quantitation limit is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. The quantitation limit is expressed as the concentration of analyte (e.g., percentage, parts per billion) in the sample. It is generally determined by the

analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be determined with acceptable accuracy and precision. In the case of procedures submitted for consideration as official compendial procedures, it is almost never necessary to determine the actual quantitation limit. Rather, the quantitation limit is shown to be sufficiently low by the analysis of samples with known concentrations of analyte. In the case of instrumental analytical procedures that exhibit background noise, the Inernational Conference of Harmonization documents describe a common approach, which is to compare measured signals from samples with known low concentrations of analyte with those of blank samples. The minimum concentration at which the analyte can reliably be quantified is established. A typically acceptable signal to noise ratio is 10:1. Other approaches depend on the determination of the slope of the calibration curve and the standard deviation of responses, which is the method applied in this study.

Limit of Quantification = 10 (SD/S)

Root-Mean-Square Error (RMSE) ≡ SD = the standard deviation of the response signal from regression line

S ≡ slope from linear regression analysis

iv. Specificity

Is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and excipients. [Note—Other reputable international authorities such as International Union of Pure and Applied Chemistry (IUPAC) and Association of Official Analytical Chemists International (AOAC), they preferred the term selectivity]. For assay, it has to provide an exact result, which allows an accurate statement on the content or potency of the analyte in a sample. In the case of the assay, demonstration of specificity requires that it can be shown that the procedure is

unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substance or product with appropriate levels of impurities or excipients (placebo) and demonstrating that the assay result is unaffected by the presence of these excipients. When chromatographic procedures are used, representative chromatograms should be presented to demonstrate the degree of selectivity, and peaks should be appropriately labeled.

v. Accuracy

The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value. The accuracy of an analytical procedure should be established across its range. In the documents of the ISO, its termed trueness. Accuracy may be determined by application of the analytical procedure to an analyte of known purity (e.g., a Reference Standard) or by comparison of the results of the procedure with those of a second, well-characterized procedure, the accuracy of which has been stated or defined. In the case of the assay of a drug in a formulated product, accuracy may be determined by application of the analytical procedure to synthetic mixtures of the drug product components to which known amounts of analyte have been added within the range of the procedure. If it is not possible to obtain samples of all drug product components, it may be acceptable either to add known quantities of the analyte to the drug product (i.e., “to spike”) or to compare results with those of a second, well characterized procedure, the accuracy of which has been stated or defined. Accuracy is calculated as the percentage of recovery by the assay of the known added amount of analyte in the sample, or as the difference between the mean and the accepted true value, together with confidence intervals. Accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels, covering the

specified range (i.e., three concentrations and three replicates of each concentration). Assessment of accuracy can be accomplished in a variety of ways, including evaluating the recovery of the analyte (percent recovery) across the range of the assay, or evaluating the linearity of the relationship between estimated and actual concentrations. The statistically preferred criterion is that the confidence interval for the slope be contained in an interval around 1.0, (not less than 0.997).

vi. Precision (Repeatability and/or Reproducibility)

The precision of an analytical procedure is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. The precision of an analytical procedure is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements. Precision may be a measure of either the degree of reproducibility or of repeatability of the analytical procedure under normal operating conditions. In this context, reproducibility refers to the use of the analytical procedure in different laboratories, as in a collaborative study. Intermediate precision (also known as ruggedness) expresses within-laboratory variation, as on different days, or with different analysts or equipment within the same laboratory. Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time using the same analyst with the same equipment. The precision of an analytical procedure is determined by assaying a sufficient number of aliquots of a homogeneous sample to be able to calculate statistically valid estimates of standard deviation or relative standard deviation (coefficient of variation). Assays in this context are independent analyses of samples that have been carried through the complete analytical procedure from sample preparation to final test result. It is recommend that repeatability should be

assessed using a minimum of nine determinations covering the specified range for the procedure (i.e., three concentrations and three replicates of each concentration) or using a minimum of six determinations at 100% of the test concentration.

vii. Robustness

Robustness is a measure of the performance of a method when small, deliberate changes are made to the method conditions, these should be suitably controlled, or a precautionary statement should be included in the procedure to ensure that the validity of the analytical procedure is maintained. Typical variations are the pH of the mobile phase, the mobile phase composition, different lots or suppliers of columns, the temperature, and the flow rate. (Stephan 2002, European Medicines Agency 1995, Feinberg 2007, United Nations Office on Drugs and Crime 2009, CIPAC 2003, Zoonen 1999, Fajgelj 2000, ICH 1994, Gustavo 2007, FDA 2015).

1.2 Literature review

British Pharmacopeia (2009) has stated a method for determination of p-Chloroaniline, which has been carried out by using gas chromatography. In the preparation of analytical samples, the method has applied heptafluorobutyric anhydride. Heptafluorobutyric anhydride has the following Potential Acute Health Effects: Hazardous in case of skin contact (corrosive, irritant), of eye contact (irritant), of ingestion, of inhalation. Liquid or spray mist may produce tissue damage particularly on mucous membranes of eyes, mouth and respiratory tract. Skin contact may produce burns. Inhalation of the spray mist may produce severe irritation of respiratory tract, characterized by coughing, choking, or shortness of breath.

United State Pharmacopeia (2016) has stated a method for determination of p-Chloroaniline, which has been carried out by using HPLC chromatography.

The chromatographic procedure was carried out using gradient elution techniques, which is very expensive and quite difficult.

Alain Nicolay et al. (2011) described an isocratic reversed-phase (RP) high-performance liquid chromatography (HPLC) method. The method was developed and validated for the simultaneous determination of chlorhexidine (CHD) and p-

Chloroaniline (pCA) in various pharmaceutical formulations. Compound separation was achieved in less than 10 min with an XBridge C18 column that was maintained at 40°C and a mobile phase consisting of 32:68 (v/v) of acetonitrile and a pH 3.0 phosphate buffer solution (a 0.05 M monobasic sodium phosphate solution containing 0.2% of triethylamine). Analyses were performed at a flow rate of 2 mL min−1 and at a detection wavelength of 239 nm.

Gavlick (1992) described a high-performance liquid chromatographic method for the separation of chlorhexidine and its known degradation product, p-Chloroaniline. These amine-containing compounds could be separated without the addition of ion-pairing reagents and/or amine modifiers if the proper specialty column was selected. A photodiode-array detector was used to acquire spectral data and demonstrate the importance of the mobile phase pH when optimizing the response of p-Chloroaniline.

Paulson (1993) described a method to quantify simultaneously chlorhexidine (CHD) and its major metabolite, para chloroaniline (pCA) was described by HPLC with UV detection without the additional need of mobile-phase amine modifiers or ion-pairing reagents. HPLC-UV analyses were performed using a Dionex® Summit liquid chromatograph (Dionex Corp, Sunnyvale, CA, USA). Chromatographic separations were carried out on a Luna® 150 mm×3 mm i.d. column packed with 3 µm CN (cyano) particles (Phenomenex®), guarded by an on-line filter. Mobile phase consisted of methanol: water with sodium chloride with 0.02% of formic acid (55:45). Wavelengths for pCA and for CHD were 238 and 255 nm respectively. Linearity of CHD was very good, from 0.5 up to 21.2 µg/l while linearity of pCA was in the range of 0.05 to 10 µg/l with correlation coefficients above 0.999. Resolution between the components was above 4,

asymmetry was about 1.3 and 1.7 for pCA and CHD respectively, and the run time was less than 5 minutes.

Marcus et al. (1984) described a high-performance liquid chromatographic method for the determination of the common antiseptic chlorhexidine in urine. The method employed Sep-Pak cartridges to remove chlorhexidine from the urine matrix. Chromatographic separation was achieved on a C18 reversed-phase column using a mobile phase of methanol-20 mM sodium acetate solution (60:40) adjusted to pH 5 with glacial acetic acid. An ion-pair agent (pentadecafluorooctanoic acid) was used at a concentration of 100 μ ml^{-1}. 3-Bromobenzophenone was used as chromatographic standard (k' = 4.0). 4-Bromobenzophenone (k' = 3.9) or dibenzal hydrazine (k' = 4.4) might also be used. A series of urine samples was analysed and no interferences were observed. The method was simple and rapid with a total analysis time of ca. 30 min.

Thomas et al. (2008) described a study to (1) establish a method for quantification of chlorhexidine (CHD) in small volumes and (2) to determine CHD release from differently concentrated CHD-containing preparations, varnishes, and a CHD gel applied on artificial fissures. CHD determination was conducted in a microplate reader using polystyrene wells. The reduced intensity of fluorescence of the microplates was used for CHD quantification. For verification of the technique, intra- and inter-assay coefficients of variation were calculated for graded series of CHD concentrations, and the lower limit of quantification (LLOQ) was determined. Additionally, artificial fissures were prepared in 50 bovine enamel samples, divided into five groups (A–E, n = 10) and stored in distilled water (7 days); *A*: CHD-varnish EC40; B: CHD-varnish Cervitec; C: CHD-gel Chlorhexamed; D: negative control, no CHD application; and E: CHD-diacetate standard (E1, n = 5) or CHD-digluconate (E2, n = 5) in the solution. The specimens

were brushed daily, and CHD in the solution was measured. The method showed intra- and inter-assay coefficients of variation of <10 and <20%, respectively; LLOQ was 0.91–1.22 nmol/well. The cumulative CHD release (mean ± SD) during the 7 days was: EC40 (217.2 ± 41.8 nmol), CHD-gel (31.3 ± 8.5 nmol), Cervitec (18.6 ± 1.7 nmol). Groups A–C revealed a significantly higher CHD release than group D and a continuous CHX-release with the highest increase from day 0 to 7 for EC40 and the lowest for Chlorhexamed. The new method was a reliable tool to quantify CHD in small volumes. Both tested varnishes demonstrated prolonged and higher CHD release from artificial fissures than the CHD-gel tested.

