**Sudan University of Science and Technology**

**College of Graduate Studies**

# Development and Validation of an Assay for Orphenadrine Citrate and Acetaminophen in Besodol Tablet – by( HPLC)

## التطوير والتحقق من طريقة لتحليل سترات الاورفينادرين والاسيتامينوفين في قرص بيزادول بجهاز كروماتوغرافيا السائل ذو الكفاءة العالية

**A Thesis submitted in partial fulfillment for the requirements of a master degree in chemistry**

**By**

**Mohammed Ahmed Dood Hamid**

**B.Sc. (Honor) - Chemistry**

**Supervisor**

**Dr . Mohamed Suleiman Ali**

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# الآية

**قال الله تعالى:**

**{وَقُلِ اعْمَلُواْ فَسَيَرَى اللّهُ عَمَلَكُمْ وَرَسُولُهُ وَالْمُؤْمِنُونَ وَسَتُرَدُّونَ إِلَى عَالِمِ الْغَيْبِ وَالشَّهَادَةِ فَيُنَبِّئُكُم بِمَا كُنتُمْ تَعْمَلُونَ}**

**صدق الله العظيم**

**التوبة: الآية 105**

# Dedication

**Dedicate**

**To**

**My Mother**

**My father**

**My wife and daughter**

Acknowledgements

I would like to thank God for giving me health to accomplish this work. Many thanks also my supervisor Dr. Mohammed Suleiman Ali for this support, encouragement and assistance throughout the provider of research he has cheerfully answered my queries, provided me with knowledge, checked my result, assisted me in willingly in writing up and helpfully commented on the earlier drafts of this project.

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# Abstract

At the present work a simple, precise, accurate and economical reverse phase HPLC with UV detector method, was developed and validated for the simultaneous determination of acetaminophen and orphenadrine citrate in pharmaceutical formulations. better separation was achieved using cyanide column (250mm × 4.6mm, 5µm), maintained at 25℃. The mobile phase was composed of (tri ethyl amine aqueous, methanol and acetonitrile) in the ratio of 50:20:30 (v/v/v), adjusted at pH 3.8 by ortho phosphoric acid. isocratic elution was used with a flow rate of 1.5 ml/min, injection volume was 10µ, and effluents were monitored by UV (220nm). The retention time of acetaminophen and orphenadrine citrate was 2.25 min and 5.9 min, respectively.

The method was tested for linearity over a concentration range of (25-200)% and (25-200)% , for acetaminophen and orphenadrine citrate, respectively, the correlation coefficient ($R^2$), was found to be 0.9988 and 0.9968, respectively.

The method successfully passed all validation tests stipulated in the validation protocol of International Conference on Harmonization (ICH) and united State Pharmacopeia (USP).

## مستخلص البحث

تم تطوير والتحقق من طريقه لتحليل خليط عقار الاسيتامينوفين وسترات الاورفينادرين في منتج البيزادول, وكانت طريقه سهلة، دقيقة واقتصادية باستخدام تقنية كروماتوغرافيا السائل ذوالضغط العالى مع كاشف الاشعة فوق البنفسجية، وقد تم الفصل بصورة جيدة باستخدام عمود السيانيد (250مم * 4.6مم* 5 مايكرومتر)عند درجة حرارة 25 درجة مئوية. الطورالمتحرك يتكون من محلول ثلاثى ايثيل امين المائى والميثانول والاسيتونايترايل بنسب 30:20:50على التوالى . تم ضبط الأس الهيدروجيني الى 3, 8 بواسطة حمض الفوسفوريك ,وتم استخدام الازاحة احادية الطور المتحرك للفصل، وكان معدل سريان الطور المتحرك 1.5مل لكل دقيقة، كمية حقن العينة 10مايكرولتر، وقد تم تقدير المادتين الفعالتين عند طول موجي 220 نانوميتر وكان زمن استبقاء الاسيتامينوفين وستراتالاورفينادرين هى 2.25 دقيقة و5.9 دقيقة على التوالي.

تمت دراسة خطية العلاقة لمادة الاسيتامينوفينوسترات الاورفينادرين في مدي التراكيز المئوية (25 - 200) % و(25 - 200)%على التوالى فكان معامل الخطية لمادة الاسيتامينوفين 0.9988 ولمادة سترات الاورفينادرين 0.9968 على التوالي.

وقد اجتازت هذه الطريقة بنجاح جميع اختبارات التحقق المنصوص عليها في بروتوكول المؤتمر الدولي للموائمة ودستور الولايات المتحدة الأمريكية للأدوية.

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# List of Abbreviations

| | |
|---|---|
| CHD | Coronary heart disease |
| CV | Cardiovascular |
| ACE | Angiotensin Converting Enzyme |
| RP-HPLC | Reversephase - High Performance Liquid Chromatography |
| FDA | Food and Drug Administration |
| UPLC-MS | Ultra-PerformanceLiquid Chromatography - Mass spectrometry |
| IUPAC | International Union of Pure and Applied Chemistry |
| UPLC | Ultra-PerformanceLiquid Chromatography |
| HPLC-UV | High Performance Liquid Chromatographywith Ultra violet detector |
| HPLC/TLC | HighPerformance thin-layer Chromatography |
| Rf | Retardation Factor |
| ICH | International Conference of Harmonization |
| USP | United State Pharmacopeia |
| LOD | Limit Of Detection |
| LOQ | Limit Of Quantitation |
| AVG | Average |
| STDEV | Standard Deviation |
| RSD | Relative StandardDeviation |

| | |
|---|---|
| NMT | Not More Than |
| Nm | Nanometer |
| µm | Micrometer |
| Mm | Millimeter |
| Cm | Centimeter |
| Ppm | Part Per Million |
| ˚C | Celsius Scale |

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# Chapter one

# Introduction and  Literature review

## 1.1 Introduction

Acetaminophen"Paracetamol" or 4-hydroxyacetanilide N-(4-hydroxyphenyl) acetamide is a para-aminophenol derivative (Fig: 1-1). It has antipyretic and analgesic properties and do not show any anti-inflammatory activities. Paracetamol is a white crystalline powder with 151.16256 g/mole molecular weight and 169-171 $^{O}$**C**melting point. Paracetamol reduces the temperature of patients suffering from fever and it is a mild painkiller. In different formulations, many of these products are available for the relief of cold and influenza. Allergic skin reactions and gastrointestinal problems are often caused by paracetamol. Nephropathy can be caused by Paracetamol with drugs combinations containing phenacetin.( Stieger, N., 2005. The formulation and evaluation of alcohol-free paediatric paracetamol preparations (Doctoral dissertation, North-West University)).

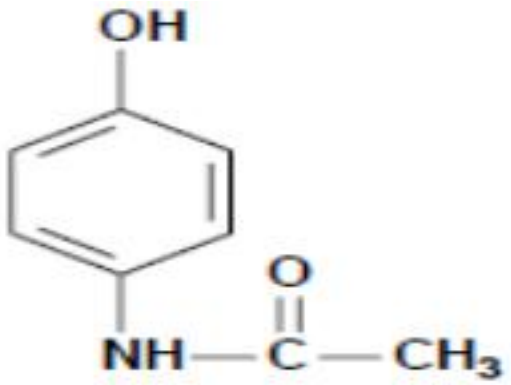



Figure:(1-1) Paracetamol structure. (USP 2017).

Orphenadrine citrate also known as dimethyl [2-(2-methylbenzhydrylox ethyl] amine dihydrogen citrate (Fig:2-1). Molecular formula of orphenadrine citrate is C18H23NO.C6 H6O. Orphenadrine is an anti-muscarinic, anticholinergic, centrally acting skeletal muscle relaxant . Orphenadrine is a white crystalline powder and its molecular weight is 461.5 gm. It is use to relieve pain due to spasm of voluntary muscle Orphenadrine citrate is used as a n alternative to quinine nocturnal leg cramps.( Nazir, A., Naseer, Y., Shahid, R. and Raza, S.,

2016.development and validation of analytical method used for simultaneous determination of paracetamol, caffeine and codeine phosphate by HPLC, in pharmaceutical formulation. Science international, 28(3),pp.2497-2497).

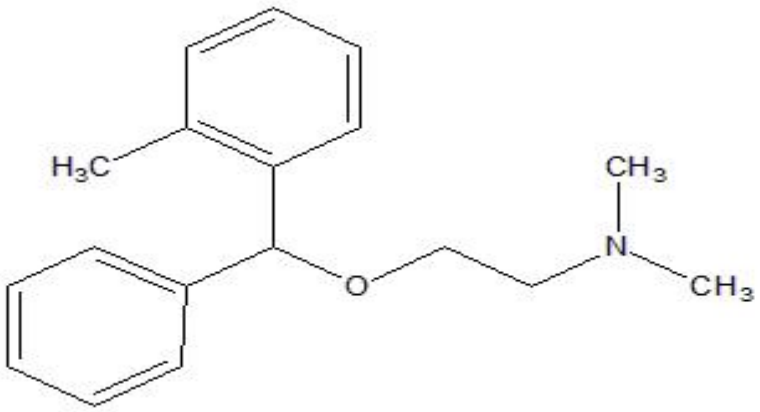



Figure: (1-2)Orphenadrine Citrate structure. (USP 2017)

The combinations of analgesic and antispasmodic drugs is widespread, yet they have been inadequately evaluated. Separately, analgesics like paracetamol and antispasmodics like orphenadrine citrate have been shown to be effective. There has,however, been no report of a double-blind comparative study of a combination of thesetwo agents assessed against one, at least in the literature from general practice. It wasdecided, therefore, to carry out a study to evaluate paracetamol and orphenadrine given synchronously against paracetamol alone. the quantity of paracetamol being the same in each dose.( McGuinness, B.W., 1983. A Double-Blind Comparison in General Practice of a Combination Tablet Containing Orphenadrine Citrate and Paracetamol (‘Norgesic') with Paracetamol Alone. Journal of International Medical Research, 11(1), pp.42-45).

### 1.1.1 Method validation

The method was validated for system suitability specificity,linearity, precision, accuracy and robustness.

