KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

COLLEGE OF HEALTH SCIENCES

DEVELOPMENT OF AN HPLC METHOD FOR THE CHEMICAL QUALITY AND CLEANING VALIDATION OF CEPHALOSPORIN PRODUCTS

A THESIS SUBMITTED TO THE DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

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DEGREE OF MASTER OF SCIENCE: PHARMACEUTICAL ANALYSIS AND QUALITY CONTROL

BY

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DECLARATION

It is hereby declared that this thesis is the outcome of research work undertaken by the author. Any assistance obtained has been duly acknowledged. It has neither in part nor whole been presented for another degree elsewhere.

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TABLE OF CONTENT

DECLARATION ..... **II**

TABLE OF CONTENT ..... **III**

LIST OF TABLES ..... **VI**

LIST OF FIGURE ..... **X**

LIST OF APPENDICES ..... **XIII**

ACKNOWLEGEMENT ..... **XVIII**

ABSTRACT ..... **XIX**

CHAPTER ONE: INTRODUCTION **1**

1.0 BACKGROUND 1

1.1 STATEMENT OF PROBLEM 4

1.2 JUSTIFICATION 6

1.3 OBJECTIVES 7

1.4 SCOPE 7

CHAPTER TWO **9**

2.0 LITERATURE REVIEW **9**

2.1 CEFUROXIME 13

2.2 CEFTRIAXONE 17

2.3 CEFEPIME 22

CHAPTER THREE **26**
3.0 MATERIALS, REAGENTS AND EXPERIMENTAL METHODS **26**
3.1 MATERIALS / REAGENTS 26
3.2 EXPERIMENTAL METHODS 27
3.2.1 IDENTIFICATION 27
3.2.1.0 CEFUROXIME 27
3.1.1.1 CEFTRIAXONE 28
3.1.1.2 CEFEPIME 28
3.1.2 METHOD DEVELOPMENT 28
3.2.3 METHOD OPTIMIZATION 31
3.2.3 METHOD VALIDATION 32
3.2.4 PREPARATION OF THE FORMULATED SAMPLES 33
CHAPTER FOUR **34**
4.0 RESULTS AND CALCULATIONS **34**
4.1 IDENTIFICATION TESTS FOR SAMPLES 34
4.2 CHROMATOGRAPHIC CONDITIONS 35
4.3 RETENTION TIMES FOR VARIOUS MOBILE PHASE CONCENTRATIONS ACROSS THE VARIOUS ANALYTES 36
4 5 REPEATABILITY AND INTERMEDIATE PRECISION STUDY 48
4.6 RUGGEDNESS / ROBUSTNESS 49
4.7 LINEARITY 49
4.8 RECOVERY STUDY 59
4.9 LIMIT OF QUANTIFICATION (LOQ) AND LIMIT OF DETECTION (LOD) 68
4.5 ASSAY OF FORMULATED PRODUCTS 71

CHAPTER FIVE **72**

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION **72**

5.1 DISCUSSION 72

5.1.1 PRODUCT SELECTION 72

5.1.2 SAMPLE CHARACTERIZATION 72

5.1.2.1 IDENTIFICATION OF PURE SAMPLES 72

5.1.3 INITIAL METHOD DEVELOPMENT 73

5.1.4. METHOD OPTIMIZATION WITH C_{18} REVERSE PHASE 74

5.1.5 METHOD VALIDATION 75

5.1.5.1 REPEATABILITY AND INTERMEDIATE PRECISION STUDY 77

5.1.5.2 RUGGEDNESS AND ROBUSTNESS 77

5.1.5.3 RECOVERY STUDY 78

5.1.5.4 LINEAR REGRESSION ANALYSIS 80

5.1.5.5 LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ) 81

5.1.6 ASSAY 83

5.3 RECOMMENDATIONS 84

CHAPTER SIX: REFERENCE **85**

CHAPTER SEVEN: **88**

7.0 APPENDIXES **88**

LIST OF TABLES

TABLE 3.0: PROFILE OF PURE SAMPLES 26

TABLE 4.1.1 IDENTIFICATION TEST RESULTS FOR CEFUROXIME SAMPLE 34

TABLE 4.1.2 IDENTIFICATION TEST RESULTS FOR CEFTRIAXONE SAMPLE 34

TABLE 4.1.3 IDENTIFICATION TEST RESULTS FOR CEFEPIME SAMPLE 35

TABLE 4.3.1 RETENTION TIMES FOR THE CEFEPIME REFERENCE STANDARD USING 75% BUFFER AND 25% METHANOL AS MOBILE PHASE; 42

TABLE 4.3.1.1 STATISTICAL ANOVA TABLE FOR COMPARISON 42

TABLE 4.3.2 RETENTION TIMES FOR THE CEFTRIAXONE REFERENCE STANDARD USING 75% BUFFER AND 25% METHANOL AS MOBILE PHASE; 43

TABLE 4.3.2 .1STATISTICAL ANOVA TABLE FOR COMPARISON 43

TABLE 4.3.3 RETENTION TIMES FOR THE CEFUROXIME REFERENCE STANDARD USING 75% BUFFER AND 25% METHANOL AS MOBILE PHASE;. 44

TABLE 4.3.3.1 STATISTICAL ANOVA TABLE FOR COMPARISON 44

TABLE 4.3.4 RETENTION TIMES FOR THE CEFEPIME REFERENCE STANDARD USING 80% BUFFER AND 20% METHANOL AS MOBILE PHASE; 45

TABLE 4.3.4.1STATISTICAL ANOVA TABLE FOR COMPARISON 45

TABLE 4.3.5 RETENTION TIMES FOR THE CEFTRIAXONE REFERENCE STANDARD USING 80% BUFFER AND 20% METHANOL AS MOBILE PHASE; 46

TABLE 4.3.5.1STATISTICAL ANOVA TABLE FOR COMPARISON 46

TABLE 4.3.6 RETENTION TIMES FOR THE CEFUROXIME REFERENCE STANDARD USING 80% BUFFER AND 20% METHANOL AS MOBILE PHASE. 47

TABLE 4.3.6.1STATISTICAL ANOVA TABLE FOR COMPARISON 47

TABLE.4.4 EFFECT OF CONCENTRATION ON RETENTION TIME 48

TABLE 4.4.1 STATISTICAL ANOVA FOR COMPARISON 48

TABLE 4.6.1 STATISTICAL COMPARISON OF RESULTS TWO DIFFERENT HPLC EQUIPMENTS (30µG/ML OF EACH ANALYTE) 49

TABLE 4.7.1 CORRELATION COEFFICIENTS FOR ALL THE ANALYTE REFERENCE STANDARDS 49

TABLE 4.7.2 PEAK AREA FOR CEFEPIME REFERENCE STANDARDS AND CEFTRIAXONE REFERENCE STANDARD WITH CEFUROXIME AS INTERNAL STANDARD AT VARIOUS CONCENTRATIONS AND THEIR RESPECTIVE CORRELATION COEFFICIENTS 51

TABLE 4.7.3 PEAK AREA FOR CEFEPIME REFERENCE STANDARDS AND CEFUROXIME REFERENCE STANDARD WITH CEFTRIAXONE AS INTERNAL STANDARD AT VARIOUS CONCENTRATIONS AND THEIR RESPECTIVE CORRELATION COEFFICIENTS 54

TABLE 4.7.4 PEAK AREA FOR CEFTRIAXONE REFERENCE STANDARDS AND CEFUROXIME REFERENCE STANDARD WITH CEFEPIME AS INTERNAL STANDARD AT VARIOUS CONCENTRATIONS AND THEIR RESPECTIVE CORRELATION COEFFICIENTS 56

TABLE 4.8.1 MASS RECOVERY STUDIES OF CEFEPIME REFERENCE STANDARD USING STANDARD ADDITION METHOD – CEFTRIAXONE INTERNAL REFERENCE STANDARD 59

TABLE 4.8.2 PERCENTAGE RECOVERY (%) OF CEFEPIME REFERENCE STANDARD USING STANDARD ADDITION METHOD – CEFTRIAXONE INTERNAL REFERENCE STANDARD 59

TABLE 4.8.2.1 STATISTICAL ANOVA TABLE FOR COMPARISON 60

TABLE 4.8.3 MASS RECOVERY STUDIES OF CEFEPIME REFERENCE STANDARD USING STANDARD ADDITION METHOD – CEFUROXIME INTERNAL REFERENCE STANDARD 60

TABLE 4.8.4 PERCENTAGE RECOVERY (%) OF CEFEPIME REFERENCE STANDARD USING STANDARD ADDITION METHOD – CEFUROXIME INTERNAL REFERENCE STANDARD 61

TABLE 4.8.4.1 STATISTICAL ANOVA TABLE FOR COMPARISON 61

TABLE 4.8.5 MASS RECOVERY STUDIES OF CEFTRIAXONE REFERENCE STANDARD USING STANDARD ADDITION METHOD – CEFEPIME INTERNAL REFERENCE STANDARD 62
TABLE 4.8.6 PERCENTAGE RECOVERY (%) OF CEFTRIAXONE REFERENCE STANDARD USING STANDARD ADDITION METHOD –CEFEPIME INTERNAL REFERENCE STANDARD 62
TABLE 4.8.6.1 STATISTICAL ANOVA TABLE FOR COMPARISON 63
TABLE 4.8.7 MASS RECOVERY STUDIES OF CEFTRIAXONE REFERENCE STANDARD USING STANDARD ADDITION METHOD –CEFUROXIME INTERNAL REFERENCE STANDARD 63
TABLE 4.8.8 PERCENTAGE RECOVERY (%) OF CEFTRIAXONE REFERENCE STANDARD USING STANDARD ADDITION METHOD –CEFUROXIME INTERNAL REFERENCE STANDARD 64
TABLE 4.8.8.1 STATISTICAL ANOVA TABLE FOR COMPARISON 64
TABLE 4.8.9 MASS RECOVERY STUDIES OF CEFUROXIME REFERENCE STANDARD USING STANDARD ADDITION METHOD – CEFEPIME INTERNAL REFERENCE STANDARD 65
TABLE 4.8.10 PERCENTAGE RECOVERY (%) OF CEFUROXIME REFERENCE STANDARD USING STANDARD ADDITION METHOD – CEFEPIME INTERNAL REFERENCE STANDARD 65
TABLE 4.8.10.1 STATISTICAL ANOVA TABLE FOR COMPARISON 66
TABLE 4.8.11 MASS RECOVERY STUDIES OF CEFUROXIME REFERENCE STANDARD USING STANDARD ADDITION METHOD – CEFTRIAXONE INTERNAL REFERENCE STANDARD 66
TABLE 4.8.12 PERCENTAGE RECOVERY (%) OF CEFUROXIME REFERENCE STANDARD USING STANDARD ADDITION METHOD – CEFTRIAXONE INTERNAL REFERENCE STANDARD 67
TABLE 4.8.12.1 STATISTICAL ANOVA TABLE FOR COMPARISON 67
TABLE 4.9.1 PEAK HEIGHT BASELINE OF CEFTRIAXONE AND CEFEPIME – CEFUROXIME AS INTERNAL STANDARD 69
TABLE 4.9.2 PEAK HEIGHT BASELINE OF CEFEPIME AND CEFUROXIME – CEFTRIAXONE AS INTERNAL STANDARD 69

TABLE 4.9.3 PEAK HEIGHT BASELINE OF CEFTRIAXONE AND CEFUROXIME - CEFEPIME AS INTERNAL STANDARD 69

TABLE 4.9.4 LIMIT OF QUANTIFICATION (LOQ) AND LIMIT OF DETECTION (LOD) WITH INTERNAL STANDARDS 70

TABLE 4.9.5 PEAK HEIGHT BASELINE OF THE VARIOUS REFERENCE STANDARDS WITH INSTRUMENT RESPONSE 70

TABLE 4.9.6 LIMIT OF QUANTIFICATION (LOQ) AND LIMIT OF DETECTION (LOD) WITH INSTRUMENT RESPONSE 70

TABLE 4.5.1 ASSAY OF FORMULATIONS 71

LIST OF FIGURES

FIG 1.0. A GENERIC STRUCTURE OF CEPHALOSPORIN AND PENICILLIN 1

FIG 2.0 CEPHALOSPORIN STRUCTURE ACTIVITY RELATIONSHIP 13

FIG. 2.1 STRUCTURE OF CEFUROXIME AXETIL 14

FIG. 2.2 STRUCTURE OF CEFUROXIME SODIUM 14

FIG 2.3 STRUCTURE OF CEFTRIAXONE SODIUM..... 17

FIG 2.3 STRUCTURE OF CEFEPIME HYDROCHLORIDE 22

FIG4.3.1 CHROMATOGRAM OF CEFEPIME REFERENCE STANDARD USING 80% BUFFER AND 20% METHANOL AS A MOBILE PHASE WITH C_{18} REVERSE PHASE 36

FIG4.3.2 CHROMATOGRAM OF CEFTRIAXONE REFERENCE STANDARD USING 80% BUFFER AND 20% METHANOL AS A MOBILE PHASE WITH C18 REVERSE PHASE 37

FIG4.3.3 CHROMATOGRAM OF CEFUROXIME REFERENCE STANDARD USING 80% BUFFER AND 20% METHANOL AS A MOBILE PHASE WITH C_{18} REVERSE PHASE 38

FIG4.3.4 CHROMATOGRAMS OF REFERENCE STANDARD MIXTURES OF CEFEPIME, CEFTRIAXONE AND CEFUROXIME USING 80% BUFFER AND 20% METHANOL AS A MOBILE PHASE WITH C_{18} REVERSE PHASE..... 39

FIG4.3.5 CHROMATOGRAMS OF REFERENCE STANDARD MIXTURES OF CEFEPIME, CEFTRIAXONE AND CEFUROXIME USING 85% BUFFER AND 15% METHANOL AS A MOBILE PHASE WITH C_{18} REVERSE PHASE..... 40

FIG4.3.6 CHROMATOGRAMS OF REFERENCE STANDARD MIXTURES OF CEFEPIME, CEFTRIAXONE AND CEFUROXIME USING 75% BUFFER AND 25% METHANOL AS A MOBILE PHASE WITH C_{18} REVERSE PHASE..... 41

TABLE 4.5.1 REPEATABILITY AND INTERMEDIATE PRECISION (REPRODUCIBILITY)..... 48

FIG 4.7 1CALIBRATION PLOTS OF CEFEPIME REFERENCE STANDARD WITH INSTRUMENT RESPONSE50

FIG 4.7.2 CALIBRATION PLOTS OF CEFTRIAXONE REFERENCE STANDARD WITH INSTRUMENT RESPONSE 50

FIG 4.7.3 CALIBRATION PLOTS OF CEFUROXIME REFERENCE STANDARD WITH INSTRUMENT RESPONSE 51

FIG. 4.7.4 CALIBRATION PLOTS FOR CEFTRIAXONE REFERENCE STANDARD USING 0.030MG/ML CEFUROXIME REFERENCE STANDARD AS INTERNAL STANDARD 52

FIG. 4.7.5 RESIDUAL PLOTS FOR CEFTRIAXONE REFERENCE STANDARD USING 0.030MG/ML CEFUROXIME REFERENCE STANDARD AS INTERNAL STANDARD 52

FIG. 4.7.6 CALIBRATION PLOTS FOR CEFEPIME REFERENCE STANDARD USING 0.030MG/ML CEFUROXIME REFERENCE STANDARD AS INTERNAL STANDARD 53

FIG. 4.7.8 RESIDUAL PLOTS FOR CEFEPIME REFERENCE STANDARD USING 0.030MG/ML CEFUROXIME REFERENCE STANDARD AS INTERNAL STANDARD 53

FIG. 4.7.9 CALIBRATION PLOTS FOR CEFEPIME REFERENCE STANDARD USING 0.030MG/ML CEFTRIAXONE REFERENCE STANDARD AS INTERNAL STANDARD 54

FIG. 4.7.10 RESIDUAL PLOTS FOR CEFEPIME REFERENCE STANDARD USING 0.030MG/ML CEFTRIAXONE REFERENCE STANDARD AS INTERNAL STANDARD 55

FIG. 4.7.11 CALIBRATION PLOTS FOR CEFUROXIME REFERENCE STANDARD USING 0.030MG/ML CEFTRIAXONE REFERENCE STANDARD AS INTERNAL STANDARD 55

FIG. 4.7.12 RESIDUAL PLOTS FOR CEFUROXIME REFERENCE STANDARD USING 0.030MG/ML CEFTRIAXONE REFERENCE STANDARD AS INTERNAL STANDARD 56

FIG. 4.7.13 CALIBRATION PLOTS FOR CEFTRIAXONE REFERENCE STANDARD USING 0.030MG/ML CEFEPIME REFERENCE STANDARD AS INTERNAL STANDARD 57

FIG. 4.7.14 RESIDUAL PLOTS FOR CEFTRIAXONE REFERENCE STANDARD USING 0.030MG/ML CEFEPIME REFERENCE STANDARD AS INTERNAL STANDARD 57

FIG. 4.7.15 CALIBRATION PLOTS FOR CEFUROXIME REFERENCE STANDARD USING 0.030MG/ML CEFEPIME REFERENCE STANDARD AS INTERNAL STANDARD 58

FIG. 4.7.16 RESIDUAL PLOTS FOR CEFUROXIME REFERENCE STANDARD USING 0.030MG/ML CEFEPIME REFERENCE STANDARD AS INTERNAL STANDARD 58

FIG 4.9.1 CHROMATOGRAMS FOR LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION..... 68

LIST OF APPENDICES

TABLE 7.1 RETENTION TIMES FOR THE CEFEPIME REFERENCE STANDARD USING 80% BUFFER AND 20% METHANOL AS MOBILE PHASE; C_{18} AS REVERSE STATIONARY PHASE USING DIFFERENT CONCENTRATION LEVELS. 88

TABLE 7.1.1 STATISTICAL ANOVA TABLE FOR COMPARISON 88

TABLE 7.2 RETENTION TIMES FOR THE CEFTRIAXONE REFERENCE STANDARD USING 80% BUFFER AND 20% METHANOL AS MOBILE PHASE; C_{18} AS REVERSE STATIONARY PHASE USING DIFFERENT CONCENTRATION LEVELS. 89

TABLE 7.2.1STATISTICAL ANOVA TABLE FOR COMPARISON 89

TABLE 7.3 RETENTION TIMES FOR THE CEFUROXIME REFERENCE STANDARD USING 80% BUFFER AND 20% METHANOL AS MOBILE PHASE; C_{18} AS REVERSE STATIONARY PHASE USING DIFFERENT CONCENTRATION LEVELS. 90

TABLE 7.3.1 STATISTICAL ANOVA TABLE FOR COMPARISON 90

TABLE 7.4 AREA UNDER THE CURVE FOR $0.0003\%^{W}/_{V}$ CEFEPIME REFERENCE STANDARD 91

TABLE 7.4.1 STATISTICAL ANOVA TABLE FOR COMPARISON 91

TABLE 7.5 AREA UNDER THE CURVE FOR $0.0003\%^{W}/_{V}$ CEFTRIAXONE REFERENCE STANDARD (50% CONCENTRATION LEVEL) USING 80% BUFFER AND 20% METHANOL AS MOBILE PHASE; C_{18} AS REVERSE STATIONARY PHASE OVER A THREE- DAY PERIOD 92

TABLE 7.5.1STATISTICAL ANOVA TABLE FOR COMPARISON 92

TABLE 7.6 STATISTICAL COMPARISON OF TWO DIFFERENT HPLC EQUIPMENTS BY DIFFERENT UNDER DIFFERENT LABORATORY CONDITIONS AT THE SAME TIME USING THE MEAN AREA UNDER THE CURVE 93

TABLE 7.6.1 F-TEST FOR ROBUSTNESS 93

TABLE 7.7 AREA UNDER THE CURVE FOR 0.0003%$^{W}/_{V}$ CEFUROXIME REFERENCE STANDARD USING 80% BUFFER AND 20% METHANOL AS MOBILE PHASE; 94
TABLE 7.7.1STATISTICAL ANOVA TABLE FOR COMPARISON 94
TABLE 7.8 AREA UNDER THE CURVE FOR 0.0003%$^{W}/_{V}$ CEFEPIME REFERENCE STANDARD 95
TABLE 7.8.1STATISTICAL ANOVA TABLE FOR COMPARISON 95
TABLE 7.9 AREA UNDER THE CURVE FOR 0.0003%$^{W}/_{V}$ CEFTRIAXONE REFERENCE 96
TABLE 7.9.1STATISTICAL ANOVA TABLE FOR COMPARISON 96
TABLE 7.10 AREA UNDER THE CURVE FOR 0.0003%$^{W}/_{V}$ CEFUROXIME REFERENCE STANDARD 97
TABLE 7.10.1STATISTICAL ANOVA TABLE FOR COMPARISON 97
TABLE 7.11 AREA UNDER THE CURVE FOR 0.00045%$^{W}/_{V}$ CEFEPIME REFERENCE STANDARD 98
TABLE 7.11.1STATISTICAL ANOVA TABLE FOR COMPARISON 98
TABLE 7.12 AREA UNDER THE CURVE FOR 0.00045%$^{W}/_{V}$ CEFTRIAXONE REFERENCE STANDARD ... 99
TABLE 7.12.1STATISTICAL ANOVA TABLE FOR COMPARISON 99
TABLE 7.13 AREA UNDER THE CURVE FOR 0.00045%$^{W}/_{V}$ CEFUROXIME REFERENCE STANDARD... 100
TABLE 7.13.1STATISTICAL ANOVA TABLE FOR COMPARISON 100
TABLE 7.14 AREA UNDER THE CURVE FOR 0.0006%$^{W}/_{V}$ CEFEPIME REFERENCE STANDARD 101
TABLE 7.14.1STATISTICAL ANOVA TABLE FOR COMPARISON 101
TABLE 7.15 AREA UNDER THE CURVE FOR 0.0006%$^{W}/_{V}$ CEFTRIAXONE REFERENCE STANDARD ... 102
TABLE 7.15.1STATISTICAL ANOVA TABLE FOR COMPARISON 102
TABLE 7.16 AREA UNDER THE CURVE FOR 0.0006%$^{W}/_{V}$ CEFUROXIME REFERENCE STANDARD..... 103
TABLE 7.16.1STATISTICAL ANOVA TABLE FOR COMPARISON 103
TABLE 7.17 AREA UNDER THE CURVE FOR 0.00075%$^{W}/_{V}$ CEFEPIME REFERENCE STANDARD 104
TABLE 7.17.1STATISTICAL ANOVA TABLE FOR COMPARISON 104
TABLE 7.18 AREA UNDER THE CURVE FOR 0.00075%$^{W}/_{V}$ CEFTRIAXONE REFERENCE STANDARD . 105

TABLE 7.18.1STATISTICAL ANOVA TABLE FOR COMPARISON 105

TABLE 7.19 AREA UNDER THE CURVE FOR 0.0075%$^{W}/_{V}$ CEFUROXIME REFERENCE STANDARD 106

TABLE 7.19.1STATISTICAL ANOVA TABLE FOR COMPARISON 106

TABLE 7.20 AREA UNDER THE CURVE FOR 0.0009%$^{W}/_{V}$ CEFEPIME REFERENCE STANDARD 107

TABLE 7.20.1STATISTICAL ANOVA TABLE FOR COMPARISON 107

TABLE 7.21 AREA UNDER THE CURVE FOR 0.0009%$^{W}/_{V}$ CEFTRIAXONE REFERENCE STANDARD ... 108

TABLE 7.21.1STATISTICAL ANOVA TABLE FOR COMPARISON 108

TABLE 7.22 AREA UNDER THE CURVE FOR 0.0009%$^{W}/_{V}$ CEFUROXIME REFERENCE STANDARD 109

TABLE 7.22.1STATISTICAL ANOVA TABLE FOR COMPARISON 109

TABLE 7.23 AREA UNDER THE CURVE FOR 0.0012%$^{W}/_{V}$ CEFEPIME REFERENCE STANDARD 110

TABLE 7.23.1STATISTICAL ANOVA TABLE FOR COMPARISON 110

TABLE 7.24 AREA UNDER THE CURVE FOR 0.0012%$^{W}/_{V}$ CEFTRIAXONE REFERENCE STANDARD ... 111

TABLE 7.24.1STATISTICAL ANOVA TABLE FOR COMPARISON 111

TABLE 7.25 AREA UNDER THE CURVE FOR 0.012%$^{W}/_{V}$ CEFUROXIME REFERENCE STANDARD 112

TABLE 7.25.1STATISTICAL ANOVA TABLE FOR COMPARISON 112

TABLE 7.26 CALIBRATION CURVE FOR CEFEPIME REFERENCE STANDARD USING 0.030MG/ML CEFTRIAXONE REFERENCE STANDARD AS INTERNAL STANDARD 113

TABLE 7.27 CALIBRATION CURVE FOR CEFEPIME REFERENCE STANDARD USING 0.030MG/ML CEFUROXIME REFERENCE STANDARD AS INTERNAL STANDARD 113

TABLE 7.27.1 LINEAR REGRESSION ANALYSIS FOR CEFEPIME REFERENCE STANDARD WITH 0.030MG/ML CEFTRIAXONE AS INTERNAL REFERENCE STANDARD 114

TABLE 7.28 CALIBRATION CURVE FOR CEFTRIAXONE REFERENCE STANDARD USING 0.030MG/ML CEFEPIME REFERENCE STANDARD AS INTERNAL STANDARD 114

TABLE 7.29 CALIBRATION CURVE FOR CEFTRIAXONE REFERENCE STANDARD USING 0.030MG/ML CEFUROXIME REFERENCE STANDARD AS INTERNAL STANDARD 115
TABLE 7.30 CALIBRATION CURVE FOR CEFUROXIME REFERENCE STANDARD USING 0.030MG/ML CEFEPIME REFERENCE STANDARD AS INTERNAL STANDARD 115
TABLE 7.31 CALIBRATION CURVE FOR CEFUROXIME REFERENCE STANDARD USING 0.030MG/ML CEFTRIAXONE REFERENCE STANDARD AS INTERNAL STANDARD..... 116
TABLE 7.32 % RSD OF CEFEPIME REFERENCE STANDARD IN RECOVERY STUDIES 116
TABLE 7.33 % RSD OF CEFEPIME REFERENCE STANDARD IN RECOVERY STUDIES 116
TABLE 7.34 % RSD OF CEFTRIAXONE REFERENCE STANDARD IN RECOVERY STUDIES..... 117
TABLE 7.35 % RSD OF CEFTRIAXONE REFERENCE STANDARD IN RECOVERY STUDIES..... 117
TABLE 7.36 % RSD OF CEFUROXIME REFERENCE STANDARD IN RECOVERY STUDIES 117
TABLE 7.37 % RSD OF CEFUROXIME REFERENCE STANDARD IN RECOVERY STUDIES 118
FIG 7.1. CHROMATOGRAMS OF CEFEPIME, CEFTRIAXONE AND CEFUROXIME MIXTURE. 119
FIG 7.2 CHROMATOGRAMS OF CEFEPIME, CEFTRIAXONE AND CEFUROXIME MIXTURE AT THEIR RESPECTIVE CONCENTRATION LEVELS OF 50% WITH DIFFERENT ANALYST AND HPLC AT STANDARD CONDITIONS 1. 120
FIG 7.3 CHROMATOGRAMS OF CEFEPIME, CEFTRIAXONE AND CEFUROXIME MIXTURE AT THEIR RESPECTIVE CONCENTRATION LEVELS OF 50% WITH DIFFERENT ANALYST AND HPLC AT STANDARD CONDITIONS 2. 121
FIG 7.4 CHROMATOGRAMS OF CEFEPIME, CEFTRIAXONE AND CEFUROXIME MIXTURE AT THEIR RESPECTIVE CONCENTRATION LEVELS OF 75% 122
FIG 7.5 CHROMATOGRAMS OF CEFEPIME, CEFTRIAXONE AND CEFUROXIME MIXTURE AT THEIR RESPECTIVE CONCENTRATION LEVELS OF 125% 123

FIG 7.6 CHROMATOGRAMS OF CEFEPIME, CEFTRIAXONE AND CEFUROXIME MIXTURE AT THEIR RESPECTIVE CONCENTRATION LEVELS OF 150% 124
FIG 7.7 CHROMATOGRAMS OF CEFEPIME, CEFTRIAXONE AND CEFUROXIME MIXTURE AT THEIR RESPECTIVE CONCENTRATION LEVELS OF 200% 125
FIG 7.8 CHROMATOGRAMS OF CEFEPIME, CEFTRIAXONE AND CEFUROXIME MIXTURE 126
7.1 PREPARATION OF SOLUTIONS 127
7.2 ABBREVIATIONS 128

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ABSTRACT

A new cost effective HPLC method of analysis has been developed to assay separately and simultaneously these compounds; **cefuroxime (injection), ceftriaxone (injection) and cefepime (injection).** 80% Phosphate buffer and 20% methanol as mobile phase with C_{18} reverse phase column; Zorbax ODS (C_{18}) - 4.6mm x 25cm was used. The detector used was UV-Vis with a wavelength of 260nm. The flow rate and the injector volume were **1.5ml/min** and **20µl** respectively. Diluents was methanol (80% phosphate buffer in the ratio 1:4).

The results were analyzed statistically using ANOVA, T-test and F-Test. At 95% confidence level, the method is robust. The average retention time for **Cefepime**, **Ceftriaxone** and **Cefepime** were **4.78min ± 0.016**, **5.87 ± 0.039** and **15.39 ± 0.026** respectively. The percentage recovery for respective cephalosporin injections in the assay performed at the three different concentration levels were greater than **98%** and their relative standard deviation were less than **2%** and within acceptable limits of accuracy. The linear regression analysis results showed a correlation coefficient of about 0.99 for each calibration plot. This gave an indication of good linear relationship between instrument response and analytes concentration in the range 30µg/ml to 120µg/ml for each cephalosporin analytes. The **limit of Quantification (LOQ)** and **limit of Detection (LOD)** based on instrument response were as follows **Cefepime: LOQ – 0.003933mg/ml** and **LOD – 0.001180mg/ml**; **Ceftriaxone: LOQ - 0.001595mg/ml** and **LOD - 0.0004786mg/ml.** and **Cefuroxime: LOQ – 0.0007239mg/ml** and **LOD – 0.0002172mg/ml**.

The HPLC method of analysis can effectively assay separately and simultaneously **cefuroxime**, **ceftriaxone** and **cefepime** products and hence had performance of the developed method as a validation procedure for cleaning of equipments after manufacturing the cephalosporin products assured at confidence level of 95%.

CHAPTER ONE: INTRODUCTION

1.0 Background

Cephalosporins are **β-lactam antibiotics** with the same fundamental structural requirements as penicillin. They are used for the treatment of infections caused by Gram-positive and Gram-negative bacteria. They act by inhibiting the synthesis of essential components of bacterial cell wall. They are among the safest and the most effective broad-spectrum antibactericidal antibiotics. [1], [2] As only **cephalosporin C** is found naturally, the remaining semi-synthetic cephalosporins are derived from 7-amino-cephalosporanic acid, a product obtained from cephalosporin C hydrolysis. [3] Their composition is accomplished by β-lactam ring fusion with a dihydrothiazine ring differing in the nature of the substituents attached at the 3- and/or 7-positions of the **cephem ring**. The substitution at the 3-position affects the pharmacokinetic properties, whereas the substitution at the 7-position affects the antibacterial spectrum of the cephalosporins. [4] 7-amino-cephalosporanic acid derivative are much more acid and stable than the corresponding 6-amino-penicillinic acid compounds. [5]

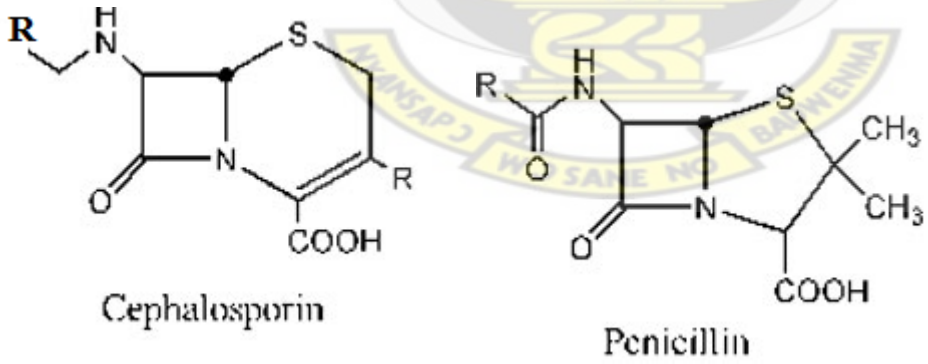


Fig 1.0. A generic structure of cephalosporin and penicillin

Cephalosporin antibiotics are divided into four generations: first, second, third, and fourth generation compounds based on their spectrum of antimicrobial activity.