Havlikova et al. (2007) described an isocratic reversed-phase HPLC method for simultaneous determination of chlorhexidine and its degradation product p-chloroaniline was developed. Zorbax SB Phenyl column (75 mm x 4.6 mm, 3.5 microm) was used for the separation. Mobile phase composed of acetonitrile and buffer solution of 0.08 M sodium phosphate monobasic containing 5 ml of triethylamine (0.5%) and adjusted with 85% phosphoric acid to pH 3.0 in ratio 35:65 (v/v) pumped isocratically at flow rate 0.6 ml $min^{(-1)}$ was used. UV detection was performed at 239 nm, the total analysis time was about 10 min.

Dave et al. (2012) described a reverse phase high performance liquid chromatographic method is for the simultaneous determination of Chlorhexidine Hydrochloride and Triamcinolone in Lozenges. Reverse phase chromatography was developed using Waters symmetry C18 column (250 X4.6 mm) with 5 µm particle size monitored at 254nm with a mobile phase MeOH: Water: Glacial acetic acid (75:25:10) used with ion pair reagent Octane-1-sulfonic acid sodium salt (0.2 gm).The method was validated with the range of 25-125µg/ml and 5-25 µg/ml and correlation coefficients were found to be 0.997 and 0.999 for Chlorhexidine Hydrochloride and Triamcinolone, respectively. Recovery studies

showed good results: 98.93% for chlorhexidine hydrochloride and 99.95% for triamcinolone. Coefficients of variation for precision ranging from 0.14% to 1.32% and 0.15% to 0.67% for chlorhexidine hydrochloride and triamcinolone, respectively.

Yuying et al. (2009) described the extraction and analysis of chlorhexidine (CHD) from whole blood using solid-phase extraction (SPE) together with high-performance liquid chromatography (HPLC). Blood samples, spiked with chlorpromazine used as an internal standard, were fortified with sodium acetate buffer and purified with Bakerbond C18 SPE columns. The columns were washed, dried, and eluted with experimental optimized solvent systems. The HPLC was performed using a Capcell Pak C18 MG column (4.6 × 250-mm) and monitored at 260 nm, using a UVdetector. Amobile phase consisting of acetonitrile: water (40:60 v/v), containing 0.05% trifluoroacetic acid, 0.05% heptafluorobutyric acid, and 0.1% triethylamine, was employed. The assay was linear over the range of 0.05 to 2.0 µg/g and the limit of detection was 0.01 µg/g for CHD in whole blood. At the concentration range of 0.05 to 2.0 µg/g, the recoveries ranged from 72% to 85%, and the intra- and interday precision, expressed as coefficient of variation, were less than 11% and 13%, respectively.

Fresenius (1997) described a titrimetric and spectrophotometric methods for the determination of chlorhexidine digluconate (CHD). The titrimetric determination is based on the precipitation of CHD as a 1: 1 complex with Cu^{2+}-ions and EDTA back-titration of the non-bonded Cu^{2+}-ions without separating the precipitate. The spectrophotometric determination is based on the formation of a soluble CHD associate with dodecylsulphate (DDS) in a mixed medium of DDS-H_2SO_4-propanol. Both methods are applied to tooth pastes. When analysing a series of identical samples, the coefficient K (absorption of 1 g of the matrix) could be determined. Standard tooth pastes and corresponding placebo-compositions were

specially prepared for the investigations and for estimating the accuracy of the methods.

Gavlick and Davis (1994) described a gas chromatographic (GC) method with flame ionization detection to separate and quantitate p-chloroaniline (pCA) from other components in a chlorhexidine digluconate (CHD)-containing alcohol foam surgical scrub product. A simple sample preparation method was developed in which 1-butanol was used to dissolve the foam and precipitate the CHD, which otherwise would interfere with the GC analysis. The method was validated with respect to linear dynamic range, precision, accuracy, selectivity, limit of detection, and limit of quantitation.

Perez (1981) described a method for determining in the parts-per-million range the 4-chloroaniline content of chlorhexidine solutions. Neither cetrimide, tartrazine, methylene blue, nor carmoisine which are commonly added to chlorhexidine solutions interfere with the method presented, which takes approximately 10 min to perform. The method involveed an ion-pairing, reversed-phase high pressure liquid chromatographic (HPLC) technique and ultraviolet (UV) detection at 260 nm.

Antonio et al. (2016) described a method to determine *p*-chloroaniline (pCA) in gel, 2 % aqueous solutions, and 0.12 % oral rinse formulas of chlorhexidine digluconate (CHD) used in dentistry treatments. The method was appropriate for ensuring that these products are in accordance with current legislation. Furthermore, the precipitate formed when 2 % CHD was added to sodium hypochlorite was investigated to verify whether this mixture forms pCA. To quantify pCA, liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) was used and the *m*/*z* ratio of 127.9/93.0 and 127.9/111.0 were used as qualifier and quantifier transitions, respectively. The LC separation, using a C18 column proved highly efficient for pCA and its isomers, i.e., *m*-chloroaniline and

o-chloroaniline. Multiple reaction monitoring provided the proper selectivity and specificity for the method. Commercial aqueous solutions, gels, and oral rinses containing CHD were analyzed, and their pCA contents complied with those recommended by the European and United States Pharmacopeias. The method was also able to detect pCA in the precipitate and its concentration is below 0.1 %.

1.3 Objectives

The main objective of this research work is to develop and validate a method for simultenous determination of an API and its degradation product using, simple common instruments, like HPLC-UV chromatographs, which are available in most laboratories. In addition, high-performance liquid chromatography (HPLC) is the most remarkable development and the technique has become very significant in the quality control of drugs and pharmaceutical formulations.

The specific objective of this study is to develop and validate an HPLC method for simultenous determination of Chlorhexidine and its degradation product p-Chloroaniline. The developed method should be simple, precise, accurate, stability-indicating and selective. The intension is also to use, in this liquid chromatographic method, the simple isocratic elution instead of the more complex gradient elution.

Chapter Two

Materials, Methods and Results

2. Material, Method and Results

2.1 Chemicals

- Chlorhexidine STD (Unilab Pharmaceutical - India)
- p-Chloroaniline STD (Unilab Pharmaceutical - India)
- Formulated Products (Yamani Medical Products - Sudan)
- All excipients were obtained from Unilab Pharmaceutical - India
- Methanol, HPLC Grade (Scharlau)
- Acetic acid, HPLC Grade (Scharlau)
- Purified Water

2.2 Instruments

The HPLC-UV system consisted of analytical apparatus (Analytical Technologies Limited Corporation, Mumbai, India) with a P2230 pump, Sr No P2304051, UV2230 UV-Vis detector, Sr No U2304633. This system was connected to a

computer loaded with A2000-Solutions software. A C18 column (200 mm x 4.6 mm, I.D. 3 µm) was used.

2.3 Glassware and Apparatus

- 50-ml volumetric flask
- 100-ml volumetric flask
- 250-ml volumetric flask
- 10-ml graduated pipette
- Glass funnel
- Aluminium foil
- Buchner system
- Syringe (polypropylene)
- Syringe filter (nylon, 0.22micrometer porous)
- Nylon membrane filter 0.45micrometer porous.

2.4 Procedures and Results

2.4.1 Optimized chromatographic conditions

A C18 column (200 mm x 4.6 mm, I.D. 3 µm), for simple isocratic elusion, was used (one pump required) with flow-rate of 1.0 ml/min; both ingredients were detected at 254 nm; injection volume was 20µl (universal loop), and analysis temperature was 25ºC (ambient temperature).

2.4.2 Buffer

1000-ml volumetric flask was half filled with deionised water; 8.2038 g of sodium acetate was added and completely dissolved; then 50 ml of acetic acid was added to the flask, and the volume was completed to the mark with deionised water.

2.4.3 Mobile Phase

Mixture of methanol and acetate buffer was prepared in 55:45 ratio, respectively. The mixture was shaken, filtered with vacuum filtration pump

through 0.45µm nylon membrane filter, and then transferred to solvent reservoir and sonicated for 5 min.

2.4.4 Standard Stock Solution

To prepare stock solutions, 0.0075 g of pCA was weighed accurately and transferred quantitatively to 25-ml volumetric solution, the flask was half-filled with the mobile phase and sonicated for 10 minutes, cooled to room temperature; then the volume was completed to the mark with the same solvent. 1.0 ml of this solution transferred to a 100-ml volumetric flask containing 0.1000 g of CHD previously weighed accurately. The flask was half-filled with the mobile phase and sonicated for 10 minutes, cooled to room temperature; then the volume was completed to the mark with the same solvent.

2.4.5 System Suitability

Subsequent dilutions were made from the stock solution with the mobile phase to make solutions with 100 µg/ml of CHD and 0.3 µg/ml of pCA. The resulting solution was filtered through a 0.45 µm membrane nylon filter. System suitability solution was injected six times.

Table 2.1 and Table 2.2 show System suitability results for CHD and pCA respectively.