### 1.1.1.1 System suitability

If measurements are susceptible to variations in analytical conditions, these should be Suitably controlled, or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness and ruggedness should be that a series of system suitability parameters is established to ensure that the validity of the analytical procedure is maintained whenever used .Typical variations are the stability of analytical solutions, different equipment, and different analysts. In the case of liquid chromatography 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. In the case of gas chromatography, typical variations are different lots or suppliers of columns, the temperature, and the flow rate .(USP 2017)

### 1.1.1.2 Specificity

The ICH documents define specificity as 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 matrix components. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures. [NOTE—Other reputable international authorities (IUPAC, AOAC-I) have preferred the term “selectivity”, reserving “specificity” for those procedures that are completely selective.] For the tests discussed below, the above definition has the following implications .(USP 2017)

### 1.1.1.3 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, in this section, “linearity” refers to the linearity of the relationship of concentration and assay measurement. In some cases, to attain linearity, the concentration and/or the measurement may be transformed.(USP 2017) 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 or parts per million) obtained by the analytical procedure.(USP 2017)

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 afunction of analyte concentration of content. 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 provid 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 the procedure is validated by verifying that the analytical procedure provides acceptable precision, accuracy, and linearity when applied to samples containing analyte at the extremes of the range as well as within the range.(USP 2017).

### 1.1.1.4 Detection limit

The detection limit is a characteristic of limit tests. It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated Experimental conditions. Thus, limit tests merely substantiate that the amount of analyte is above or below a certain level. The detection limit is usually expressed as the concentration of analyte (e.g., percentage or parts per billion) in the sample. For non instrumental procedures, 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.(USP 2017)

### 1.1.1.5 Quantitation limit

The quantitation limit is a characteristic of quantitative assays for low levels of compound s in sample matrices, such as impurities in bulk drug substances and degradation products in finished pharmaceuticals. It 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 or parts per billion) in the sample. for non instrumental procedures, the quantitation 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 determined with acceptable Accuracy and Precision.

(USP 2017)

### 1.1.1.6 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. [A note on terminology: The definition of accuracy in this chapter and ICH Q2 corresponds to unbiasedness only. In the International Vocabulary of Metrology (VIM) and documents of the International Organization for Standardization (ISO), "accuracy" has a different meaning. In ISO, accuracy combines the concepts of unbiasedness (termed "trueness") and precision]. In the case of the assay of a drug substance, 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 th e 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. (USP 2017)

### 1.1.1.7 Precision

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. 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. The ICH documents 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. (USP 2017)

### 1.1.1.8 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain Unaffected by small but deliberate variations in procedural parameters listed in the procedure documentation and provides an indication of its suitability during normal usage. Robustness may be determined during development of the analytical procedure.

(USP 2017)

## 1.2 Literature review

Karuna B. Singh et al. (2012), validated reverse-phase HPLC method for the simultaneous estimation of paracetamol and naproxen in bulk and in tablet formulation. The proposed RP-HPLC method utilizes Eclipse XDB C18 column (150 ×4.6 mm i.d., 5µm), optimum mobile phase consisted of gradient run of initial ratio of water (pH-2.5 adjusted with ortho phosphoric acid): acetonitrile (87:13) with the effluent flow rate of 1.0 ml/min, and UV detection wavelength 263 nm. The linearity range was 5-80 µg/ml for paracetamol and 3-4 8µg/ml for naproxen.

Tabbouche O.S.*, Soukkariyyeh I., Naji M., & Alwan T.(2013),compared the purity and quantity of the Active Pharmaceutical Ingredient (API) “Paracetamol” in five different brands of Paracetamol solution for IV infusion present in the Lebanese market.The **λ** max was 244nm in all five brands which confirms purity of the API, however **λ** max was the highest in Bofalgan (Bosch Pharma) and lowest in Perfumol (Hikma Pharmaceuticals). The highest concentration was observed in Perfalgan (Bristol Myers Squibb), while the lowest concentration was observed in Perfumol (Hikma Pharmaceuticals).

RP-HPLC method has been developed and validated for the simultaneous estimation of

a three component mixture - aspirin, caffeine and orphenadrine citrate.Sanjay Pai P. N. et al. (2013) used The chromatographic separation which was achieved with methanol: phosphate buffer (pH 3) in the ratio of 65:35 (v/v) as mobile phase, on ACCLAIMTM 120 C18 column (5 µm, 4.6 X 250 mm), at a flow rate of 1ml/m using isocratic elution. Detection was carried out at 215 nm. The linearity range was found to be 10-100 µg/ml for aspirin and 2-20 µg/ml for both caffeine and orphenadrine citrate.

D. Samson et al. (2013). developed and validated a RP-HPLC method for the analysis of Paracetamol, Orphenedrine Citrate and Ibuprofen tablet. Column C18 (150 x 4.6 mm, 3.5 µm) was used, using a mobile phase containing mixture of Ortho phosphoric acid, water and acetonitrile in gradient mode was used. The linearity was observed over the concentration range of 112.65 to 675.90 µg/mL for Paracetamol 8.788 to 52.725µg/mL for Orphenedrine and 100.15 to 600.90 µg/mL for Ibuprofen. The lower limit of quantification of Paracetamol, Orphenedrine and Ibuprofen was 6 µg/ml, 7µg/ml and 8µg/ml, and lower limit of detection of Paracetamol, Orphenedrine and Ibuprofen was 1.8 µg/ml, 2.1 µg/ml and 2.4 µg/ml, respectively.

Dammalapati Srikantha and Rudra Raju Ramesh Raju (2014). reported and validated a reversed phase liquid chromatographic (RP-HPLC) method for the determination of orphenedrine citrate in tablet; using C18 column (250x 4.6 mm x 5µm). A mobile phase containing mixture of methanol: acetonitrile: water (40:30:30, v/v/v) and isocratic elution was used with 1ml/min flow rate. The eluents were monitored at 217nm.The linearity range measured is from 10-70µg/mL and limit of detection is 0.3µg/ml.

sensitive, fast, limit of detection (LOD), robustness and ruggedness, drug recovery and the system suitability parameters have been validated for the developed method Dammalapati Srikantha & Rudra Raju Ramesh Raju* (2014). The HPLC conditions are methanol: acetonitrile: water (40:30:30, v/v/v) mobile phase, Zodiac $C_{18}$ column (250x 4.6 mm x 5 m),t, precise, reversed phase- high performance liquid chromatography (RP-HPLC) method is developed for the determination of orphenadrine citrate in tablet dosage form. Linearity, precisio pump pressure (10.6 MPa), flow rate (1.0 ml/min) and the wavelength of detection is 217 nm. The measured retention time of orphenadrine citrate is 5.35 minutes. The limit of detection is 0.3µg/ml . The linearity range measured is from 10 -70 µg/mL with a correlation coefficient ($R^2$ = 0.998). The above measured parameters indicate the developed method is useful in determination of orphenadrine citrate in tablet dosage forms.

Sanjay Pai P. N.[1], et.al(2014) have reported a simple, precise and accurate RP-HPLC method for the simultaneous estimation of a three component mixture - aspirin, caffeine and orphenadrine citrate.The chromatographic separation was achieved with methanol: phosphate buffer (pH 3) in the ratio of 65:35 (v/v) as mobile phase, on ACCLAIM$^{TM}$ 120 $C_{18}$ column (5 µm, 4.6 X 250 mm), at a flow rate of 1ml/m using isocratic elution. Detection was carried out at 215 nm. The retention time for aspirin, caffeine and orphenadrine citrate was found to be 4.2, 3.3 and 6.5 m respectively. The linearity range was found to be 10-100 µg/ml for aspirin and 2-20 µg/ml for both caffeine and orphenadrine citrate with $r^2$ value 0.997, 0.994 and 0.997 respectively. The mean percent recovery was found to be 97.3-97.80% for aspirin, 95-104.3% for caffeine and 96.36-103.4% for orphenadrine citrate.

Mohammad Anas Alfeel et al. (2017); invistigated a high-performance liquid chromatography method for Orphenadrine Citrate and Paracetamol was developed and validated; using C18 column (250 x 3.4mm, 5µm) at ambient temperature, injection volume was 20µl. The mobile phase was 1% Trimethylamine aqueous: Methanol: Acetonitrile in the ratio of (35:20:45 v/v) and using isocratic elution with 2ml/min flow rate, the eluents were monitored at 220nm. The Linearity ranges (20-140) µg/ml and (0.1-50) µg/ml for PA and ORC respectively. The lower limit of quantification of PA and ORC was 0.3097, 0.1063 ppm, and lower limit of detection of PA and ORC was 0.0153 and 0.0135, respectively.

Electronic absorption measurements were carried out on a Shimadzu UV160 spectrophotometer connected to a computer loaded with Shimadzu UVPC software using quarts cells with a cm path length .The absorption spectra of all test and reference solution were recorded each 1 nm in the range 200_ 300 nm .Kawan SRATTHAPHUT* and Non Gluck RUANGWISES(2007).The obtained data were processed by spentium IV computer having 512 MB for RAM . The PLS was performed by PLS Toolbox 2.0 under MATLAB 7.0 using the additional Neutral network The highly interference of the individual spectra of two drugs at equal concentrations

(12 mg L－1) are shown in . The spectra at the same solvent, but with analyte concentrations corresponding to sample diluted 1/15000: PAR, 33 mg L－1,and

OPC,2.3mgL－1 , the minor analyte's concentration (OPC) is near to the noise level (absorbance is under 0.05), while that for the major analyte (PAR).

## 1.3 Objectives and Research Purposes

- Development of assay methods for Orphenadrine Citrate and Paracetamol, determining system suitability, specificity, linearity, accuracy, precision and robustness for the developed method comparing the obtained results with the acceptance criteria of USP and ICH guidelines.
- Application of the developed method for real sample assay.

# Chapter Two
# Materials and Methods

## 2.1 Chemicals

Paracetamol(acetaminophen) (purity: 99.50%),orphenadrine citrate (purity: 99.39%), tri ethylamine for HPLC grade (made in Spain), Methanol multisolvent HPLC grade ACS ISO(made in Spain) , Acetonitrile gradient240nm/far UV- HPLC grade(made in Spain), all other chemicals used were of analytical grade and Purified water .