First generation cephalosporins are moderate spectrum agents. They are effective alternatives for treating staphylococcal and streptococcal infections and therefore are alternatives for skin and soft-tissue infections, as well as for streptococcal **pharyngitis.** Examples of first generation cephalosporin are as follows:

- **Cefadroxil**
- **Cephalexin**
- **Cephaloridine**
- **Cephalothin**
- **Cephapirin**
- **Cefazolin**
- **Cephradine**

The second generation cephalosporins have a greater gram-negative spectrum while retaining some activity against gram-positive bacteria. They are useful agents for treating upper and lower respiratory tract infections**, sinusitis** and **otitis media**. These agents are also active against *E. coli, Klebsiella and Proteus*, which makes them potential alternatives for treating urinary tract infections caused by these organisms. Examples of second generation cephalosporin are:

- **Cefaclor**
- **Cefoxitin**
- **Cefprozil**
- **Cefuroxime** [1]

Third generation cephalosporins have a broad spectrum of activity and further increased activity against gram-negative organisms. Some members of this group (particularly those available in an oral formulation) have decreased activity against gram-positive organisms. Examples of third generation cephalosporin are as follows:

- **Cefdinir**
- **Cefixime**
- **Cefpodoxime**
- **Ceftibuten**
- **Ceftriaxone**
- **Cefotaxime** [1]

Fourth generation cephalosporins are extended spectrum agents with similar activity against gram-positive organisms as first generation cephalosporins. They also have a greater resistance to beta-lactamases than the third generation cephalosporins. Many can cross blood brain barrier and are effective in meningitis. Examples of fourth generation cephalosporin are as follows:

- **Cefepime**
- **Cefluprenam**
- **Cefozopran**
- **Cefpirome**
- **Cefquinome** [1]

In Ghana, cephalosporins are used to treat a wide variety of bacterial infections, such as **respiratory tract infections (pneumonia, strep throat, tonsillitis, and bronchitis), skin infections and urinary tract infections**. They are sometimes given with other antibiotics. Cephalosporins are also commonly used for surgical prophylaxis - prevention of bacterial infection before, during, and after surgery. **Bacterial meningitis** is a threat of an epidemic in the northern part of the country therefore travelers are advised to get vaccinated. Ministry of Health has adopted the use of **ceftriaxone** as first line treatment for bacterial meningitis.[2]

1.1 Statement of Problem

According to **British Pharmacopoeia (BP)** and **United States Pharmacopoeia (USP)**, all cephalosporins have specific HPLC method of assay and identification. Each cephalosporin has its mobile phase, column and method optimization different from each other.

In HPLC analysis of cephalosporins, each product has its own way of analysis using the BP and USP. So far there is no reported method for analyzing more than one product either separately or simultaneously. This makes the local manufacturers spend more on their budget when they want to produce more cephalosporin products. So they resort to produce those with relatively simple method of analysis thus **cephalexin** and **cefuroxime** products. In Ghana, the cephalosporins currently listed among the essential medicine are **cephalexin**, **cefuroxime**, **ceftriaxone** and **cefepime** products. The above challenge has a lot of effects on the operations of regulatory bodies and other quality control laboratories in monitoring of quality of these antibiotics. These cephalosporins have varied sources of import.

Most regulatory bodies have to run a lot of analysis on these antibiotics in order to monitor their respective quality. Most of the reagents are not readily available and are also expensive.

This makes other quality control laboratories not able to perform thorough analysis on these cephalosporin products. There is no reported common method of analysis for these cephalosporin products which will enhance routine work of regulatory bodies and other quality control laboratories.

In this regard, different mobile phase, column and buffers are needed for analysis of any two or more given cephalosporins. This becomes labour intensive when two or more cephalosporins are being manufactured. Checking cross contamination will involve all the methods of analysis of cephalosporins in question.

Additionally, the official methods involve the use of reagents which are not readily available and are also relatively expensive. These methods involve the use of specific or specialized column which cannot be used for other products hence making method of analysis very expensive and limited in our local setting with the possibility of compromise on drug product quality.

1.2 Justification

This project therefore seeks to develop methods which can be used to analyse cephalosporins currently in the essential medicine list of Ghana and then validated against their respective methods of analysis according to either the **British Pharmacopoeia** or **United States Pharmacopoeia.**

As numerous new molecules of cephalosporin are being developed, purity of the product is important. As cross-contamination may occur between these cephalosporins or other products during their production, a simple separation technique is required to determine contamination of one cephalosporin with another. This method offers the following benefits:

- The use of water and methanol mixture is relatively cheaper than any other mobile phase system for assay of any cephalosporin. Moreover, the ability of an analyst to run a mixture of the cephalosporins which gives the same result as the cephalosporins being run individually. This depicts a means of saving time and solutions.
- A combination product of cephalosporin and any other related group of antibiotic can be assayed easily. This will encourage combination product of antibiotics involving cephalosporin to be manufactured. Hence having broader spectrum of action and enhancing patient adherence.
- Among cephalosporins and cleaning of manufacturing equipment.
- The cost of cephalosporin product will be reduced. This is due to the fact that local manufacturers can produce more generations of cephalosporins than they do now. The cost of most cephalosporins is high because they are imported as finished products.
- The above benefit will then create employment for local manufacturers for packaging and more factory hands at all levels of production.

- This will be of great help to the laboratories of regulatory bodies in the country who needs to run a lot of analysis of chemical compounds and offer a guideline for selection of cephalosporins to be imported into the country.

1.3 Objectives

The Main Objective of this project seeks to develop a new HPLC method of analysis that can be used to assay separately or simultaneously at least a **cephalosporin** from each of the four generations. The specific objectives of this research are to:

- ❖ Develop and validate a cost effective HPLC method for **cefuroxime** (injection), **ceftriaxone** (injection) and **cefepime** (injection)
- ❖ Evaluate the performance of the developed method as a validation procedure for cleaning of equipments after manufacturing the above **cephalosporin** products
- ❖ Use this method as a validation to check cross contamination among the above cephalosporins.

1.4 Scope

The work involves HPLC method development which comprises of initial method development, method optimization and application of method for the assay of three cephalosporin products separately and simultaneously.

Initial method development involves column, detector and mobile phase selection. The focus will be on reversed- phase methods for quantitative analysis and uv- vis detection. The use of water and methanol mixture as the mobile phase is of great importance in this research.

These factors are defined by the physio-chemical properties of the analytes. **Standard reverse-phase high-performance liquid chromatography** is used because **cephalosporin** products are relatively polar compounds which are poorly retained on C_{18} and C_8 columns.

Method optimization and fine-tuning will involve the use of phosphate buffers, having right pH range for adequate resolution with sufficient precision and sensitivity in a reasonable time. Method validation process will ensure that, the test procedure is accurate, reproducible and robust. The analytical performance parameters shall be:

- **specificity,**
- **linearity,**
- **accuracy,**
- **limit of detection,**
- **limit of quantitation,**
- **precision,**
- **range,**
- **robustness, and**
- **system suitability**.

CHAPTER TWO

2.0 LITERATURE REVIEW

Quality monitoring is an essential operation of pharmaceutical industries, regulatory institutions, research and educational institutions. Drugs must be manufactured as safe and therapeutically active formulations whose performance is consistent and predictable. New medications are being produced at accelerated rate hence more exacting and sophisticated analytical methods are developed for their quality monitoring. Globally, regulatory bodies consistently review requirements for quality monitoring, this makes quality monitoring a vital operation and needs continuous validation. Hence it involves quite a large percentage capital expenditure of an institutions' budget. [3]

Quality monitoring means checking and directing the degree or grade of excellence of processes and products. To the ethical Pharmaceutical player, it implies a detailed system of inspection and control covering production, evaluation and distribution of every drug in question. It is the purpose of this operation to produce medications of superior efficacy, safety and quality which will provide assurance to the physician, pharmacist and the general public that a given product performs uniformly and for the purpose it is intended. [4]

In Ghana, the personal and professional role played by Pharmacists for many years in preparing and compounding drugs has been gradually taken over by Pharmaceutical Industry which now provides an impressive finished dosage form. Ninety-six percent or more of all medications the Pharmacist dispenses are now compounded and packaged by the Industry. This brings into light the issue of the control of quality of the drugs.

This good deal of responsibility such as to conduct and actions and well-defined codes of performance has shifted from a recognized profession to a competitive free-enterprise industry. They are slightly subjected to restrictions.

Its business is to produce and supply medicines to Pharmacist as finished dosage forms. Although groups within Pharmaceutical industry organized themselves into trade association governed by professional codes including requirements for quality control of products manufactured and marketed, membership in such association remain voluntary. Requirements for the conduct Pharmaceutical companies are not restricted to professional bodies as in the case of practice of medicine or pharmacy. Legally individuals without relevant profession background may engage in and carry on Pharmaceutical business in our free-enterprise economy. [5]

The Food and Drugs Board of Ghana has various operational guidelines to govern the quality of drugs. Some of these guidelines have a platform for inclusion of recognized professions to control the quality of drugs. These guidelines do not compromise the free-enterprise economy but to avoid any restrictions as to conduct and well-defined codes of performance by recognized profession. One of the guidelines is the selection of authorized personnel in pharmaceutical or chemical industry. In the guideline 'a qualified person' means a key personnel among the manufacturing establishment responsible for the release of batches of finished products for sale.

The Board shall confer the status of 'qualified person' with a degree or its equivalent in the following disciplines and possesses requisite years of experience in pharmaceutical or chemical industry. The disciplines are **pharmacy**, **chemistry**, **pharmaceutical sciences**, **chemical engineering**, **biological science** and **biomedical science**. [5]

Notwithstanding the above requirements, one needs to have deep knowledge in pharmaceutical quality system regulation, products manufacturing, current Good Manufacturing Practice (cGMP) guidelines, standard control technology and general work practices.[7] All these measures are in place in order to ensure quality monitoring in all endeavours.

Quality monitoring is very important in the pharmaceutical industry. This ensures products of superior efficacy and safety. Requirements pertaining to quality monitoring of pharmaceuticals must be examined in the light of the importance of the industry to the health and the welfare of Ghana. If proper quality monitoring is not done, resistance to these medications may develop which is life threatening and will increase the cost of health. Hence, social responsibility imposes important moral obligation on the manufacturer to market drugs of continuing uniformity and safety.

The manufacturer's reputation is indeed heavily invested in the quality monitoring it exercise over its products on the market.The government plays an important role of protecting the consuming public against health hazards and fraud. It is the objective of this communication to outline some of the requirements set forth in the Food and Drug Act and Regulations for manufacture and quality control of drugs distributed in Ghana. Regulatory requirements cover preparations manufactured in foreign countries and imported into Ghana as well as preparations manufactured locally.

Quality monitoring is capital intensive and mandatory operational procedures. It involves in-process and post-market procedure of drug analysis. It is essential that technically qualified personnel are employed to supervise formulation, processing, testing, packaging and labelling of the drug.

A competent staff is placed in charge of the maintenance of machinery, equipment and sanitation. A simple, precise and cost-effective method of analysis needs to be developed and validated against the method of analysis in the official compendia. These measures will help cut down on the cost of quality monitoring.

The single most important medicine ever discovered is the antibiotic. Life saving antibiotics is losing power due to resistant germs. Germ resistance can result from drug misuse, substandard drug and rapid degradation of drug. Quality monitoring needs to be done to check substandard drugs, to check on excipients which will cause degradation of the active pharmaceutical ingredients in formulations, to check on related compounds in a formulation which can easily cause cross resistance and to check on how long a formulation can remain wholesome for consumption.

Drug resistance is both a public health and global security threat. [8]

Resistance has emerged for almost all known antibiotics in use. One of the ways to minimize this threat is to understand that antibiotics are precious medications that need to be preserved for life threatening infections. In Ghana, Cephalosporins are preserved either as monotherapy or in combination with other antibiotics for life threatening infections.

Another way is funding for research on drug resistance and simple method development for quality monitoring of these lifesaving antibiotics.

Most of the methods of analysis in the official compendia are complex and expensive to run for routine quality monitoring. The Food and Drugs Board should have priority regulatory review for applications of these lifesaving antibiotics – **cephalosporins.**

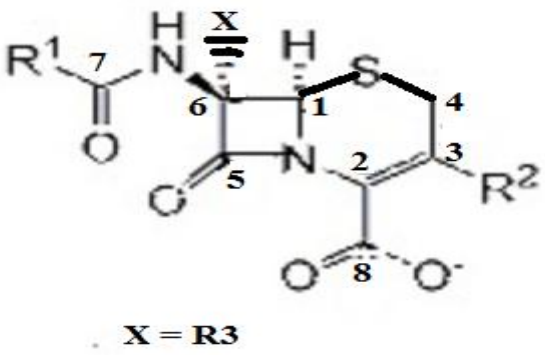


Fig 2.0 Cephalosporin structure activity relationship

Beta lactam ring is required for penicillin binding protein reactivity and antibacterial activity. The penicillin binding protein is reduced as compared to pencillins.

The 2- carboxyl groups indicate acidic formation and enhance product formulation. These help with prodrug formulation and make the drug to undergo renal elimination.

The X-substituent is normally the removal of the hydrogen atom on carbon -6 and this enhances resistance to beta-lactamase.

2.1 Cefuroxime

Cefuroxime come in two forms; cefuroxime sodium (Sodium (6*R*,7*R*)-3-[(carbamoyloxy)methyl]-7-[[(*Z*)-(furan-2-yl)(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate) and cefuroxime axetil (a mixture of the two diastereoisomers:(1*RS*)-1-(acetyloxy)ethyl(6*R*,7*R*)-3-[(carbamoyloxy)methyl]-7-[[(*Z*)-2-(furan-2-yl)-2-(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate) [9]

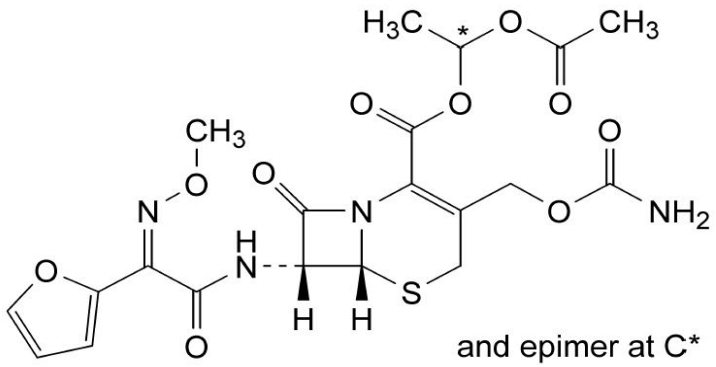


Fig. 2.1 Structure of Cefuroxime axetil

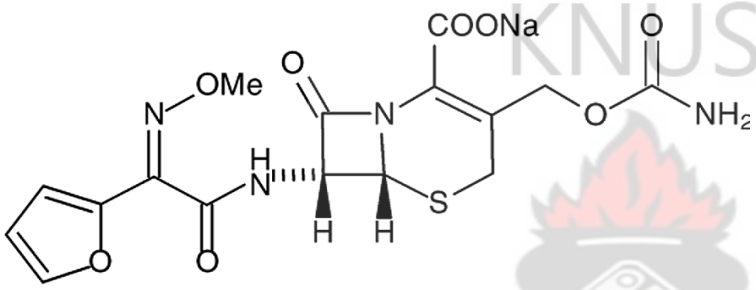


Fig. 2.2 Structure of Cefuroxime sodium

According to the **British Pharmacopoeia**, the assays of the two forms of Cefuroxime are different from each other in terms of column and mobile phase. For the assay of cefuroxime sodium, the British Pharmacopoeia (2005) adopts a mixture of tetradecylammonium bromide, tetraheptylammonium bromide, water, citric acid, phosphate buffer solution pH 7 and acetonitrile (25:75) as a mobile phase. The stationary phase is octadecylsilyl silica gel with uv detection at 254nm and a flow rate of 1.5ml/min.

According to the British Pharmacopoeia, the assay of cefuroxime axetil involves the use of methanol and ammonium dihydrogen phosphate mixture (80:20) as a mobile phase with trimethylsilyl silica gel as a stationary phase.

Its flow rate is 1.0ml/min and uv detection at 278nm. These are salts of the same molecule but their methods of analysis differ from each other. They have a different wavelength of detection. Moreover, the reagents needed for their respective analyse is relatively expensive and not readily available in this part of the World for routine work. [9]

CAN Nafiz Ö et al were able to determine cefuroxime axetil in tablets and biological fluids using liquid chromatography and flow injection analysis. Cefuroxime axetil is the pro-drug of the cephalosporin cefuroxime that is used in the treatment of common community-acquired infections. A simple and precise liquid chromatographic method for the determination of cefuroxime axetil in pharmaceutical tablets, human serum and urine has been developed and validated. Cefuroxime axetil and indapamide (internal standard) were separated by a reversed phase column (Supelco Hypersil 5 μm, 150 mm x 4.6 mm i.d., C_{18}) using a mobile phase consisting of KH_2PO_4 (0.1 M) and acetonitrile (70:30 v/v) (at pH 4.0). The mobile phase was pumped at 1.0 mL min^{-1} flow rate and cefuroxime axetil was detected by ultraviolet detection at 281 nm within an average analysis time of 11 min.

Flow injection analysis was performed for pharmaceutical tablet analysis using a carrier stream of methanol: water (10:90 v/v) with a flow rate of 1.0 mL min^{-1}. The LOD and LOQ concentrations of the HPLC method were 1.35 x 10^{-7} and 4.08 x 10^{-7} M for the HPLC analysis and 1.31 x 10^{-7} and 4.00 x 10^{-7} M for FIA. [10]

The results of the analysis of the tablet formulation obtained by using these methods were statistically comparable with each other and with an additional spectrophotometric method. This method of analysis is quite simple and precise compared to the ones in the official compendia.

There was no further analysis done to check whether this method can be used for other cephalosporin molecules. There is a possibility for this method to analyze other cephalosporin molecules because of the same chemical structure. Most of the known cephalosporins are soluble in methanol and possess a chromophore and other conjugated double bonds. The mobile phase of methanol: water (10:90 v/v) is one of the cheapest mobile phases and readily available for routine work. Acetonitrile is relatively expensive but readily available. [10]

Y.J. Lee and H.S. Lee (College of Pharmacy, Sungkyunkwan – Korea) did a work on cefuroxime-"simultaneous determination of cefoxitin, cefuroxime, cephalexin, and cephaloridine in plasma using HPLC and a column-switching technique". A new high performance liquid chromatographic method was developed using a column-switching technique for the simultaneous determination of cephalexin, cefuroxime, cefoxitin and cephaloridine in plasma. The plasma samples were injected onto a precolumn packed with Corasil RP C_{18} (37–50 µm) after simple dilution with an internal standard solution in 0.01 M acetate buffer (pH 3.5). Polar plasma components were washed out using 0.01 M acetate buffer (pH 3.5).

After valve switching, the concentrated drugs were desorbed in back-flush mode and separated on a Partisil ODS-3 column using acetonitrile in 0.02 M acetate buffer (pH 4.3) (15 :85, v/v) as the mobile phase.

The method showed excellent precision with good sensitivity and speed with a detection limit of 0.5 µg/ml. The total analysis time per sample was less than 25 min, and the mean coefficients of variation for intra- and inter-assay were both less than 4.9 %.

This method has been successfully applied to plasma from rats after subcutaneous injection of cefuroxime. [11]Although this method is good for simultaneous analysis, two different brands of columns were used and quite expensive. It may be unavailable and the method of analysis quite cumbersome.

2.2 Ceftriaxone

This is defined as a sterile solution of ceftriaxone sodium in Water for Injections. (6*R*,7*R*)-7-{[(2*Z*)-2-(2-amino-1,3-thiazol-4-yl)->2-(methoxyimino)acetyl]amino}-3-{[(2-methyl-5,6-dioxo-1,2,5,6-tetrahydro-1,2,4-triazin-3-yl)thio]methyl}-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid [9]

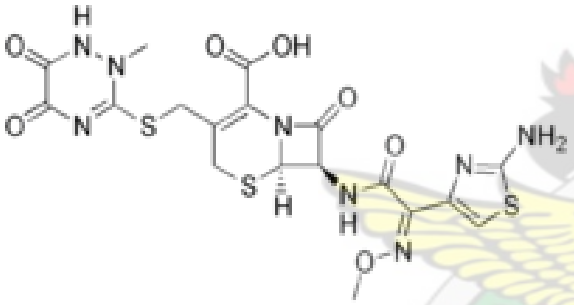


Fig 2.3 Structure of ceftriaxone sodium

For the assay of ceftriaxone sodium, the British Pharmacopoeia (2005) - the chromatographic procedure may be carried out using a stainless steel column (25 cm × 4.6 mm) packed with *stationary phase C* (5 µm) (Lichrosphere RP-18 is suitable), as the mobile phase with a flow rate of 1.5 ml per minute, a mixture prepared in the following manner: dissolve 2 g of *tetradecylammonium bromide* and 2 g of *tetraheptylammonium bromide* dissolved in a mixture of 440 ml of *water*, 55 ml of *0.067*M *mixed phosphate buffer pH 7.0*,

5 ml of a citrate buffer solution of pH 5.0 was prepared by dissolving 20.17 g of *citric acid* in 800 ml of *water*. The solution was adjusted to pH 5.0 with 10M *sodium hydroxide*.

This was diluted to 1000 ml with 500 ml of *acetonitrile* and water. A detection wavelength of 254 nm was used. [9] The mobile phase preparation involves many processes. Caution needs to be taken because of the buffers. Their salts may easily crystallize out and block the tubes and the pumps if proper washing is not done.

This method of analysis is specific for ceftriaxone. There is no evidence to prove that this method can be used for other cephalosporins as the case should be.

Mohammed E. Abdel-Hamid from the Department of Pharmaceutical and Medical Sciences, College of Health Sciences, Public Authority for Applied Education and Training, 72853, Faiha, Kuwait did a work in 1997 on FSQ spectrophotometric and HPLC analysis of some cephalosporins in the presence of their alkali-induced degradation products which involved ceftriaxone sodium. Accurate and precise spectrophotometric full spectrum quantitation (FSQ) and high-performance liquid chromatography (HPLC) procedures for the quantitation of some selected cephalosporins, namely, cefotaxime, ceftazidime and ceftriaxone in the presence of their alkali-induced degradation products and in commercial injections have been described. The degradation products or formulation excipients did not interfere in the analysis.

The HPLC procedure was based on resolution of cefotaxime or ceftazidime or ceftriaxone from the alkali-induced degradation products using an ODS column and mobile phase composed of acetonitrile-ammonium acetate buffer solution (0.1 M) in a ratio 10:90 (pH 7.5) with peak detection at 270 nm using a diode array detector. The collected data proved that both procedures were of comparable accuracy and precision, however, the HPLC method was of higher sensitivity ($\ll$1 μg/ml) compared to the FSQ method.

Accelerated stability studies of cefotaxime, ceftazidime and ceftriaxone in aqueous solutions (pH 2–10) using the HPLC method indicated that the degradation of the antibiotics followed pseudo-first-order kinetics. The rate constant-pH profiles showed that the antibiotics were relatively stable over the pH range 4–6 with optimum stability at pH 5.The extrapolated shelf-life (t_{90}) values as determined from Arrhenius plots at pH 5 and 25°C were 6.56, 2.14 and 0.88 h for cefotaxime, ceftazidime and ceftriaxone, whereas these values were found to be 15.0, 3.62 and 2.14 h, respectively at 4°C. [12] This is quite complex and based on FSQ software which will make the method of analysis quite expensive. This method of analysis cannot be used for routine work because it is involving.

Sue J. Kohlhepp, David N. Gilbert, and James E. Leggett from *Earle A. Chiles Research Institute, Providence Portland Medical Center and Oregon Health Sciences University,*2 *Portland, Oregon respectively have a work on ceftriaxone* - The influence of assay methodology on the measurement of the active free fraction of ceftriaxone in plasma was determined.

The free fraction was measured by three methods: agar diffusion bioassay, precipitation of plasma protein with methanol followed by high-performance liquid chromatography (HPLC) of the supernatant and ultrafiltration of plasma followed by HPLC of the filtrate.

In human serum, the free ceftriaxone levels were significantly lower (*P* 5 0.03) when measured on ultrafiltrates compared to the other two methods.

This difference disappeared when dolphin serum was studied. After ultrafiltration, human serum was shown, by Scatchard plot analysis, to have two ceftriaxone binding sites. Species differences were also demonstrated. Hence, in humans, determination of free plasma ceftriaxone varies with the assay method employed. HPLC ceftriaxone concentration analysis was based on the methods

of Granich and Krogstad . Chromatography was carried out with Beckman 110A pump; an Econosphere C18 5-mm silica gel column purchased from Alltech Associates, Inc., Deerfield, Ill.; a Hitachi L-4250 UV-VIS detector set at 280 nm and either 0.02 or 0.002 absorbance unit full scale; and a Hewlett-Packard HP 3396 series II integrator.

The mobile phase consisted of 3 g of hexadecyltrimethylammonium bromide, 10 ml of 1 M potassium phosphate buffer (pH 7.0), 600 ml of acetonitrile, and double-distilled water to make 1 liter. Chromatography was carried out at room temperature and a 1-ml/min flow rate. Ceftriaxone eluted at approximately 5 min under these conditions. Samples were prepared for HPLC analysis by either cold-methanol precipitation or ultrafiltration. [13] This method of analysis was only used for free plasma ceftriaxone. This method cannot be used for routine work for the drug.

George G. Granich and Donald J. Krogstad from Microbiology and Therapeutic Drug Monitoring Laboratories, Barnes Hospital, and Departments of Pathology and Medicine, Washington University School of Medicine, St. Louis, Missouri 63110 respectively developed a high-performance liquid chromatographic assay to measure ceftriaxone in serum, urine, and cerebrospinal fluid.

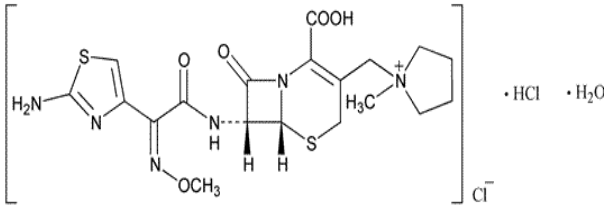
Ion pairing was used because **ceftriaxone** is a relatively polar compound which is poorly retained on C_{18} columns in standard reverse-phase high-performance liquid chromatography and which produces trailing peaks in the absence of ion-pairing agents.

The mobile phase was a combination of acetonitrile and water (46:54), adjusted to pH 9.0 with 10 mM K_2HPO_4, which contained 10 mM hexadecyltrimethylammonium bromide as the ion-pairing agent. Moxalactam (200ug/ml) was used as the internal standard.

A silica-packed precolumn (3 cm long) was used to prevent rapid deterioration of the analytical column (30 by 0.4cm) by the alkaline pH of the mobile phase, and it significantly extended the life of the analytical column. The assay was linear with ceftriaxone concentrations of 1 to 250 ug/ml (r = 0.999) and correlated well with an agar diffusion bioassay (r = 0.990).

Reproducibility was good, with intrarun coefficients of variation from 2.3 to 6.4% and interrun coefficients of variation from 3.2 to 21.4%. The absolute recoveries of ceftriaxone and moxalactam were 91 to 97 and 96 to 98%, respectively. No interferences were observed with more than 40 commonly prescribed drugs, including 10 cephalosporins (cefotaxime, cefoperazone, ceftazidime, ceftizoxime, cefoxitin, cefamandole, cephalothin, cefazolin, cephapirin, and cephalexin), or with sera from patients with renal or hepatic disease. [14]This high-performance liquid chromatographic assay measures ceftriaxone in serum, urine, and cerebrospinal fluid. There is no evidence of data on assay for the drug.

2.3 Cefepime
Cefepime Hydrochloride



$C_{19}H_{25}ClN_6O_5S_2 \cdot HCl \cdot H_2O$ 571.50

Fig 2.3 Structure of Cefepime Hydrochloride

Pyrrolidinium,1-[[7-[[(2-amino-4-thiazolyl)(methoxyimino)acetyl]amino]-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl]methyl]-1-methyl,chloride,monohydrochloride,monohydrate,[6*R*-[6a,7b(*Z*)]]-1-[[(6*R*,7*R*)-7-[2-(2-Amino-4-thiazolyl)glyoxylamido]-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl]methyl]-1-methylpyrrolidiniumchloride,7^2-(*Z*)-(*O*-methyloxime),monohydrochloride,monohydrate [9]

F. J. Jimenez Palacios, M. Callejon Mochon, J. C. Jimenez Sanchez, M.A. Bello Lopez, and A. Guiraum Perez; they are from Department of Analytical Chemistry, Faculty of Chemistry, 41012 Seville, Spain. They have publication on 'Validation of an HPLC Method for Determination of Cefepime (a Fourth-Generation Cephalosporin) determination in Human Serum, Cerebrospinal Fluid, and Urine -Pharmacokinetic Profiles.'A high-performance liquid-chromatographic method with detection at 256 nm has been developed and validated for analysis of cefepime in several biological matrices. Serum samples were deproteinized with acetonitrile and extracted once with dichloromethane. For urine and cerebrospinal fluid samples, only a microfiltration step was necessary.

The method was validated in accordance with the recommendations of the International Conference on Harmonization (ICH), the Food and Drug Administration (FDA), and the Center for Drug Evaluation and Research (CDER). The method was used to determine levels of the drug in the serum, cerebrospinal fluid, and urine of twelve patients treated with Maxipime. The results obtained were compared with those from previously published HPLC methods. The HPLC equipment (Merck–Hitachi– Lachrom, Barcelona; Spain) comprised an L-7100 pump, a LiChrospher 100 RP-18 column (250 mm _ 4 mm LichroCart,5 lm particles), and a L-7455 diode-array detector. The injector was a Rheodyne (model 7725i) manual injection valve, fitted with a 20-lL sample loop. Chromatograms were processed by means of the HSM D-7000 HPLC system manager (Merck–Hitachi). The mobile phase, phosphate buffer (pH 7) 10 mM–methanol, 75:25, was always freshly prepared. Flow rates were 1 mL min) 1 for serum and urine analysis and 0.5 mL min)1 for analysis of cerebrospinal fluid. UV detection was performed at 256 nm. [15] A. Isla, et al from Laboratory of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, University of the Basque Country, Paseo de la Universidad no. 7, 01006 Vitoria-Gasteiz, Spain. J. Maynar and E. Corral are workers of Intensive Care Unit, Santiago Apóstol Hospital, Vitoria-Gasteiz, Spain. These people developed and validated a new, rapid and reproducible HPLC method for the determination of cefepime and ceftazidime in plasma and dialysate-ultrafiltrate samples obtained from intensive care unit (ICU) patients undergoing continuous veno-venous hemodiafiltration (CVVHDF).

The method for plasma samples involved protein precipitation with acetonitrile, followed by washing with dichloromethane to remove apolar lipophilic compounds. Dialysate-ultrafiltrate samples did not require any preparation. Separation was performed on a μBondapak C_{18} (30 cm × 3.9 mm × 10 μm) with UV detection. The mobile phase contained acetate buffer: ACN and was delivered at 2 ml/min. The coefficients of determination of the calibration curves were always ≥0.998 and % R.S.D of the response factors <10%. The intra and inter-assay precision and accuracy of the quality controls (QC) and limit of quantification (LOQ) were satisfactory in all cases. Plasma and dialysate-ultrafiltrate samples were stable at −20 and −80 °C for 2 months and also after three freeze/thaw cycles. Dialysate-ultrafiltrate samples were stable in the chromatographic rack for 24 h at room temperature, but we recommend storing processed plasma samples at 4 °C until the analysis. The described method has proved to be useful to give accurate measurements of ceftazidime and cefepime in samples obtained from patients undergoing CVVHDF. [16]

A study was done to describe a high-performance liquid chromatography (HPLC) assay for the determination of cefepime and cefpirome in human serum without changing chromatographic conditions. The assay consisted to measure cefepime and cefpirome which were unbound to proteins having a molecular mass of 10 000 or more by ultrafiltration followed by HPLC with a Supelcosil ABZ+ column and UV detection at a wavelength of 263 nm.

The assay was found to be linear and has been validated over the concentration range 200 to 0.50 μg/ml for both cefepime and cefpirome, from 200 μl serum, extracted.