Table 2.1 System suitability results for CHD

	Area	Retention time	Resolution	Theoretical plate	Asymmetry factor
STD 1	5659567	5.18	4.71	13420	1.13
STD 2	5651240	5.18	4.71	13619	1.14
STD 3	5656282	5.17	4.69	13580	1.13
STD 4	5650005	5.17	4.69	13576	1.13
STD 5	5660625	5.17	4.68	13576	1.12

STD 6	5648577	5.16	4.65	13516	1.14
Average	5654383	5.171666667	4.68833333	13547.83333	1.131666667
STDEV	5141.937	0.007527727	0.02228602	70.76840161	0.007527727
RSD	0.090937	0.145557071	0.47535058	0.522359553	0.665189384

Table 2.2 System suitability results for pCA

	Area	Retention time	Resolution	Theoretical plate	Asymmetry factor
STD 1	46534	3.77	4.71	15760	1.18
STD 2	46519	3.78	4.71	15801	1.09
STD 3	46500	3.78	4.69	15994	1.1
STD 4	45611	3.78	4.69	16001	1.14
STD 5	46566	3.78	4.68	16008	1.12
STD 6	45688	3.78	4.65	15994	1.11
Average	46236.33	3.778333333	4.68833333	15926.33333	1.123333333
STDEV	455.723	0.004082483	0.02228602	113.8220834	0.032659863
RSD	0.985638	0.108049834	0.47535058	0.71467852	2.907406223

2.4.6

Linearity, limt of detection and limit of quantitation

Subsequent dilutions were made from the stock solution with mobile phase to give concentrations of 20, 40, 60, 80, 100, 120, 140 and 160 µg/ml CHD solutions and 0.3, 0.45, 0.6, 0.75, 0.9, 1.05 and 1.2 µg/ml pCA. Each solution was injected three times and results were collected; LOD and LOQ were calculated from the linear regression analysis according to ICH guidelines.

i) Chlorhexidine

Table 2.3 shows linearity results for CHD which then treated by XLSTAT-2016 program to predict linearity data that shown in Table 2.4, Table 2.5, Figure 2.1 and Figure 2.2.

Table 2.3 linearity result for CHD

µg/ml	40	60	80	100	120	140	160
1	2251910	3389419	4485536	5644243	6778806	7842425	9050787
2	2281477	3385917	4465321	5654360	6789928	7897321	9060847
3	2253076	3387766	4497874	5657822	6793164	7934013	9042708
Avg	2262154	3387701	4482910	5652142	6787299	7891253	9051447

Figure 2.1 shows the plot of average area versus concentrations for CHD in µg/ml, the linear regression equation:

$$\text{Area} = -12044.167 + 56570.308 \times \mu g/ml$$

According to ICH guidelines, acceptance criteria is $R2 \geq 0.997$.

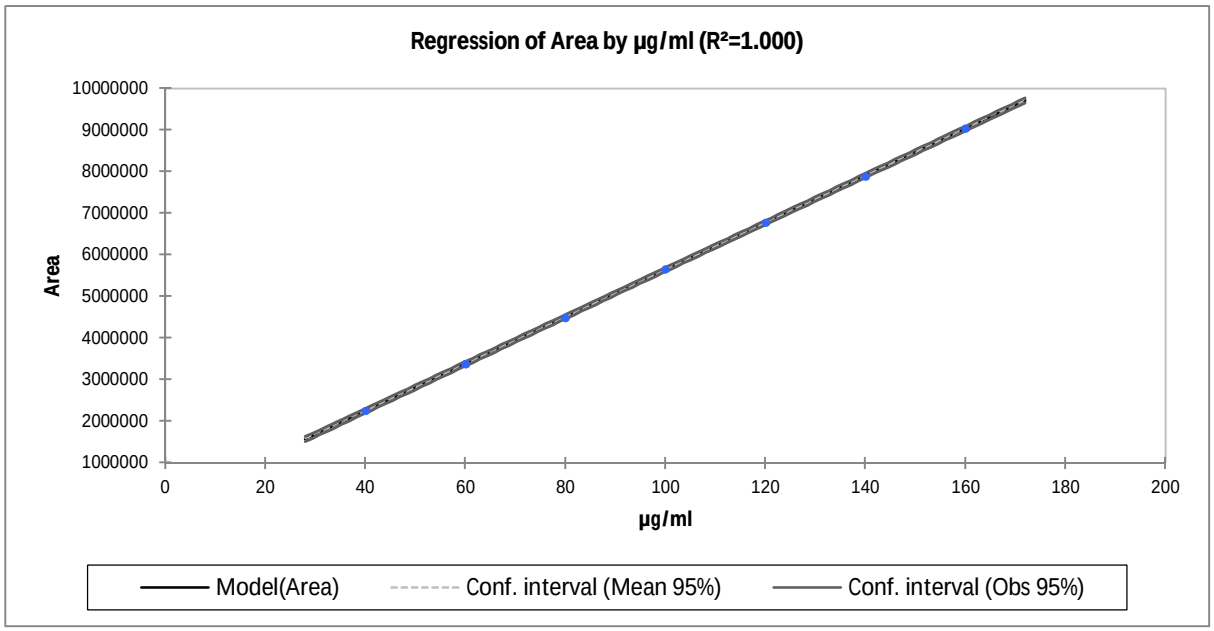


Figure 2.1 XL- STAT 2016 Graph of conc. in µg/ml Vs average area of CHD

Table 2.4 XL- STAT 2016 Goodness of fit statistics for CHD

Observations	7.000
Sum of weights	7.000

R^2	1.000
Adjusted R^2	1.000
MSE	338926160.554
RMSE	18409.947

Table 2.5 XL STAT 2016 predicted area for CHD

Observation	Weight	µg/ml	Area	Pred(Area)
Obs1	1	40.000	2262154.333	2250768.167
Obs2	1	60.000	3387700.667	3382174.333
Obs3	1	80.000	4482910.333	4513580.500
Obs4	1	100.000	5652141.667	5644986.667
Obs5	1	120.000	6787299.333	6776392.833
Obs6	1	140.000	7891253.000	7907799.000
Obs7	1	160.000	9051447.333	9039205.167

Figure 2.2 is a plot of average area versus predicted area for CHD, i.e. concentration Vs predicted concentration of CHD, acceptance limit for this graph is that slope ≥ 0.997

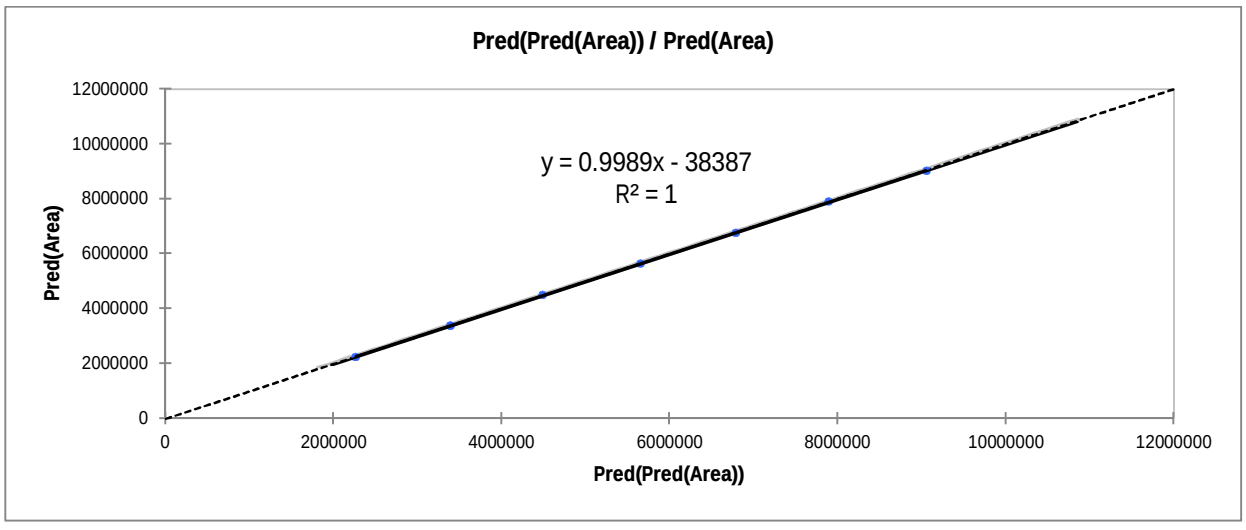


Figure 2.2 XL- STAT 2016 Graph of (area) Vs (Predicted area) for CHD

Limit of detection and limit of quantitation

LOD = 3.3 x (SD/S).

LOD = 3.3 x (18409/56570) =

LOD = **1.073885 µg/ml**

Percentage =1.073885x100/100 = 1.07 %

LOQ = 10 x (SD/S).

LOQ = 10 x (18409/56570)

LOQ = **3.254198 µg/ml**

Percentage =3.254198 x 100/100 = 3.25%

ii) Chloroaniline

Table 2.6 shows linearity results for pCA which then treated by XLSTAT-2016 program to predict linearity data that shown in Table 2.7, Table 2.8, Figure 2.3 and Figure 2.4.

Table 2.6 linearity result for pCA

µg/ml	0.3	0.45	0.6	0.75	0.9	1.05	1.2
1	18613	27924	37022	46516	56388	65242	74486
2	18605	27939	37061	45737	55253	65741	74491
3	18594	27935	37028	47122	55585	62456	74329
Avg	18604	27932.67	37037	46458.33	55742	64479.67	74435.33

Figure 2.3 shows the plot of average area versus concentrations for valsartan in µg/ml, the linear regression equation:

$$\text{Area} = 81.821 + 61736.429 \times \mu g/ml$$

According to ICH guidelines, acceptance criteria is $R^2 \geq 0.997$.