## 2.2Instruments

- **High Performance Liquid Chromatography HPLC**

Type: HPLC SYKAM

Model No: S 3210

Serial No: 010704

Origin : Germany

- **Analytical Balance**

Type: ATX224

Serial No: D307036374

Capacity: 220 g

Readability: 0.1 mg

Company: Shimadzu Corporation

Origin JAPAN

- **Ultrasonic**

Model: SC-152TH

SERIAL NUMBER:11589-1206

Origin: U.S.A

❖ **pH-meter**

Model: HANAA

CODE NO:PH -01

Origin: MAURITIUS.

❖ **Magnetic Stirrer**

Model: LMS, 1001

Serial No: 2016017862

Company: QAIHAN LAB TECH Co-LTD

Origin: Korea

## 2.3Methods

### 2.3.1 Optimized chromatographic conditions

Cyanide column (250 × 4.6mm, 5µm), and simple isocratic elusion, were used (one pump required) with flow-rate of 1.5/min, both active ingredients were detected at 220nm, injection volume was 10µl (universal loop) and analysis temperature was 25°C.

### 2.3.2 Buffer Solution

5ml of tri ethylamine was mixed with 450ml of deionized water in 500 ml volumetric flak and completed to the mark.

### 2.3.3 Mobile PhasepH 3.8

Mixture of buffer (tri ethyl amine aqueous50% , methanol20% and acetonitrile30%) were prepared in 500:200:300 v/v/v ratio, respectively. The mixture was shaken and adjusted to pH 3.8 with ortho phosphoric acid, filtered with vacuum filtration pump through 0.45µm

nylon membrane filter, and then transferred to solvent reservoir and sonicated for 15 min.

### 2.3.4 Standard Stock Solution

450 mg acetaminophen and 35mg orphenadrine citrate were weighed accurately, transferred quantitatively to 100ml volumetric flask, 50ml of Mobile phase was added, shaked by mechanical means for 10 min and sonicated to 5 min, cooled, and completed to the mark with mobile phase.

### 2.3.5 System Suitability

Subsequent dilutions were made from the stock solution with mobile phase to give the concentrations of 450µg/ml orphenadrine citrate and 35µg/ml acetaminophen.
System suitability solution was injected five times.

### 2.3.6 Specificity

#### i. Standard

Subsequent dilutions were made from the stock solution with mobile phase to give the concentrations of 450µg/ml orphenadrine citrate and 35µg/ml acetaminophen.
System suitability solution was injected six times.

#### ii. Placebo

A placebo equivalent to average weight of one tablet was transferred to 50-ml volumetric flask, the flask was half filled with mobile phase, sonicated for 15minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent. Subsequent dilutions were made in mobile phase with similar to those made for standard preparation.

### iii. Sample

Ten tablets were taken in to clean and dry 100 ml volumetric flask, shaked with 10 ml methanol, sonicated for 10 min, cooled, then 50 ml mobile phase were added, sonicated for 20 min, left to reach room temperature, and then completed to required volume with mobile phase. Then 5ml were diluted with mobile phase to 25ml volumetric flask, passed through a suitable filter 0.45µm pore size.

## 2.3.7 Linearity

Subsequent dilutions were made from the stock solution with mobile phase to give concentrations of 25%, 50%, 75%, 100%, 150% and 200% (0.00343,0.00686,0.01053, 0.01372 and 0.02744mg/ml) Orphenadrine citrate and 25%, 50%, 75%, 100%, 150% and 200% (0.04482, 08964,013644, 0.17928, 0.2691 and 0.35856mg/ml acetaminophen. Each solutions were injected three times and results were collected, LOD and LOQ were calculated from the linear regression analysis.

## 2.3.8 Accuracy

### ❖ Standard

Subsequent dilutions were made from the stock solution with mobile phase to give the concentrations of 450 mg/ml Orphenadrine citrate and 35 mg/ml acetaminophen . System suitability solution was injected five times.

### ❖ Preparation of Test Solution

Three 100-ml volumetric flasks were labeled; a placebo equivalent to tablets weight was transferred to each flask. A volume of standard stock solution required to produce 60%, 100%, and 120% tablets content of orphenadrine citrate and acetaminophen were added

each to different flask. The flasks were half filled with mobile phase,shaked by mechanical means for 10 minutes, sonicated for 5 minutes, cooled to room temperature and completed to the mark with the mobile phase. Subsequent dilutions were made with mobile phase like those made for the standard preparation. Each solution was injected three times. The results were collected and subjected to statistical treatments.

### 2.3.9Precision

- **Precision Standard**

Subsequent dilutions were made from the stock solution with mobile phase to give the concentrations of 450mg/ml Orphenadrine citrate and 35mg/ml acetaminophen. System suitability solution was injected five times.

- **Preparation of working Standard Solution**

5ml standard stock solution were taken in to 25ml volumetric flask, then Completed to the required volume with mobile, passed through a suitable filter 0.45µm pore size.

- **Precision sample**

Five tablets were taken in to clean and dry 100 ml volumetric flask and shaked with 10 ml methanol, sonicated for 10 min, cooled, then 50 ml mobile phase was added, sonicated to 20 min, leave to reach room temperature, and then completed to required volume with mobile phase. Then 5ml was diluted with mobile phase in to 25ml volumetric flask, passed through a suitable filter 0.45µm pore size.

### 2.3.10 Robustness

Accuracy sample solution of target concentration was used. The sample was injected three times at each of the different conditions relative to optimum condition, PH(+0.1) more for PH 3.8,PH(-0.1) less for PH3.8 ,0.5 more flow rate of mobile phase, 0.5 less flow rate of mobile phase, 2nm above the detection wavelength and 2nm below the detection wavelength. The results were collected and subjected to statistical treatments

# Chapter Three

# Results and Discussion

## 3.1.**Purpose**

The purpose of this report is to summarize the results of the validation of test method "Determination of Orphenadrine citrate and Acetaminophen " following Validation Protocol

### 3.1.1 **Test Method procedure:**

The test procedure done according the method of developmenting report which attached to validation protocol

## 3.2 Results

### 3.2.1 accuracy

Table(3.1) shows the accuracy results for orphenadrine citrate of % recovery & difference between mean and accepted value

| Orphenadrine Citrate level | Actual concentration | Individual % recovery | Mean % recovery | Pass/ Fail (P/F) | %RSD | P/F |
|---|---|---|---|---|---|---|
| 60% | 0.0084 mg/ml | 98.76 | 100.71 | P | 1.68 | p |
| | | 101.75 | | | | |
| | | 101.63 | | | | |
| | Acceptance Citeria | 100 ±2.0% | 100±2% | P | ≤2% | p |
| 100% | 0.014 mg/ml | 97.71 | 98.30 | P | 0.80 | p |
| | | 99.20 | | | | |
| | | 98.00 | | | | |
| | Acceptance Citeria | 100 ±2.5% | 100±2% | P | ≤2% | p |
| 120% | 0.0168 mg/ml | 99.70 | 101.04 | P | 1.34 | P |
| | | 100.04 | | | | |
| | | 102.35 | | P | ≤2% | p |
| | Acceptance Citeria | 100±2.5% | 100±2% | | | |

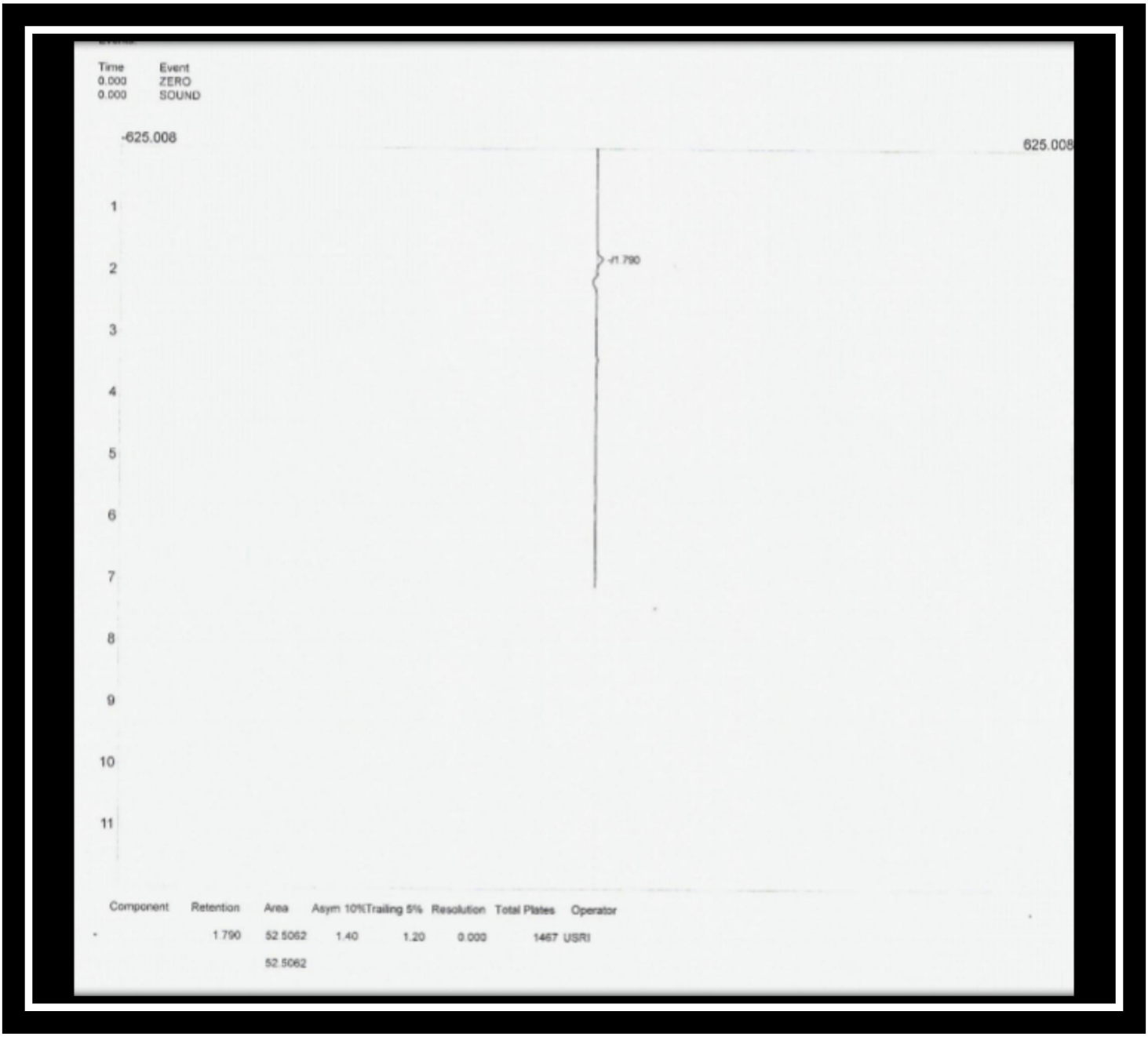
Table(3.2) shows the accuracy results for acetaminophen of % recovery & difference between mean and accepted value