In future, the assay will support therapeutic drug monitoring for cefepime and cefpirome in neutropenic patients in correlation with microbiological parameters such as MIC_{90} (minimal inhibitory concentration of antibiotic which kills 90% of the initial bacterial inoculum) and clinical efficacy. [17]

All the research done respective to cefepime was on biological matrices. With the exception of the USP, no work has yet been reported on the drug analysis of the injection.

All the works done do not involve the use of methanol and water for the analysis of **cefuroxime**, **cefepime** and **ceftriaxone.** The analysis involving combinations of cephalosporin products was done on biological matrices. These methods of analysis cannot be used for routine work because they are involving.

CHAPTER THREE

3.0 MATERIALS, REAGENTS AND EXPERIMENTAL METHODS

3.1 Materials / Reagents

Methanol (BDH Analar grade), Potassium dihydrogen orthophosphate-KH_2PO_4 (BDH), Sodium hydroxide pellets, 99% (BDH), Ethanol (BDH Analar grade), were provided by the Department of Pharmaceutical Chemistry, KNUST-Kumasi, Methanol (HPLC grade), Deionised and distilled water (HPLC grade) were provided by Phyto-Riker (GIHOC) Pharmaceutical Company - Quality Control Department.

The following pure samples were obtained from the source indicated in the table below.

Table 3.0: Profile of Pure Samples

Name	Company / Institution	Batch Number	Date of Manufacture	Date of Expiry*	Assay (%)
Ceftriaxone	Food & Drugs Board, Ghana – Quality Control Laboratory	B3059	09/2006	02/2010*	97.23
Cefuroxime	Kinapharma Ghana	200703002	01/2007	01/2011*	99.0

* Analysis was done in the month of November, 2008.

The following lists of equipments below were used:

- Digital pH/mV/Temp. Meter
- Analytical Balance (Radwag USA) serial number- 279419/10
- DU series 640B spectrophotometer (Beckman Coulter)
- Hewlett Packard Ti-series 1050 auto-sampler HPLC with uv detector,
- Polarimeter
- Cecil CE 2041 2000 series-UV spectrophotometer

3.2 Experimental Methods

This involved characterizing the samples, development of the HPLC procedure, optimization and validation of the method with official methods. The characterization of the samples involved the various identification tests as described in the pharmacopoeias. Development of the HPLC procedure comprises information on the physic-chemical properties of the samples and their respective solubilities and the ways of ensuring efficient separation. Optimization entails analytical performance, application of method to formulated samples and validation of the new methods with official methods.

3.2.1 Identification

3.2.1.0 Cefuroxime

Small amount of the powder was dissolved in the following solvents in 25ml beaker; water, methanol, ethanol, hexane and chloroform. There were clear solutions of water, methanol and ethanol observed.

A solution labeled "S" was prepared by dissolving 2.0g of the pure sample in deionised water and then diluted to 20.0ml with the same solvent. The following tests below were performed with solution S.

Solution S was opalescent and its absorbance was measured at 450nm and recorded.

2ml of solution S was diluted to 20ml with deionised water. The pH of the resultant solution was determined and recorded.

Another solution was prepared by dissolving 0.50g in acetate buffer of pH 4.6 R. This was then diluted to 25.0ml with the same buffer. The specific optical rotation of the resultant solution was determined and recorded. [5]

3.1.1.1 Ceftriaxone

Small amount of the powder was dissolved in water, methanol and ethanol. A clear and yellowish solution were observed in each solvent.

A solution labelled"S" was prepared by dissolving 2.40g in deionised water and diluted to 20ml with the same solvent. The solution is clear and coloured. 2ml of the solution was then diluted to 20ml then the pH of the diluted solution was recorded.

The specific optical rotation of this solution was taken. The solution was prepared by dissolving 0.25g in deionised water and then diluted to 25.0ml with water. [5]

3.1.1.2 Cefepime

Small amount of the powder was dissolved in the following solvents; water, methanol, ethanol, hexane and chloroform. The sample was soluble in water, methanol and ethanol.

Three solutions were prepared from this respective solvents water, methanol and ethanol. These were done by dissolving 2.0g of the pure powder in the respective solvents and then diluted to 20ml with the same respective solvents. The pH of the respective solutions was determined. [6]

3.1.2 Method development

Different solvents of HPLC grade were selected in order to ascertain the solubilities of the pure samples and their respective formulated products. The solvents are water, methanol, ethanol, acetonitrile, isopropyl alcohol and hexane. Small amount of each sample was dissolved in the above solvents in 25ml volumetric flask. The flasks were stoppered and shaken for about two (2) minutes. They were allowed to stand on the bench for ten (10) minutes. Clarity of each solution was checked. All the samples were readily soluble in water, methanol and acetonitrile. All the samples were soluble in combination (variable ratios) of water and any other HPLC grade solvents selected.

The main objective of this new method is based on the use of simple ,readily available and cost-effective reagents and equipments, this then influenced the selection of the mobile phase to start with.

Most of the known cephalosporins are soluble in methanol and possess a chromophore and other conjugated double bonds.

The other solvent combinations – water: hexane was totally ignored because all the formulated product samples were slightly soluble which may need filtration. These cephalosporin products are relatively polar compound which are poorly retained on C_{18} columns and C_8 columns in standard reverse-phase high-performance liquid chromatography. These columns are readily available in our part of the world. The detector was selected based on availability and the chemical structure of the samples. The commonest detector for HPLC application is the UV-Visible. The samples also possessed chromophores that made them suitable for UV detection. Chromophore absorbs in UV-Visible region of the electromagnetic spectrum and auxochromes enhance the absorbance. UV/VIS detectors are reliable, sensitive, easy-to-use and very precise for chromophoric analytes.

Small amount of the various samples were dissolved in deionised water; each in 25ml volumetric flask. A quantity of each sample solution was poured into a cuvette and it was scanned within a wavelength range of 200 to 400nm using a Cecil CE 2041 2000 series-UV spectrophotometer. There were UV absorbances at wavelength of 260nm, 263nm and 265nm for all the three analytes. These three wavelengths were chosen for the initial development.

The mobile phase was prepared by trying different ratios of water to any other solvents such as ethanol, methanol, acetonitrile and isopropyl alcohol.

90ml of deionised water was measured and mixed with 10ml of HPLC grade ethanol in 250ml volumetric flask. The resulting solution was filtered and degassed. Preparations of the ratios were prepared using HPLC grade ethanol and deionised water. The ratios of HPLC grade ethanol to deionised water were 15:85, 20:80, 25:75, 30:70, 35:65, 40:60 and 45:55.

Other mobile phase preparations were done using HPLC grade methanol to deionised water; acetonitrile to deionised water; isopropyl alcohol to deionised water. These solutions were prepared according to the procedure described above. The mobile phase preparations of HPLC grade ethanol to deionised water gave splitting peaks especially when the three analytes are mixed together. The mobile phase of HPLC grade isopropyl alcohol to deionised water (30:70) did not elute ceftriaxone but gave ghost peaks for cefuroxime sample and tailing peak for cefepime sample. The other mobile phase preparation ratios did not yield a peak. Both mobile phase preparations involving either acetonitrile or HPLC grade methanol to deionised water gave various peaks with poor resolutions when the analytes are mixed up. Their respective peaks were almost of the same intensity

With the acetonitrile, the peak area was severely tailing when its percentage is lowered below 20%. This may be due possibly to solubility issues in the weaker mobile phase. This then gives very poor peak resolution relative to the methanol. Moreover, this project aims at a more cost-effective mobile phase preparation. All these mobile phases used C_{18} columns and C_8 columns in standard reverse-phase high-performance liquid chromatography with UV-Vis detector.

Based on the above initial method development, HPLC grade methanol to deionised water in various ratios was adopted as the mobile phase, C_{18} columns as stationary phase and UV –Vis as detector.

3.2.3 Method Optimization

The basic goal of the chromatographic method now is to achieve adequate resolution of all the three analytes mixed up with sufficient selectivity, precision and sensitivity in a reasonable time. Buffers were introduced in the mobile phase. Various phosphate buffers were tried. Phosphate buffer was selected due to availability at the time of the project. The pH of the phosphate buffer was first selected on the pH range of the solution of the pure samples which is pH 6±1.5. A phosphate buffer was prepared around this pH range of 6.2±1.5. 89.5 ml of 0.2M NaOH and 200ml of 0.2M KH_2PO_4 were measured separately and mixed together. Then diluted with distilled water to 1.0L and the final pH was within the range of 6.60 -6.80. This phosphate buffer modified one of the initial mobile phase components; water.

90ml of phosphate buffer (pH 6.70±0.1) was measured and mixed with 10ml of methanol R in 250ml volumetric flask. The resulting solution was filtered and degassed. Preparations of solutions in the ratios below were prepared using HPLC grade methanol and phosphate buffer (pH6.70±0.1).

The ratios of HPLC grade methanol to phosphate buffer (pH 6.70±0.1) were 15:85, 20:80, 25:75, 30:70, 35:65, 40:60 and 45:55. The resulting solution was filtered and degassed.

The reference samples and their respective products were dissolved in deionised water and made up to the mark with the same water to prepare a stock solution of $0.1\%^{w}/_{v}$. The respective solutions were sonicated for 10 minutes. They were then filtered through 45mm membrane filter and degassed.

3.2.3 Method Validation

A stock solution of 0.1%$^{w}/_{v}$ of all the reference standards were prepared and serially diluted to different concentrations 30µg/ml to 120µg/ml. Twenty micro-litres of the resultant solutions were injected into the column. The peak areas were measured electronically and plotted against their respective concentrations in order to identify regions of detector linearity. Linearity data of this assay method show the peak area responses at 50, 75, 100, 125 and 150% of the pure sample concentrations.

Stock solutions of 0.1%w/v of all the reference standards were prepared. Appropriate concentrations were taken out of the stock (30µg/ml to 120µg/ml) and five injections for each concentration were made to get the peak area responses. The above procedure was repeated for two (2) consecutive days. This helped to get enough peak area responses for three consecutive days with different analysts and different instruments. These peak area responses are used to test for the precision and reproducibility of this method.

The accuracy of this method was determined by recovery studies using standard addition method. 75%, 100% and 125% of all the pure sample concentrations were selected. 1mg of each pure sample was added to their respective pure sample solutions at the various percentages above. Three injections for each resultant solution were made to get the peak area responses. Their respective concentrations were obtained on their calibration curve using the peak area responses. The amount recovered was then calculated to get the percentage of recovery.

A stock solution of 0.1% w/v of all the samples were prepared and serially diluted to ten different concentrations. Twenty micro-litres (20µl) of the resultant solutions were injected into the column. The peak areas were measured electronically. The Limit of Detection (LOD) and the Limit of Quantification (LOQ) were determined using the following formulae; LOD = 3.3 x S/N and LOQ = 10 x S/N; where **S** is the standard deviation of the injected concentrations and **N** is the slope of the calibration curve. [18]

3.2.4 Preparation of the Formulated samples

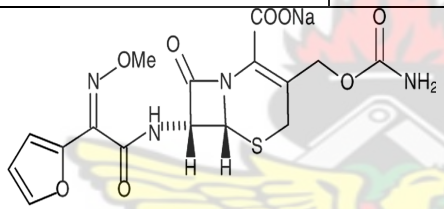
An amount of formulated sample equivalent to 60µg/ml of the respective reference sample was weighed and dissolved in 25ml of methanol in 100ml volumetric flask. The solution was then made up to the mark of 100ml with methanol. The respective solution was sonicated for 10 minutes. It was then filtered through 45mm membrane filter and degassed. Twenty micro-litres (20µl) of the resultant solution was injected into the column. The peak areas were measured electronically.

CHAPTER FOUR

4.0 RESULTS AND CALCULATIONS

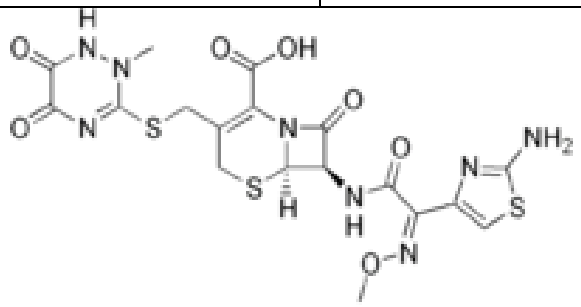
4.1 Identification Tests for Samples

Table 4.1.1 Identification test results for Cefuroxime sample

Test	Literature Value**	Experimental Value	Inference
UV –Vis Test	The absorbance is not greater than 0.25	The absorbance was 0.18	The sample is Cefuroxime
pH	5.5 to 8.5	6.6	The sample is Cefuroxime
Polarimetry	+59 to +66	+62	The sample is Cefuroxime
			

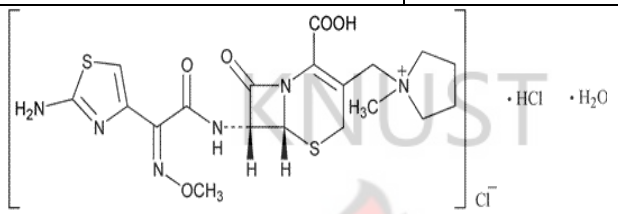
**Source: The British Pharmacopoeia 2004

Table 4.1.2 Identification test results for Ceftriaxone sample

Test	Literature Value**	Experimental Value	Inference
pH	6.0 to 8.0	7.5	The sample is Ceftriaxone Sodium
Polarimetry	-155 to -170	-162	The sample is Ceftriaxone Sodium
			

**Source: The British Pharmacopoeia 2004

Table 4.1.3 Identification test results for Cefepime sample

Test	Literature Value**	Experimental Value	Inference
pH Determination	A pH between 4.0 and 6.0 in a solution containing about 100mg of cefepime per ml	pH in water – 4.5 pH in methanol – 5.6 pH in ethanol – 6.0	The sample is Cefepime powder
			

**Source: The United States Pharmacopoeia 2007

4.2 Chromatographic conditions

- the Analyte : **Cefuroxime**, **Ceftriaxone**, **Cefepime** and their respective formulated products simultaneously
- Mobile Phase: 20 methanol:80 phosphate buffer (pH 6.7±0.15)
- Column: Zorbax ODS (C18) and Zorbax C_8 - 4.6mm x 25cm; surface area of 300m^2g; pore size of 70A; manufactured by Agilent Company, USA.
- Flow rate: 1.5ml/min
- Detector / Wavelength: UV-visible detector; 260nm
- Diluents: 20 methanol:80 phosphate buffer(pH 6.7±0.15)
- Injector Volume: 20µl

4.3 Retention times for various mobile phase concentrations across the various analytes

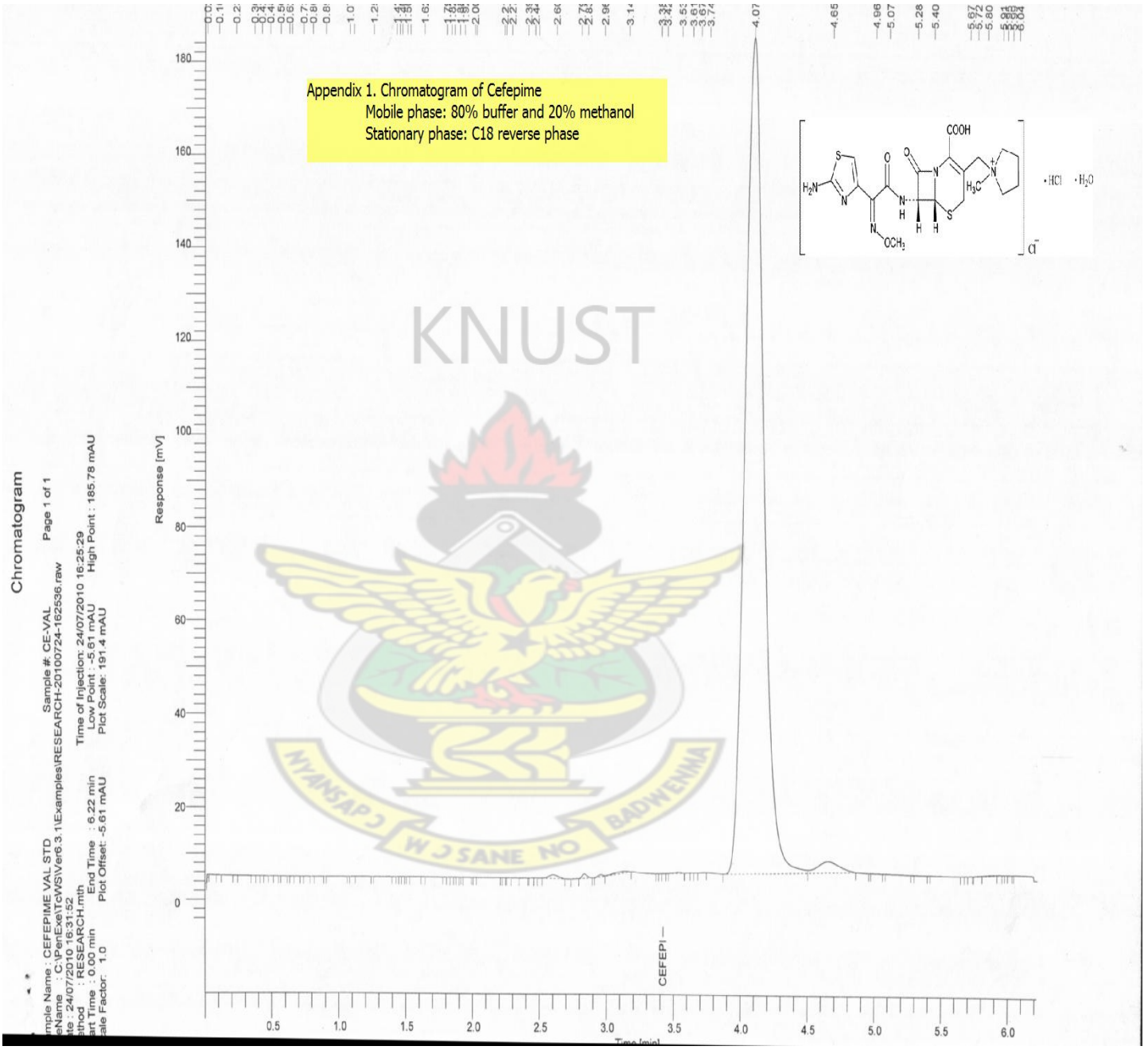


Fig4.3.1 Chromatogram of Cefepime Reference standard using 80% buffer and 20% methanol as a mobile phase with C_{18} reverse phase

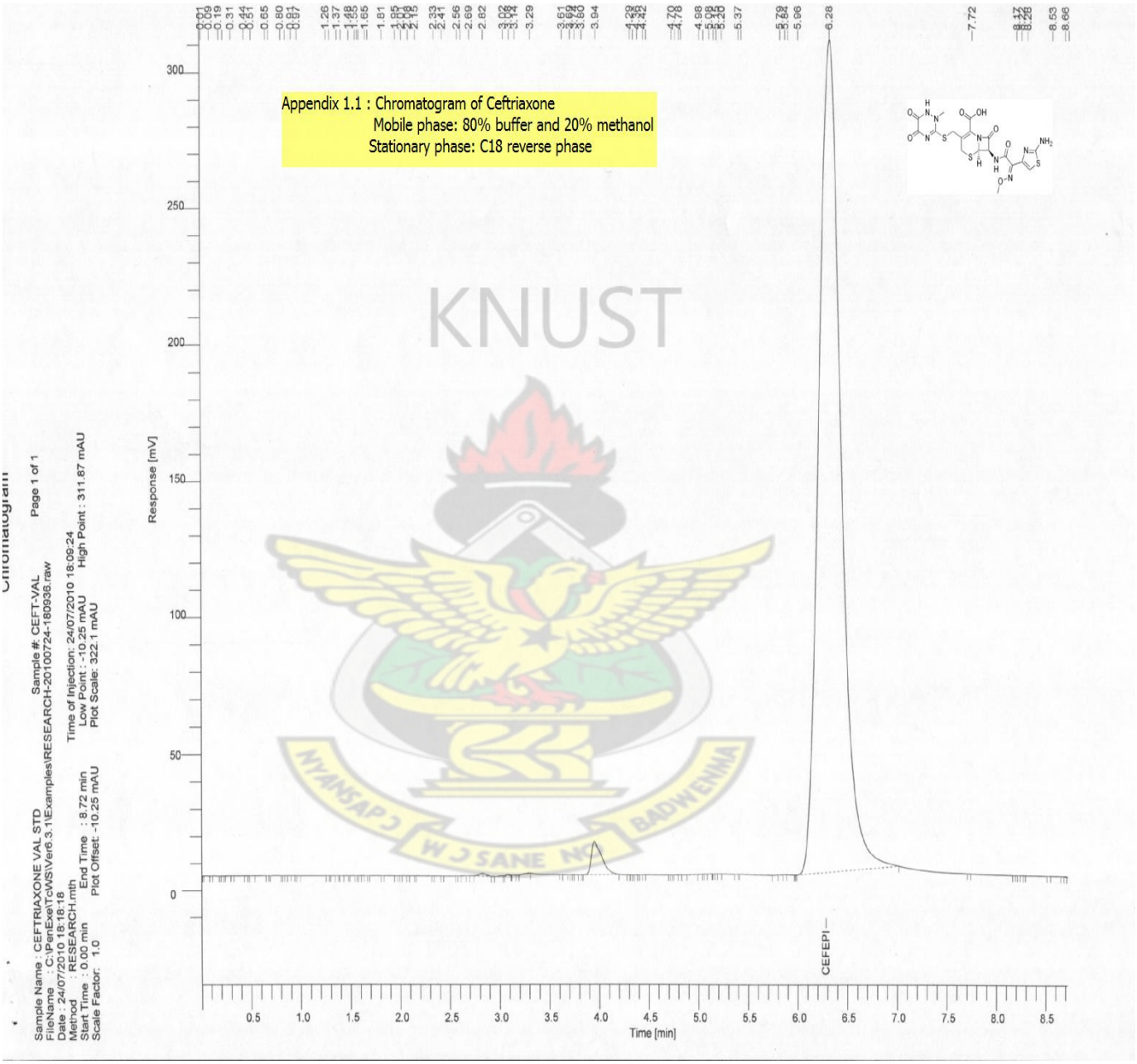


Fig4.3.2 Chromatogram of Ceftriaxone Reference standard using 80% buffer and 20% methanol as a mobile phase with C18 reverse phase

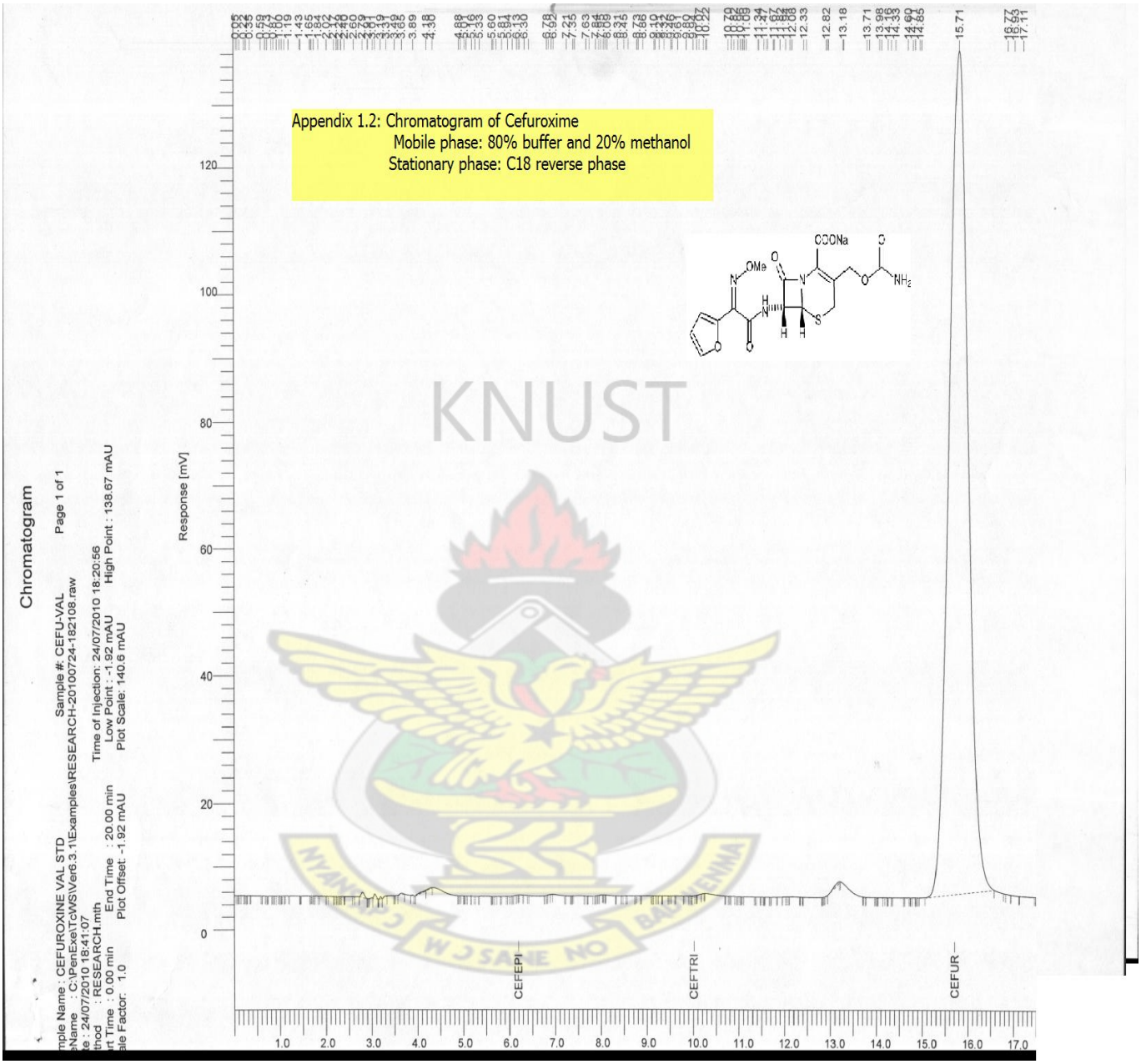


Fig4.3.3 Chromatogram of Cefuroxime Reference standard using 80% buffer and 20% methanol as a mobile phase with C_{18} reverse phase

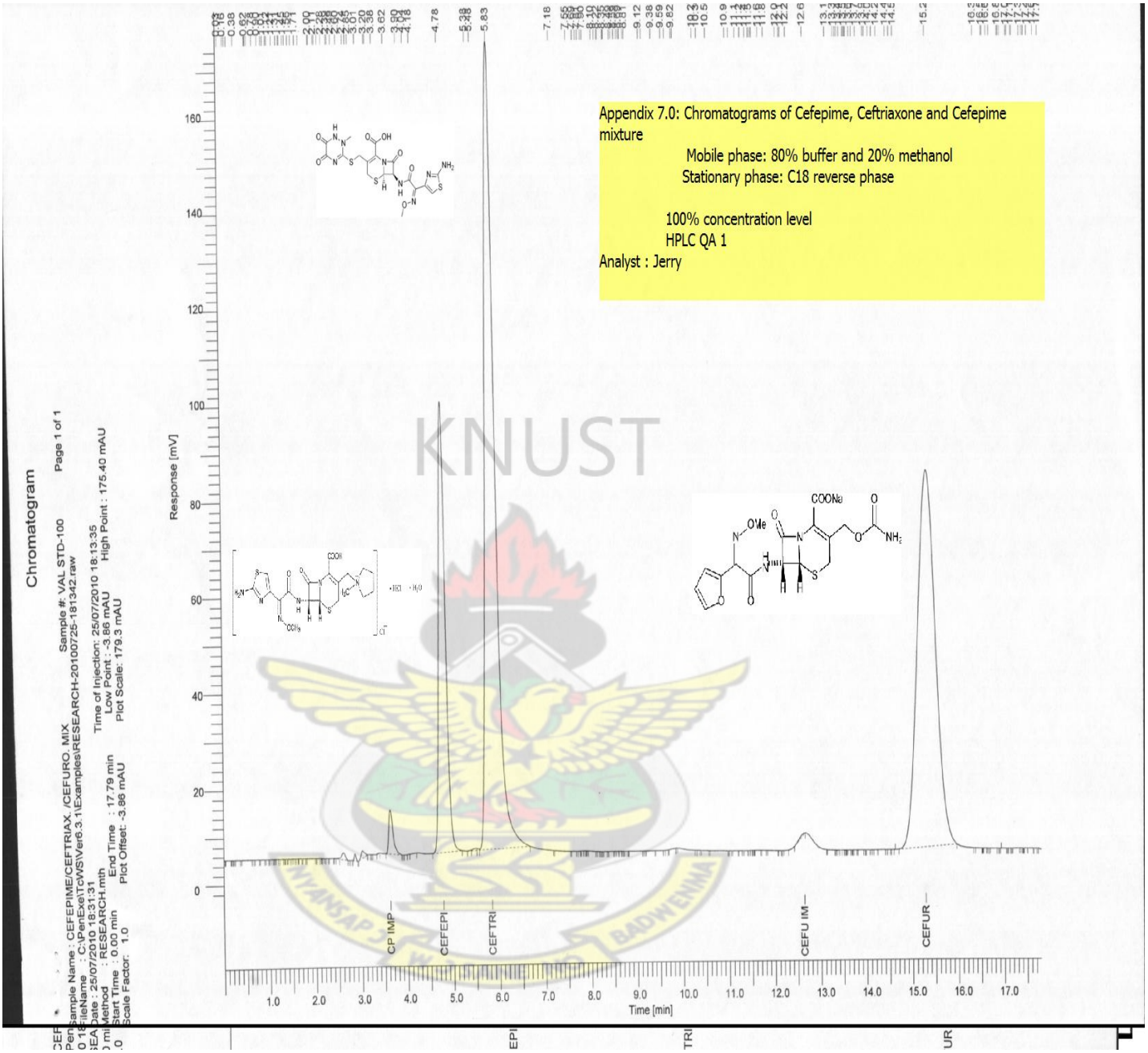


Fig4.3.4 Chromatograms of Reference standard mixtures of Cefepime, Ceftriaxone and Cefuroxime using 80% buffer and 20% methanol as a mobile phase with C_{18} reverse phase

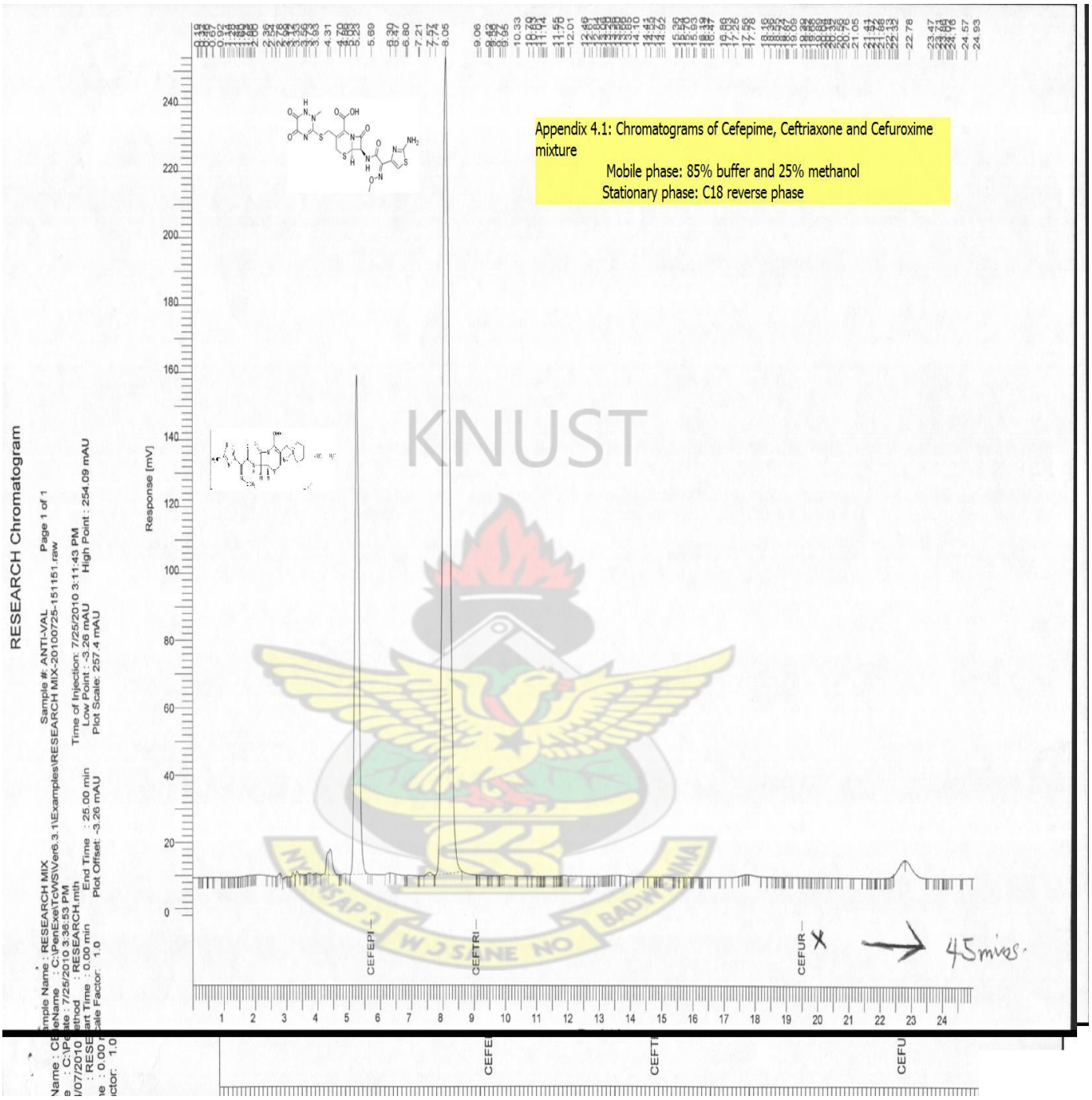


Fig4.3.5 Chromatograms of Reference standard mixtures of Cefepime, Ceftriaxone and Cefuroxime using 85% buffer and 15% methanol as a mobile phase with C_{18} reverse phase