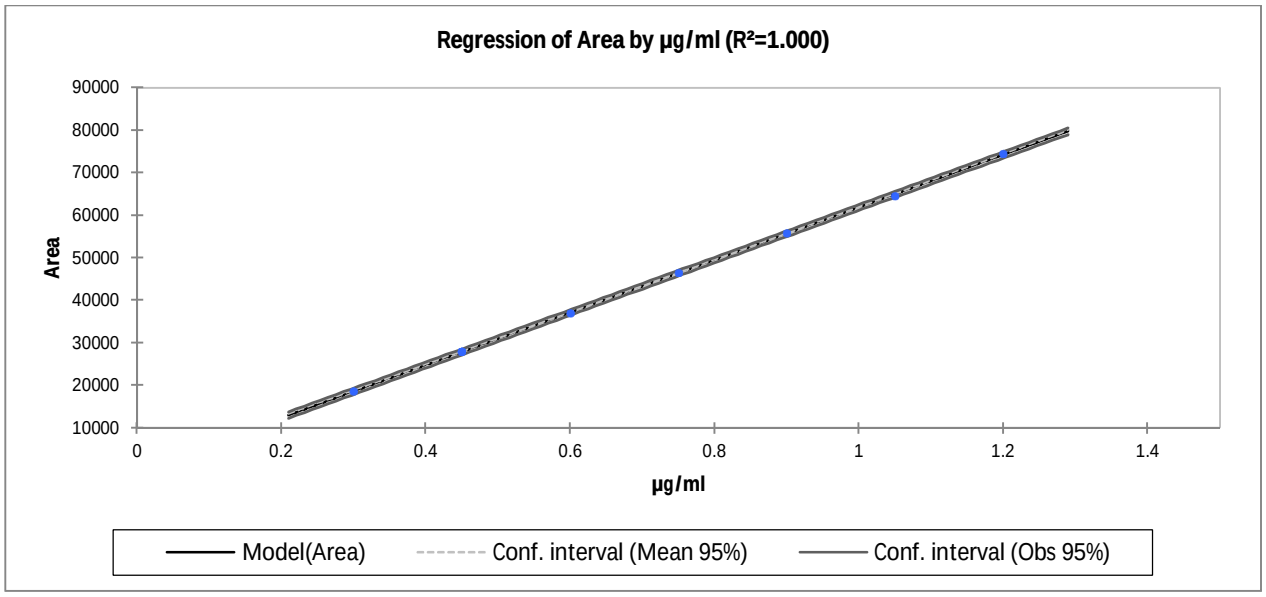


Figure 2.3 XL- STAT 2016 Graph of conc. in µg/ml Vs average area of pCA

Table 2.7 XL- STAT 2016 Goodness of fit statistics of pCA

Observations	7.000
Sum of weights	7.000
R^2	1.000
Adjusted R^2	1.000
MSE	56217.587
RMSE	237.102

Table 2.8 XL- STAT 2016 predicted area for pCA

Observation	Weight	µg/ml	Area	Pred(Area)
Obs1	1	0.300	18604.000	18602.750
Obs2	1	0.450	27932.667	27863.214
Obs3	1	0.600	37037.000	37123.679
Obs4	1	0.750	46458.333	46384.143
Obs5	1	0.900	55742.000	55644.607
Obs6	1	1.050	64479.667	64905.071
Obs7	1	1.200	74435.333	74165.536

Figure 2.4 is the plot of average area versus predicted area for pCA, i.e. concentration versus predicted concentration of pCA, acceptance limit for this graph is that slope ≥ 0.997

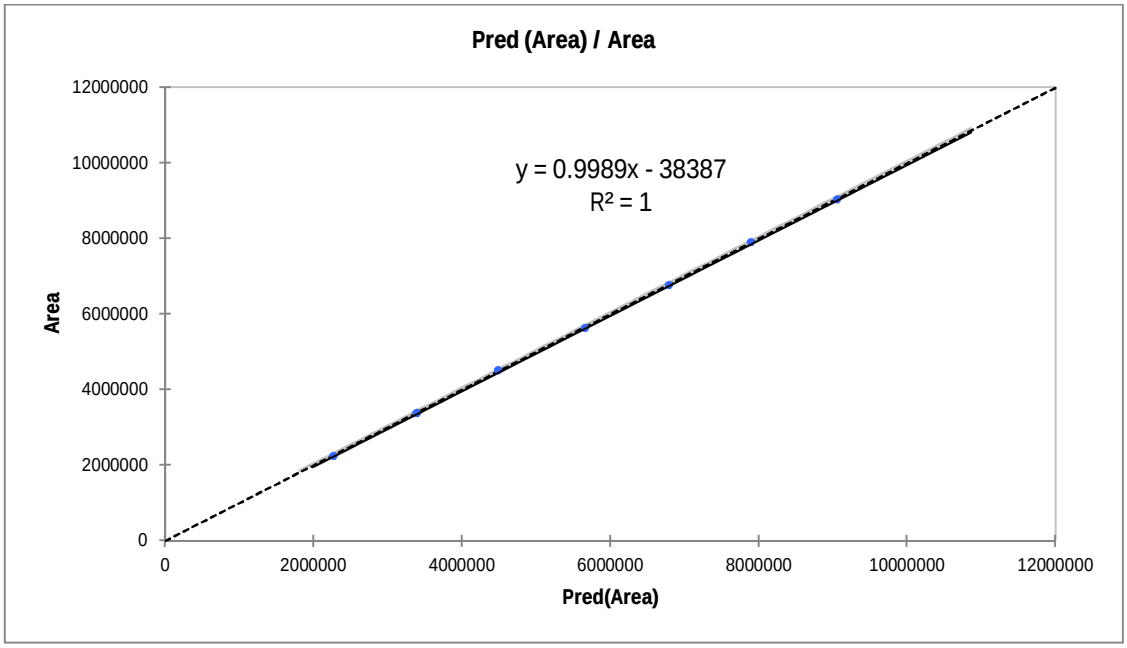


Figure 2.4 XL- STAT 2016 Graph of (area) versus (Predicted area) for pCA

Limit of detection and limit of quantitation

LOD = 3.3 x (SD/S).

LOD = 3.3 x (237/61736) =

LOD = **0.012668 µg/ml**

LOQ = 10 x (SD/S).

LOQ = 10 x ((237/61736)

LOQ = **0.038389µg/ml**

2.4.7 Specificity

(a) Standard

Subsequent dilutions were made from the stock solution with the mobile phase to make solutions with 100 µg/ml of CHD and 0.3 µg/ml of pCA. The resulting solution was filtered through a 0.45 µm membrane nylon filter. This solution was injected six times.

(b) Placebo

A placebo equivalent to average of 100 ml solution was transferred to 100-ml volumetric flask. The flask was half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature, and then the volume was completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase similar to those made for standard preparation.

(c) Sample

A placebo equivalent to average of 100 ml solution was transferred to 100-ml volumetric flask; 0.01 g of CHD and 0.03 g of pCA were weighed accurately and transferred quantitatively to the same flask which was then half filled with mobile phase, sonicated for 10 minutes, cooled at room temperature, and then the volume was completed to the mark with the same solvent.

Figure 2.5, Figure 2.6 and Figure 2.7 shows the specificity chromatograms for placebo, sample and standard respectively for CHD and pCA.

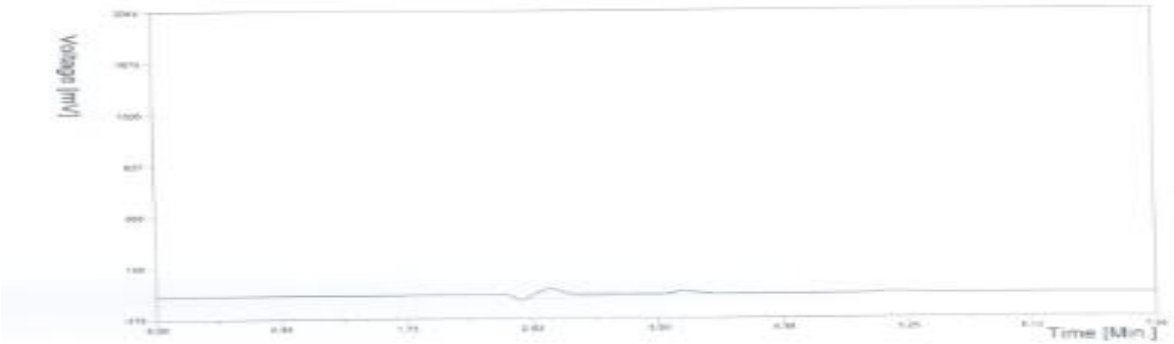


Figure 2.5 chromatogram for the Placebo of CHD and pCA

Figure 2.6 chromatogram for the sample of CHD and pCA

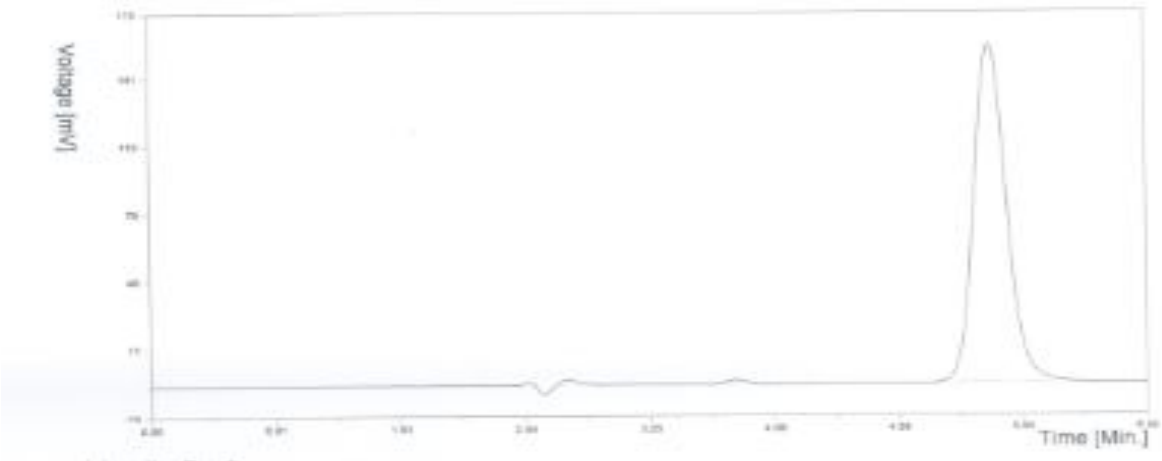


Figure 2.7 chromatogram for mixed standard of CHD and pCA

2.4.8 Accuracy

(a) Standard

Subsequent dilutions were made from the stock solution with the mobile phase to make solutions with 100 µg/ml of CHD and 0.3 µg/ml of pCA. The resulting solution was filtered through a 0.45 µm membrane nylon filter. This solution was injected six times.