| Acetaminophen level | Actual concentration | Individual % recovery | Mean % recovery | Pass/ Fail (P/F) | %RSD | P/F |
|---|---|---|---|---|---|---|
| 60% | 0.108 mg/ml | 100.53 | 101.33 | P | 0.68 | P |
| | | 101.71 | | | | |
| | | 101.76 | | | | |
| | Acceptance Citeria | 100 ± 2.0% | 100 ± 2% | P | ≤2% | P |
| 100% | 0.18mg/ml | 99.78 | 100.53 | P | 0.66 | P |
| | | 101.04 | | | | |
| | | 100.77 | | | | |
| | Acceptance Citeria | 100 ± 2.5% | 100 ± 2% | P | ≤2% | P |
| 120% | 0.216mg/ml | 97.97 | 98.51 | P | 1.04 | P |
| | | 97.87 | | | | |
| | | 99.69 | | P | ≤2% | P |
| | Acceptance Citeria | 100 ± 2.5% | 100 ± 2% | | | |

## 3.2.2 specificity

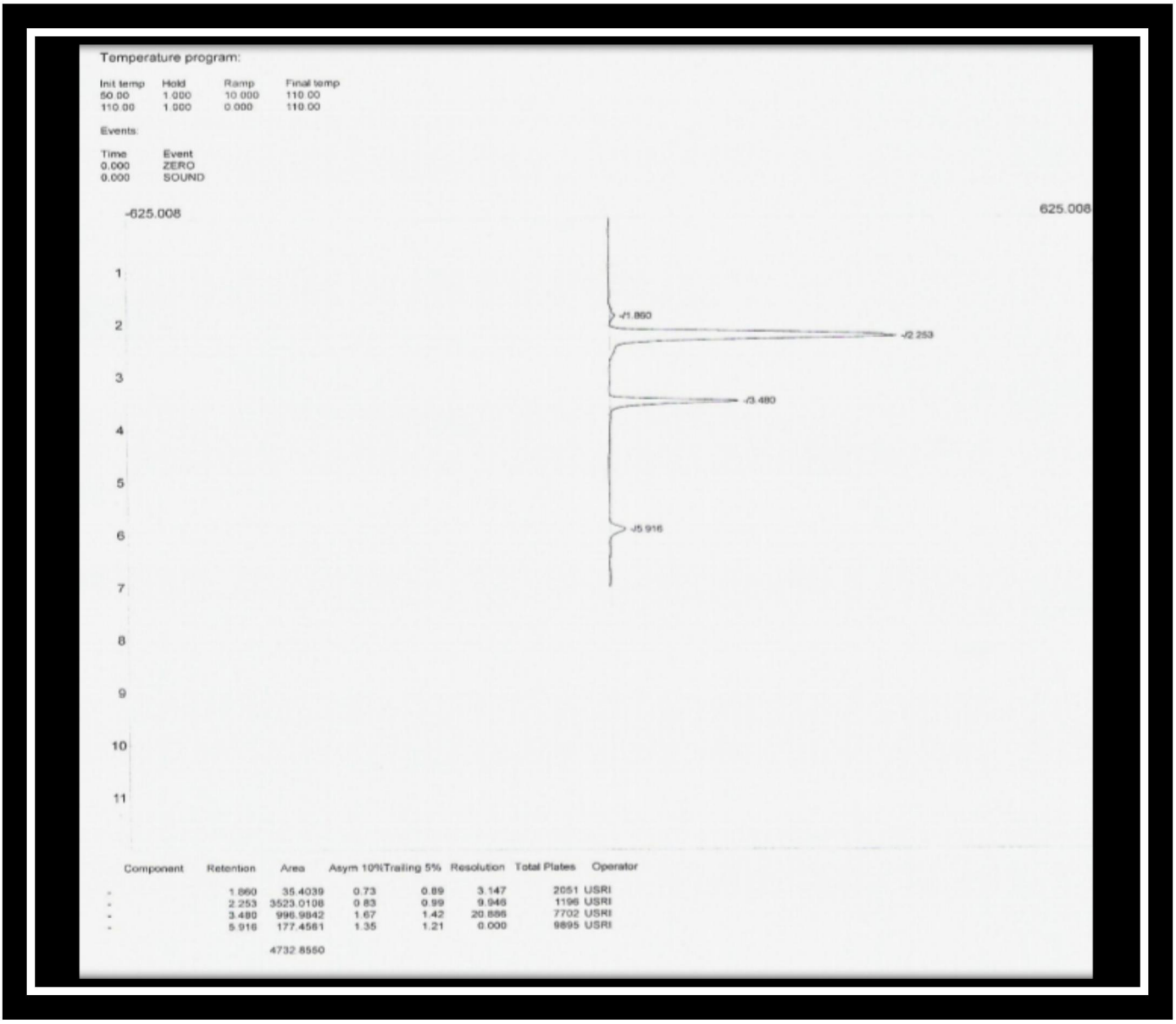
Representative figures (Chromatogram) of a placebo , Representative sample , each analyte alone ,internal standard and mobile are attached .

✧ Figure( 3.1) : placebo sample



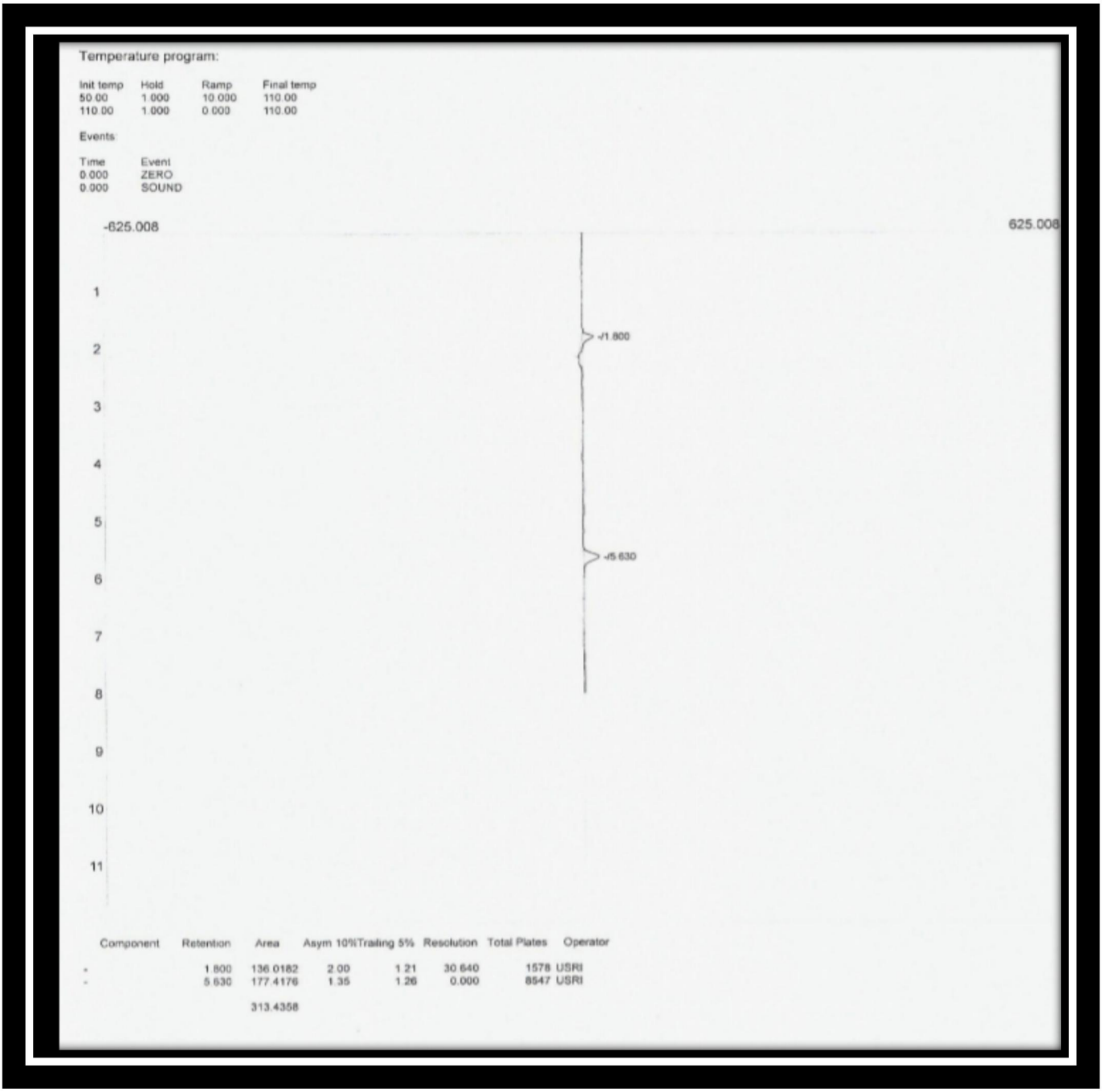

Figures (3.1) : placebo sample chromatogram.