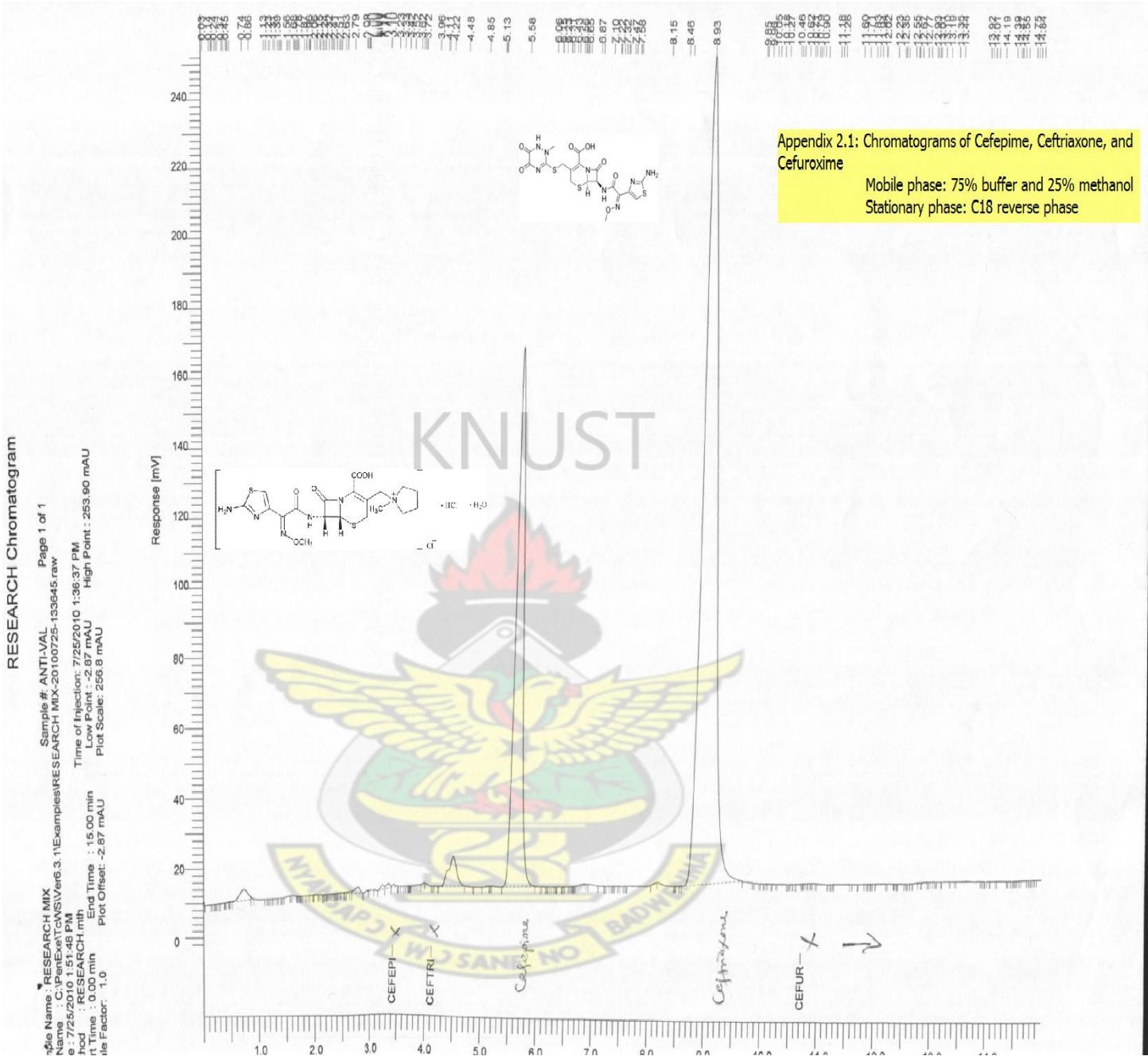


Fig4.3.6 Chromatograms of Reference standard mixtures of Cefepime, Ceftriaxone and Cefuroxime using 75% buffer and 25% methanol as a mobile phase with C_{18} reverse phase

Table 4.3.1 Retention times for the cefepime reference standard using 75% buffer and 25% methanol as mobile phase;

Injection Number	Day 1/min	Day 2/min	Day 3/min	Day 4/min	Day 5/min
1	3.126	3.154	3.486	3.307	3.541
2	3.950	3.430	3.560	3.348	3.233
3	3.453	3.232	3.611	3.334	3.240
4	3.411	3.332	3.450	3.468	3.533
5	3.300	3.212	3.378	3.330	3.447
6	3.387	3.348	3.430	3.243	3.506
Average/min	3.437833	3.284667	3.485833	3.338333	3.416667
Variance	0.076417	0.0105	0.007439	0.005413	0.020567

Total mean = 3.39min

Total variance = 0.026109

Therefore the **actual mean = 3.39±0.162**

Table 4.3.1.1 Statistical ANOVA table for comparison

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0	0	65535	65535		
Columns	0.155473	4	0.038868	1.614968	0.20163	2.75871
Interaction	0	0	65535	65535		
Within	0.601688	25	0.024068			
Total	0.757161	29				

There is no significant difference between the various retention times; $F(2,75)=1.614, p>0.05$

(Confidence level of 95%)

Table 4.3.2 Retention times for the ceftriaxone reference standard using 75% buffer and 25% methanol as mobile phase;

Injection Number	Day 1/min	Day 2/min	Day 3/min	Day 4/min	Day 5min
1	3.177	3.250	3.525	4.007	3.626
2	3.754	3.860	3.890	3.790	3.780
3	3.880	3.900	4.150	3.820	3.790
4	3.990	4.010	3.500	3.840	3.800
5	3.840	3.750	3.750	3.870	3.870
6	3.387	3.730	4.015	3.780	3.650
Average/min	3.671333	3.75	3.805	3.851167	3.752667
Variance	0.101117	0.07052	0.06896	0.006908	0.008947

Total mean = 3.76min

Total variance = 0.047975

Therefore the **actual mean = 3.76±0.219**

Table 4.3.2 .1Statistical ANOVA table for comparison

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0	0	65535	65535		
Columns	0.109019	4	0.027255	0.531384	0.713796	2.75871
Interaction	0	0	65535	65535		
Within	1.282258	25	0.05129			
Total	1.391277	29				

There is no significant difference between the various retention times; F (2,75)=1.614,p>0.05 F (2,75)=1.614,p>0.05 (Confidence level of 95%)

Table 4.3.3 Retention times for the cefuroxime reference standard using 75% buffer and 25% methanol as mobile phase;.

Injection Number	Day 1/min	Day 2/min	Day 3/min	Day 4/min	Day 5/min
1	7.822	7.717	7.860	7.770	7.670
2	7.830	7.860	7.900	7.790	7.780
3	7.880	7.900	8.150	7.820	7.790
4	7.990	8.010	8.500	7.840	8.800
5	7.840	8.750	8.750	7.870	7.870
6	7.987	8.030	7.715	8.100	8.050
Average/min	7.8915	8.0445	8.145833	7.865	7.993333
Variance	0.006044	0.132261	0.163644	0.01451	0.172107

Therefore the **actual mean = 7.99±0.308**

Table 4.3.3.1 Statistical ANOVA table for comparison

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0	0	65535	65535		
Columns	0.31544	4	0.07886	0.807055	0.532386	2.75871
Interaction	0	0	65535	65535		
Within	2.442831	25	0.097713			
Total	2.758271	29				

There is no significant difference between the various retention times; $F(2,75)=0.807, p>0.05$

(Confidence level of 95%)

Table 4.3.4 Retention times for the cefepime reference standard using 80% buffer and 20% methanol as mobile phase;

Injection Number	Day 1/min	Day 2/min	Day 3/min	Day 4/min	Day 5/min
1	4.756	4.757	4.766	4.770	4.790
2	4.773	4.768	4.781	4.790	4.780
3	4.788	4.790	4.750	4.782	4.790
4	4.799	4.780	4.759	4.784	4.801
5	4.784	4.750	4.750	4.787	4.777
6	4.787	4.800	4.768	4.800	4.750
Average/min	4.781167	4.774167	4.762333	4.7855	4.781333
Variance	0.000221	0.000374	0.000142	9.75×10^{-5}	0.000308

Therefore the **actual mean = 4.78±0.0163**

Table 4.3.4.1Statistical ANOVA table for comparison

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0	0	65535	65535		
Columns	0.001989	4	0.000497	2.176257	0.100981	2.75871
Interaction	0	0	65535	65535		
Within	0.005712	25	0.000228			
Total	0.007701	29				

There is no significant difference between the various retention times; $F(2,75)=2.176, p>0.05$

(Confidence level of 95%)

Table 4.3.5 Retention times for the ceftriaxone reference standard using 80% buffer and 20% methanol as mobile phase;

Injection Number	Day 1/min	Day 2/min	Day 3/min	Day 4/min	Day 5/min
1	5.822	5.917	5.860	5.870	5.970
2	5.830	5.860	5.900	5.890	5.880
3	5.880	5.900	5.850	5.820	5.890
4	5.990	5.887	5.850	5.840	5.880
5	5.840	5.850	5.875	5.870	5.870
6	5.887	5.830	5.850	5.810	5.850
Average/min	5.874833	5.874	5.864167	5.85	5.89
Variance	0.003891	0.00108	0.000404	0.001	0.00172

Therefore the **actual mean = 5.870±0.0397**

Table 4.3.5.1Statistical ANOVA table for comparison

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0	0	65535	65535		
Columns	0.00523	4	0.001307	0.807512	0.532112	2.75871
Interaction	0	0	65535	65535		
Within	0.040476	25	0.001619			
Total	0.045705	29				

There is no significant difference between the various retention times; $F(2, 75) = 2.176, p > 0.05$

(Confidence level of 95%)

Table 4.3.6 Retention times for the cefuroxime reference standard using 80% buffer and 20% methanol as mobile phase.

Injection Number	Day 1/min	Day 2/min	Day 3/min	Day 4/min	Day 5/min
1	15.126	15.154	15.486	15.307	15.541
2	15.950	15.430	15.560	15.348	15.233
3	15.453	15.232	15.611	15.334	15.240
4	15.411	15.332	15.450	15.468	15.533
5	15.300	15.212	15.378	15.330	15.447
6	15.387	15.348	15.430	15.243	15.506
Average/min	15.43783	15.28467	15.48583	15.33833	15.41667
Variance	0.076417	0.0105	0.007439	0.005413	0.020567

Total mean = 15.39min

Total variance = 0.026109

Therefore the **actual mean = 15.39±0.161**

Table 4.3.6.1Statistical ANOVA table for comparison

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0	0	65535	65535		
Columns	0.155473	4	0.038868	1.614968	0.20163	2.75871
Interaction	0	0	65535	65535		
Within	0.601688	25	0.024068			
Total	0.757161	29				

There is no significant difference between the various retention times; $F(2,75)=1.615, p>0.05$

(Confidence level of 95%)

Table.4.4 Effect of Concentration on Retention time

Analytes	Retention Times (min)						
	30µg/ml	45µg/ml	60µg/ml	75µg/ml	90µg/ml	Mean Retention times (min)	Variance
Cefepime Reference Standard	4.767167	4.779	4.7695	4.772167	4.785333	4.7746334	$5.54327x10^{-5}$
Ceftriaxone Reference Standard	5.871167	5.84	5.853333	5.875333	5.8545	5.8588666	0.00020702
Cefuroxime Reference Standard	15.28467	15.48583	15.33833	15.41667	15.39267	15.383634	0.005865844

Table 4.4.1 Statistical ANOVA for comparison

Source of Variation	SS	df	MS	F	P-value	F crit
Comparing Analytes	340.7461	2	170.373	79540.5304	$6.x10^{-18}$	4.45897
Varying Concentrations of an analyte	0.007377	4	0.001844	0.86106248	0.526247	3.837853
Error	0.017136	8	0.002142			
Total	340.7706	14				

There is no effect of concentration on the retention time; as F (3.83) =0.8611, p>0.05

(confidence level of 95%)

4 5 Repeatability and Intermediate Precision Study

Table 4.5.1 Repeatability and Intermediate Precision (Reproducibility)

	Peak Area uV*sec			% Relative Standard Deviation (RSD)		
	Analyte			Analyte		
Day	Cefepime Reference Standard	Ceftriaxone Reference Standard	Cefuroxime Reference Standard	Cefepime Reference Standard	Ceftriaxone Reference Standard	Cefuroxime Reference Standard
1	489770.6	1228402	1127745.2	0.292763	0.844338	1.742214
2	491553.2	1212938.7	1147987.2			
3	492617.3	1208952.6	1167739.3			
Mean	491313.7	1216764.4	1147823.9			
Variance	2068945	105546903	399901849			

4.6 Ruggedness / Robustness

Table 4.6.1 Statistical Comparison of results two different HPLC equipments (30µg/ml of each Analyte)

	Peak Area uV*sec			**% Relative Standard Deviation (RSD)**		
	Analyte			Analyte		
HPLC Equipment	Cefepime Reference Standard	Ceftriaxone Reference Standard	Cefuroxime Reference Standard	Cefepime Reference Standard	Ceftriaxone Reference Standard	Cefuroxime Reference Standard
1	491313.69	1216764.503	1147823.9	0.678459	0.782484	0.609431
2	486622.12	1203373.885	1137973.645			
Mean	488967.91	1210069.194	1142898.773			
Variance	11005449	89654325.21	48513761.78			

4.7 Linearity

Table 4.7.1 Correlation coefficients for all the analyte reference standards

	Mean Peak Area uV*sec			**Correlation Coefficient**		
	Analyte			Analyte		
Concentration	Cefepime Reference Standard	Ceftriaxone Reference Standard	Cefuroxime Reference Standard	Cefepime Reference Standard	Ceftriaxone Reference Standard	Cefuroxime Reference Standard
30µg/ml	476622	1203374	1117974	0.996	0.995	0.994
45µg/ml	706517	1828133	1628708			
60µg/ml	931626	2465074	2144236			
75µg/ml	1191913	3119338	2724980			
90µg/ml	1363374	3737904	3397808			
120µg/ml	1921736	5042527	4509022			

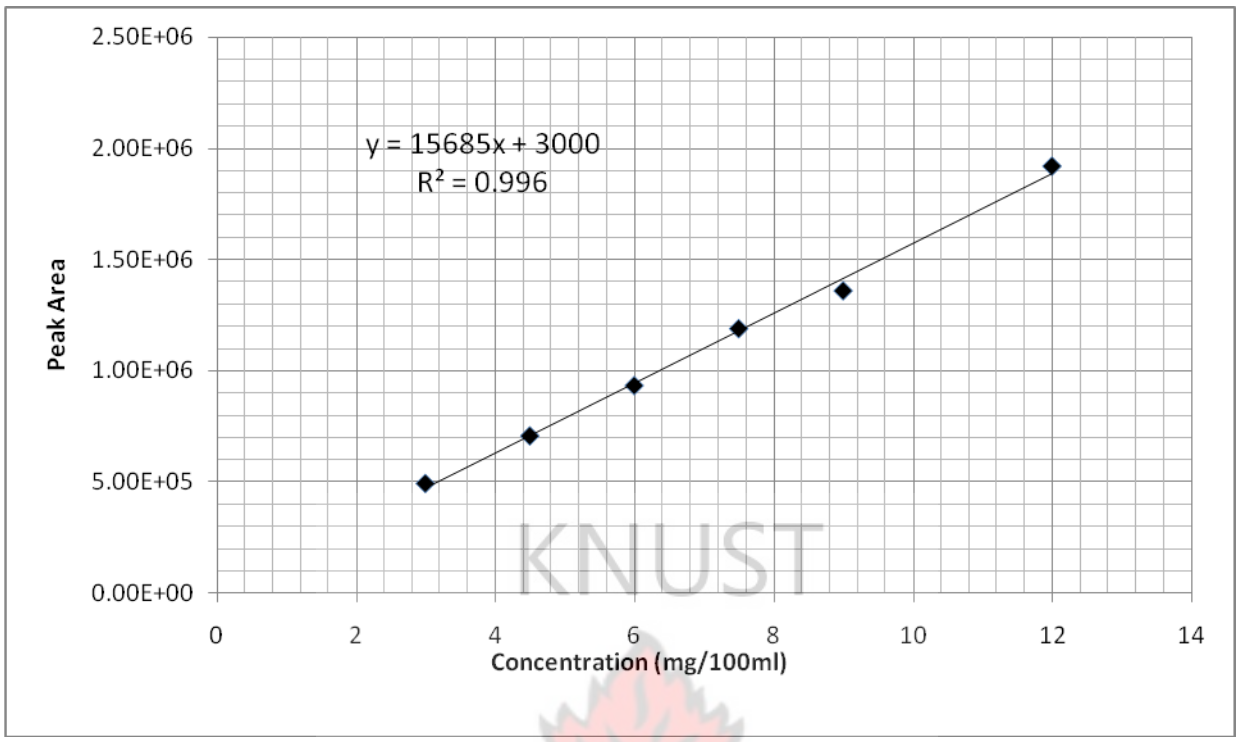


Fig 4.7 1Calibration plots of Cefepime reference standard with instrument response

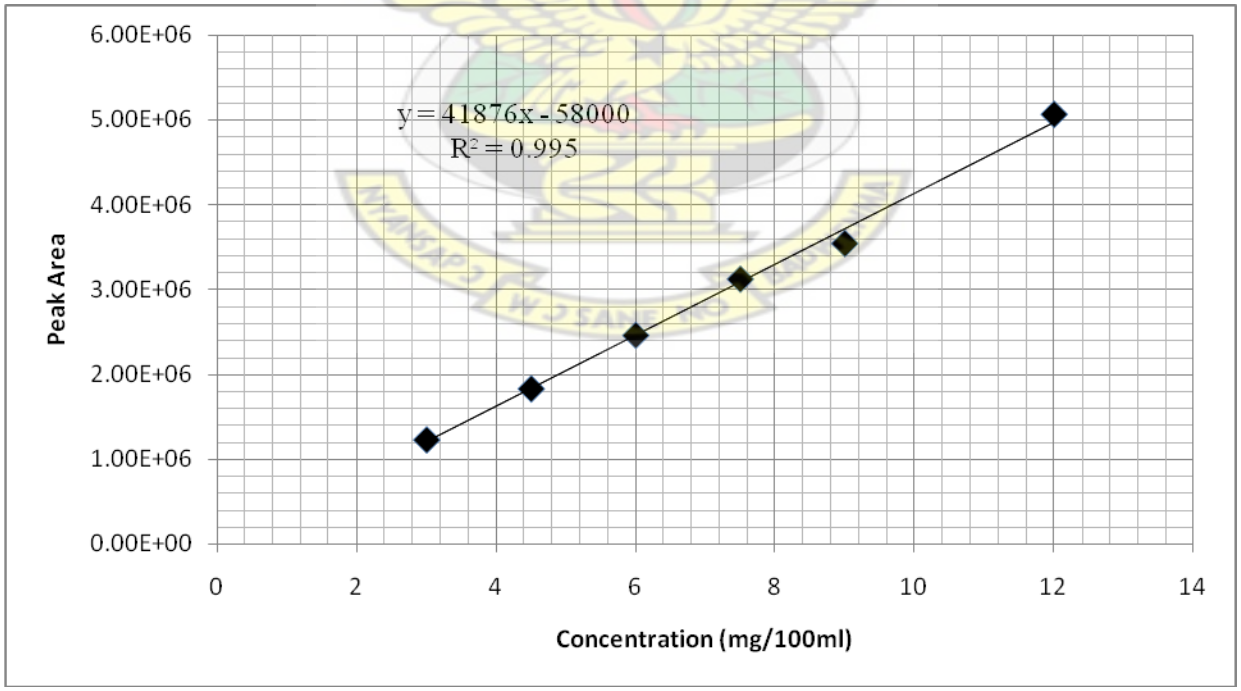


Fig 4.7.2 Calibration plots of Ceftriaxone reference standard with instrument response

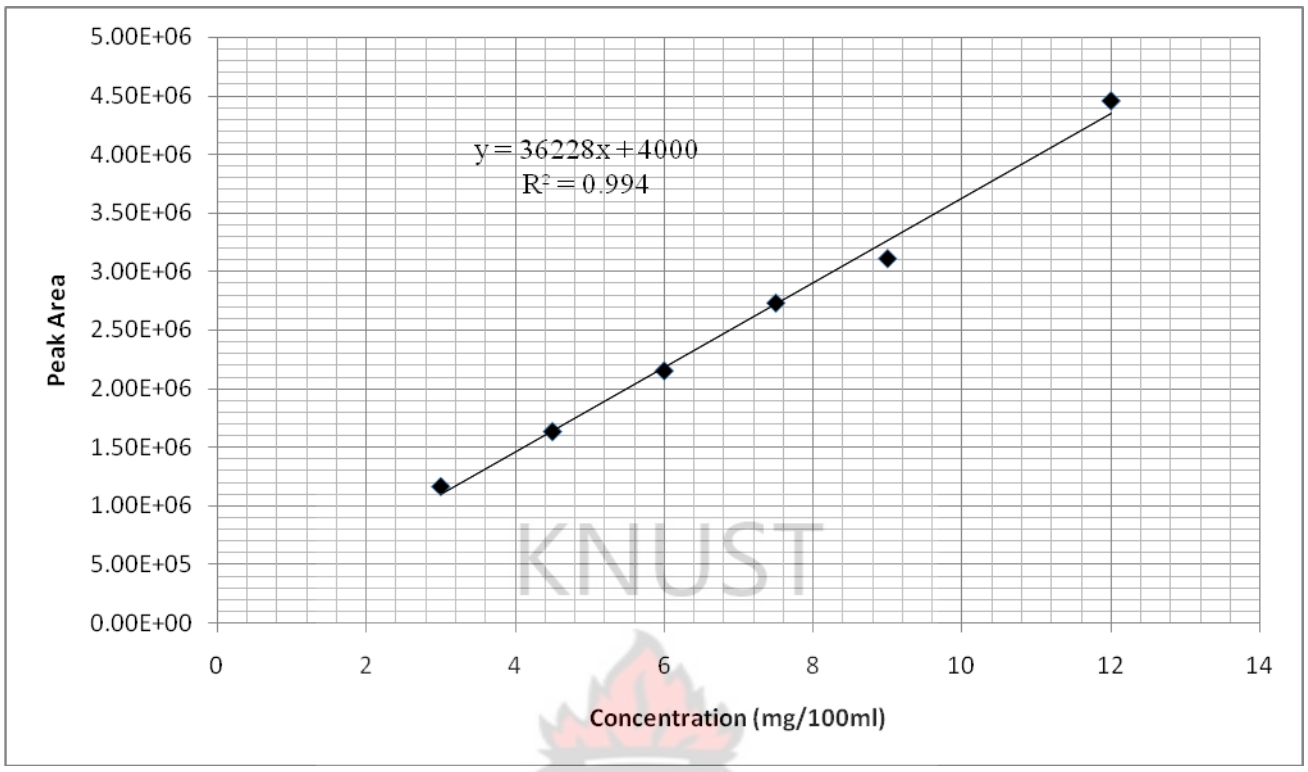


Fig 4.7.3 Calibration plots of Cefuroxime reference standard with instrument response

Table 4.7.2 Peak Area for Cefepime reference standards and Ceftriaxone reference standard with Cefuroxime as internal standard at various concentrations and their respective correlation coefficients

	Mean Peak Area uV*sec Analyte					**Correlation Coefficient** Analyte	
Concentration	Cefepime Reference Standard	Ceftriaxone Reference Standard	30µg/ml Cefuroxime as Internal Standard	Peak area ratio - Cefepime	Peak area ratio - Ceftriaxone	Cefepime Reference Standard	Ceftriaxone Reference Standard
30µg/ml	476622	1203374	1103721	0.43183	1.09029	0.998	0.999
45µg/ml	706517	1828133	1109446	0.63682	1.64779		
60µg/ml	931626	2465074	1111828	0.83792	2.21714		
75µg/ml	1191913	3119338	1116670	1.06738	2.79343		
90µg/ml	1363374	3737904	1116751	1.22084	3.34712		
120µg/ml	1921736	5042527	1149426	1.67191	4.387		

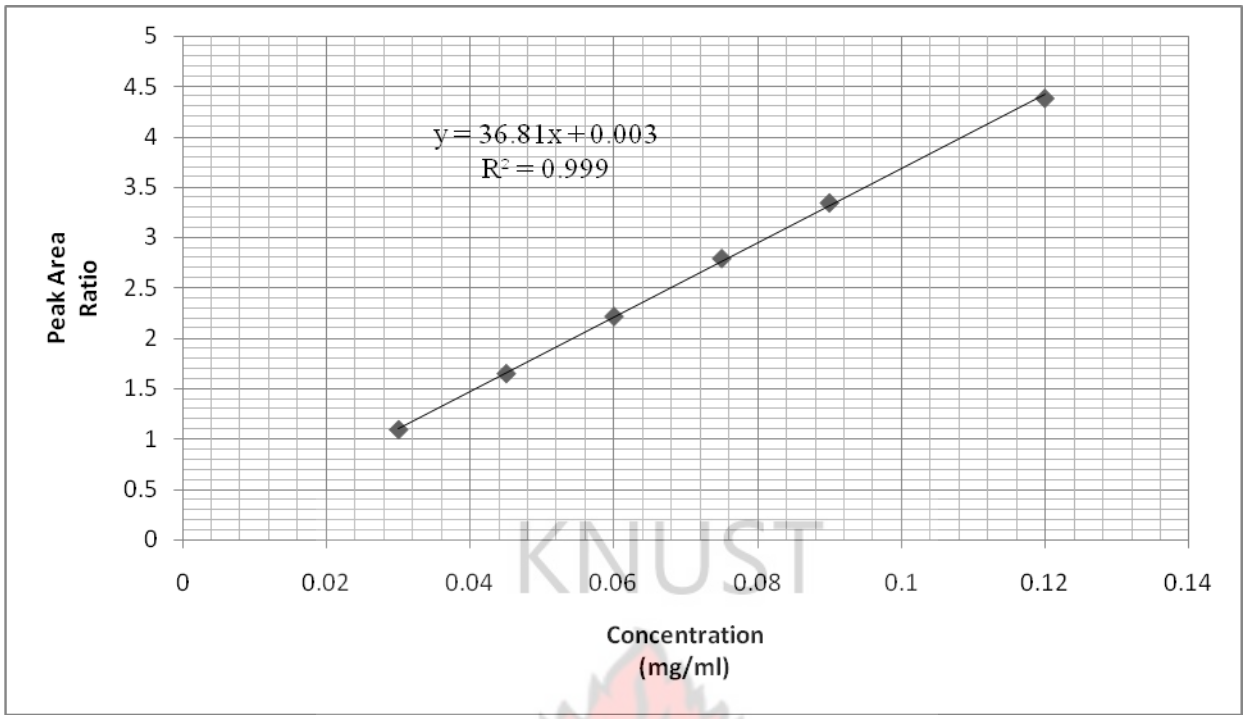


Fig. 4.7.4 Calibration plots for Ceftriaxone reference standard using 0.030mg/ml Cefuroxime reference standard as internal standard

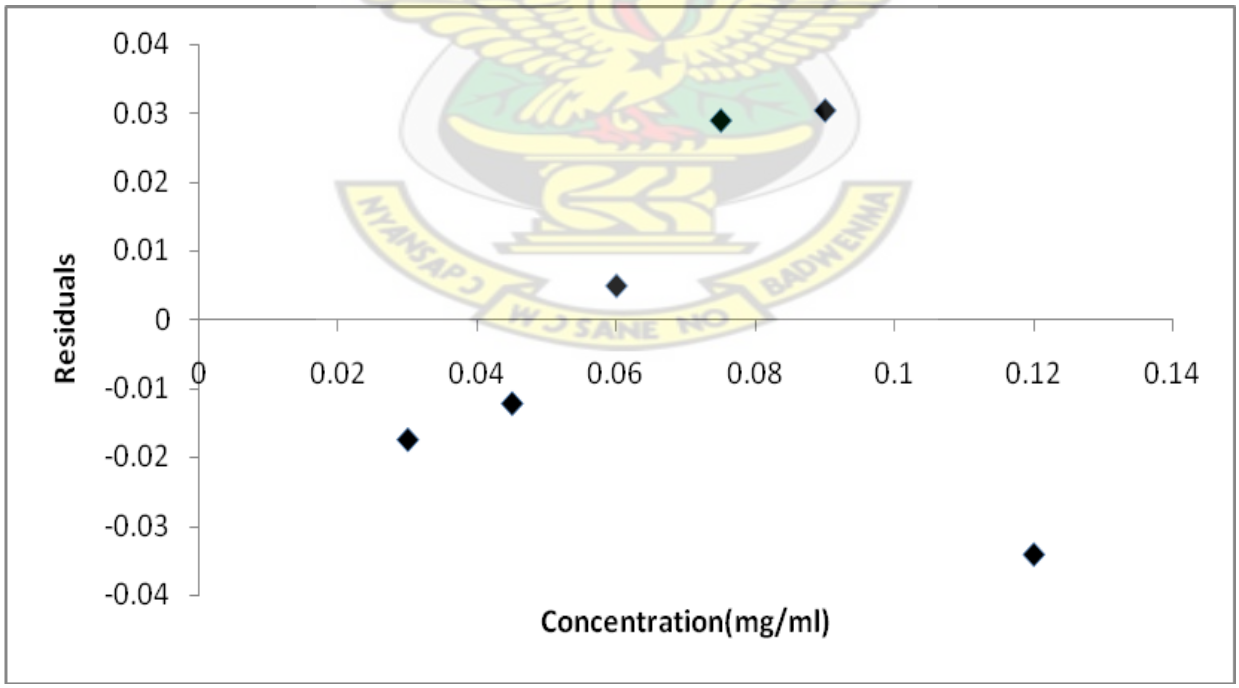


Fig. 4.7.5 Residual plots for Ceftriaxone reference standard using 0.030mg/ml Cefuroxime reference standard as internal standard