(b) Samples

Seven 100-ml volumetric flasks were labeled, and the placebo equivalent to a 100 ml solution was transferred to a different flask. The volume of the mixed standard stock solution required to produce 40%, 60%, 80%, 100%, 120%, 140% and 160% of the target concentration of both CHD and pCA was added to each to different flasks. The flasks were half-filled with the mobile phase, sonicated for 10 minutes, cooled to room temperature, and then completed to the mark with the same solvent. Subsequent dilutions were made with the mobile phase in the same manner as the standard preparation. Each solution was injected three times.

The results were collected and subjected to statistical treatments.

Table 2.9 shows the results of mixed standard of CHD and pCA, while the accuracy results for CHD and pCA samples are shown in Table 2.10 and Table 2.11, respectively; summary of accuracy results for both components is shown in Table 2.12.

Table 2.9 Results of CHD and pCA standard for accuracy test

No	CHD	pCA
STD1	5659567	46534
SDT2	5651240	46519
STD3	5656282	46500
STD4	5650005	45611
STD5	5660625	46566
STD6	5646912	45688
Avg	5654105.167	46236.33
STDEV	5546.902682	455.723
RSD	0.098103988	0.985638

Table 2.10 Accuracy results for CHD

µg/ml	40	60	80	100	120	140	160
%	40	60	80	100	120	140	160
Trial 1	2251910	3389419	4485536	5644243	6778806	7842425	9050787
Trial 2	2281477	3385917	4465321	5654360	6789928	7897321	9060847
Trial 3	2253076	3387766	4497874	5657822	6793164	7934013	9042708
Age	2262154	3387701	4482910	5652142	6787299	7891253	9051447
STDEV	16744.07	1751.914	16434.57	7056.066	7531.299	46094.53	9087.511
RSD	0.740183	0.051714	0.366605	0.124839	0.110962	0.584122	0.100398
Recovery	40.00906	59.91577	79.28594	99.96527	120.042	139.5668	160.0863
Recovery %	100.0227	99.85962	99.10742	99.96527	100.035	99.69056	100.0539

Table 2.11 Accuracy results for pCA

µg/ml	0.3	0.45	0.6	0.75	0.9	1.05	1.2
%	40	60	80	100	120	140	160
Trial 1	18613	27924	37022	46516	56388	65242	74486
Trial 2	18605	27939	37061	45737	55253	65741	74491
Trial 3	18594	27935	37028	47122	55585	62456	74329
Age	18604	27932.67	37037	46458.33	55742	64479.67	74435.33
STDEV	9.539392	7.767453	21	694.2984	583.5606	1770.218	92.1213
RSD	0.051276	0.027808	0.0567	1.494454	1.046896	2.745389	0.12376
Recovery	40.23675	60.41281	80.10367	100.4801	120.5589	139.4567	160.9888
Recovery %	100.5919	100.688	100.1296	100.4801	100.4657	99.61193	100.618

Table 2.12 Summary of accuracy results for CHD and pCA

Amount added%	CHD		pCA	
	Recovery	Recovery%	Recovery	Recovery%
40	40.00906	100.0227	40.23675	100.5919
60	59.91577	99.85962	60.41281	100.688
80	79.28594	99.10742	80.10367	100.1296
100	99.96527	99.96527	100.4801	100.4801
120	120.042	100.035	120.5589	100.4657
140	139.5668	99.69056	139.4567	99.61193
160	160.0863	100.0539	160.9888	100.618
Av		99.81921		100.36932
STDEV		0.3389236		0.3798261
RSD		0.3395374		0.3784285

2.4.9 Precision

(a) Precision standard

Subsequent dilutions were made from the stock solution with the mobile phase to make solutions with 100 µg/ml of CHD and 0.3 µg/ml of pCA. The resulting solution was filtered through a 0.45 µm membrane nylon filter. This solution was injected six times.

(b) Precision samples

Three 25-ml volumetric flasks were labeled, and the placebo equivalent to target concentration was transferred to each flask. The volume of the standard stock solution required to produce 80%, 100% and 120% of the product content of both CHD and pCA was added. The flasks were half-filled with the mobile phase, sonicated for 10 minutes, cooled at room temperature and completed to the mark with the same solvent.

i) Intraday Precision

Table 2.13 shows results of CHD and pCA mixed standard for intraday precision test.

Table 2.13 CHD and pCA mixed standard for intraday precision

No.	CHD	pCA
STD1	5659567	46534
SDT2	5651240	46519
STD3	5656282	46500
STD4	5650005	45611
STD5	5660625	46566
STD6	5646912	45688
Avg	5654105	46236.33
STDEV	5546.903	455.723
RSD	0.098104	0.985638

Tables numbered 2.14, 2.15 and 2.16 show intraday precision for 80%, 100% and 120% of CHD, respectively, while tables numbered 2.17, 2.18 and 2.19 show intraday precision for 80%, 100% and 120% of pCA, respectively. Table 2.20 show the summary of the previous six tables and the average and RSD of each five assays of the three concentrations for each active ingredient.

Table 2.14 Intraday results for 80% CHD

	1st	2nd	3rd	4th	5th
1 Trial	4485536	4476068	4485555	4482204	4481642
2 Trial	4465321	4479345	4494803	4481271	4481878
3 Trial	4497874	4478720	4483635	4480925	4484165
Avg	4482910	4478044	4487998	4481467	4482562
Recovery	79.28594	79.19988	79.37591	79.26041	79.27977
Recovery %	99.10742	98.99985	99.21989	99.07551	99.09971

Table 2.15 Intraday results for 100% CHD

	1st	2nd	3rd	4th	5th
1 Trial	5644243	5652653	5649985	5654895	5652342
2 Trial	5654360	5652365	5633245	5652954	5650356
3 Trial	5657822	5657562	5642760	5648350	5657919
Avg	5652142	5654193	5641997	5652066	5653539
Recovery	99.96527	100.0016	99.78585	99.96394	99.98999
Recovery %	99.96527	100.0016	99.78585	99.96394	99.98999

Table 2.16 Intraday results for 120% CHD

	1st	2nd	3rd	4th	5th
1 Trial	6778806	6785083	6784072	6785791	6780716
2 Trial	6789928	6785376	6726165	6788634	6780498
3 Trial	6793164	6784475	6766498	6781565	6752618
Avg	6787299	6784978	6758912	6785330	6771277
Recovery	120.042	120.0009	119.5399	120.0071	119.7586
Recovery %	100.035	100.0008	99.61658	100.006	99.79884

Table 2.17 Intraday results for 80% pCA

	1st	2nd	3rd	4th	5th
1 Trial	37022	37027	37085	37092	37091
2 Trial	37061	37066	37077	37008	37041
3 Trial	37028	37055	37010	37078	37110
Avg	37037	37049.33	37057.33	37059.33	37080.67
Recovery	80.10367	80.13034	80.14765	80.15197	80.19811
Recovery %	100.1296	100.1629	100.1846	100.19	100.2476

Table 2.18 Intraday results for 100% pCA

	1st	2nd	3rd	4th	5th
1 Trial	46516	46255	46212	46294	46284
2 Trial	45737	46210	46271	46264	46239
3 Trial	47122	46241	46245	46263	46204
Avg	46458.33	46235.33	46242.67	46273.67	46242.33
Recovery	100.4801	99.99784	100.0137	100.0807	100.013
Recovery %	100.4801	99.99784	100.0137	100.0807	100.013

Table 2.19 Intraday results for 120% pCA

	1st	2nd	3rd	4th	5th
1 Trial	56388	55778	55745	55742	55709
2 Trial	55253	55816	55749	55748	55250
3 Trial	55585	55770	55743	55767	55525
Avg	55742	55788	55745.67	55752.33	55494.67
Recovery	120.5589	120.6584	120.5668	120.5812	120.0239
Recovery %	100.4657	100.5486	100.4723	100.4843	100.0199

Table 2.20 Summery of intraday precession for CHD and pCA

	80 %		100 %		120 %	
	CHD	pCA	CHD	pCA	CHD	pCA
1st trial	99.10742	100.1296	99.96527	100.4801	100.035	100.4657
2nd trial	98.99985	100.1629	100.0016	99.99784	100.0008	100.5486
3rd trial	99.21989	100.1846	99.78585	100.0137	99.61658	100.4723
4th trial	99.07551	100.19	99.96394	100.0807	100.006	100.4843
5th trial	99.09971	100.2476	99.98999	100.013	99.79884	100.0199
Avg	99.10048	100.1829	99.94132	100.1171	99.89142	100.3982
STDEV	0.07915	0.043258	0.088395	0.205474	0.180131	0.213999
RSD	0.079869	0.043179	0.088447	0.205234	0.180327	0.21315

ii) Interday Precision

Table 2.21 shows results of CHD and pCA mixed standard for interday precision test.