✧ Figure(3. 2) : representative sample



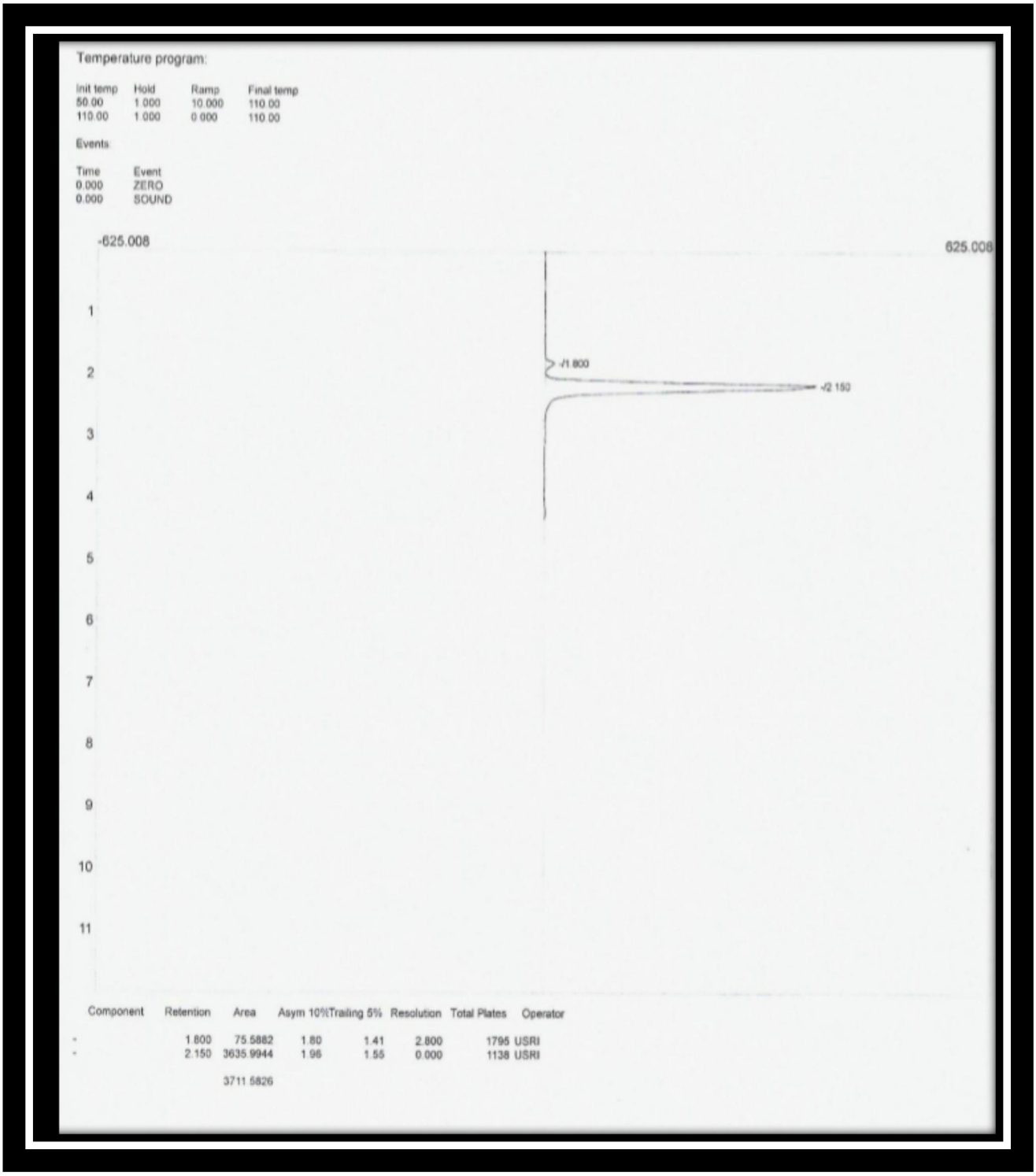

Figure( 3.2): Representative sample chromatogram.

✧ Figure(3. 3) :Orphenadrine citrate alone



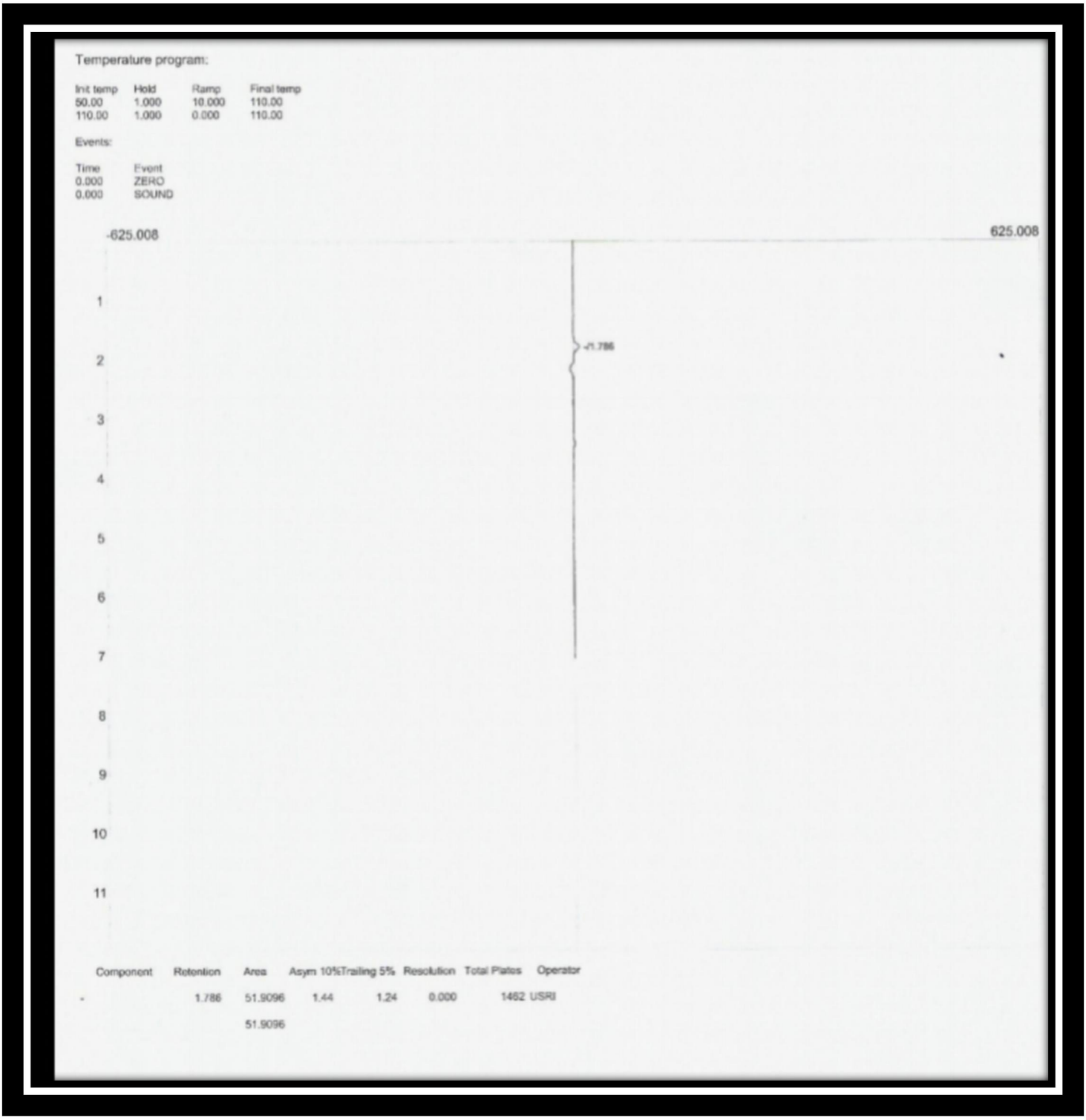

Figure(3. 3): orphenadrine citrate alone chromatogram.

✧ Figure( 3. 4) :Acetaminophen alone



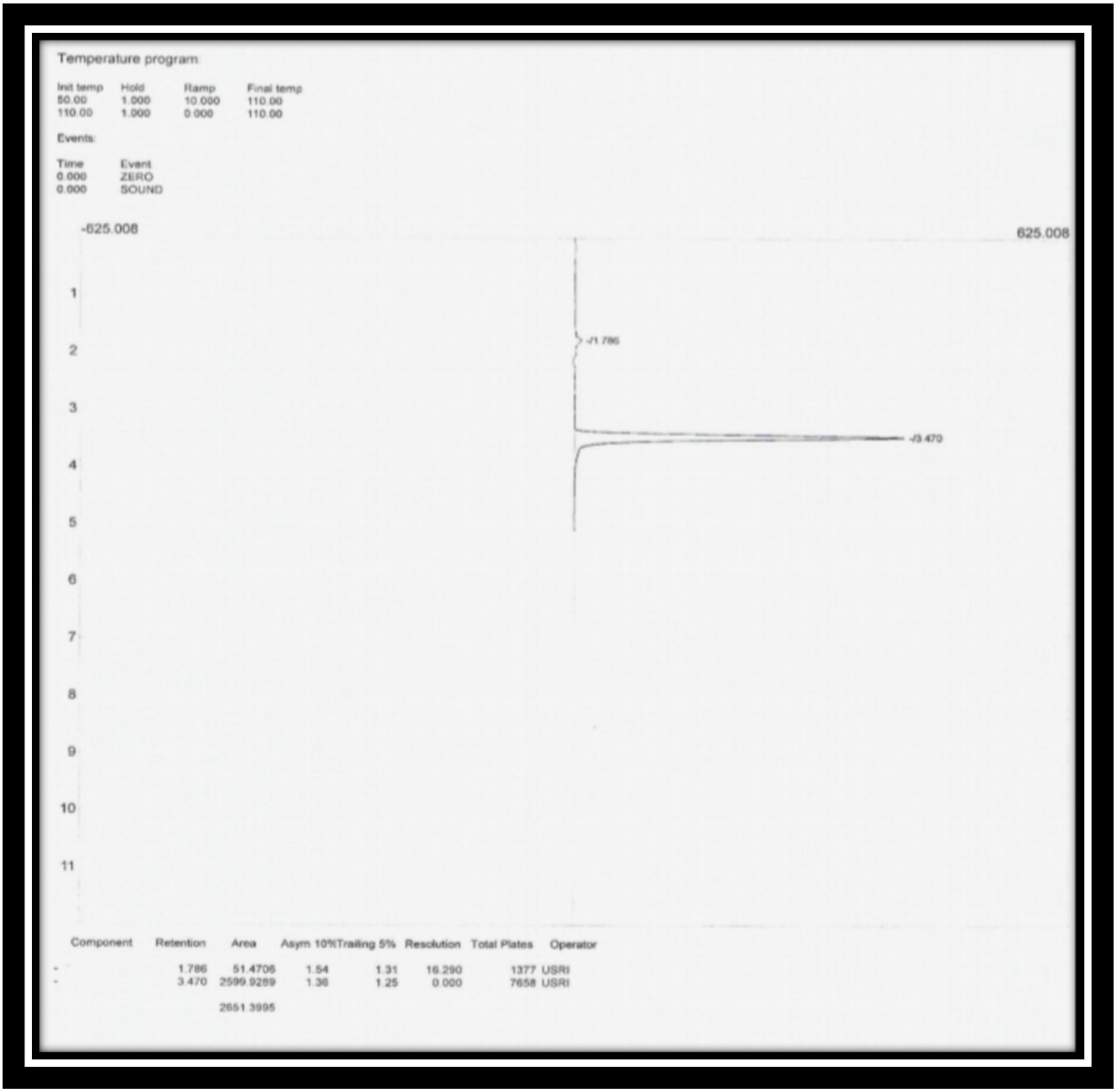

Figure(3. 4) : acetaminophen alone chromatogram.