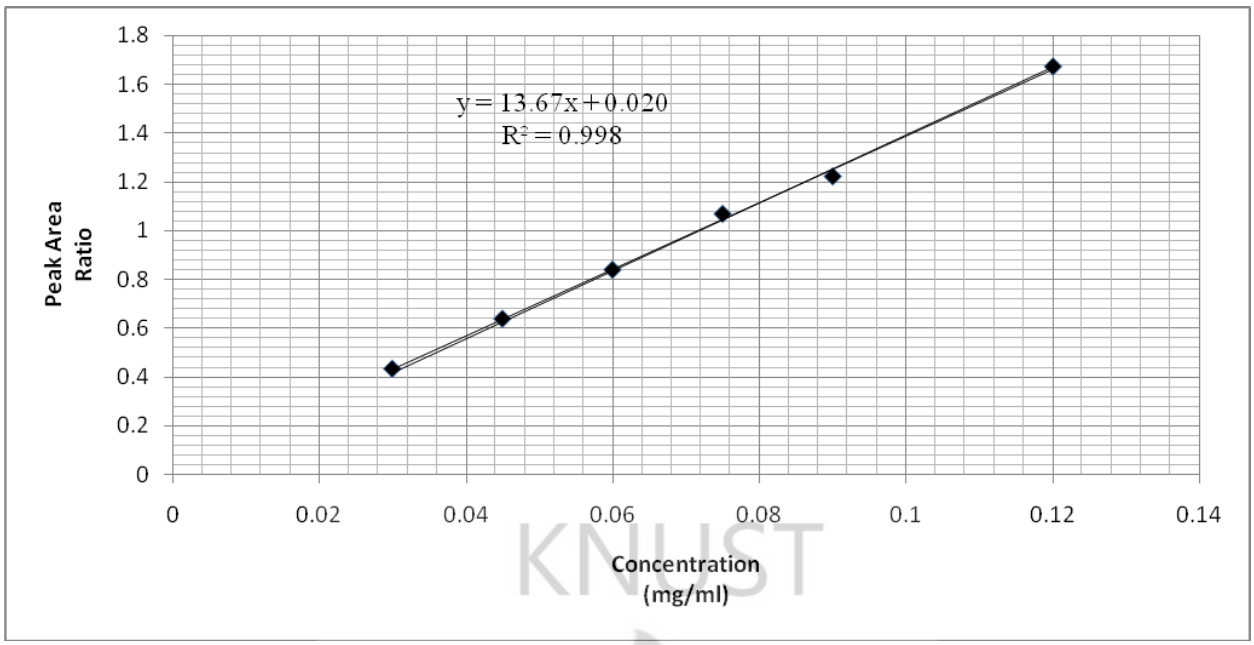


Fig. 4.7.6 Calibration plots for Cefepime reference standard using 0.030mg/ml Cefuroxime reference standard as internal standard

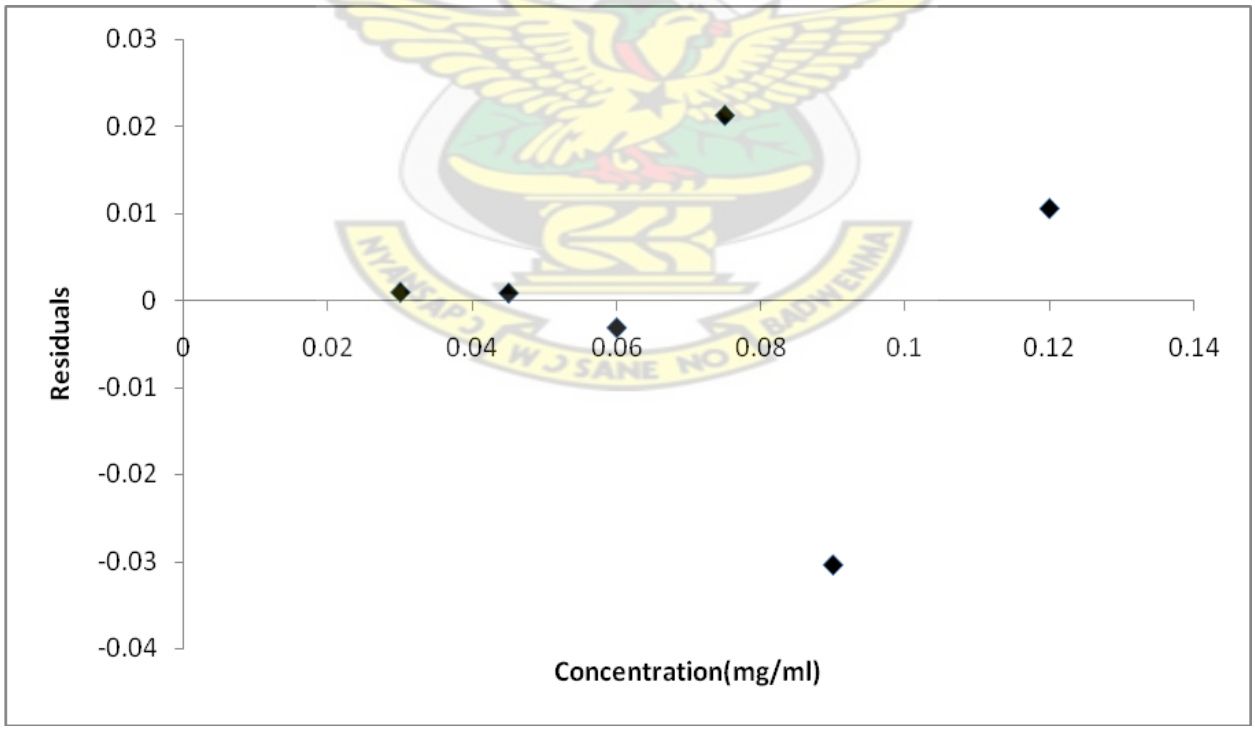


Fig. 4.7.8 Residual plots for Cefepime reference standard using 0.030mg/ml Cefuroxime reference standard as internal standard

Table 4.7.3 Peak Area for Cefepime reference standards and Cefuroxime reference standard with Ceftriaxone as internal standard at various concentrations and their respective correlation coefficients

	Peak Area uV*sec Analyte					**Correlation Coefficient** Analyte	
Concentration	Cefepime Reference Standard	Cefuroxime Reference Standard	30µg/ml Ceftriaxone as Internal Standard	Peak area ratio - Cefepime	Peak area ratio - Cefuroxime	Cefepime Reference Standard	Cefuroxime Reference Standard
30µg/ml	476622	1117974	1189490	0.4007	0.93988	0.995	0.967
45µg/ml	706517	1628708	1186510	0.59546	1.37269		
60µg/ml	931626	2144236	1227862	0.75874	1.74632		
75µg/ml	1191913	2724980	1222625	0.97488	2.22879		
90µg/ml	1363374	3397808	1202392	1.13389	2.82587		
120µg/ml	1921736	4509022	1191364	1.61306	3.78476		

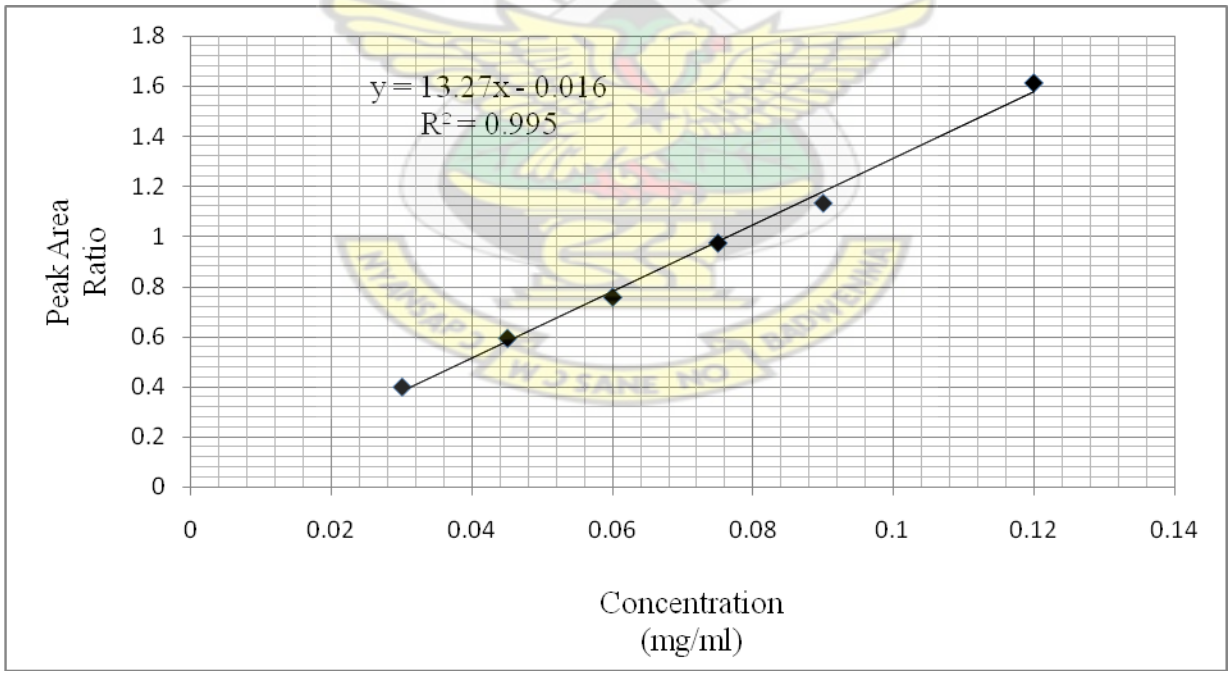


Fig. 4.7.9 Calibration plots for Cefepime reference standard using 0.030mg/ml Ceftriaxone reference standard as internal standard

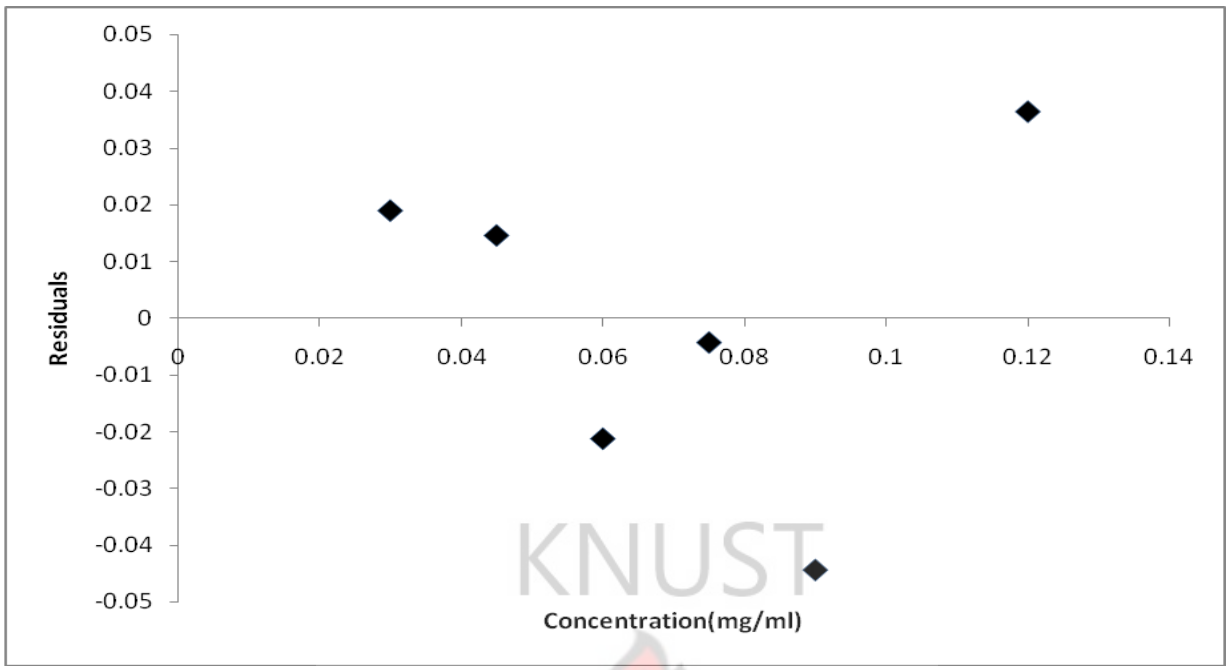


Fig. 4.7.10 Residual plots for Cefepime reference standard using 0.030mg/ml Ceftriaxone reference standard as internal standard

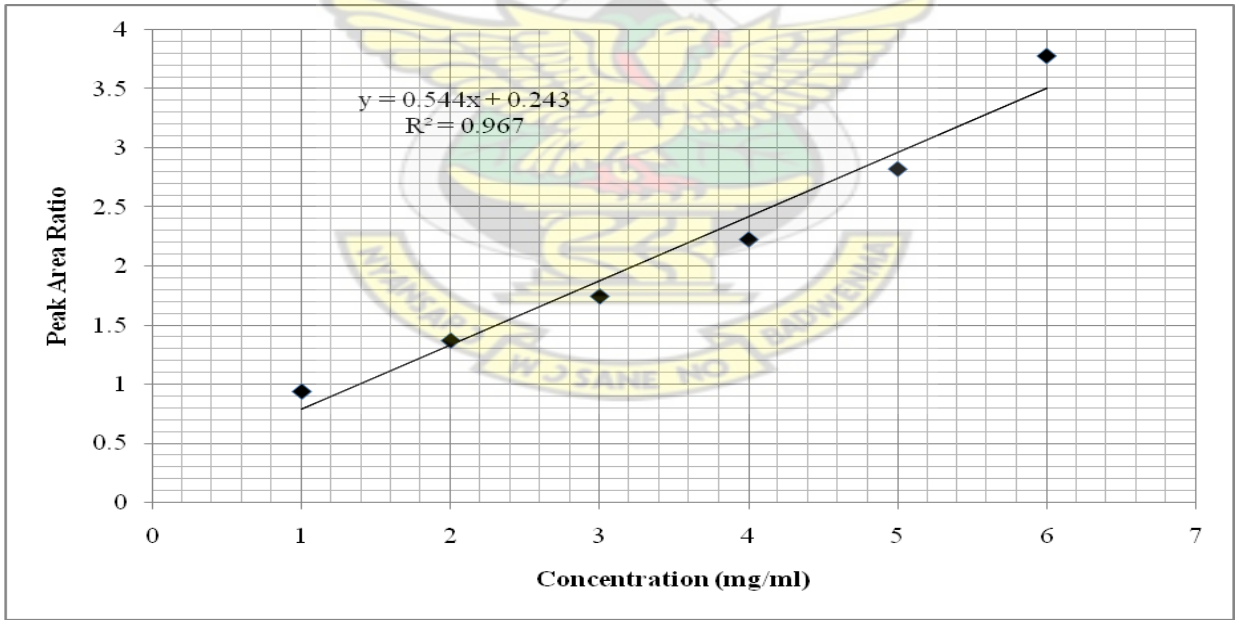


Fig. 4.7.11 Calibration plots for Cefuroxime reference standard using 0.030mg/ml Ceftriaxone reference standard as internal standard

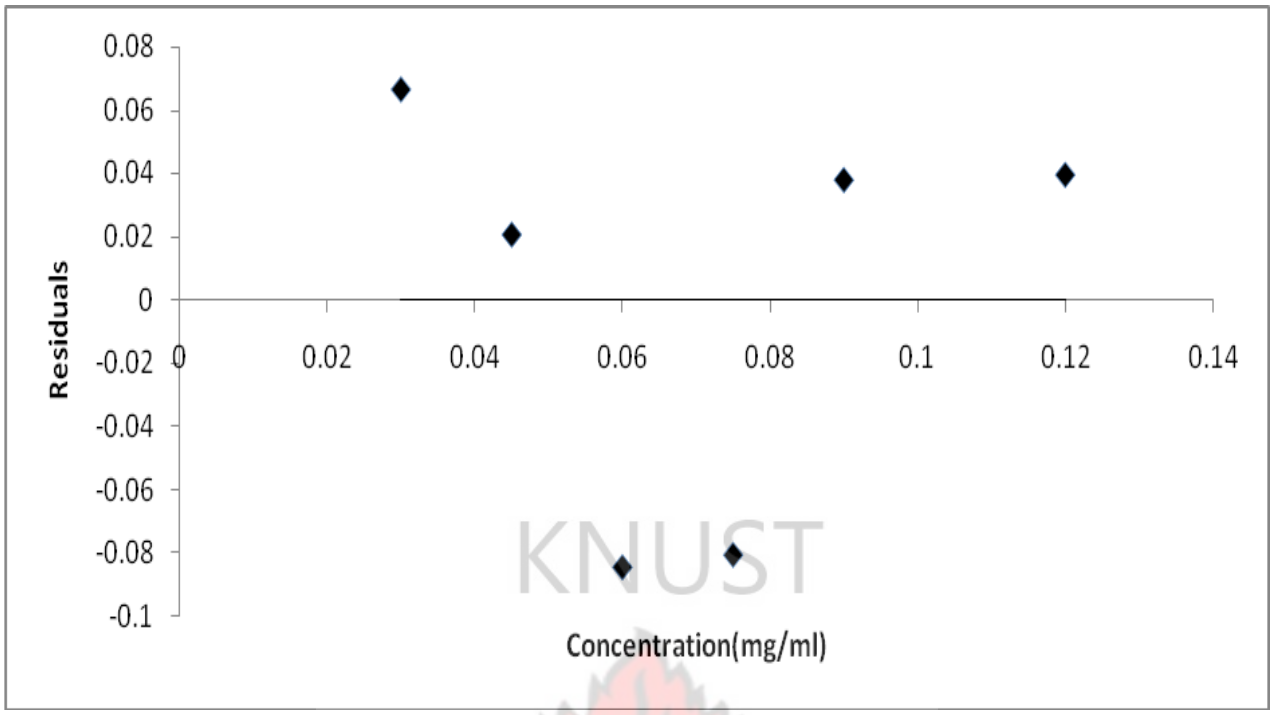


Fig. 4.7.12 Residual plots for Cefuroxime reference standard using 0.030mg/ml Ceftriaxone reference standard as internal standard

Table 4.7.4 Peak Area for Ceftriaxone reference standards and Cefuroxime reference standard with Cefepime as internal standard at various concentrations and their respective correlation coefficients

	Peak Area uV*sec Analyte					**Correlation Coefficient** Analyte	
Conc.	Ceftriaxone Reference Standard	Cefuroxime Reference Standard	30µg/ml Cefepime Internal Standard	Peak area ratio - Ceftriaxone	Peak area ratio - Cefuroxime	Ceftriaxone Reference Standard	Cefuroxime Reference Standard
30µg/ml	1203374	1117974	473272	2.54267	2.36222	0.999	0.997
45µg/ml	1828133	1628708	473072	3.86439	3.44284		
60µg/ml	2465074	2144236	475929	5.1795	4.50537		
75µg/ml	3119338	2724980	475279	6.56317	5.73344		
90µg/ml	3737904	3397808	472774	7.90632	7.18696		
120µg/ml	5042527	4509022	489407	10.3033	9.21324		

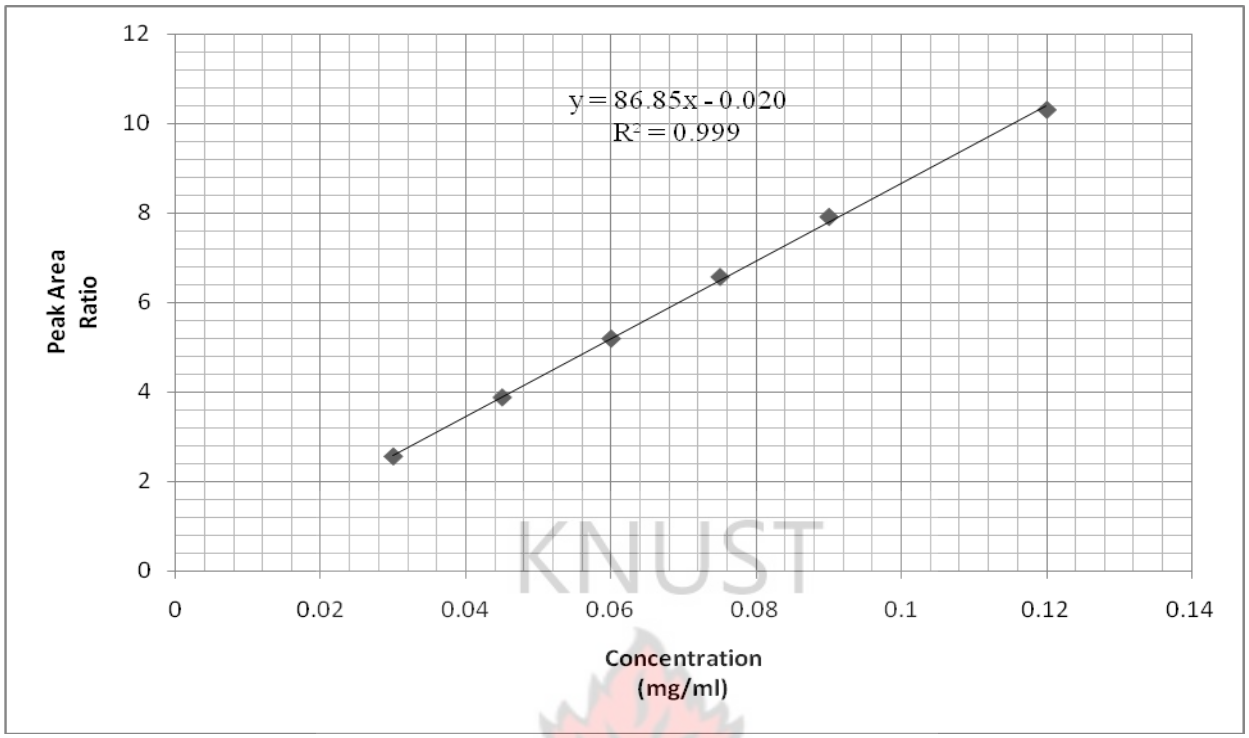


Fig. 4.7.13 Calibration plots for Ceftriaxone reference standard using 0.030mg/ml Cefepime reference standard as internal standard

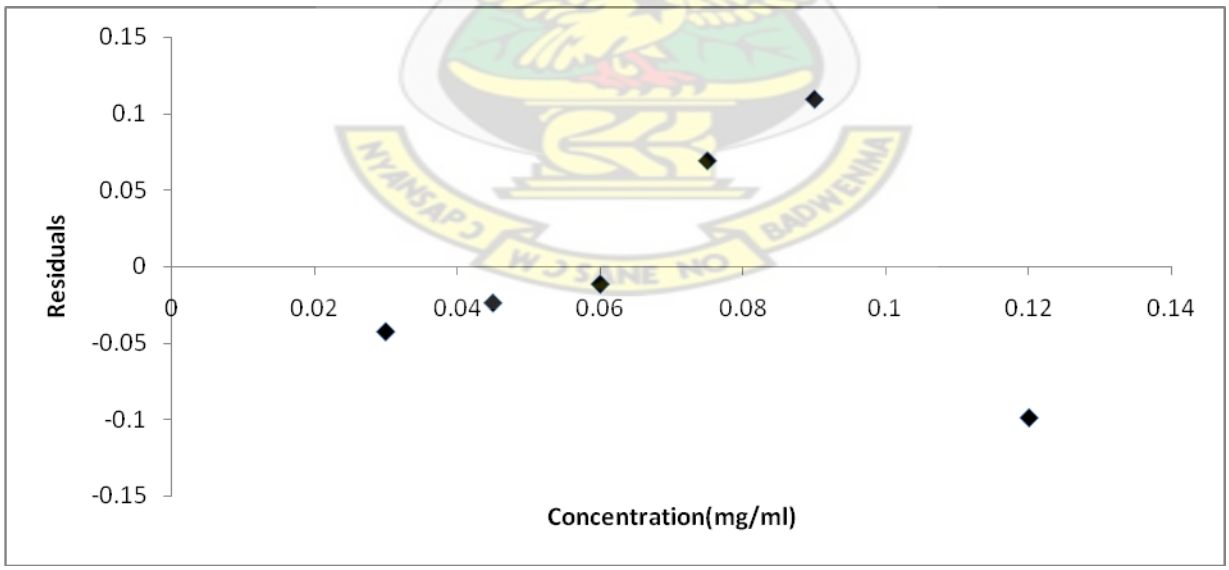


Fig. 4.7.14 Residual plots for Ceftriaxone reference standard using 0.030mg/ml Cefepime reference standard as internal standard

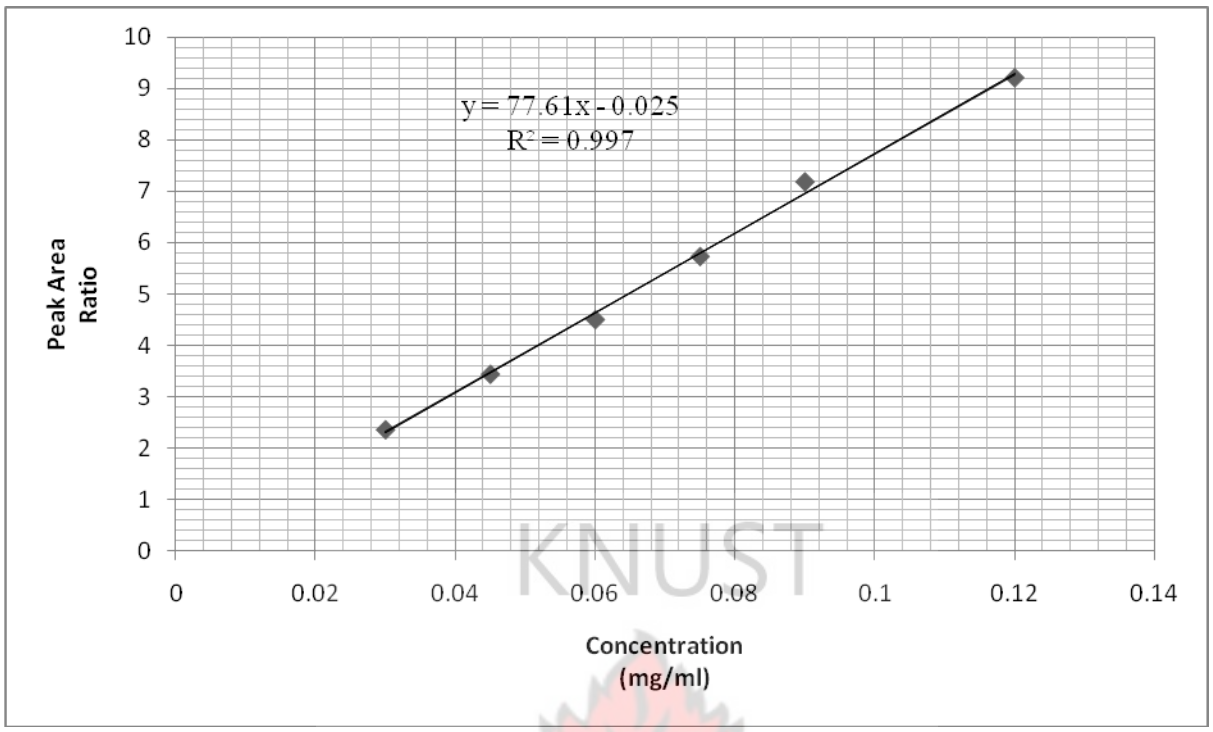


Fig. 4.7.15 Calibration plots for Cefuroxime reference standard using 0.030mg/ml Cefepime reference standard as internal standard

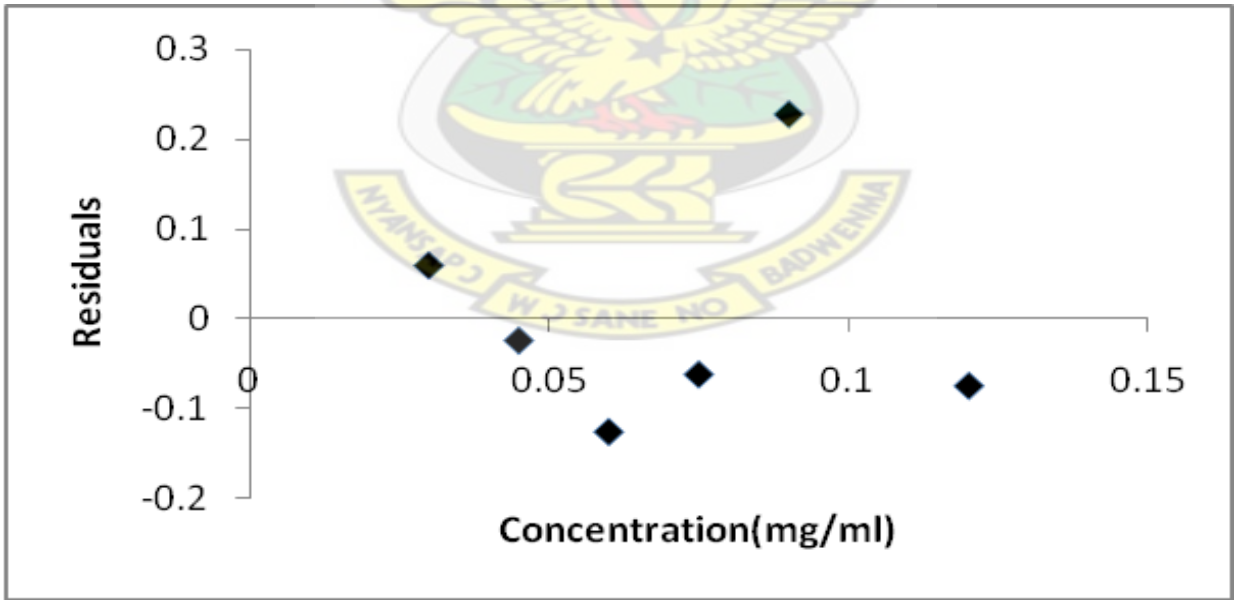


Fig. 4.7.16 Residual plots for Cefuroxime reference standard using 0.030mg/ml Cefepime reference standard as internal standard

4.8 Recovery Study

Table 4.8.1 Mass Recovery Studies of Cefepime reference standard using Standard Addition Method – Ceftriaxone internal reference standard

Amount	Standard Addition	Injections (Peak Area Ratio)					
(mg/ml)	(mg/ml)	1	2	3	4	5	6
0.045	0.010	0.694832	0.710869	0.696897	0.699164	0.708557	0.699784
0.060	0.010	0.895648	0.898873	0.914728	0.897769	0.898689	0.920415
0.075	0.010	1.086942	1.084723	1.084821	1.084372	1.083780	1.084794

y =Amount + Standard Addition Amount

Table 4.8.2 Percentage Recovery (%) of Cefepime reference standard using Standard Addition Method – Ceftriaxone internal reference standard

Expected Amount / y	Mean Mass Recovery /x	Injections (mass recovery)						% Recovery
(mg/ml)	(mg/ml)	1	2	3	4	5	6	(x/y) x100
0.055	0.055095	0.05480	0.05570	0.05485	0.05492	0.05530	0.05500	100.20
0.070	0.07000	0.06980	0.06983	0.07021	0.06992	0.06996	0.07028	100.00
0.085	0.084908	0.08501	0.08490	0.08495	0.08486	0.08480	0.08493	99.89

Table 4.8.2.1 Statistical ANOVA table for comparison

Expected Amt	*Count*	*Sum*	*Average*	*Variance*		
0.05	6	0.33057	0.055095	1.1895×10^{-7}		
0.07	6	0.42	0.07	3.988×10^{-8}		
0.085	6	0.50945	0.0849083	5.33667×10^{-9}		
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	0.002667	2	0.0013333	24364.00091	4.59×10^{-27}	3.68232
Within Groups	8.21×10^{-7}	15	5.472×10^{-8}			
Total	0.002667	17				

There is no significant difference between the peak area ratios within each concentration level.

Table 4.8.3 Mass Recovery Studies of Cefepime reference standard using Standard Addition Method – Cefuroxime internal reference standard

Amount	**Standard Addition**	**Injections (Peak Area Ratio)**					
(mg/ml)	(mg/ml)	1	2	3	4	5	6
0.045	0.010	0.751098	0.749671	0.756118	0.754928	0.753991	0.749878
0.060	0.010	0.96883	0.96759	0.97970	0.98006	0.969841	0.975082
0.075	0.010	1.19026	1.18975	1.18877	1.18938	1.19018	1.18891

y =Amount + Standard Addition Amount

Table 4.8.4 Percentage Recovery (%) of Cefepime reference standard using Standard Addition Method – Cefuroxime internal reference standard

Expected Amount / y	**Mean Mass Recovery /x**	**Injections (mass recovery)**						**% Recovery**
(mg/ml)	(mg/ml)	1	2	3	4	5	6	(x/y) x100
0.055	0.05493	0.05495	0.0549	0.0551	0.05496	0.05479	0.05488	99.87
0.070	0.069938	0.06910	0.06906	0.0707	0.07150	0.06949	0.06978	99.91
0.085	0.084913	0.08507	0.08491	0.08479	0.08487	0.08502	0.08482	99.90

Table 4.8.4.1 Statistical ANOVA table for comparison

Expected Amt	***Count***	***Sum***	***Average***	***Variance***		
0.055	6	0.32958	0.05493	1.064×10^{-8}		
0.07	6	0.41963	0.069938	9.4386×10^{-7}		
0.085	6	0.50948	0.084913	1.2347×10^{-8}		
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	0.002697	2	0.001349	4184.23831	2.48×10^{-21}	3.68232
Within Groups	4.83×10^{-6}	15	3.22×10^{-7}			
Total	0.002702	17				

There is no significant difference between the peak area ratios within each concentration level.