Table 2.21 CHD and pCA mixed standard for interday precision

	CHD			pCA		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
STD1	5659567	5676276	5762888	46534	46429	47126
SDT2	5651240	5679889	5765943	46519	46459	47163
STD3	5656282	5677492	5766162	46500	46328	47160
STD4	5650005	5678364	5764854	45611	46398	47161
STD5	5660625	5672683	5756054	46566	46307	47183
STD6	5648577	5674893	5765526	45666	46438	47120
Avg.	5654382.67	5676600	5763571	46232.67	46393.17	47152.17
STDEV	5141.93717	2572.415	3867.287	461.0743	62.16564	24.19435
RSD	0.0909372	0.045316	0.067099	0.997291	0.133997	0.051311

Tables numbered 2.22, 2.23 and 2.24 shows intraday precision for 80%, 100% and 120% for both components, respectively. Table 2.25 shows the summary of interday precision, the average and RSD of each three assays of the three concentrations for each active ingredient.

Table 2.22-interday precision results for 80% of CHD and pCA

	CHD			pCA		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Trial 1	4485536	4480400	4624475	37022	37345	37813
Trial 2	4465321	4486604	4624424	37061	37374	37804
Trial 3	4497874	4487054	4623611	37028	37300	37830
Avg	4482910.33	4484686	4624170	37037	37339.67	37815.67
STDEV	16434.5686	3718.598	484.7793	21	37.28717	13.20353
RSD	0.36660489	0.082918	0.010484	0.0567	0.099859	0.034916
Recovery	79.2820472	79.00304	80.23099	80.11002	80.48527	80.19921
Recovery%	99.102559	98.7538	100.2887	100.1375	100.6066	100.249

Table 2.23-interday precision results for 100% of CHD and pCA

	CHD			pCA		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Trial 1	5644243	5662172	5651960	46516	46305	47181
Trial 2	5654360	5662888	5772715	45737	46325	47468
Trial 3	5657822	5664765	5775269	47122	46341	47376
Avg	5652141.67	5663275	5733315	46458.33	46323.67	47341.67
STDEV	7056.06564	1339.119	70466.78	694.2984	18.037	146.5481
RSD	0.1248388	0.023646	1.229076	1.494454	0.038937	0.309554
Recovery	99.960367	99.76527	99.47504	100.4881	99.85019	100.4019
Recovery%	99.960367	99.76527	99.47504	100.4881	99.85019	100.4019

Table 2.24-interday precision for 120% of CHD and pCA

	CHD			pCA		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Trial 1	6778806	6759614	6962419	56388	55729	56564
Trial 2	6789928	6756075	6946311	55253	55790	56495
Trial 3	6793164	6760515	6956669	55585	55780	56530
Avg	6787299.33	6758735	6955133	55742	55766.33	56529.67
STDEV	7531.29852	2346.981	8163.111	583.5606	32.71595	34.50121
RSD	0.11096164	0.034725	0.117368	1.046896	0.058666	0.061032
Recovery	120.036081	119.0631	120.674	120.5684	120.2038	119.8877
Recovery%	100.030067	99.21924	100.5617	100.4737	100.1698	99.90645

Table 2.25-interday precision summery for both CHD and pCA

	80%		100%		120%	
	CHD	pCA	CHD	pCA	CHD	pCA
Day 1	99.102559	100.1375	99.960367	100.4881	100.03007	100.4737
Day 2	98.753796	100.6066	99.765273	99.85019	99.21924	100.1698
Day 3	100.28873	100.249	99.475039	100.4019	100.56168	99.90645
Avg.	99.381696	100.331	99.73356	100.2467	99.890462	100.1833
STDEV	0.8046402	0.245054	0.2442133	0.346107	0.9492505	0.283862
RSD	0.8096463	0.244245	0.2448657	0.345256	0.9502914	0.283343

2.4.10 Robustness

(a) Standard

Subsequent dilutions were made from the stock solution with the mobile phase to make solutions with 100 µg/ml of CHD and 0.3 µg/ml of pCA. The resulting solution was filtered through a 0.45 µm membrane nylon filter. This solution was injected six times at each different condition.

(b) Samples

A placebo equivalent to a target concentration was transferred to 100-ml volumetric flask. The volume required to prepare 100 µg/ml of CHD and 0.3 µg/ml of pCA was transferred quantitatively from standard stock solution to the placebo flask which was then half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature and the volume was completed to the mark with the same solvent.

The method was examined for robustness test under nine different conditions comparing the method output under each condition with that of the optimized conditions and with permissible limits according to ICH, lastly the variation in method output was evaluated through calculation of RSD of the nine results obtained under the different nine conditions, the results shown in the followings.

i) Optimized conditions

Standard solution was injected six times while sample solution was injected three times under optimized conditions. Results of CH and pCA standards were shown in Table 2.26 and 2.27, respectively; results of samples for both components were shown in Table 2.28.

Table 2.26 Robustness results at optimum conditions for CHD Standards

No.	Ret. Time	Area	Asymmetry factor	Theoretical plate	Resolution
STD1	5.18	5659567	1.13	13420	4.71
SDT2	5.18	5651240	1.14	13619	4.71
STD3	5.17	5656282	1.13	13580	4.69
STD4	5.17	5650005	1.13	13576	4.69
STD5	5.17	5660625	1.12	13576	4.68
STD6	5.16	5648577	1.14	13516	4.65
Avg	5.171666667	5654383	1.131666667	13547.83333	4.688333333
STDEV	0.007527727	5141.937	0.007527727	70.76840161	0.02228602
RSD	0.145557071	0.090937	0.665189384	0.522359553	0.475350577

Table 2.27 Robustness results at optimum conditions for pCA Standards

No.	Ret. Time	Area	Asymmetry factor	Theoretical plate	Resolution
STD1	3.77	46534	1.18	15760	4.71
SDT2	3.78	46519	1.09	15801	4.71
STD3	3.78	46500	1.1	15994	4.69
STD4	3.78	45611	1.14	16001	4.69
STD5	3.78	46566	1.12	16008	4.68
STD6	3.78	45688	1.11	15994	4.65
Avg	3.778333333	46236.33	1.123333333	15926.33333	4.688333333
STDEV	0.004082483	455.723	0.032659863	113.8220834	0.02228602
RSD	0.108049834	0.985638	2.907406223	0.71467852	0.475350577

Table 2.28 Results of CHD and pCA sample at optimum conditions

No	CHD	pCA
1st trial	5644243	46516
2nd trial	5654360	45737
3rd trial	5657822	47122
Avg.	5652141.667	46458.33333
STDEV	7056.065641	694.2984469
RSD	0.124838797	1.494454056
Recovery %	99.96036703	100.4801419

ii) 5^0C Less

Standard solution was injected six times while sample solution was injected three times after the column temperature was decreased five degrees Celsius, Results of CHD and pCA standards are shown in Table 2.29 and 2.30, respectively; results of samples for both components are shown in Table 2.31.

Table 2.29 Results of CHD standard at decreased temperature

No.	Ret. Time	Area	Asymmetry factor	Theoretical plate	Resolution
STD1	5.89	5705797	1.64	11327	5.83
SDT2	5.89	5707949	1.6	11327	5.84
STD3	5.89	5705280	1.64	11327	5.83
STD4	5.89	5709271	1.58	11399	5.85
STD5	5.89	5716512	1.6	11327	5.84
STD6	5.89	5705025	1.65	11327	5.81
Avg	5.89	5708306	1.618333333	11339	5.833333333
STDEV	0	4350.031	0.02857738	29.39387691	0.013662601
RSD	0	0.076205	1.765852544	0.259228123	0.234216018

Table 2.30 Results of pCA standard at decreased temperature

No.	Ret. Time	Area	Asymmetry factor	Theoretical plate	Resolution
STD1	3.86	47212	1.09	14935	5.83
SDT2	3.86	47277	1.08	14929	5.84
STD3	3.86	47293	1.09	14935	5.83
STD4	3.86	47233	1.08	14929	5.85
STD5	3.86	47269	1.07	14929	5.84
STD6	3.86	47251	1.08	14610	5.81
Avg	3.86	47255.83	1.081666667	14877.83333	5.833333333
STDEV	0	29.89593	0.007527727	131.2439205	0.013662601
RSD	0	0.063264	0.695937737	0.882144043	0.234216018

Table 2.31 Results of hydrochlorothiazide and valsartan sample at decreased temperature

No	CHD	pCA
1st trial	5708449	47292
2nd trial	5698686	47220
3rd trial	5702632	47224
Avg.	5703255.667	47245.33333
STDEV	4911.289274	40.46397575
RSD	0.086113784	0.085646503
Recovery %	99.91153242	99.97778052

iii) 5^0C More

Standard solution was injected six times while sample solution was injected three times after the column temperature was increased five celsius degrees. Results of CHD and pCA standards are shown in Table 2.32 and 2.33, respectively; results of samples for both components are shown in Table 2.34.