✧ Figure(3. 5) :mobile phase alone




Figure( 3.5): mobile phase alone chromatogram.

✧ Figure( 3. 6) : internal standard alone




Figure( 3.6): internal standard alone chromatogram

## 3.2.3 Precision

### 3.2.3.1 Repeatability (method precision):

Table(3.3) shows results of orphenadrine citrate and acetaminophen repeatability using multiple determinations at test concentration

| Determination | Orphenadrine citrate recovery % | Acetaminophen recovery % |
|---|---|---|
| Sample 1 | 97..71 | 99.78 |
| Sample 2 | 99.2 | 101.04 |
| Sample 3 | 98 | 100.77 |
| Sample 4 | 101.86 | 99.51 |
| Sample 5 | 100. 96 | 101.01 |
| Sample 6 | 100.18 | 101.75 |
| Mean | 99.82 | 100.64 |
| %RSD | 1.84% | 0.84 |
| Acceptance Criteria | %RSD $\leq$2.0 | %RSD $\leq$2.0 |
| P/F | PASS | PASS |

### 3.2.3.2 intermediate precision :

Table(3.4) shows results for intermediate precision of orphenadrine citrate

| (orphenadrine citrate) recovery % sample No. | Analyst 1 /day 1 | Analyst 2 /day 2 |
|---|---|---|
| Sample 1 | 97.71 | 101.86 |
| Sample 2 | 99.20 | 101.90 |
| Sample 3 | 98.00 | 100.18 |
| Sample 4 | 98.70 | 98.76 |
| Sample 5 | 100.52 | 101.80 |
| Sample 6 | 101.63 | 101.6 |
| Mean (N=6) | 99.29 | 101.01 |
| %RSD (N=12) | 1.61 | |
| Acceptance criteria (n =12) | %RSD (N=12)< 3 | |
| P/F | PASS | |

Table(3.5) shows results for intermediate precision of acetaminophen

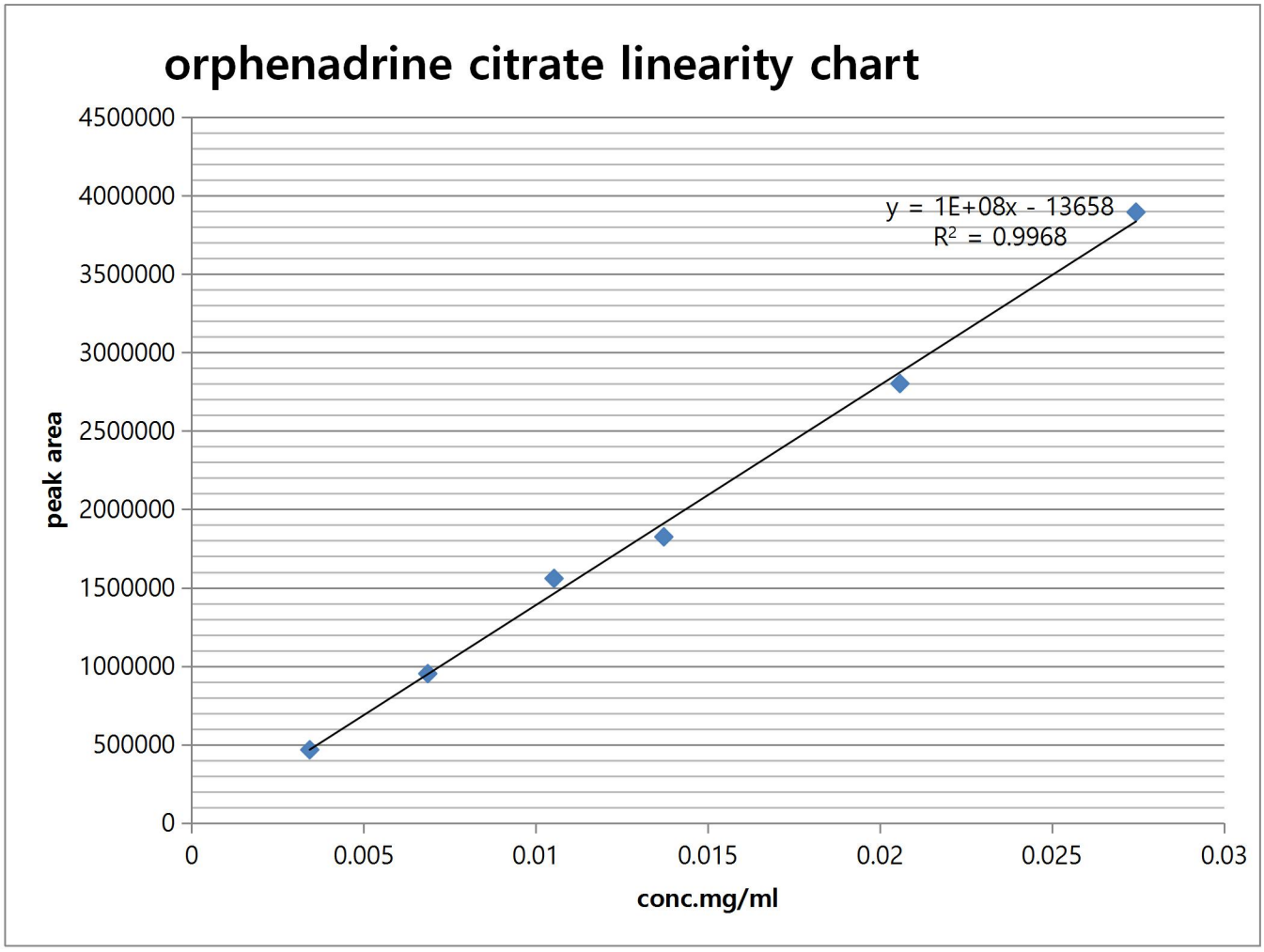
| (Acetaminophen) recovery % sample No | Analyst 1 /day 1 | Analyst 2 /day 2 |
|---|---|---|
| Sample 1 | 97.78 | 99.51 |
| Sample 2 | 101.04 | 101.01 |
| Sample 3 | 100.77 | 101.75 |
| Sample 4 | 101.86 | 100.53 |
| Sample 5 | 101.88 | 101.71 |
| Sample 6 | 101.74 | 101.76 |
| Mean (N=6) | 101.17 | 101.04 |
| %RSD (N=12) | 0.81 | |
| Acceptance criteria (n =12) | %RSD (N=12)< 3 | |
| P/F | PASS | |

### 3.2.4 Linearity and Range :

Table(3.6)shows linearity results for orphenadrine citrate

| (orphenadrine citrate) level | | | Area |
|---|---|---|---|
| Concentration level relative to nominal standard Concentration in % | Actual Concentration | | Average of 3 Injections |
| 25% | 0.00343 mg/ml | | 486849.3 |
| 50% | 0.00686 mg/ml | | 952387.7 |
| 75% | 0.01053 mg/ml | | 1559616.0 |
| 100% | 0.01372 mg/ml | | 1824690.0 |
| 150% | 002058 mg/ml | | 2802306 |
| 200% | 0.02744 mg/ml | | 3895465 |
| Slope | 1E +08 | | |
| Y intercept | -3188.8 | | |
| Correlation coefficient | 0.9967 | | |
| Correlation coefficient acceptance criteria | ≥0.995 | P/F | PASS |
| Range | (0.00343-0.02744)mg/ml | | |

Set attachment figure(3. 7): linearity chart for orphenadrine  citrate.