Table 4.8.5 Mass Recovery Studies of Ceftriaxone reference standard using Standard Addition Method – Cefepime internal reference standard

Amount	Standard Addition	Injections (Peak Area Ratio)					
(mg/ml)	(mg/ml)	1	2	3	4	5	6
0.045	0.010	4.55074	4.54959	4.549778	4.55065	4.54971	4.55128
0.060	0.010	6.00578	5.98351	5.98599	5.99072	5.99718	5.99800
0.075	0.010	7.41974	7.48721	7.50631	7.49298	7.51609	7.49961

y =Amount + Standard Addition Amount

Table 4.8.6 Percentage Recovery (%) of Ceftriaxone reference standard using Standard Addition Method –Cefepime internal reference standard

Expected Amount / y	Mean Mass Recovery /x	Injections (mass recovery)						% Recovery
(mg/ml)	(mg/ml)	1	2	3	4	5	6	(x/y) x100
0.055	0.05502	0.05512	0.05492	0.05496	0.05507	0.05490	0.05515	100.04
0.070	0.070273	0.07180	0.06922	0.06981	0.07020	0.07001	0.07060	100.39
0.085	0.084963	0.084877	0.08490	0.085009	0.08497	0.08507	0.08495	99.96

Table 4.8.6.1 Statistical ANOVA table for comparison

Expected Amt	*Count*	*Sum*	*Average*	*Variance*		
0.055	6	0.33012	0.05502	$1.148x10^{-8}$		
0.07	6	0.42164	0.070273	$7.6727x10^{-7}$		
0.085	6	0.509776	0.084963	$5.0295x10^{-9}$		
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	0.00269	2	0.001345	5148.16893	$5.26x10^{-22}$	3.68232
Within Groups	$3.919x10^{-6}$	15	$2.61x10^{-7}$			
Total	0.0026939	17				

There is no significant difference between the peak area ratios within each concentration level.

Table 4.8.7 Mass Recovery Studies of Ceftriaxone reference standard using Standard Addition Method –Cefuroxime internal reference standard

Amount	**Standard Addition**	**Injections (Peak Area Ratio)**					
(mg/ml)	(mg/ml)	1	2	3	4	5	6
0.045	0.010	1.95886	1.98749	1.99104	2.05184	1.99846	1.98786
0.060	0.010	2.55150	2.54098	2.55708	2.59874	2.58729	2.58408
0.075	0.010	3.20971	3.10752	3.15048	3.10984	3.18352	3.10852

y =Amount + Standard Addition Amount

Table 4.8.8 Percentage Recovery (%) of Ceftriaxone reference standard using Standard Addition Method –Cefuroxime internal reference standard

Expected Amount / y	**Mean Mass Recovery /x**	**Injections (mass recovery)**						**% Recovery**
(mg/ml)	(mg/ml)	1	2	3	4	5	6	(x/y) x100
0.055	0.05467	0.05401	0.05418	0.05487	0.05520	0.05513	0.05463	99.40
0.070	0.07033167	0.06975	0.06923	0.07013	0.07150	0.07081	0.07057	100.47
0.085	0.08496117	0.08512	0.08453	0.08503	0.08492	0.08510	0.085067	99.95

Table 4.8.8.1 Statistical ANOVA table for comparison

Expected Amt	***Count***	***Sum***	***Average***	***Variance***
0.055	6	0.32802	0.05467	2.4196×10^{-7}
0.07	6	0.42199	0.07033167	6.48657×10^{-7}
0.085	6	0.509767	0.08496117	4.96082×10^{-8}

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	0.00275373	2	0.00137686	4393.19874	1.72×10^{-21}	3.68232
Within Groups	4.70112×10^{-6}	15	3.1341×10^{-7}			
Total	0.002758431	17				

There is no significant difference between the peak area ratios within each concentration level.

Table 4.8.9 Mass Recovery Studies of Cefuroxime reference standard using Standard Addition Method – Cefepime internal reference standard

Amount	Standard Addition	Injections (Peak Area Ratio)					
(mg/ml)	(mg/ml)	1	2	3	4	5	6
0.045	0.010	4.27295	4.15399	4.29622	4.11497	4.28139	4.29418
0.060	0.010	5.30873	5.40027	5.31790	5.39622	5.37420	5.35829
0.075	0.010	6.68490	6.59752	6.65931	6.67463	6.70379	6.62992

y =Amount + Standard Addition Amount

Table 4.8.10 Percentage Recovery (%) of Cefuroxime reference standard using Standard Addition Method – Cefepime internal reference standard

Expected Amount / y	Mean Mass Recovery /x	Injections (mass recovery)						% Recovery
(mg/ml)	(mg/ml)	1	2	3	4	5	6	(x/y) x100
0.055	0.055022	0.05497	0.05490	0.0553	0.05483	0.05504	0.05509	100.04
0.070	0.069662	0.06923	0.07012	0.06950	0.06986	0.06966	0.06960	99.52
0.085	0.084872	0.08502	0.084720	0.084862	0.084879	0.08510	0.08465	99.85

Table 4.8.10.1 Statistical ANOVA table for comparison

Expected Amt	*Count*	*Sum*	*Average*	*Variance*		
0.055	6	0.33013	0.055022	2.73367×10^{-8}		
0.07	6	0.41797	0.069662	9.31367×10^{-8}		
0.085	6	0.509231	0.084872	2.9285×10^{-8}		
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	0.0026734	2	0.001337	26777.37167	2.26×10^{-27}	3.68232
Within Groups	7.488×10^{-7}	15	4.99×10^{-8}			
Total	0.0026742	17				

There is no significant difference between the peak area ratios within each concentration level

Table 4.8.11 Mass Recovery Studies of Cefuroxime reference standard using Standard Addition Method – Ceftriaxone internal reference standard

Amount	**Standard Addition**	**Injections (Peak Area Ratio)**					
(mg/ml)	(mg/ml)	1	2	3	4	5	6
0.045	0.010	1.65031	1.64791	1.65002	1.63855	1.60996	1.64583
0.060	0.010	2.09562	2.11062	1.99521	1.99748	2.07329	2.05264
0.075	0.010	2.69370	2.70038	2.66945	2.66941	2.65992	2.67341

y =Amount + Standard Addition Amount

Table 4.8.12 Percentage Recovery (%) of Cefuroxime reference standard using Standard Addition Method – Ceftriaxone internal reference standard

Expected Amount / y	**Mean Mass Recovery /x**	**Injections (mass recovery)**						**% Recovery**
(mg/ml)	(mg/ml)	1	2	3	4	5	6	(x/y) x100
0.055	0.0549715	0.0553	0.0549	0.0551	0.05487	0.05477	0.054889	99.95
0.070	0.06996333	0.0700	0.0710	0.06940	0.06979	0.06981	0.06978	99.95
0.085	0.08502283	0.0853	0.0857	0.08495	0.08407	0.085037	0.08508	100.03

Table 4.8.12.1 Statistical ANOVA table for comparison

Expected Amt	***Count***	***Sum***	***Average***	***Variance***
0.055	6	0.329829	0.0549715	3.745E-08
0.07	6	0.41978	0.06996333	2.9611E-07
0.085	6	0.510137	0.08502283	2.9041E-07

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	0.002709252	2	0.00135463	6512.99843	$9.033x10^{-23}$	3.6823203
Within Groups	$3.11982x10^{-6}$	15	$2.0799x10^{-7}$			
Total	0.002712372	17				

There is no significant difference between the peak area ratios within each concentration level

4.9 Limit of Quantification (LOQ) and Limit Of Detection (LOD)

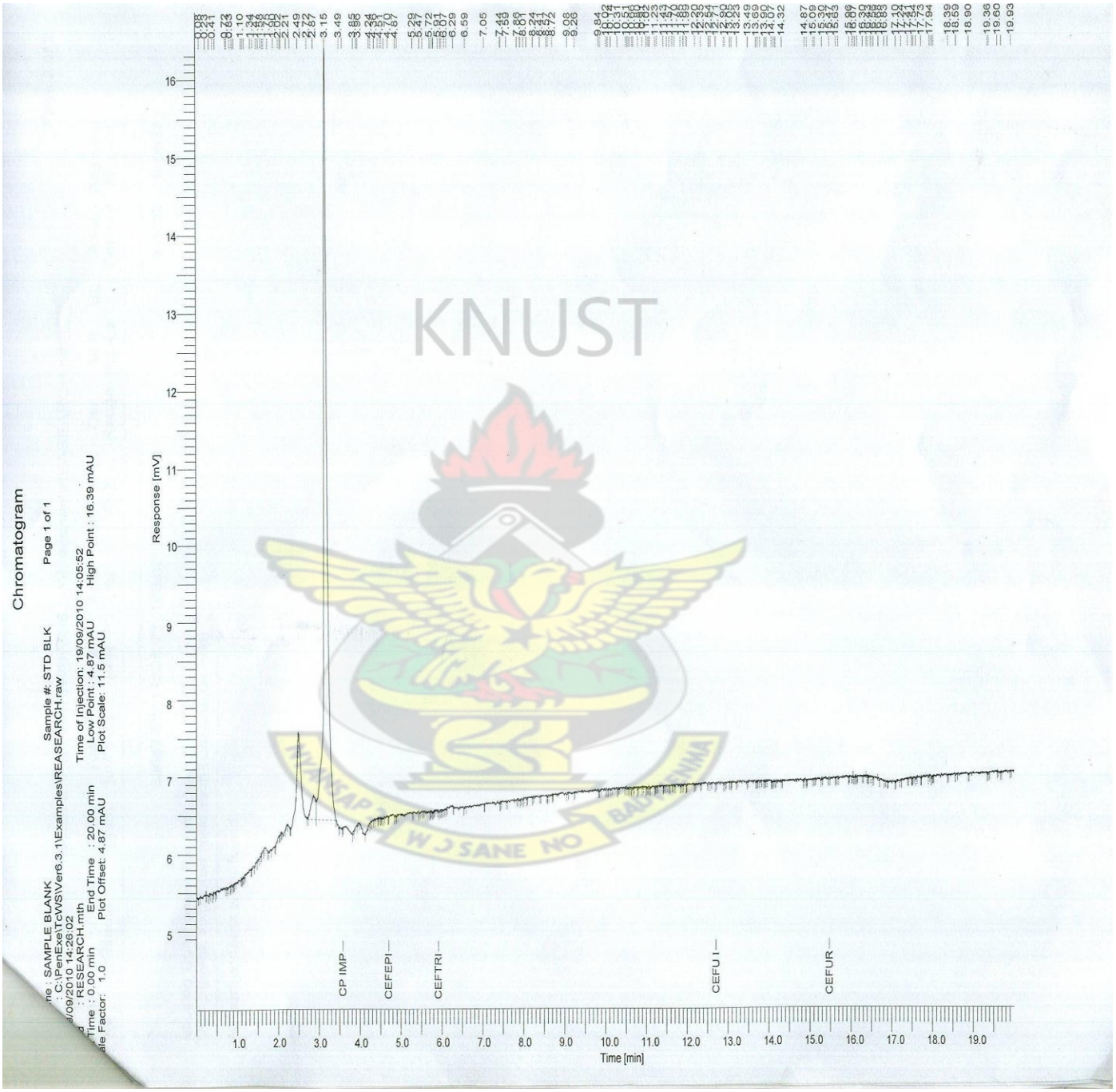


Fig 4.9.1 Chromatograms for Limit of detection and Limit of quantification

Table 4.9.1 Peak Height Baseline of Ceftriaxone and Cefepime – Cefuroxime as internal standard

	Injections (Peak Height Ratio)							
Reference Standard	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**Standard Deviation**
Cefepime	0.9143	0.9312	0.9540	1.0013	0.9953	1.0716	0.97795	0.057319
Ceftriaxone	0.9601	1.0000	1.0000	1.0058	1.0445	1.1730	1.030567	0.074738

Table 4.9.2 Peak Height Baseline of Cefepime and Cefuroxime – Ceftriaxone as internal standard

	Injections (Peak Height Ratio)							
Reference Standard	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**Standard Deviation**
Cefepime	0.952381	0.931195	0.954035	0.995537	0.995252	0.913589	0.956998	0.033253
Cefuroxime	1.041548	1.0000	1.0000	0.994215	0.95743	0.852544	0.974289	0.065344

Table 4.9.3 Peak Height Baseline of Ceftriaxone and Cefuroxime - Cefepime as internal standard

	Injections (Peak Height Ratio)							
Reference Standard	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**Standard Deviation**
Ceftriaxone	1.0500	1.073889	1.04818	1.004483	1.00477	1.094584	1.045984	0.036284
Cefuroxime	1.093625	1.073889	1.04818	0.998672	0.961997	0.933181	1.018257	0.063965

LOQ = 10 x standard deviation/ Slope of Calibration curve

LOD = 3 3x standard deviation/ Slope of Calibration curve

Table 4.9.4 Limit of Quantification (LOQ) and Limit of Detection (LOD) with internal standards

Reference . Standard	Slope		Standard Deviation		LOQ		LOD	
	$Slope_1$	$Slope_2$	STD_1	STD_2	LOQ_1	LOQ_2	LOD_1	LOD_2
Cefepime	13.27	13.67	0.05732	0.03325	0.04319442	0.02432553	0.01295833	0.00729766
Ceftriaxone	86.85	36.81	0.07474	0.03628	0.00860541	0.009857104	0.00258162	0.00295713
Cefuroxime	77.61	0.544	0.06534	0.06397	0.00841953	1.175827206	0.00252586	0.35274816

Table 4.9.5 Peak Height Baseline of the various reference standards with instrument response

	Injections (Peak Height)							
Reference Standard	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**Standard Deviation**
Cefepime	50.2	51.97	55.21	60.23	62.89	65.55	57.675	6.168285823
Ceftriaxone	52.71	55.81	57.87	60.5	63.19	71.75	60.305	6.680831535
Cefuroxime	54.9	55.81	57.87	60.15	60.5	61.17	58.4	2.622380598

LOQ = 10 x standard deviation/ Slope of Calibration curve

LOD = 3.3 x standard deviation/ Slope of Calibration curve

Table 4.9.6 Limit of Quantification (LOQ) and Limit of Detection (LOD) with instrument response

Reference Standard	Slope	Standard Deviation	LOQ	LOD
Cefepime	15685	6.168285823	0.0039326	0.001179781
Ceftriaxone	41876	6.680831535	0.00159538	0.000478615
Cefuroxime	36228	2.622380598	0.00072385	0.000217156

4.5 Assay of Formulated products

Table 4.5.1 Assay of Formulations

Tests	**Con. @0.060mg/ml**	**Con. @0.060mg/ml**	**Con. @0.060mg/ml**	**% Assay A**	**% Assay A**	**% Assay A**
Formulations	Cefepime	Ceftriaxone	Cefuroxime	Cefepime	Ceftriaxone	Cefuroxime
A1	0.05982	0.0615	0.05988	99.7	102.5	99.8
A2	0.05874	0.06042	0.06012	97.9	100.7	100.2
A3	0.060042	0.0606	0.05988	100.07	101.0	99.8
B4	0.05928	0.05976	0.05784	98.8	99.6	96.4
B5	0.06066	0.05988	0.06030	101.1	99.8	100.5
B6	0.06918	0.0609	0.06672	115.3	101.5	111.2

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 DISCUSSION

5.1.1 Product Selection

Cephalosporins are β-lactam antibiotics with the same fundamental structural requirements as penicillin. They are used for the treatment of infections caused by Gram-positive and Gram-negative bacteria. In Ghana, cephalosporins are used to treat a wide variety of bacterial infections, such as respiratory tract infections (pneumonia, strep throat, tonsillitis, bronchitis), skin infections and urinary tract infections.[2] They are sometimes given with other antibiotics. Cephalosporins are also commonly used for surgical prophylaxis - prevention of bacterial infection before, during, and after surgery. Currently, cephalosporins are one of the leading drugs imported into the country. They are widely used antibiotics; caution needs to be taken to avoid drug resistance. Based on the above facts, cephalosporins were selected for this project to enhance the products' integrity.

5.1.2 Sample characterization

5.1.2.1 Identification of pure samples

Cefuroxime was identified based on British Pharmacopoeia standards.

The pH of the resultant solution was determined to be 6.6 which is within the literature value range 5.5 to 8.5 which gives an indication of cefuroxime. Another B.P. test was done on the test sample. A solution was prepared by dissolving 0.50g in acetate buffer solution of pH 4.6. This was then diluted to 25.0ml with the same buffer. The specific optical rotation of the resultant solution was determined to be +62; which falls within the literature range of +59 to +66.[5]

Cefepime was identified based on United States Pharmacopoeia standard. As stated in the Pharmacopoeia, any solution containing 100mg of cefepime per ml should a pH in the range of 4.0 to 6.0.This was done with three solvents: methanol, water and ethanol. These gave a respective pH of 4.5, 5.6 and 6.0. This implied the presence of cefepime. [19]

Ceftriaxone was identified based on British Pharmacopoeia standard. This states that a solution S which is prepared by dissolving 2.40g in deionised water and diluted to 20ml with the same solvent. The solution needs to be clear and coloured. Then if 2ml of the solution is diluted to 20ml, the pH of the diluted solution must be between the ranges of 6.0 to 8.0. Another B.P. test was done. A solution was prepared by dissolving 0.25g of the sample in water R and then diluted to 25.0ml with water R. The specific optical rotation of this solution was taken and recorded as -162. This fell within the British Pharmacopoeia literature value range of -155 to -170; this confirmed the presence of Ceftriaxone. [5]

5.1.3 Initial method development

The method was initially developed for an analytical task and optimized before validation. Method development and validation are, therefore, an iterative process. Initial method development involved column, detector and mobile phase selection. All the cephalosporins were soluble in water and methanol.

This made them relatively polar; this reverse phase column was adopted as the stationary phase using **octadecylsilylsilane (ODS – C_{18})**

The mobile phase selection was based on cost, environment friendliness, good retention time and ease of preparation. Solvents such as isopropyl alcohol, methanol and water were tried.

All these solvents did not give peaks within thirty (30) minutes hence mixtures of the solvents were prepared. The water content was maintained in each mixture with variation in its percentage content. The mixture of water and isopropyl alcohol did not give any good peak

irrespective of percentage content variations in the mixture. There was peak shape distortion which may be due to poor solubility of the analytes in the lower strength mobile phases. This can be due to poor selectivity of the separation.[20] The use of water and methanol mixture as the mobile phase has been of great importance in this research. There was appearance of peaks with mixture of water and methanol as mobile phase and the stationary phase being either C_{18} or C_8 column. The range for the water content is between 75% and 85%. Different ratios of water: methanol mixture -75/25, 80/20 and 85/15 respectively were selected because they gave relatively good peaks. The problem associated with these combinations was with good resolution when the cephalosporins were mixed. Some of the peaks did overlap making identification to be difficult. Currently, there are no combination formulations of any these products hence it is possible to use these above procedures to analyse these products individually.

5.1.4. Method Optimization with C_{18} reverse phase

The use of C_{18} reverse phase with mobile phase of 75% buffer and 25% methanol showed the various average retention times being less than 9.00min. There was a problem of resolution when cefepime and ceftriaxone were mixed up hence this conditions cannot be used in preparation involving these two cephalosporins especially in cross-contamination and cleaning validation. The average retention time for cefepime, ceftriaxone and cefuroxime were 3.43min, 3.6min and 8.5min respectively. As show in Fig.4.3.6

When the individual cephalosporins were run using 85% buffer and 15% methanol as a mobile phase, the average retention time for cefepime, ceftriaxone and cefuroxime were 5.40min, 8.06min and 45.00min respectively. As shown in Fig. 4.3.5

The average retention times for cefepime and ceftriaxone were good and more economical hence this method would be suitable for identification and quantification tests involving cefepime and

ceftriaxone. Although the chromatograms had good resolution, the average retention time for cefuroxime was greater than 20min making it less significant for the objective of this project.

The use of 80% buffer and 20% methanol as mobile phase with C_{18} reverse phase had shown to be more suitable condition. From the data collected, the specific objectives of developing a simple, precise and cost-effective method have been achieved with this condition. This makes 80% buffer and 20% methanol as mobile phase with C_{18} reverse phase a suitable condition for cefepime, ceftriaxone and cefuroxime HPLC analysis whether individually or in a mixture. This suitable condition comes with a good resolution and a better retention time which is less than 20min. As shown in Fig. 4.3.4

Hence this developed method can be successfully be used as a validation procedure for cross-contamination and cleaning manufacturing equipments. The average retention time for cefepime, ceftriaxone and cefuroxime were 4.77min, 5.85min and 15.32min respectively.

5.1.5 Method validation

Since 80% buffer and 20% methanol as mobile phase with C_{18} reverse phase is a suitable condition for cefepime, ceftriaxone and cefuroxime HPLC analysis, its data needs to be validated. Validation refers to the procedures involved in checking data or programs for correctness and compliance with standards for the purpose of achieving consistent conformance with specific requirement specifications. Validation may be performed for several methods and processes such as manufacturing procedures, product formulation process, sterilization, cleaning or purification procedure and analytical methods for consistency and traceability of results.

The most important, priceless and indispensable tool in the hands of the analytical chemist in the laboratory is the analytical method. Without the analytical method and the ability to develop one, the analytical chemist is essentially useless and every other person whose activities depends

upon analytical report is crippled. The analytical method is an embodiment of all the scientific procedures, equipment and reagents used in performing a scientifically valid analytical test.

The objective of validation of this method is to demonstrate that the procedure, when correctly applied, produces results that are reliable and accurate and that the procedure is suitable for its intended use. According to the ICH, accuracy, any type of precision and limits of detection and quantification are not required if the analytical task is identification.[20]

For assays in USP category 1 tests, the major component or active ingredient to be measured is normally present at high concentrations and therefore for assay of actives or other key for potency, validation for limits of detection and quantification is not necessary but for assay of analytes that are expected in minute quantities in a bulk matrix, validation for specificity and detection limits may be required.

There is several validation parameters of which the typical ones which should be considered are: selectivity (specificity), linearity, range, accuracy, precision, limit of detection and quantification. [6]

It is clear from the list above that there are several validation parameters. However, it is not always necessary to validate all analytical parameters that are available for a specific technique. The type of validation parameter to determine which subset of each parameter is required to demonstrate validity and is based on the intended use of the method; the analytical task and the scope of the method. This project work is method development based on the assay method.

This will be intended to detect trace quantities of the active ingredient for purposes of a cleaning validation study. Hence knowledge of the detection and quantification limits is appropriate and necessary.

5.1.5.1 Repeatability and Intermediate Precision Study

Quantification was done based on the internal standard peak area ratio and the preparation of calibration curves for all the compounds assayed. The peak area ratio is the peak area of the sample drug divided by the peak area of the internal standard. The internal standard provides protection against fluctuation of results due to injection problems, day to day variation of environmental conditions, power fluctuations and inherent deficiencies in the instruments. With the elimination of these variations, the precision of the method was improved.

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision - the variation arising when all efforts are made to keep conditions constant by using the same instrument and operator, and repeating during a short time period; [21]

According to the ICH guidelines, repeatability requires precision from at least six (6) replications to be measured at 100% of the test target concentration or from at least nine (9) replications covering the complete specified range but the former was chosen. [22]

The HPLC method had a high precision. As indicated in Table 4.5.1, all had RSD values of less than 2.0% and therefore acceptable limits of precision. [22] Cefepime reference standard had the highest precision by having the least relative standard deviation.

5.1.5.2 Ruggedness and Robustness

Reproducibility expresses the precision between laboratories (collaborative studies usually applied to standardization of methodology) — the variation arising using the same measurement process among different instruments and operators, and over longer time periods. Ruggedness is not addressed in the ICH documents (4,5) but rather addressed in the USP which also does not address reproducibility on its own.

In the ICH documents ruggedness has been replaced by reproducibility, which has the same meaning as ruggedness, defined by the USP as the degree of reproducibility of results obtained under a variety of conditions, such as different laboratories, analysts, instruments, environmental conditions, operators and materials. [22]

HPLC QA1 is the equipment located in the main laboratory at ordinary room temperature of about 30^{o}C; HPLC QA2 is the equipment located in a strictly controlled laboratory at room temperature of 25^{o}C.The method is applicable on both instruments under their respective operational conditions with acceptable level of precisions.

As indicated in Table 4.6.1, all had RSD values of less than 2.0% and therefore the new method is robust. [22]

5.1.5.3 Recovery Study

In general terms, accuracy refers to the degree of closeness to the truth or the degree of veracity in a process. The accuracy of an analytical method(or instrument) is the extent to which test results generated by the method agrees with the conventional true value or the extent to which test results generated by the method is within the range of the true value. [22] It is a measure of analyte recovery from a matrix by an analytical procedure.

Generally, assessment of accuracy of a method measures essentially the effectiveness of sample preparation, reagents used and equipment specifications, hence care should be taken to mimic the actual sample preparation as closely as possible.

The accuracy (or bias) of an analytical method may be measured in many different ways but dependent on availability of known reference, sample components are known and available, analyte is available but sample components are not known, available or defined, reference analyte, matrix and extraneous substances are either available or not .

For this new method developed, analyte is available but sample components are not known. The respective manufacturers are not willing to define the sample components hence standard addition method was adopted. This refers to the accuracy determination method in which a real sample is spiked at varying levels with known amounts of the reference standard sample of the analyte. Afterwards, a known amount of the pure analyte(s) is added, and the sample is again assayed with the proposed method. This method is of general convenience and most suitable where it's difficult to obtain matrix components or manufacture control samples or when developing a method for a product obtained on the market, the excipients of which are unknown to the analyst.[22]

The percentage recovery for cefepime in the assay performed at the three different concentration levels was greater than 98% and the relative standard deviation was less than 2% and within acceptable limits. Tables 4.8.2 and 4.8.4

The percentage recovery for ceftriaxone in the assay performed at the three different concentration levels was greater than 98% and the relative standard deviation was less than 2% and within acceptable limits. Tables 4.8.6 and 4.8.8

The percentage recovery for cefuroxime in the assay performed at the three different concentration levels was greater than 98% and the relative standard deviation was less than 2% and within acceptable limits. Tables 4.8.10 and 4.8.12

5.1.5.4 Linear regression analysis

The linearity is the ability of analytical procedure to produce test results which are proportional to the concentration (amount) of analyte in samples within a given concentration range, either directly or by means of a well-defined mathematical transformation. Linearity should be determined by using a minimum of six standards whose concentration span 80 –120% of the expected concentration range. [22]

The linearity of a method should be established by visual inspection of a plot of analytical response as a function of analyte concentration. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of the regression line by the method of least squares. In some cases, the test data may need to be subjected to a mathematical transformation prior to regression analysis.

Reports submitted must include the slope of the line, intercept and correlation coefficient data.

The measured slope should demonstrate a clear correlation between response and analyte concentrations. The results should not show a significant deviation from linearity, which is taken to mean that the correlation coefficient, $r > 0.99$, over the working range (80 –120%).

If this is not the case (i.e. r is < 0.99), the submitter must provide an explanation of how accurate calibration is to be maintained. In cases where a non-linear response is deliberately used, an explanation must be provided.

The linear regression analysis results showed a correlation coefficient of about 0.99 for each calibration plots. This gives an indication of good linear relationship between instrument response and analytes concentration in the range 30µg/ml to 120µg/ml for each cephalosporin analyte. Tables 4.7.1 – 4 and Figures 4.7.1-16

5.1.5.5 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection is the point at which a measured value is larger than the uncertainty associated with it. It is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified. The limit of quantification is the lowest amount of the analyte in the sample that can be quantitatively determined with defined precision under the stated experimental conditions.

In case the lower working limit is more than one decade above the limit of detection, we may estimate these limits from signal-to-noise ratio. This is especially useful for chromatographic methods, yielding a simple way to check real samples in routine work. Usually, three times standard deviation per the slope of the calibration curve gives the limit of detection (LOD) while ten times standard deviation per the slope of the calibration curve gives the limit of quantification (LOQ).[22]

In this method of analysis, two different categories of calibration curve were plotted. First the calibration plot of the analytes involved their respective internal standard while the second was based on the instrument response of the analytes. The data obtained from these calibration plots are significantly different hence there were two different data each for limits of quantification and limit of detection. The limit of Quantification and limit of Detection based on instrument response had injections of only the analytes reference standard hence the peak area was used for data processing. The limit of Quantification and limit of Detection based on internal standards had injections of preparations of both the analytes reference standard and its respective internal standard hence peak area ratio was used for data processing.

Cefepime reference standard's limit of Quantification and limit of Detection based on internal standard were: LOQ_1 – 0.04319mg/ml, LOQ_2 -0.02433mg/ml and LOD_1 – 0.012958mg/ml, LOD_2 -0.007298mg/ml. Table 4.9.4

The cefepime reference standard's limit of Quantification and limit of Detection based on instrument response were: LOQ – 0.003933mg/ml and LOD – 0.001180mg/ml .Table 4.9.6

The LOQ_1 of the Cefepime reference standard was greater than the lowest concentration level of its calibration plots (0.03mg/ml). This concentration gave a good peak with an intensity of 472774. This indicates further dilutions of this concentration will still result in having good peaks. Even the LOD_1 and LOD_2 were greater than the LOQ of the instrument response. On the issue of the internal standard, it will be prudent to use ceftriaxone reference standard as internal standard as its LOQ_2 and LOD_2 are relatively closer to the LOQ and LOD of the instrument response.

The ceftriaxone reference standard's LOQ_1 – 0.008605mg/ml; LOQ_2 - 0.009857mg/ml and LOD_1 - 0.002582mg/ml; LOD_2 - 0.002957mg/ml; these are due to internal standard responses. The LOQ and LOD due to instrument response are 0.001595mg/ml and 0.0004786mg/ml respectively .From the results this means either cefepime or cefuroxime can be an internal standard for ceftriaxone reference standard.

The cefuroxime reference standard's limit of Quantification and limit of Detection based on instrument response were: LOQ – 0.0007239mg/ml and LOD – 0.0002172mg/ml. Table 4.9.6

Cefepime reference standard's limit of Quantification and limit of Detection based on internal standard were: LOQ_1 – 0.008420mg/ml, LOQ_2 - 1.1758mg/ml and LOD_1 – 0.002526mg/ml, LOD_2 - 0.35275mg/ml. Table 4.9.4. The suitable internal standard for cefuroxime reference standard will be cefepime. The best LOQ and LOD data should be based on instrument responses.

5.1.6 Assay

This method of analysis was applied to six formulations which were all injections. Percentage assay for Cefepime were: A - 99.22% ±0.013 and B – 105.07% ±0.026

Percentage assay for Ceftriaxone were: A – 101.40%±0.096 and B – 100.30%±0.103. Percentage assay for Cefepime were: A – 99.93%±0.065 and B – 102.70%±0.006

These above assays fell within the literature values of the British Pharmacopoeia and United States Pharmacopoeia. For the British Pharmacopoeia, the literature value for ceftriaxone injection is 96.00% to 102.0% and cefuroxime injection is 96.0% to 102.0% [5]

The United States Pharmacopoeia has the literature values of 90% to 115% for ceftriaxone injection, 90% to 120% for cefuroxime injection and 90% to 115% for cefepime injection. [19]

5.2 Conclusion

A simple and cost-effective HPLC method for chemical quality and clean validation of Cephalosporin products Analysis has been developed. The three cephalosporin injections products were **cefuroxime**, **ceftriaxone** and **cefepime**.

This HPLC method of analysis is relatively cost-effective. The use of water and methanol mixture is relatively cheaper than any other mobile phase system for assay of any cephalosporin. This method of analysis will be able to help an analyst to run a mixture of the cephalosporins because the cephalosporins can be run individually and simultaneously hence there is time saving.

The HPLC method had a high sensitive, precision and accuracy so the performance of the developed method as a validation procedure for cleaning of equipments after manufacturing the cephalosporin products is assured.

This method is a simple separation technique required to determine contamination of one cephalosporin in another. This will be of great help to the laboratories of regulatory bodies in the country who needs to run a lot of analysis of chemical compounds and a guideline for selection of cephalosporins to be imported into the country.

5.3 Recommendations

This method of Analysis can be used to analysis other cephalosporin formulations such as infusions, tablets and suspension.

Research can be done using the above method of analysis to validate cleaning procedure in various production floors that produce any the cephalosporin products.

This HPLC method of analysis can be adopted in bioavailability studies of the these cephalosporin products.

CHAPTER SIX: REFERENCE

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CHAPTER SEVEN:

7.0 APPENDIXES

Table 7.1 Retention times for the cefepime reference standard using 80% buffer and 20% methanol as mobile phase; C_{18} as reverse stationary phase using different concentration levels.