Table 2.32 Results of CHD standard at increased temperature

No.	Ret. Time	Area	Asymmetry factor	Theoretical plate	Resolution
STD1	5.89	5731861	1.47	11844	5.9
SDT2	5.89	5750245	1.44	11844	5.9
STD3	5.89	5759073	1.3	12236	5.97
STD4	5.89	5735029	1.3	12156	5.96
STD5	5.89	5739666	1.4	11998	5.93
STD6	5.89	5730750	1.22	12398	5.99
Avg	5.89	5741104	1.355	12079.33333	5.941666667
STDEV	0	11296.5	0.096695398	223.3156212	0.037638633
RSD	0	0.196765	7.136191736	1.848741276	0.633469273

Table 2.33 Results of pCA standard at increased temperature

No.	Ret. Time	Area	Asymmetry factor	Theoretical plate	Resolution
STD1	3.86	47796	1.1	14586	5.9
SDT2	3.86	47776	1.1	14592	5.9
STD3	3.86	47689	1.08	14573	5.97
STD4	3.86	47719	1.07	14555	5.96
STD5	3.86	47752	1.1	14567	5.93
STD6	3.86	47776	1.06	14567	5.99
Avg	3.86	47751.33	1.085	14573.33333	5.941666667
STDEV	0	40.35674	0.017606817	13.603921	0.037638633
RSD	0	0.084514	1.622748098	0.09334804	0.633469273

Table 2.34 Results of CHD and pCA sample at increased temperature

No	CHD	pCA
1st trial	5734888	47788
2nd trial	5746301	47587
3rd trial	5740140	47382
Avg.	5740443	47585.66667
STDEV	5712.53	203.003284
RSD	0.099513748	0.426605947
Recovery %	99.98848653	99.65306379

iv) 5% Less flow

Standard solution was injected six times while sample solution was injected three times after decreasing the flow rate 5% of its optimized value. Results of CHD and pCA standards are shown in Table 2.35 and 2.36, respectively; results of samples for both components are shown in Table 2.37.

Table 2.35 Results of CHD standard at decreased flow rate

No.	Ret. Time	Area	Asymmetry factor	Theoretical plate	Resolution
STD1	5.86	5462786	1.17	12267	5.27
SDT2	5.83	5442904	1.16	13619	5.19
STD3	5.85	5435263	1.16	12289	5.23
STD4	5.87	5464238	1.16	12369	5.29
STD5	5.82	5458215	1.16	12179	5.19
STD6	5.86	5475196	1.16	12246	4.65
Avg	5.848333333	5456434	1.161666667	12494.83333	5.136666667
STDEV	0.019407902	14749.29	0.004082483	554.1712431	0.241881514
RSD	0.331853557	0.27031	0.351433249	4.435203162	4.708919799

Table 2.36 Results of pCA standard at decreased flow rate

No.	Ret. Time	Area	Asymmetry factor	Theoretical plate	Resolution
STD1	4.04	55354	1.11	14680	5.27
SDT2	4.04	55050	1.1	14492	5.19
STD3	4.04	55430	1.14	14348	5.23
STD4	4.04	55363	1.14	14527	5.87
STD5	4.03	55464	1.16	14620	5.19
STD6	4.04	55328	1.13	14680	5.26
Avg	4.038333333	55331.5	1.13	14557.83333	5.335
STDEV	0.004082483	146.9772	0.021908902	128.8633643	0.264253666
RSD	0.101093262	0.26563	1.938840912	0.885182303	4.95320836

Table 2.37 Results of CHD and pCA sample at decreased flow rate

No	CHD	pCA
1st trial	5463840	55325
2nd trial	5477115	55465
3rd trial	5466166	55314
Avg.	5469040.333	55368
STDEV	7088.91743	84.18432158
RSD	0.129619037	0.152045083
Recovery %	100.2310422	100.065966

v) 5% More flow

Standard solution was injected six times while sample solution was injected three times after increasing the flow rate 5% of its optimized value. Results of CHD and pCA standards are shown in table 2.38 and 2.39, respectively; results of samples for both components are shown in Table 2.40.

Table 2.38 Results of CHD standard at increased flow rate

No.	Ret. Time	Area	Asymmetry factor	Theoretical plate	Resolution
STD1	5.19	5046532	1.18	12220	4.64
SDT2	5.18	5029495	1.18	12233	4.58
STD3	5.18	5018299	1.17	12154	4.56
STD4	5.2	5038272	1.17	12239	4.66
STD5	5.18	5030944	1.18	12230	4.58
STD6	5.19	5033491	1.17	12201	4.63
Avg	5.186666667	5032839	1.175	12212.83333	4.608333333
STDEV	0.008164966	9419.403	0.005477226	31.74534087	0.040207794
RSD	0.15742222	0.187159	0.466146857	0.259934284	0.87250185

Table 2.39 Results of pCA standard at increased flow rate

No.	Ret. Time	Area	Asymmetry factor	Theoretical plate	Resolution
STD1	3.7	51761	0.61	12322	4.64
SDT2	3.7	51737	0.6	12068	4.58
STD3	3.7	51782	0.59	11822	4.56
STD4	3.7	51771	0.62	12452	4.66
STD5	3.7	51779	0.59	12068	4.58
STD6	3.7	51741	0.63	12322	4.63
Avg	3.7	51761.83	0.606666667	12175.66667	4.608333333
STDEV	4.86475E-16	19.16681	0.016329932	231.2796287	0.040207794
RSD	1.3148E-14	0.037029	2.69174697	1.899523328	0.87250185

Table 2.40 Results of CHD and pCA sample at increased flow rate

No	CHD	pCA
1st trial	5026490	51749
2nd trial	5034537	51775
3rd trial	5023992	51705
Avg.	5028339.667	51743
STDEV	5510.46335	35.38361203
RSD	0.109588129	0.068383379
Recovery %	99.9106038	99.96361541

vi) 5% Less organic solvent

Standard solution was injected six times while sample solution was injected three times after decreasing of organic solvent in mobile phase 5% less than optimized value. Results of CHD and pCA standards are shown in Table 2.41 and 2.42, respectively; results of samples for both components are shown in Table 2.43.

Table 2.41 Results of CHD standard at decreased organic solvent

No.	Ret. Time	Area	Asymmetry factor	Theoretical plate	Resolution
STD1	6.29	5812862	1.15	12443	6.48
SDT2	6.31	5876804	1.15	12518	6.58
STD3	6.3	5872650	1.15	12335	6.52
STD4	6.31	5870936	1.15	12370	6.55
STD5	6.32	5876715	1.17	12409	6.59
STD6	6.33	5862994	1.15	12359	6.58
Avg	6.31	5862160	1.153333333	12405.66667	6.55
STDEV	0.014142136	24675.26	0.008164966	67.03332505	0.042895221
RSD	0.224122593	0.420924	0.707945012	0.540344399	0.654888873

Table 2.42 Results of pCA standard at decreased organic solvent

No.	Ret. Time	Area	Asymmetry factor	Theoretical plate	Resolution
STD1	3.94	55670	0.83	13273	6.48
SDT2	3.94	55795	0.95	13832	6.58
STD3	3.94	55623	0.94	13689	6.52
STD4	3.94	55603	0.94	13689	6.55
STD5	3.94	55664	1.15	13832	6.59
STD6	3.94	55778	0.97	13689	6.58
Avg	3.94	55688.83	0.963333333	13667.33333	6.55
STDEV	0	79.86843	0.103858879	205.4932278	0.042895221
RSD	0	0.143419	10.78119847	1.503535641	0.654888873

Table 2.43 Results of CHD and pCA sample at decreased organic solvent

No	CHD	pCA
1st trial	5778694	54984
2nd trial	5871446	55782
3rd trial	5878260	55767
Avg.	5842800	55511
STDEV	55621.86689	456.4570078
RSD	0.951972802	0.822282084
Recovery %	99.66974347	99.68066608

vii) 5% More organic solvent

Standard solution was injected six times while sample solution was injected three times after increasing of organic solvent in mobile phase 5% more than optimized value. Results of CHD and pCA standards are shown in Table 2.44 and 2.45, respectively; results of samples for both components are shown in Table 2.46.

Table 2.44 Results of CHD standard at increased organic solvent

No.	Ret. Time	Area	Asymmetry factor	Theoretical plate	Resolution
STD1	8.37	5759653	1.13	13404	10.09
SDT2	8.37	5777985	1.14	13404	10.09
STD3	8.37	5759653	1.13	13404	10.09
STD4	8.37	5773435	1.13	13404	10.09
STD5	8.37	5782434	1.13	13404	10.09
STD6	8.37	5778389	1.12	13404	10.09
Avg	8.37	5771925	1.13	13404	10.09
STDEV	0	9924.012	0.006324555	0	1.9459E-15
RSD	0	0.171936	0.559695161	0	1.92854E-14

Table 2.45 Results of pCA standard at increased organic solvent

No.	Ret. Time	Area	Asymmetry factor	Theoretical plate	Resolution
STD1	4.14	44640	1.12	16270	10.09
SDT2	4.14	44736	1.1	16270	10.09
STD3	4.14	44603	1.12	16270	10.09
STD4	4.14	44689	1.11	16270	10.09
STD5	4.14	44663	1.11	16270	10.09
STD6	4.14	44656	1.12	16270	10.09
Avg	4.14	44664.5	1.113333333	16270	10.09
STDEV	0	45.09878	0.008164966	0	1.9459E-15
RSD	0	0.100972	0.733380163	0	1.92854E-14

Table 2.46 Results of CHD and pCA sample at increased organic solvent

No	CHD	pCA
1st trial	5778572	44655
2nd trial	5779442	44653
3rd trial	5775942	44655
Avg.	5777985.333	44654.33333
STDEV	1822.260501	1.154700538
RSD	0.031537991	0.002585864
Recovery %	100.1049996	99.9772377

viii) 3nm Less

Standard solution was injected six times while sample solution was injected three times after decreasing the wavelength 3nm less than the optimized detection wavelength. Results of CHD and pCA standards are shown in Table 2.47 and 2.48, respectively; results of samples for both components are shown in Table 2.49.