Figure(3. 7):Orphenadrine citrate linearity chart

Where

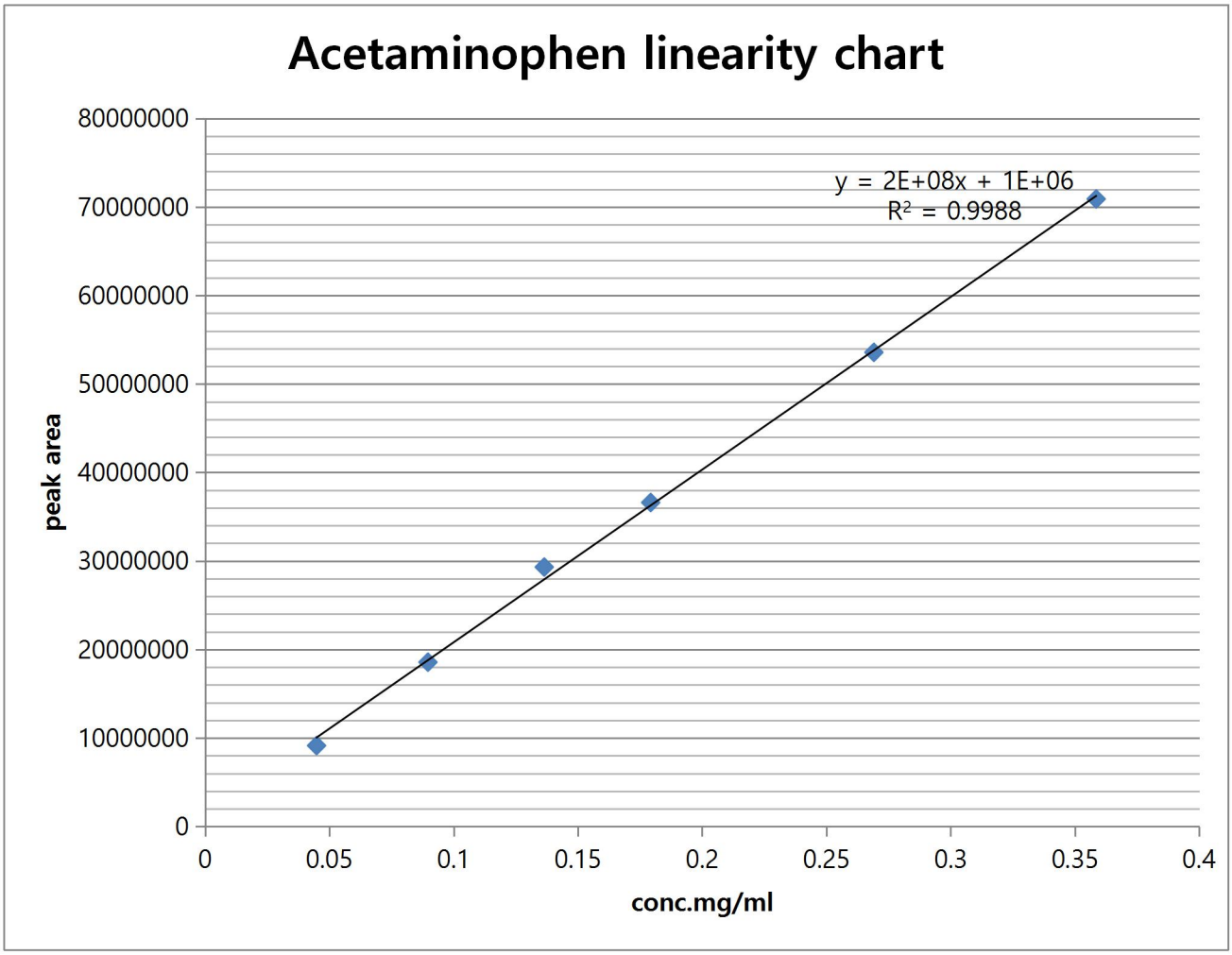
X: axis is orphenadrine citrate concentration in mg/ml

Y: axis is peak area in units

Table(3.7) shows linearity results for acetaminophen

| (acetaminophen ) level | | | Area |
|---|---|---|---|
| Concentration level relative to nominal standard Concentration in % | Actual Concentration | | Average of 3 Injections |
| 25% | 0.04482 mg/ml | | 9141225 |
| 50% | 0. 08964 mg/ml | | 18565319 |
| 75% | 13644 mg/ml | | 29312594 |
| 100% | 0.17928 mg/ml | | 36604824 |
| 150% | 0.2691 mg/ml | | 53576324 |
| 200% | 0.35856mg/ml | | 70901926 |
| Slope | 2E +08 | | |
| Y intercept | IE+06 | | |
| Correlation coefficient | 0.9988 | | |
| Correlation coefficient acceptance criteria | ≥0.995 | P/F | PASS |
| Range | (0.04482-0.35856)mg/ml | | |

Set attachment figure( 3. 8): linearity chart for acetaminophen .




Figure( 3. 8): Acetaminophen linearity chart

where

X: axis is acetaminophen concentration in mg/ml
Y: axis is peak area in units

## 3.2.5 Robustness :

Table(3.8) shows results of robustness for orphenadrine citrate

| Method parameters (orphenadrine citrate) | %RSD |
|---|---|
| Flow rate nominal (1.5 ml/min) | 0.39% |
| Flow rate 1ml/min | 0.39% |
| Flow rate 2ml/min | 2.3% |
| Mobile phase PH nominal (3.8) | 0.39% |
| Mobile phase PH+0.1 | 1.90% |
| Mobile phase PH-0.1 | 1.54% |
| Detector wave length nominal (220nm) | 0.39% |
| Detector wave length + 2nm | 0.77% |
| Detector wave length -2nm | 0.39% |
| Acceptance criteria | NMT 5% |
| Pass/fail | PASS |

**NMT** : Not more than

Table(3.9) shows results of robustness for acetaminophen

| Method parameters (Acetaminophen) | %RSD |
|---|---|
| Flow rate nominal (1.5 ml/min) | 0.52% |
| Flow rate 1ml/min | 1.15 |
| Flow rate 2ml/min | 1.04 |
| Mobile phase PH nominal (3.8) | 0.52 |
| Mobile phase PH+0.1 | 0.74% |
| Mobile phase PH-0.1 | 2.69% |
| Detector wave length nominal(220nm) | 0.52% |
| Detector wave length + 2nm | 0.79% |
| Detector wave length -2nm | 0.77% |
| Acceptance criteria | NMT 5% |
| Pass/fail | PASS |

## 3.2.6 system suitability

Fill below table for each analyte .

Table(3.10) shows results of system suitability for orphenadrine citrate

| No | Analyte (1) parameter | Upper limit | Lower limit | Acceptance criteria | P/F |
|---|---|---|---|---|---|
| 1 | Capacity factor | 1.99 | 0.05 | ≤0.2 | pass |
| 2 | Tailing factor | 1.90 | 0.05 | ≤2.0 | pass |
| 3 | Resolution | 30 | 2.3 | >1.5 | pass |
| 4 | Number of theoretical plates | 2000 | 1001 | >1000 | pass |
| 5 | Percentage relative standard deviation | 1.99 | 0.00 | ≤2.0 | pass |

Table(3.11) shows results of system suitability for acetaminophen

| No | Analyte (2) parameter | Upper limit | Lower limit | Acceptance criteria | P/F |
|---|---|---|---|---|---|
| 1 | Capacity factor | 1.9 | 0.05 | ≤0.2 | pass |
| 2 | Tailing factor | 1.9 | 0.05 | ≤2.0 | pass |
| 3 | Resolution | 30 | 2.3 | >1.5 | pass |
| 4 | Number of theoretical plates | 1000 | 401 | >1000 | pass |
| 5 | Percentage relative standard deviation | 1.99 | 0.00 | ≤2.0 | pass |

## 3.2.7 forced degradation :

There is no any interference between acetaminophen , orphenadrine cirtate , internal standard and their degradation products peaks as the minimum resolution obtained is more than 2 , the chromatograms are attached as fallow :

- Figure(3.9) : Thermal degradation zero time .
- Figure(3. 10):Thermal degradation after 6 hours .

### 3.2.8 analytical solution stability

**Standard solution stability**

Table(3.12) shows the results of standard analysis for orphenadrine citrate at zero time, day 1, day 2 and day 3 in stability.

| Time | room temperature | | | 2-8°c | | |
|---|---|---|---|---|---|---|
| | %of orp henadrine citrate | % difference | P/F | % of analyte | % difference | P/F |
| 0 time | 104.29 | 0% | pass | 104.29 | 0% | pass |
| Day 1 | 103.94 | 0.34 | pass | 106.79 | -2.40 | fail |
| Day 2 | 102.75 | 1.48 | pass | 106.96 | -2.56 | fail |
| Day 3 | 109.76 | -5.25 | fail | - | - | - |

Table(3.13) shows the results of standard analysis for acetaminophen at zero time,day 1, day 2 and day 3 in stability.

| Time | room temperature | | | 2-8°c | | |
|---|---|---|---|---|---|---|
| | %of acetaminophen | % difference | P/F | % of analyte | % difference | P/F |
| 0 time | 99.71 | 0% | pass | 99.71 | 0% | pass |
| Day 1 | 99.28 | 0.34 | pass | 100.33 | -0.63 | pass |
| Day 2 | 99.49 | 0.22 | pass | 99.30 | 0.41 | pass |
| Day 3 | 98.57 | 1.14 | pass | - | - | - |

- The standard solution is stable for 48 hrs at room temperature

## Sample solution stability

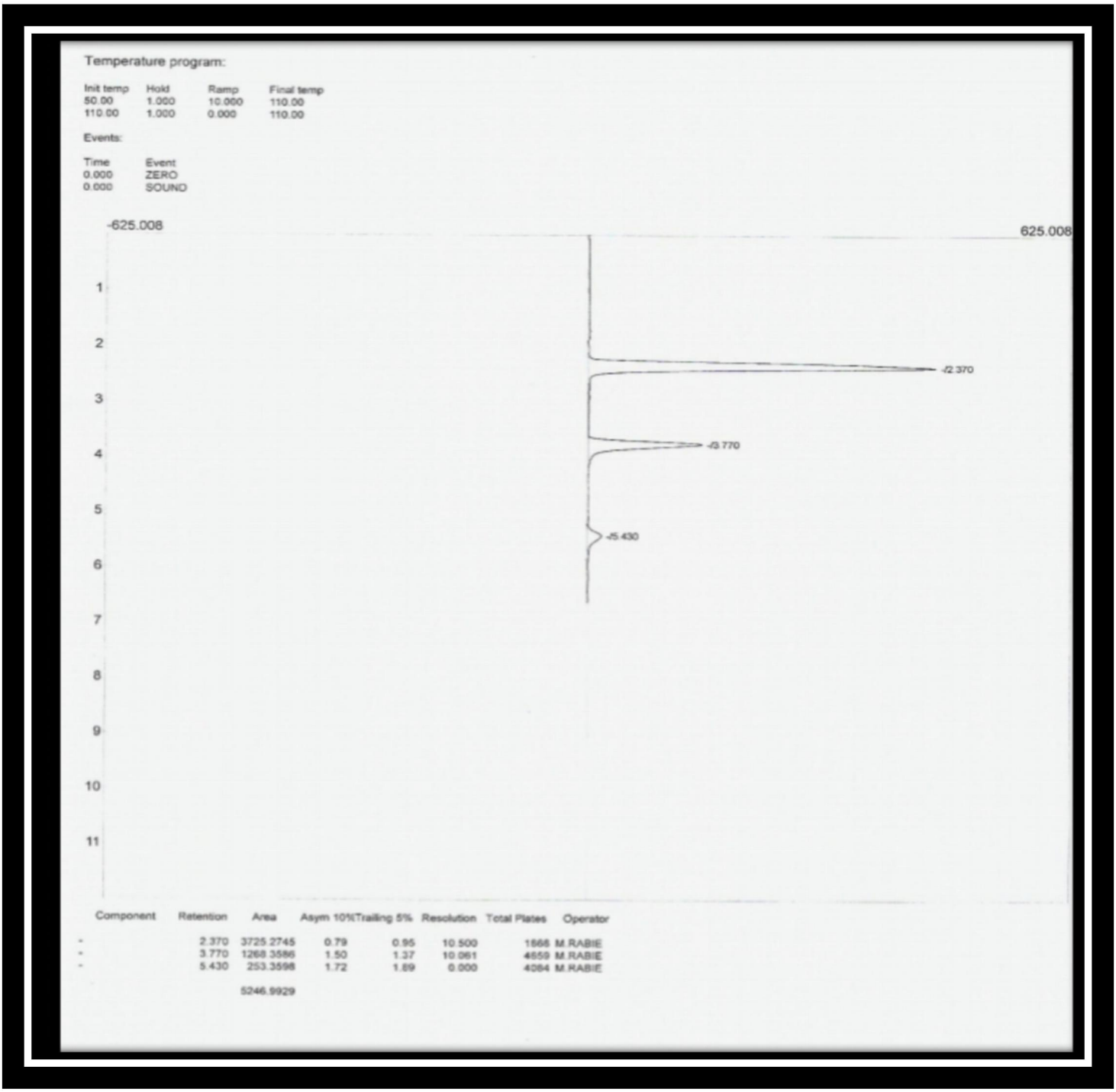
Table(3.14) shows the results of sample analysis for orphenadrine citrate at zero time, day 1, day 2 and day 3 in stability.