Injection	$0.0003\%^{w}/_{v}$	$0.00045\%^{w}/_{v}$	$0.0006\%^{w}/_{v}$	$0.00075\%^{w}/_{v}$	$0.0009\%^{w}/_{v}$
1	4.759	4.756	4.757	4.766	4.770
2	4.750	4.790	4.750	4.782	4.790
3	4.768	4.788	4.773	4.768	4.781
4	4.790	4.799	4.784	4.766	4.788
5	4.780	4.784	4.787	4.781	4.799
6	4.756	4.757	4.766	4.770	4.784
Mean/min	4.767167	4.779	4.7695	4.772167	4.785333
Variance	0.000235	0.000328	0.000215	$5.46x10^{-5}$	$9.43x10^{-5}$

Table 7.1.1 Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Sample	0	0	65535	65535		
Columns	0.00133	4	0.000333	1.794242	0.161568	2.75871
Interaction	0	0	65535	65535		
Within	0.004635	25	0.000185			
Total	0.005965	29				

There is no significant difference between the various retention times; $F(2,75)=1.794, p>0.05$

Table 7.2 Retention times for the ceftriaxone reference standard using 80% buffer and 20% methanol as mobile phase; C_{18} as reverse stationary phase using different concentration levels.

Injection Number	0.0003%$^{w}/_{v}$ (50%)	0.00045%$^{w}/_{v}$ (75%)	0.0006%$^{w}/_{v}$ (100%)	0.00075%$^{w}/_{v}$ (125%)	0.0009%$^{w}/_{v}$ (150%)
1	5.900	5.850	5.820	5.890	5.840
2	5.860	5.830	5.850	5.810	5.850
3	5.900	5.840	5.850	5.875	5.870
4	5.887	5.840	5.880	5.917	5.887
5	5.850	5.870	5.870	5.860	5.850
6	5.830	5.810	5.850	5.900	5.830
Mean/min	5.871167	5.84	5.853333	5.875333	5.8545
Variance	0.000836	0.0004	0.000427	0.001413	0.000429

Table 7.2.1Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Sample	0	0	65535	65535		
Columns	0.004968	4	0.001242	1.771921	0.166085	2.75871
Interaction	0	0	65535	65535		
Within	0.017525	25	0.000701			
Total	0.022493	29				

There is no significant difference between the various retention times; F (2,75)=1.77,p>0.05

Table 7.3 Retention times for the cefuroxime reference standard using 80% buffer and 20% methanol as mobile phase; C_{18} as reverse stationary phase using different concentration levels.

Injection	0.0003%$^{w}/_{v}$	0.00045%$^{w}/_{v}$	0.0006%$^{w}/_{v}$	0.00075%$^{w}/_{v}$	0.0009%$^{w}/_{v}$
1	15.232	15.611	15.334	15.240	15.411
2	15.332	15.450	15.468	15.533	15.300
3	15.212	15.378	15.330	15.447	15.387
4	15.348	15.430	15.243	15.506	15.450
5	15.154	15.486	15.307	15.541	15.378
6	15.430	15.560	15.348	15.233	15.430
Mean/min	15.28467	15.48583	15.33833	15.41667	15.39267
Variance	0.0105	0.007439	0.005413	0.020567	0.00277

Table 7.3.1 Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Sample	0	0	65535	65535		
Columns	0.140785	4	0.035196	1.769102	0.015639	2.75871
Interaction	0	0	65535	65535		
Within	0.233452	25	0.009338			
Total	0.374237	29				

There is no significant difference between the various retention times; $F(2,75)=1.77, p>0.05$

Table 7.4 Area under the curve for 0.0003%$^{w}/_{v}$ cefepime reference standard

Injection Number/	Day 1 / uV x sec	Day 2 / uV x sec	Day 3 / uVxsec	Average/uV x sec	Variance
1	470965.26	477745.34	509433.72	486048.1	4.22×10^{8}
2	474801.95	488013.76	497081.03	486632.20	1.26×10^{8}
3	536014.40	484353.54	475992.08	498786.70	1.06×10^{9}
4	494353.00	512456.49	485134.64	497314.70	1.93×10^{8}
5	485634.72	473890.29	478320.96	479282.00	3.51×10^{7}
6	476854.23	512859.48	509741.61	499818.40	3.98×10^{8}
Mean/uV x sec	489770.6	491553.2	492617.3		
Variance	5.84×10^{8}	2.92×10^{8}	2.27×10^{8}		

Table 7.4.1 Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	1.08×10^{9}	5	2.15×10^{8}	0.485023197	0.780321154	3.325834529
Within Groups	2.48×10^{7}	2	1.24×10^{7}	0.027984712	0.972479106	4.102821015
Standard Error	4.44×10^{9}	10	4.44×10^{8}			
Total	5.54×10^{9}	17				

There is no significant difference between the area under the curves; $F(3.33)=0.485, p>0.05$;

$F(4.10)=0.028, p>0.05$.

Table 7.5 Area under the curve for 0.0003%$^{w}/_{v}$ ceftriaxone reference standard (50% concentration level) using 80% buffer and 20% methanol as mobile phase; C_{18} as reverse stationary phase over a three- day period

Injection	Day 1 / uV x sec	Day 2 / uV x sec	Day 3 / uVxsec	Average/uV x sec	Variance
1	1184505.89	1189453.94	1305342.85	1226434	$4.68x10^{9}$
2	1198605.31	1259476.21	1183103.88	1213728	$1.63x10^{9}$
3	1312243.97	1197563.41	1195715.37	1235174	$4.46x10^{9}$
4	1289672.45	1189764.61	1184510.87	1221316	$3.51x10^{9}$
5	1197743.60	1242732.30	1195615.61	1212031	$7.08x10^{8}$
6	1187641.89	1198641.93	1189426.97	1191904	$3.49x10^{7}$
Mean/uV x sec	1228402	1212938.733	1208952.592		
Variance	$3.24x10^{9}$	$9.17x10^{8}$	$2.26x10^{9}$		

Table 7.5.1Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	$3.31x10^{9}$	5	$6.62x10^{8}$	0.230037	0.940764423	3.325834529
Within Groups	$1.27x10^{9}$	2	$6.33x10^{8}$	0.220163	0.806175955	4.102821015
Standard Error	$2.88x10^{10}$	10	$2.88x10^{9}$			
Total	$3.33x10^{10}$	17				

There is no significant difference between the area under the curves; $F(3.33)=0.230, p>0.05$; $F(4.10)=0.220, p>0.05$.

Table 7.6 Statistical Comparison of two different HPLC equipments by different under different laboratory conditions at the same time using the mean area under the curve

PEAK AREA uV*sec	
HPLC: Prgqalab 1	**HPLC: Prgqalab 2**
486048.1067	473272.3967
486632.2467	473071.5867
498786.6733	475928.8567
497314.71	475278.8067
479281.99	472774.2933
499818.44	489406.73
489770.5933	480875.3517
491553.15	474602.8783
492617.34	474388.105

Table 7.6.1 F-Test for Robustness

	HPLC: Prgqalab 1	***HPLC: Prgqalab 2***
Mean	491313.6944	476622.1117
Variance	45341321.07	28940275.08
Observations	9	9
df	8	8
F	1.56672046	
P(F<=f) one-tail	0.269937547	
F Critical one-tail	3.438101233	

Table 7.7 Area under the curve for 0.0003%$^{w}/_{v}$ cefuroxime reference standard using 80% buffer and 20% methanol as mobile phase;

Injection	Day 1 uV x sec	Day 2 uV x sec	Day3 / uVxsec	MeanuVx sec	Variance
1	1101729.96	1117023.98	1253799.89	1157517.943	$7.01x10^{9}$
2	1115059.87	1252859.90	1130634.88	1166184.883	$5.7x10^{9}$
3	1146307.88	1153119.87	1101789.00	1133738.917	$7.77x10^{8}$
4	1164761.93	1125721.88	1149219.78	1146567.863	$3.86x10^{8}$
5	1120765.86	1118435.86	1117191.02	1118797.58	$3.29x10^{6}$
6	1117845.79	1120761.79	1253801.27	1164136.283	$6.03x10^{9}$
Mean/uV x sec	1127745.215	1147987.213	1167739.307		
Variance	$5.4x10^{8}$	$2.82x10^{9}$	$4.69x10^{9}$		

Table 7.7.1Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	$5.22x10^{9}$	5	1043811403	0.298137	0.903069	3.325835
Within Groups	$4.8x10^{9}$	2	2399411056	0.685328	0.526105	4.102821
Standard Error	$3.5x10^{10}$	10	3501114093			
Total	$4.5x10^{10}$	17				

There is no significant difference between the area under the curves; F (3.33)=0.298,p>0.05;

F(4.10)=0.685,p>0.05

Table 7.8 Area under the curve for 0.0003%$^{w}/_{v}$ cefepime reference standard

Injection Number/	Day 1 / uV x sec	Day 2 / uV x sec	Day 3 / uV x sec	Average/uV x sec	Variance
1	468389.94	474685.02	476742.23	473272.3967	18936816
2	465710.86	475313.56	478190.34	473071.5867	42704189
3	475994.40	474575.74	477216.43	475928.8567	1746533
4	485453.06	475600.11	464783.25	475278.8067	$1.07x10^{8}$
5	476449.68	462218.44	479654.76	472774.2933	86137664
6	513254.17	485224.40	469741.62	489406.7300	$4.86x10^{8}$
Me/uV anx sec	480875.3517	474602.8783	474388.105		
Variance	$3.0x10^{8}$	53539612	33915807		

Table 7.8.1Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	$6.13x10^{8}$	5	122618436.6	0.926971	0.502633	3.325835
Within Groups	$1.63x10^{8}$	2	81474418.62	0.615931	0.559424	4.102821
Standard Error	$1.32x10^{9}$	10	132278577.2			
Total	$2.1x10^{9}$	17				

There is no significant difference between the area under the curves; F (3.33)=0.927,p>0.05; F(4.10)=0.616,p>0.05

Table 7.9 Area under the curve for 0.0003%$^{w}/_{v}$ ceftriaxone reference

Injection Number/	Day 1 / uV x sec	Day 2 / uV x sec	Day 3 / uVxsec	Average/uV x sec	Variance
1	1157569.45	1204809.89	1206090.17	1189489.837	$7.65x10^{8}$
2	1186751.76	1189673.64	1183103.88	1186509.760	10834360
3	1312243.97	1187563.70	1183778.73	1227862.133	$5.34x10^{9}$
4	1289672.45	1189767.52	1188436.23	1222625.400	$3.37x10^{9}$
5	1197743.60	1213256.97	1196175.98	1202392.183	89147050
6	1187641.89	1198761.03	1187689.07	1191363.997	41037633
Mean/uV x sec	1221937.187	1197305.458	1190879.01		
Variance	$3.98x10^{9}$	$1.05x10^{8}$	77289359		

Table 7.9.1Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	$4.78x10^{9}$	5	955597081.4	0.596599	0.704045	3.325835
Within Groups	$3.23x10^{9}$	2	1612631617	1.006799	0.399608	4.102821
Standard Error	$1.6x10^{10}$	10	1601741211			
Total	$2.4x10^{10}$	17				

There is no significant difference between the area under the curves; F (3.33)=0.597,p>0.05; F(4.10)=1.007,p>0.05

Table 7.10 Area under the curve for 0.0003%$^{w}/_{v}$ cefuroxime reference standard

Injection Number/	Day 1 / uV x sec	Day 2 / uV x sec	Day 3 / uVxsec	Average/uV x sec	Variance
1	1097511.46	1104124.87	1109526.11	1103720.813	36210400
2	1108754.71	1118648.89	1100934.79	1109446.13	78805881
3	1119307.48	1109847.52	1106329.22	1111828.073	45050752
4	1114822.83	1116938.81	1118247.63	1116669.757	2986606
5	1113298.92	1117738.56	1119215.77	1116751.083	9483611
6	1118478.34	1118296.81	1211502.89	1149426.013	$2.89x10^{9}$
Mean/uV x sec	1112028.957	1114265.91	1127626.068		
Variance	65093677	35405045	$1.74x10^{9}$		

Table 7.10.1Statistical ANOVA table for comparison

Source of Variation	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	5	783645617.6	1.486468	0.277193	3.325835
Within Groups	2	426767683.2	0.80952	0.472225	4.102821
Standard Error	10	527186353.5			
Total	17				

There is no significant difference between the area under the curves; F (3.33)=1.486,p>0.05; F(4.10)=0.810,p>0.05

Table 7.11 Area under the curve for 0.00045%$^{w}/_{v}$ cefepime reference standard

Injection Number/	Day 1 / uV x sec	Day 2 / uV x sec	Day 3 / uVxsec	Average/uV x sec	Variance
1	704708.62	708416.52	709490.46	707538.5333	6294643.9
2	705562.60	706612.32	706218.98	706131.3	281243.86
3	706749.69	704816.70	707562.49	706376.2933	1989409.5
4	709071.74	704585.96	709610.72	707756.14	7610155.8
5	704487.51	706943.72	705548.71	705659.98	1517527.7
6	704699.11	706739.32	705478.20	705638.8767	1059977
Mean/uV x sec	705879.8783	706352.4233	707318.26		
Variance	3149993.7	2061240.2	3551803.7		

Table 7.11.1Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	12759435	5	2551886.933	0.8217115	0.5614	3.325835
Within Groups	6450162	2	3225081.063	1.038481	0.389235	4.102821
Standard Error	31055753	10	3105575.311			
Total	50265350	17				

There is no significant difference between the area under the curves; F (3.33)=0.823,p>0.05; F(4.10)=1.038,p>0.05

Table 7.12 Area under the curve for 0.00045%$^{w}/_{v}$ ceftriaxone reference standard

Injection Number/	Day 1 / uV x sec	Day 2 / uV x sec	Day 3 / uVxsec	Average/uV x sec	Variance
1	1818077.40	1823049.99	1848152.54	1829759.977	$2.6x10^{8}$
2	1840048.45	1817842.57	1826055.79	1827982.27	$1.26x10^{8}$
3	1818143.50	1846734.62	1817088.70	1827322.273	$2.83x10^{8}$
4	1812019.33	1827446.59	1837017.68	1825494.533	$1.59x10^{8}$
5	1817395.18	1836078.48	1828849.56	1827441.073	88754301
6	1826385.23	1819073.52	1846937.22	1830798.657	$2.09x10^{8}$
Mean/uV x sec	1822011.515	1828370.962	1834016.915		
Variance	99210704	$1.25x10^{8}$	$1.5x10^{8}$		

Table 7.12.1Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	53619134	5	10723826.74	0.05899	0.996999	3.325835
Within Groups	$4.33x10^{8}$	2	216448980.1	1.19064	0.343694	4.102821
Standard Error	$1.82x10^{9}$	10	181792112.4			
Total	$2.3x10^{9}$	17				

There is no significant difference between the area under the curves; F (3.33)=0.059,p>0.05; F(4.10)=1.191,p>0.05

Table 7.13 Area under the curve for 0.00045%$^{w}/_{v}$ cefuroxime reference standard

Injection Number/	Day 1 / uV x sec	Day 2 / uV x sec	Day 3 / uVxsec	Average/uV x sec	Variance
1	1620045.65	1639454.81	1643673.68	1634391.38	$1.59x10^{8}$
2	1618958.73	1627518.95	1632148.37	1626208.68	44779250
3	1621736.59	1631276.72	1608749.77	1620587.69	$1.28x10^{8}$
4	1637345.78	1641172.97	1641030.08	1639849.61	4706978
5	1625382.85	1637388.71	1617792.66	1626854.74	97626139
6	1624418.33	1617261.84	1631386.33	1624355.5	49878265
Mean/uV x sec	1624647.99	1632345.67	1629130.15		
Variance	44770419	81171031	$1.82x10^{8}$		

Table 7.13.1Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	753003224	5	150600645	1.911357	0.179298	3.325835
Within Groups	179367136	2	89683568	1.138224	0.358621	4.102821
Standard Error	787925197	10	78792520			
Total	1720295557	17				

There is no significant difference between the area under the curves; F (3.33)=1.911,p>0.05; F(4.10)=1.382,p>0.05

Table 7.14 Area under the curve for 0.0006%$^{w}/_{v}$ cefepime reference standard

Injection Number/	Day 1 / uV x sec	Day 2 / uV x sec	Day 3 / uVxsec	Average/uV x sec	Variance
1	928587.42	939341.61	929704.11	932544.38	34963501
2	928669.14	929844.40	939561.74	932691.76	35742778
3	929336.29	938990.58	928149.77	932158.88	35356051
4	928516.75	928973.57	927025.53	928171.95	1037880
5	929331.32	938589.72	938870.61	935597.22	29465821
6	931005.91	927409.40	927367.58	928594.3	4362346
Mean/uV x sec	929241.14	933858.21	931779.89		
Variance	882174.8	32070723	34078910		

Table 7.14.1Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	117466424	5	23493284.75	1.079195	0.427417	3.325835
Within Groups	64164139	2	32082069.46	1.473733	0.274838	4.102821
Standard Error	217692615	10	21769261.53			
Total	399323178	17				

There is no significant difference between the area under the curves; F (3.33)=1.079,p>0.05; F(4.10)=1.474,p>0.05

Table 7.15 Area under the curve for 0.0006%$^{w}/_{v}$ ceftriaxone reference standard

Injection Number/	Day 1 / uV x sec	Day 2 / uV x sec	Day 3 / uVxsec	Average/uV x sec	Variance
1	2457422.85	2468188.76	2467544.74	2464385.45	36462040
2	2474850.82	2462925.48	2467609.82	2468462.04	36098143
3	2458321.65	2458194.00	2471719.87	2462745.17	60412959
4	2470312.70	2464115.97	2466871.08	2467099.92	9639140
5	2467481.33	2459362.68	2465139.90	2463994.64	17461841
6	2465742.93	2464915.43	2460609.88	2463756.08	7595120
Average/uV x sec	2465688.71	2462950.39	2466582.55		
Variance	46227997	13631202	13242085		

Table 7.15.1Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	73147879	5	14629575.83	0.500398	0.769751	3.325835
Within Groups	42979945	2	21489972.31	0.735055	0.503688	4.102821
Standard Error	$2.92x10^{8}$	10	29235853.9			
Total	$4.08x10^{8}$	17				

There is no significant difference between the area under the curves; F (3.33)=0.500,p>0.05; F(4.10)=0.735,p>0.05

Table 7.16 Area under the curve for 0.0006%$^{w}/_{v}$ cefuroxime reference standard

Injection Number/ HPLC: Prgqalab 2	Day 1 / uV x sec	Day 2 / uV x sec	Day 3 / uVxsec	Average/uV x sec	Variance
1	2141649.16	2151212.51	2145600.87	2146154.18	23094029.77
2	2143621.36	2145432.63	2144742.67	2144598.89	835679.9873
3	2145395.30	2142536.67	2141500.23	2143144.07	4069590.61
4	2142673.71	2145453.56	2147342.58	2145156.62	5515718.278
5	2144231.21	2140749.75	2143627.73	2142869.56	3461253.454
6	2141992.04	2141781.63	2146704.83	2143492.83	7748760.03
Average/uV x sec	2143260	2144528	2144920		
Variance	2035919.752	14428161.37	4582866.055		

Table 7.16.1Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Rows	24811201.65	5	4962240.331	0.617013462	0.690332	3.325835
Columns	9026530.056	2	4513265.028	0.561187104	0.587506	4.102821
Error	80423534.21	10	8042353.421			
Total	114261265.9	17				

There is no significant difference between the area under the curves; F (3.33)=0.617,p>0.05; F(4.10)=0.561,p>0.05

Table 7.17 Area under the curve for 0.00075%$^{w}/_{v}$ cefepime reference standard

Injection Number/	Day 1 / uV x sec	Day 2 / uV x sec	Day 3 / uVxsec	Average/uV x sec	Variance
1	1187162.69	1192999.67	1195583.99	1191915.45	18611223.18
2	1194273.87	1193971.77	1193917.26	1194054.3	36901.07349
3	1189371.92	1187581.59	1195112.61	1190688.707	15479510.9
4	1191742.52	1194782.83	1193158.86	1193228.07	2314463.742
5	1194720.88	1189279.72	1189671.75	1191224.117	9208937.238
6	1188570.72	1195185.55	1187336.90	1190364.39	17813265.76
Average/uV x sec	1190973.767	1192300.188	1192463.562		
Variance	9677474.683	9832136.13	10685616.18		

Table 7.17.1Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	32058680	5	6411736.005	0.539175347	0.743119	3.325835
Within Groups	8011148.9	2	4005574.426	0.336836542	0.721823	4.102821
Standard Error	118917455	10	11891745.49			
Total	158987284	17				

There is no significant difference between the area under the curves; F (3.33)=0.539,p>0.05; F(4.10)=0.337,p>0.05

Table 7.18 Area under the curve for 0.00075%$^{w}/_{v}$ ceftriaxone reference standard

Injection Number/	Day 1 / uV x sec	Day 2 / uV x sec	Day 3 / uVxsec	Average/uV x sec	Variance
1	3113292.85	3114527.18	3143207.69	3123675.907	286498812.8
2	3115011.20	3117741.23	3117910.12	3116887.517	2647554.131
3	3121151.69	3116251.68	3116121.19	3117841.52	8222175.98
4	3116919.57	3115023.15	3131011.10	3120984.607	76297028.63
5	3114971.72	3117528.54	3116513.62	3116337.96	1657474.459
6	3134491.51	3113914.37	3112492.11	3120299.33	151569185.7
Average/uV x sec	3119306.423	3115831.025	3122875.972		
Variance	62618119.14	2546726.225	139629988.7		

Table 7.18.1Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	119092392	5	23818478.32	0.263221991	0.923159	3.325835
Within Groups	148902685	2	74451342.42	0.822774247	0.466875	4.102821
Standard Error	904881779	10	90488177.86			
Total	1172876855	17				

There is no significant difference between the area under the curves; F (3.33)=0.263,p>0.05; F(4.10)=0.823,p>0.05

Table 7.19 Area under the curve for 0.0075%$^{w}/_{v}$ cefuroxime reference standard

Injection Number/ HPLC: Prgqalab 2	Day 1 / uV x sec	Day 2 / uV x sec	Day 3 / uVxsec	Average/uV x sec	Variance
1	2718402.37	2721287.24	2737333.01	2725674.207	104026390.1
2	2720417.19	2722731.62	2735921.15	2726356.653	69948843.92
3	2719228.26	2719538.37	2733511.31	2724092.647	66557456.29
4	2718031.39	2717899.87	2724377.32	2720102.86	13707580.6
5	2727451.98	2718697.33	2723742.24	2723297.183	19309530.73
6	2727597.88	2735955.72	2727521.21	2730358.27	23500054.45
Average/uV x sec	2721854.845	2722685.025	2730401.04		
Variance	19962912.73	45353635.45	35423168.57		

Table 7.19.1Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	176125942.2	5	35225188.43	1.075339755	0.429182	3.325835
Within Groups	266527070.6	2	133263535.3	4.068213225	0.050961	4.102821
Standard Error	327572641.6	10	32757264.16			
Total	770225654.4	17				

There is no significant difference between the area under the curves; F (3.33)=1.075,p>0.05; F(4.10)=4.068,p>0.05

Table 7.20 Area under the curve for 0.0009%$^{w}/_{v}$ cefepime reference standard

Injection Number/	Day 1 / uV x sec	Day 2 / uV x sec	Day 3 / uVxsec	Average/uV x sec	Variance
1	1209595.28	1423254.16	1433586.57	1355478.67	15988162283
2	1375396.54	1295885.42	1374592.33	1348624.763	2086240440
3	1385783.79	1418463.75	1284737.49	1362995.01	4860174524
4	1382955.61	1330986.94	1405883.89	1373275.48	1472666967
5	1408839.55	1327396.30	1309892.61	1348709.487	2788313178
6	1385722.51	1395521.76	1392240.72	1391161.663	24879597.61
Average/uV x sec	1358048.88	1365251.388	1366822.268		
Variance	5414901010	2906174010	3333730128		

Table 7.20.1Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	4095784027	5	819156805.4	0.151196639	0.974958	3.325835
Within Groups	262632266.2	2	131316133.1	0.024237799	0.976111	4.102821
Standard Error	54178241713	10	5417824171			
Total	58536658006	17				

There is no significant difference between the area under the curves; F (3.33)=0.1511,p>0.05; F(4.10)=0.0242,p>0.05

Table 7.21 Area under the curve for 0.0009%$^{w}/_{v}$ ceftriaxone reference standard

Injection Number/	Day 1 / uV x sec	Day 2 / uV x sec	Day 3 / uVxsec	Average/uV x sec	Variance
1	3703651.12	3791721.05	3695772.16	3730381.443	2837430012
2	3709275.03	3782661.31	3775933.45	3755956.597	1645692525
3	3725711.40	3709419.37	3709618.77	3714916.513	87407123.7
4	3744826.09	3715286.47	3740051.27	3733387.943	251447229.1
5	3732734.28	3731662.59	3783126.15	3749174.34	864831181.6
6	3748628.31	3722283.19	3759917.38	3743609.627	372973451
Average/uV x sec	3727471.038	3742172.33	3744069.863		
Variance	335621846.3	1279214307	1265504108		

Table 7.21.1Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Rows	3272637464	5	654527492.8	0.588124484	0.709769	3.325835
Columns	990499201.6	2	495249600.8	0.445005625	0.652918	4.102821
Error	11129063843	10	1112906384			
Total	15392200509	17				

There is no significant difference between the area under the curves; F (3.33)=0.5881,p>0.05;

F(4.10)=0.445,p>0.05

Table 7.22 Area under the curve for 0.0009%$^{w}/_{v}$ cefuroxime reference standard

Injection Number/	Day 1 / uV x sec	Day 2 / uV x sec	Day 3 / uVxsec	Average/uV x sec	Variance
1	3203792.11	3422946.49	3403618.27	3343452.29	14722119430
2	3407392.31	3416377.30	3400628.33	3408132.647	62418587.8
3	3418511.23	3401067.37	3406399.37	3408659.323	79902604.73
4	3417266.74	3397264.81	3403387.66	3405973.07	105032559.6
5	3373788.61	3420893.50	3422830.21	3405837.44	771283339.7
6	3405822.93	3415552.09	3423001.02	3414792.013	74204981.42
Average/uV x sec	3371095.655	3412350.26	3409977.477		
Variance	6980232962	113322043	103776003.8		

Table 7.22.1Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Rows	10795469220	5	2159093844	0.857083052	0.541037	3.325835
Columns	6438737181	2	3219368591	1.277974214	0.320444	4.102821
Error	25191185826	10	2519118583			
Total	42425392227	17				

There is no significant difference between the area under the curves; F (3.33)=0.857,p>0.05;

F(4.10)=1,278,p>0.05

Table 7.23 Area under the curve for 0.0012%$^{w}/_{v}$ cefepime reference standard

Injection Number/	Day 1 / uV x sec	Day 2 / uV x sec	Day 3 / uVxsec	Average/uV x sec	Variance
1	1903838.61	1912424.60	1935281.70	1917181.637	264139025.6
2	1904846.43	1918853.42	1933092.27	1918930.707	199461349.3
3	1917492.87	1953881.11	1932944.51	1934772.83	333533068.1
4	1930774.65	1930072.32	1927752.44	1929533.137	2501477.32
5	1925792.40	1923599.00	1911207.25	1920199.55	61848845.36
6	1903471.33	1917492.30	1908428.30	1909797.31	50552541.22
Average/uV x sec	1914369.382	1926053.792	1924784.412		
Variance	145945129.4	221486715	141294335.4		

Table 7.23.1Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	1212777510	5	242555502.1	1.822556144	0.196027	3.325835
Within Groups	493219225.1	2	246609612.5	1.853018632	0.206748	4.102821
Standard Error	1330853389	10	133085338.9			
Total	3036850124	17				

There is no significant difference between the area under the curves; F (3.33)=1.823,p>0.05;

F(4.10)=1.853,p>0.05

Table 7.24 Area under the curve for 0.0012%$^{w}/_{v}$ ceftriaxone reference standard

Injection Number/	Day 1 / uV x sec	Day 2 / uV x sec	Day 3 / uVxsec	Average/uV x sec	Variance
1	5002420.94	5078630.74	5085357.16	5055469.613	2121932488
2	5018477.32	5052281.66	5015836.29	5028865.09	412995572.8
3	5007499.07	5009361.48	5031852.51	5016237.687	183734173.5
4	5027558.19	5037428.33	5057355.59	5040780.703	230400066.9
5	5068886.29	5083364.21	5007438.51	5053229.67	1625025292
6	5077311.36	5047199.54	5057221.52	5060577.473	235127243
Average/uV x sec	5033692.195	5051377.66	5042510.263		
Variance	1014869101	748817368.5	865555558.4		

Table 7.24.1Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	4466109913	5	893221982.7	1.029045702	0.450936	3.325835
Within Groups	938329450.1	2	469164725	0.540506115	0.598553	4.102821
Standard Error	8680100224	10	868010022.4			
Total	14084539588	17				

There is no significant difference between the area under the curves; F (3.33)=1.029,p>0.05; F(4.10)=0.541,p>0.05

Table 7.25 Area under the curve for 0.012%$^{w}/_{v}$ cefuroxime reference standard

Injection Number/	Day 1 / uV x sec	Day 2 / uV x sec	Day 3 / uVxsec	Average/uV x sec	Variance
1	4597620.99	4414813.87	4773761.43	4595398.763	32214541426
2	4479961.90	4589447.44	4630057.63	4566488.99	6027499861
3	4607318.22	4460569.79	4458308.73	4508732.247	7290673702
4	4453183.70	4406847.38	4399684.94	4419905.34	843412069.9
5	4447400.48	4453974.21	4407552.61	4436309.1	631005269.4
6	4479377.74	4498683.59	4603833.47	4527298.267	4486406973
Average/uV x sec	4510810.505	4470722.713	4545533.135		
Variance	5226226886	4493364507	22109978409		

Table 7.25.1Statistical ANOVA table for comparison

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	72979352939	5	14595870588	1.693875518	0.223481	3.325835
Within Groups	16818582530	2	8409291265	0.975912503	0.410042	4.102821
Standard Error	86168496072	10	8616849607			
Total	$1.75966x10^{11}$	17				

There is no significant difference between the area under the curves; F (3.33)=1.694,p>0.05;

F(4.10)=0.976,p>0.05

Table 7.26 Calibration curve for Cefepime reference standard using 0.030mg/ml Ceftriaxone reference standard as internal standard

% Concentration Level	Concentration (mg/ml)	Peak Area of sample	Peak area of internal standard	Peak area ratio
50	0.030	476622.1117	1189489.837	0.400695
75	0.045	706516.8539	1186509.760	0.595458
100	0.060	931626.4139	1227862.133	0.758739
125	0.075	1191912.506	1222625.400	0.97488
150	0.090	1363374.179	1202392.183	1.133885
200	0.120	1921735.862	1191363.997	1.613055

Table 7.27 Calibration curve for Cefepime reference standard using 0.030mg/ml Cefuroxime reference standard as internal standard

% Concentration Level	Concentration (mg/ml)	Peak Area of sample	Peak area of internal standard	Peak area ratio
50	0.030	476622.1117	1103720.813	0.431832132
75	0.045	706516.8539	1109446.13	0.636819432
100	0.060	931626.4139	1111828.073	0.837923089
125	0.075	1191912.506	1116669.757	1.06738138
150	0.090	1363374.179	1116751.083	1.220839809
200	0.120	1921735.862	1149426.013	1.671909144

Table 7.27.1 Linear Regression Analysis for Cefepime reference standard with 0.030mg/ml Ceftriaxone as internal reference standard

y=13.27x-0.016

ANOVA	***df***	***SS***	***MS***	***F***	***Significance F***
Regression	1	0.92543571	0.925436	851.7343358	$8.20637x10^{-6}$
Residual	4	0.00434612	0.001087		
Total	5	0.92978184			
	Coefficients	***Standard Error***	***t Stat***	***P-value***	
Intercept	-0.0165908	0.034571434	-0.4799	0.656361449	
Concentration(mg/ml)	13.2768019	0.45492667	29.18449	$8.20637x10^{-6}$	

There is no significant difference between the peak area ratios.