Table 2.47 Results of CHD standard at decreased wavelength detection

No.	Ret. Time	Area	Asymmetry factor	Theoretical plate	Resolution
STD1	5.57	5104061	1.17	12189	5.24
SDT2	5.55	5108513	1.17	12172	5.18
STD3	5.57	5102776	1.17	12252	5.23
STD4	5.55	5104073	1.17	12172	5.18
STD5	5.55	5105740	1.17	12190	5.18
STD6	5.55	5107851	1.17	12172	5.18
Avg	5.556666667	5105502	1.17	12191.16667	5.198333333
STDEV	0.010327956	2288.675	0	31.01236313	0.02857738
RSD	0.185866027	0.044828	0	0.254383883	0.549741205

Table 2.48 Results of pCA standard at decreased wavelength detection

No.	Ret. Time	Area	Asymmetry factor	Theoretical plate	Resolution
STD1	3.84	49328	0.79	14299	5.24
SDT2	3.84	49325	0.79	14299	5.18
STD3	3.84	49378	0.81	14299	5.23
STD4	3.84	49444	0.78	14299	5.18
STD5	3.84	49323	0.76	14145	5.18
STD6	3.84	49228	0.79	14299	5.18
Avg	3.84	49337.67	0.786666667	14273.33333	5.198333333
STDEV	0	71.31526	0.016329932	62.87023673	0.02857738
RSD	0	0.144545	2.075838765	0.440473401	0.549741205

Table 2.49 Results of CHD and pCA sample at decreased wavelength detection

No	CHD	pCA
1st trial	5102051	49363
2nd trial	5102421	49377
3rd trial	5109911	49394
Avg.	5104794.333	49378
STDEV	4435.023487	15.5241747
RSD	0.086879572	0.031439456
Recovery %	99.98613261	100.0817496

ix) 3nm More

Standard solution was injected six times while sample solution was injected three times after increasing the wavelength 3nm more than the optimized detection wavelength. Results of CHD and pCA standards are shown in Table 2.50 and 2.51, respectively; results of samples for both components are shown in Table 2.52.

Table 2.50 Results of CHD standard at increased wavelength detection

No.	Ret. Time	Area	Asymmetry factor	Theoretical plate	Resolution
STD1	5.58	5748618	1.16	12295	5.24
SDT2	5.55	5768596	1.17	12358	5.19
STD3	5.55	5749518	1.17	12265	5.17
STD4	5.55	5762628	1.16	12358	5.17
STD5	5.54	5740476	1.16	12378	5.15
STD6	5.57	5748067	1.17	12367	5.25
Avg	5.556666667	5752984	1.165	12336.83333	5.195
STDEV	0.015055453	10473.24	0.005477226	45.63076448	0.040865633
RSD	0.270943966	0.182049	0.470148118	0.369874207	0.786633946

Table 2.51 Results of pCA standard at increased wavelength detection

No.	Ret. Time	Area	Asymmetry factor	Theoretical plate	Resolution
STD1	3.84	45160	0.77	13993	5.24
SDT2	3.84	45097	0.78	13993	5.19
STD3	3.84	45089	0.73	13993	5.17
STD4	3.84	45036	0.74	13697	5.17
STD5	3.84	45043	0.75	13993	5.15
STD6	3.84	45045	0.77	14145	5.25
Avg	3.84	45078.33	0.756666667	13969	5.195
STDEV	0	47.50439	0.019663842	146.4677439	0.040865633
RSD	0	0.105382	2.598745587	1.048519893	0.786633946

Table 2.52 Results of CHD and pCA sample at increased wavelength detection

No	CHD	pCA
1st trial	5744255	45133
2nd trial	5737933	45058
3rd trial	5739613	45021
Avg.	5740600.333	45070.66667
STDEV	3274.605523	57.06429123
RSD	0.057042911	0.12661071
Recovery %	99.78474648	99.98299257

Summary of recovery for both components at the nine different conditions, average and RSD are shown in Table 2.53.

Table 2.53 CHD and pCA recovery at all robustness conditions

No	Condition	CHD	pCA
1	Optimized conditions	99.96036703	100.4801419
2	less 5 degree Celsius	99.91153242	99.97778052
3	Mor 5 degree Celsius	99.98848653	99.65306379
4	5% less flow rate	100.2310422	100.065966
5	5% More flow rate	99.9106038	99.96361541
6	5% less Organic solvent	99.66974347	99.68066608
7	5% more Organic solvent	100.1049996	99.9772377
8	3nm less	99.98613261	100.0817496
9	3nm more	99.78474648	99.98299257
	Avg	99.94973935	99.98480151
	STDEV	0.163854928	0.240950102
	RSD %	0.163937324	0.240986728

2.4.11 Assay of Real Samples

(a) Standard Preparation

Subsequent dilutions were made from the stock solution with the mobile phase to make solutions with 100 µg/ml of CHD and 0.3 µg/ml of pCA. The resulting solution was filtered through a 0.45 µm membrane nylon filter. This solution was injected six times.

(b) Assay Preparation

Volume required to prepare 100 µg/ml of CHD was transferred to 100-ml volumetric flask which was then half-filled with mobile phase and sonicated for 10 minutes, cooled to room temperature and then the volume was completed to the mark with the same solvent, Subsequent dilutions were made with mobile phase similar to those made for standard preparation to achieve target concentration.

Standard solution was injected six times, while sample solution was injected three times, the average of each was used for assay calculations as shown in table 2.54 and 2.55

Table 2.54 Results of mixed standard for assay

	CHD	pCA
STD1	5667731	46473
STD2	5667406	46440
STD3	5667390	46420
STD4	5662102	46437
STD5	5665250	46479
STD6	5669489	46431
Avg	5666561.333	46446.66667
STDEV	2566.895063	23.80476143
RSD	0.04529899	0.051251819

Table 2.55 Assay results for CHD and pCA

	CHD	pCA
1st trial	5644639	36207
2nd trial	5640389	36139
3rd trial	5648741	36183
AVG	5644589.667	36176.33333
STDEV	4176.218545	34.48671242
RSD	0.07398622	0.095329485
Assay	99.6122575	77.8879001

Chapter Three

Discussion

3. Discussion and Coclusion

A simple and sensitive RP-HPLC method was developed for the determination of chlorhexidine (CHD) and para chloroaniline (pCA) in their pharmaceutical formulations. The separation was achieved using analytical – C18 column (200 × 4.6 mm, 5 μm particle size), both components were determined by UV detector (available general detector) at fixed wavelength at 254nm. For simplicity of the method an isocratic elution was selected (only one pump is required); the optimized mobile phase was composed of methanol and acetate buffer solution at 55: 45 ratio, with flow rate of 1.0 ml/min; injection volume was 20 μl (universal loop), and the separation was performed at ambient temperature (column oven is not required). Linearity of this method was checked using seven solutions centered with the target concentration, the concentrations range was (20–160) μg/ml for chlorhexidine and (0.3–1.2) μg/ml for p-chloroaniline. Each solution was injected in triplicate. Plot of average area versus prepared concentrations indicates a very good linearity correlation, (R^2 =1) for both components. The limit of detection for chlorhexidine and p-chloroaniline was found to be 1.07 μg/ml and 0.012 μg/ml, respectively; the percentage of limit of detection for chlorhexidine and p-chloroaniline was 1.07% and 4.3%, respectively; whereas the limit of quantitation was found to be 3.25 μg/ml and 0.038 μg/ml, respectively, and percentage of limit of quantitation for chlorhexidine and p-chloroaniline was 3.25% and 12.7%, respectively. Limit of detection and limit of quantitation were within the acceptance limits since the percentage of limit of detection relative to target concentration was not more than 5% and percentage of limit of quantitation relative to target concentration was not more than 20%. In specificity tests, none of placebo peaks had same retention time of active ingredients peaks. This indicates that the excipients used in the formulation did not interfere in the estimation when we used this method for assay in finished

product. Accuracy was evaluated for chlorhexidine and p-chloroaniline using seven concentrations in content of 40%, 60%, 80%, 100%, 120%, 140% and 160% of target concentration. The recovery percentage for chlorhexidine at the above concentrations was found to be 100.02, 99.85, 99.11, 99.96, 100.03, 99.69 and 100.05% respectively; while for p-chloroaniline it was 100.59, 100.69, 100.13, 100.48, 100.47, 99.61 and 100.62% respectively. The average of recovery percentage for chlorhexidine and p-chloroaniline was 99.82% and 100.37%, respectively. The precision of the methods was examined by estimating the corresponding recovery percentages five times on the same day in intraday precision and three times at three different days for inter day precision. The concentrations used was 80%, 100% and 120% of target concentration as per ICH. For chlorhexidine intraday precision, the RSD for the recovery percentage of five assay repetitions was 0.08%, 0.09% and 0.18% for 80%, 100% and 120%, respectively; whereas for p-chloroaniline RSD was 0.04, 0.20 and 0.20, for 80%, 100% and 120%, respectively. For the interday , the RSD for the recovery percentage of chlorhexidine three assay repetitions was 0.80%, 0.24% and 0.95% for 80%, 100% and 120%, respectively; whereas for p-chloroaniline RSD was 0.24, 0.35 and 0.28 for 80%, 100% and 120%, respectively. The RSD values was found to be not more than 2.0% so it is acceptable according to USP and ICH. The robustness of the method was assessed by assaying test solutions under different analytical conditions deliberately changed from the original conditions such as flow rate, mobile phase composition, detection wavelength and column temperature. RSD for the recovery at all different conditions for target concentration was calculated and were found to be 0.16 for chlorhexidine and 0.24% for p-chloroaniline. System suitability parameters at all different conditions were found to be within the accepted limit of USP and ICH guidelines. This indicates that this analytical method gives results with high reality even if

slight but deliberate changes occur in the analytical conditions; therefore it is recommended for the analysis of this drug for quality control routine work and for research purposes.

Finally, the method was found to be stability-indicating method since para chloroaniline is a degradation product of chlorhexine and both were analyzed together simultaneously without any interference in retention time, and it proved to give good analytical results.

For further research work, it will be of much benefit if other analytical techniques are attempted especially those using available instrument such as gas chromatography to validate and determine chlorhexine and para chloroaniline in their pharmaceutical formulations.

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