| Time | room temperature | | | 2-8°c | | |
|---|---|---|---|---|---|---|
| | %of orphenadrine citrate | % difference | P/F | % of analyte | % difference | P/F |
| 0 time | 103 | 0% | pass | 103 | 0% | pass |
| Day 1 | 102.02 | 0.95 | pass | 100.51 | 2.42 | fail |
| Day 2 | 101.35 | 1.60 | pass | 99.26 | 3.63 | fail |
| Day 3 | 120.73 | -17.22 | fail | - | - | - |

Table(3.15) shows the results of sample analysis for acetaminophen at zero time,day 1, day 2 and day 3 in stability.

| Time | room temperature | | | 2-8°c | | |
|---|---|---|---|---|---|---|
| | %of acetaminophen | % difference | P/F | % of analyte | % difference | P/F |
| 0 time | 101.51 | 0% | Pass | 101.51 | 0% | Pass |
| Day 1 | 100.59 | 0.90 | Pass | 100.46 | 1.03 | Pass |
| Day 2 | 99.61 | 1.87 | Pass | 98.59 | 2.87 | Fail |
| Day 3 | 99.86 | 1.62 | Pass | - | - | - |

- ❖ the sample solution is stable for 72 hrs at room temperature .




Figure(3. 9): thermal degradation zero time chromatogram.

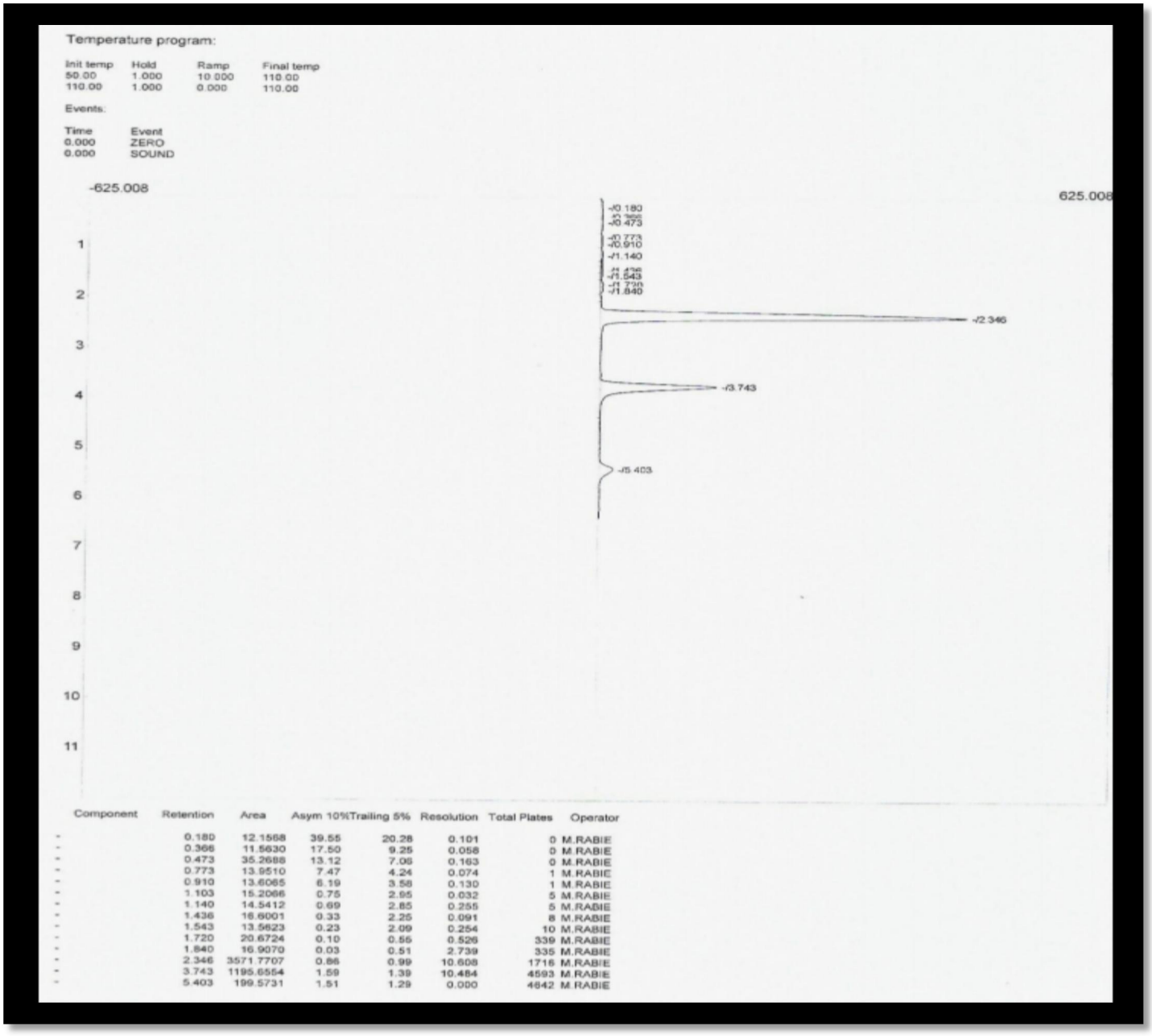



Figure (3.10): thermal degradation after 6 hours chromatogram.

### 3.2.9 Deviation from protocol :

Relative standard deviation of response for repeated injections from the sample limits increase to be NMT 2.5 % because the method validated using manual injector that affect consistency of the results.

**NMT** : Not more than

## ❖ figures:

- Figures(3. 1) : placebo sample chromatogram.
- Figure(3. 2): Representative sample chromatogram.
- Figure(3. 3): orphenadrine citrate alone chromatogram.
- Figure(3. 4): acetaminophen alone chromatogram.
- Figure(3. 5): mobile phase alone chromatogram.
- Figure(3. 6): internal standard alone chromatogram.
- Figure(3. 7): Linearity chart for Orphenadrine citrate
- Figure(3. 8):Linearity chart for acetaminophen
- Figure(3. 9): thermal degradation zero time chromatogram.
- Figure(3. 10): thermal degradation after 6 hours chromatogram.

## 3. 3 Discussion

A simple and sensitive RP-HPLC method was developed for the determination of Paracetamol(Acetaminophen) and Orphenadrine citrate in their combined pharmaceutical formulations. The separation was achieved using Cyanide column (250 × 4.6mm, 5µm particle size), both of components were determined by UV detector at fixed wave length at 220nm, for simplicity of the method an isocratic elution was selected, the optimized mobile phase was composed of triethylamin equeus ,Methanol and acetonitrile (50:20:30) ratio, with flow rate of 1.5ml/min, injection volume was 10µl, and the separation was performed at 25C. Linearity of this method was checked ° using six solutions centered with the target concentration, the concentrations range was (25–200)% for Orphenadrine citrat and (25–200)% for acetaminophen. Each solution was injected in triplicate.Plot of average area versus prepared concentrations indicates a good linearity correlation ($R^2$ =0.9978) for components.

In specificity tests, none of placebo peaks had same retention time of active ingredients peaks.There is no any interference between acetaminophen , orphenadrine cirtate , internal standard and their degradation products peaks as the minimum resolution obtained is more than 2 , the chromatograms are attached as fallow{(3.1)(3.2)(3.3)(3.4)(3.5)(3.6)}. Accuracy was evaluated for orphenadrine citrate and acetaminophen using three concentrations in content of 60%, 100%, and 120% of target concentration. The recovery percentage for orphenadrine citrate at the above concentrations was found to be 98.76, 101. 75 and 101.63, respectively; while for acetaminophen, it was 100.53, 101.71 and 101.76 respectively. The average of recovery percentage for orphenadrine citrate and acetaminophen was 100.71% and 101.33%, respectively. The precision of the methods was examined by estimating the corresponding recovery percentages six times on the same day in intermediate precision the RSD for orphenadrine citrate and acetaminophen was 1.61% and 0.81%. and three times at three different days for analytical (standard and sample )solution stability all this results was pass. The robustness of the method was assessed by assaying test solutions under different analytical conditions deliberately changed from the original conditions such as PH , flow rate and detection wavelength. RSD for the area at all different conditions for target.

## 3.4 Conclusion

The proposed method is simple, sensitive and reproducible and hence the method can be n used in routine for simultaneous determination of orphenadrine citrate and acetaminophe n in tablet as well as in pharmaceutical preparations. Statistical analysis of the results has b een carried out revealing high accuracy and good precision. The RSD for all parameters was found to be less than two, which indicates the validity of method and assay results obtained by this method are in fair agreement. The developed method can be used for routine quantitative simultaneous estimation of orphenadrine citrate and acetaminophen in Multicomponent pharmaceutical preparation .

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