Table 7.28 Calibration curve for Ceftriaxone reference standard using 0.030mg/ml Cefepime reference standard as internal standard

% Concentration Level	Concentration (mg/ml)	Peak Area of sample	Peak area of internal standard	Peak area ratio
50	0.030	1203373.885	473272.3967	2.542666535
75	0.045	1828133.131	473071.5867	3.864390047
100	0.060	2465073.883	475928.8567	5.179500777
125	0.075	3119337.807	475278.8067	6.563174631
150	0.090	3737904.411	472774.2933	7.90631907
200	0.120	5042526.706	489406.7300	10.30334566

Table 7.29 Calibration curve for Ceftriaxone reference standard using 0.030mg/ml Cefuroxime reference standard as internal standard

% Concentration Level	Concentration (mg/ml)	Peak Area of sample	Peak area of internal standard	Peak area ratio
50	0.030	1203373.885	1103720.813	1.090288296
75	0.045	1828133.131	1109446.13	1.647789002
100	0.060	2465073.883	1111828.073	2.217135853
125	0.075	3119337.807	1116669.757	2.793429111
150	0.090	3737904.411	1116751.083	3.347124053
200	0.120	5042526.706	1149426.013	4.386995465

Table 7.30 Calibration curve for Cefuroxime reference standard using 0.030mg/ml Cefepime reference standard as internal standard

% Concentration Level	Concentration (mg/ml)	Peak Area of sample	Peak area of internal standard	Peak area ratio
50	0.030	1117973.645	473272.3967	2.362220262
75	0.045	1628707.934	473071.5867	3.442836095
100	0.060	2144236.024	475928.8567	4.505370905
125	0.075	2724980.303	475278.8067	5.733435332
150	0.090	3397807.797	472774.2933	7.186955478
200	0.120	4509022.118	489406.7300	9.213240934

Table 7.31 Calibration curve for Cefuroxime reference standard using 0.030mg/ml Ceftriaxone reference standard as internal standard

% Concentration Level	Concentration (mg/ml)	Peak Area of sample	Peak area of internal standard	Peak area ratio
50	0.030	1117973.645	1189489.837	0.939876584
75	0.045	1628707.934	1186509.760	1.372688189
100	0.060	2144236.024	1227862.133	1.746316599
125	0.075	2724980.303	1222625.400	2.22879412
150	0.090	3397807.797	1202392.183	2.825873159
200	0.120	4509022.118	1191363.997	3.784756069

Table 7.32 % RSD of Cefepime Reference standard in Recovery Studies

Expected Amt	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**Standard Deviation**	**% RSD**
0.055095	0.0548	0.0557	0.05485	0.05492	0.0553	0.055	0.055095	0.000344891	0.625994
0.07	0.0698	0.06983	0.07021	0.06992	0.06996	0.07028	0.07	0.0001997	0.285285
0.084908	0.08501	0.0849	0.08495	0.08486	0.0848	0.08493	0.084908	7.30525E-05	0.086037

Table 7.33 % RSD of Cefepime Reference standard in Recovery Studies

Expected Amt	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**Standard Deviation**	**% RSD**
0.055	0.05495	0.0549	0.0551	0.05496	0.05479	0.05488	0.05493	0.00010315	0.187785
0.07	0.0691	0.06906	0.0707	0.0715	0.06949	0.06978	0.069938	0.000971523	1.389114
0.085	0.08507	0.08491	0.08479	0.08487	0.08502	0.08482	0.084913	0.000111116	0.130858

Table 7.34 % RSD of Ceftriaxone Reference standard in Recovery Studies

Expected Amt	1	2	3	4	5	6	Mean	Standard Deviation	% RSD
0.055	0.05512	0.05492	0.05496	0.05507	0.0549	0.05515	0.05502	0.000107145	0.194738
0.07	0.0718	0.06922	0.06981	0.0702	0.07001	0.0706	0.070273	0.000875938	1.246472
0.085	0.08488	0.0849	0.08501	0.08497	0.08507	0.08495	0.084963	7.09187E-05	0.08347

Table 7.35 % RSD of Ceftriaxone Reference standard in Recovery Studies

Expected Amt	1	2	3	4	5	6	Mean	Standard Deviation	% RSD
0.055	0.05401	0.05418	0.05487	0.0552	0.05513	0.05463	0.05467	0.000492	0.899752
0.07	0.06975	0.06923	0.07013	0.0715	0.07081	0.07057	0.070332	0.000805	1.145135
0.085	0.08512	0.08453	0.08503	0.08492	0.0851	0.08507	0.084961	0.000223	0.262154

Table 7.36 % RSD of Cefuroxime Reference standard in Recovery Studies

Expected Amt	1	2	3	4	5	6	Mean	Standard Deviation	% RSD
0.055	0.05497	0.0549	0.0553	0.05483	0.05504	0.05509	0.055022	0.000165	0.300496
0.07	0.06923	0.07012	0.0695	0.06986	0.06966	0.0696	0.069662	0.000305	0.438093
0.085	0.08502	0.08472	0.08486	0.08488	0.0851	0.08465	0.084872	0.000171	0.201632

Table 7.37 % RSD of Cefuroxime Reference standard in Recovery Studies

Expected Amt	1	2	3	4	5	6	Mean	Standard Deviation	% RSD
0.055	0.0553	0.0549	0.0551	0.05487	0.05477	0.05489	0.054972	0.000194	0.352035
0.07	0.07	0.071	0.0694	0.06979	0.06981	0.06978	0.069963	0.000544	0.777774
0.085	0.0853	0.0857	0.08495	0.08407	0.08504	0.08508	0.085023	0.000539	0.633824

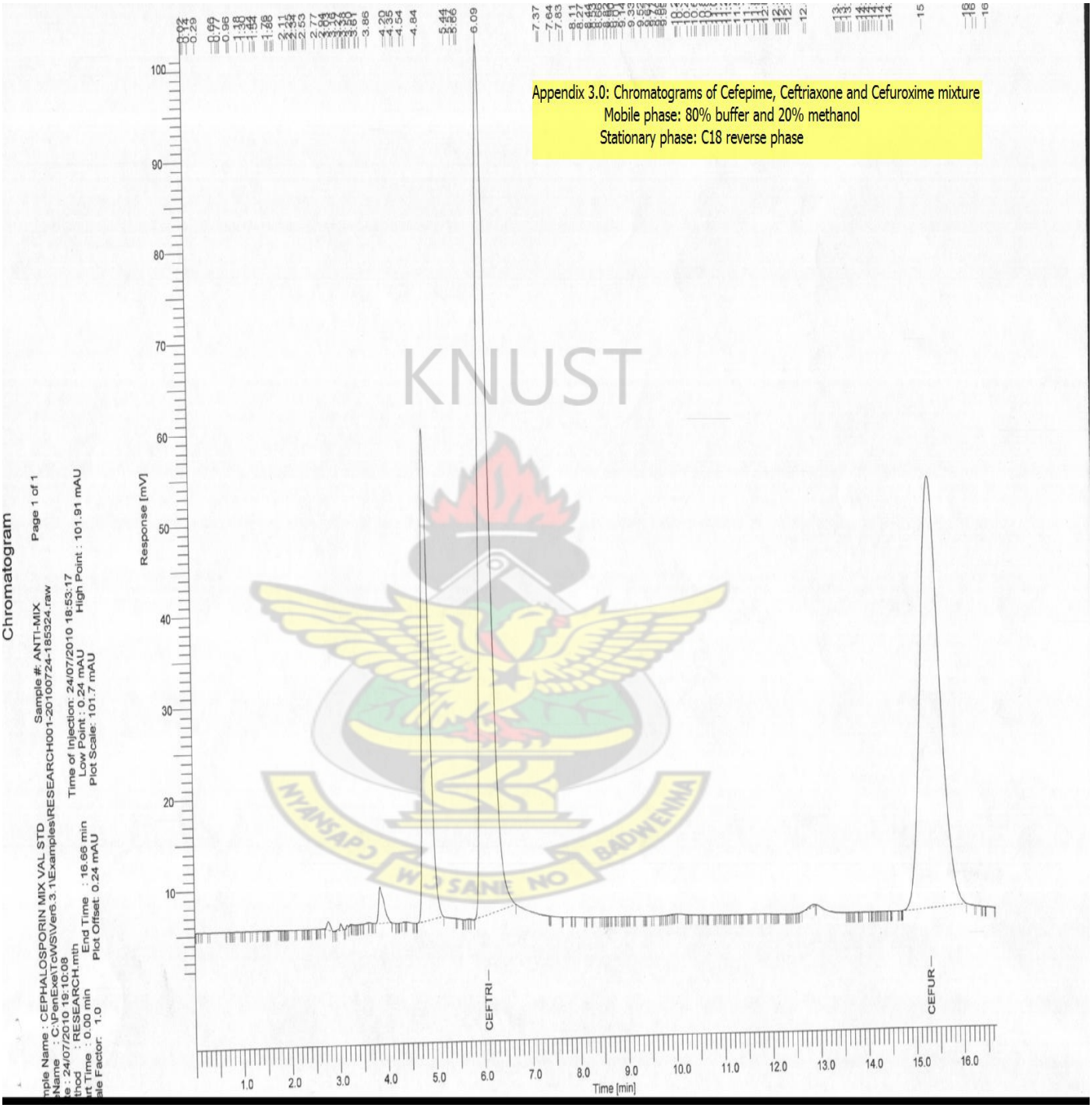

Fig 7.1. Chromatograms of Cefepime, Ceftriaxone and Cefuroxime mixture.

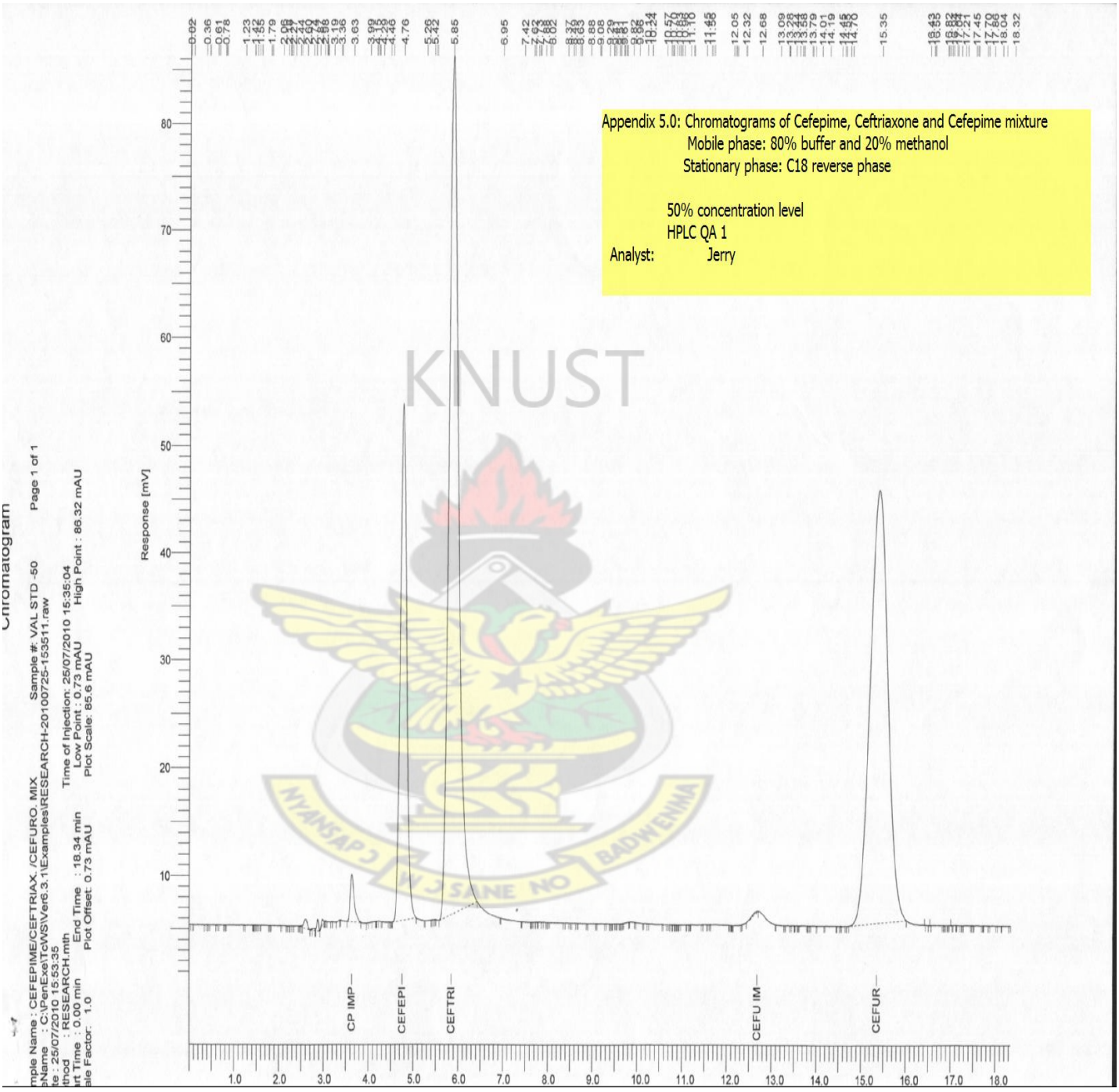

Fig 7.2 Chromatograms of Cefepime, Ceftriaxone and Cefuroxime mixture at their respective concentration levels of 50% with different analyst and HPLC at standard conditions 1.

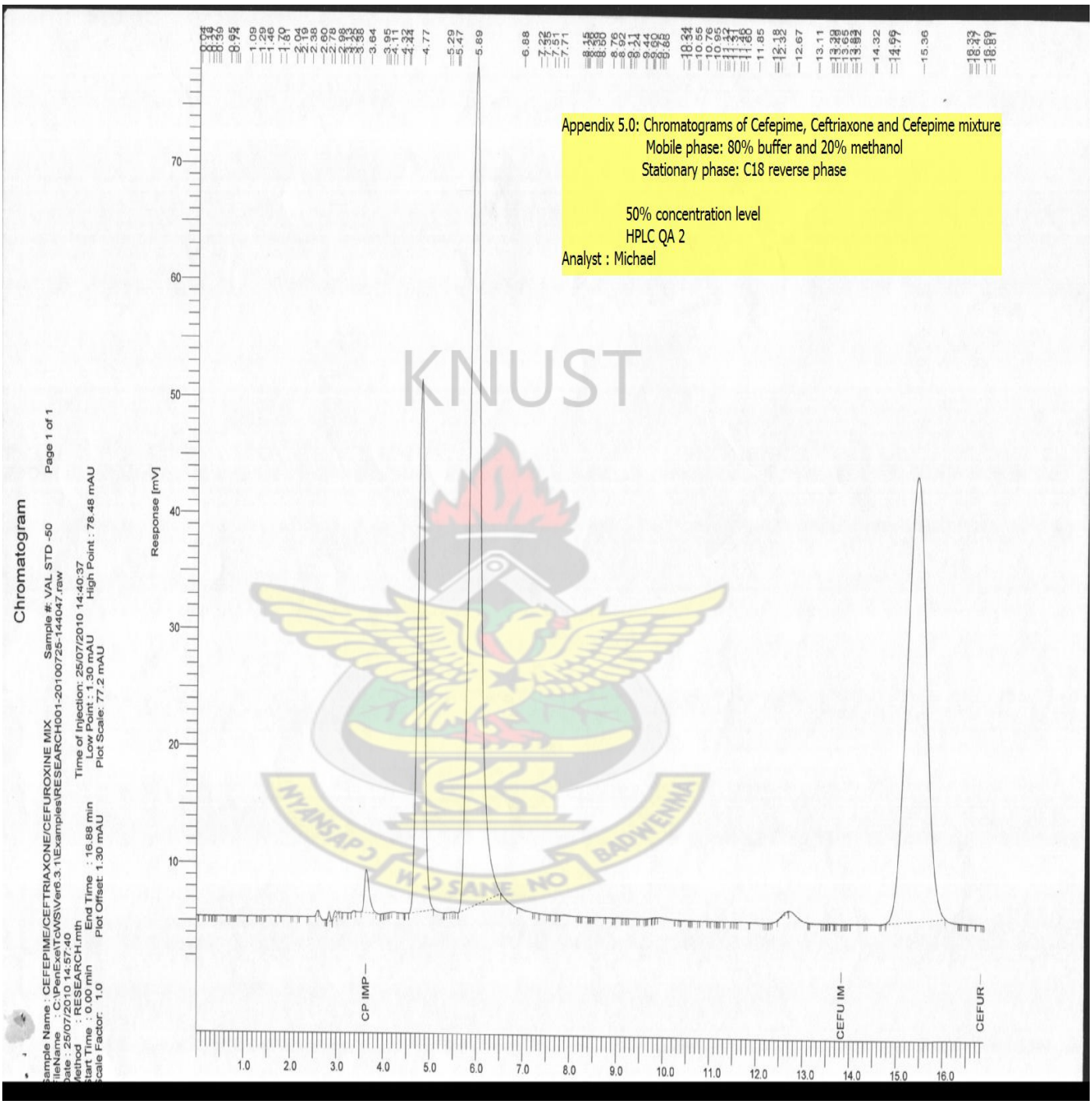

Fig 7.3 Chromatograms of Cefepime, Ceftriaxone and Cefuroxime mixture at their respective concentration levels of 50% with different analyst and HPLC at standard conditions 2.

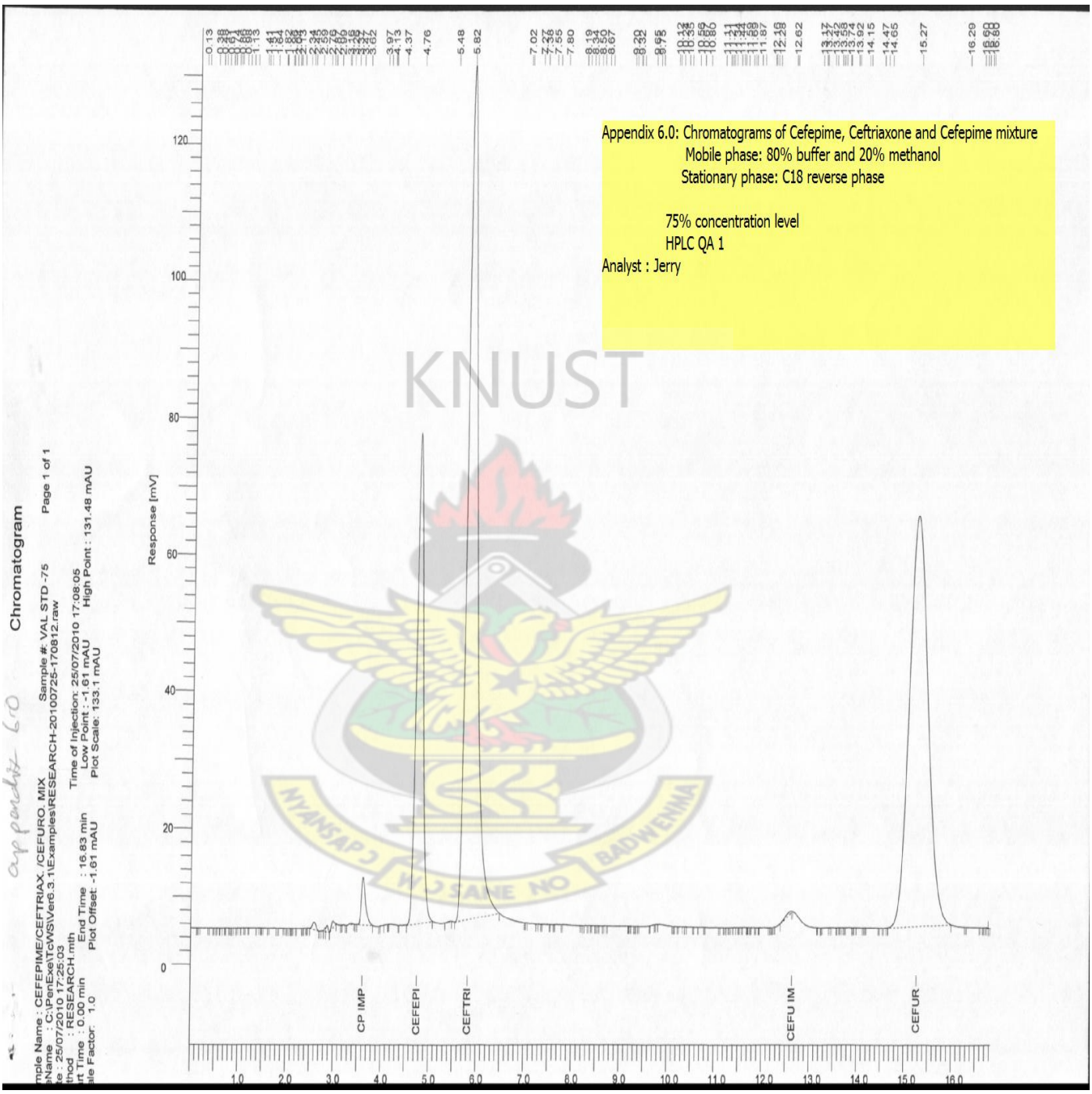

Fig 7.4 Chromatograms of Cefepime, Ceftriaxone and Cefuroxime mixture at their respective concentration levels of 75%

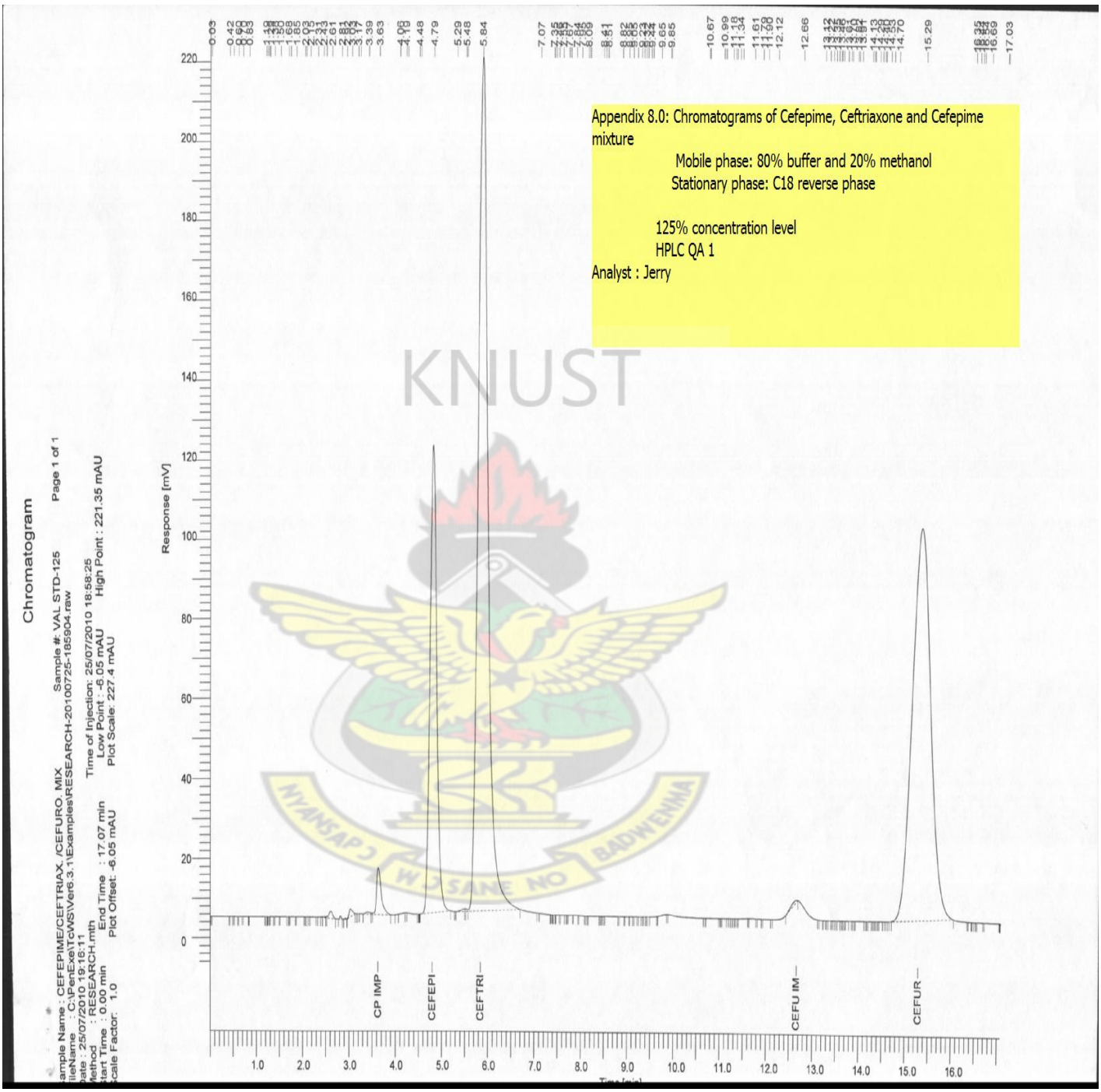

Fig 7.5 Chromatograms of Cefepime, Ceftriaxone and Cefuroxime mixture at their respective concentration levels of 125%

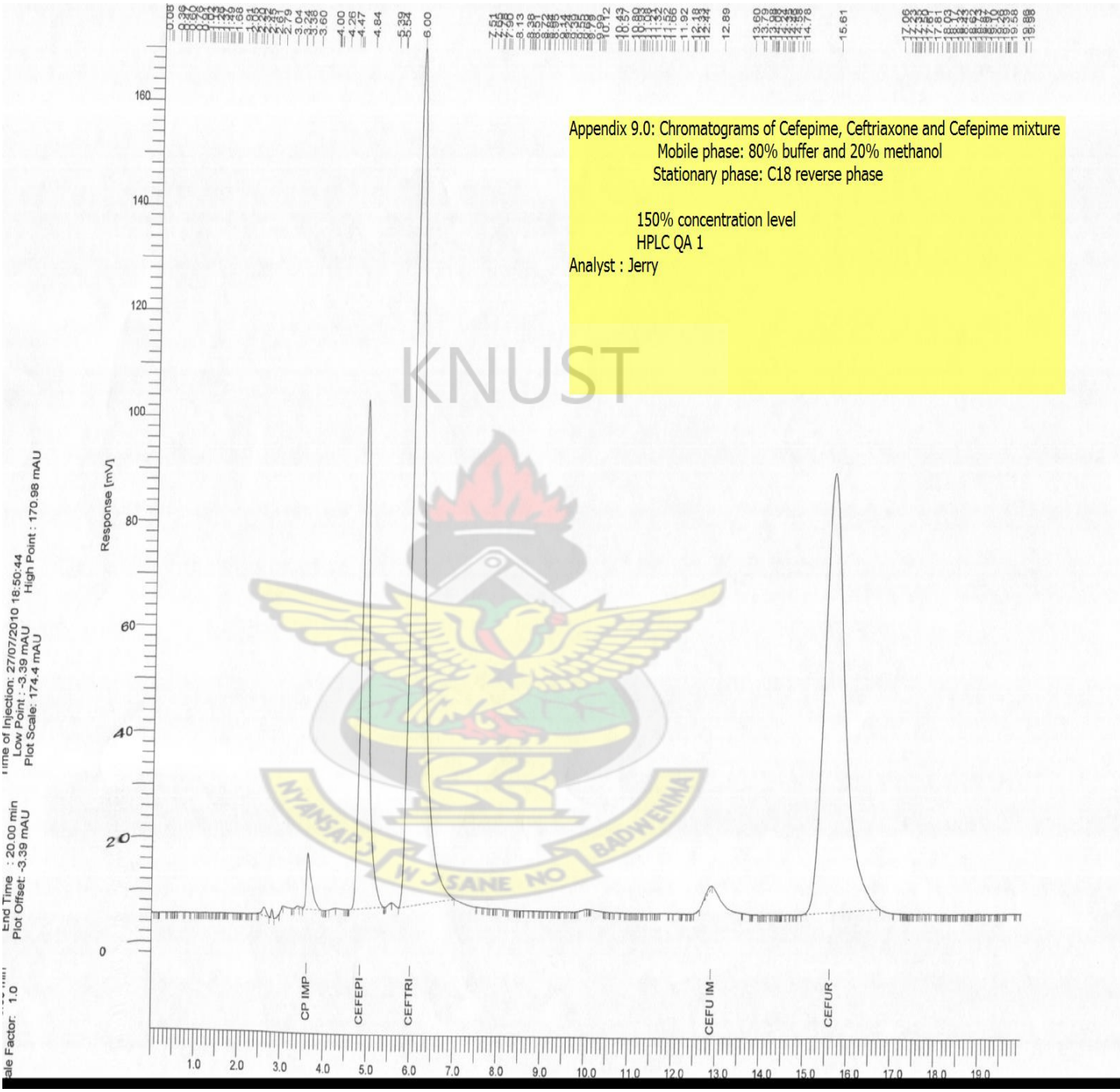

Fig 7.6 Chromatograms of Cefepime, Ceftriaxone and Cefuroxime mixture at their respective concentration levels of 150%

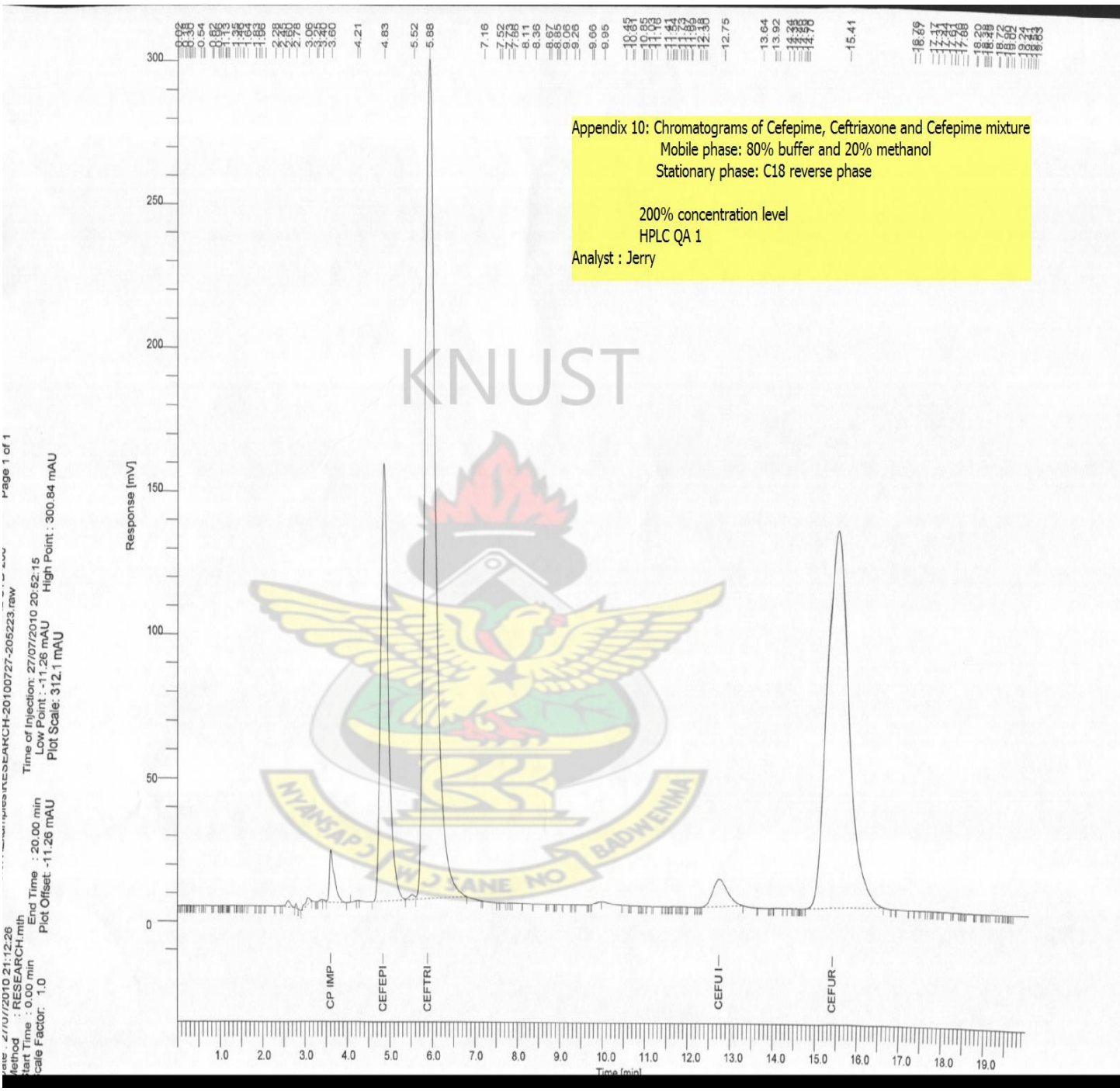

Fig 7.7 Chromatograms of Cefepime, Ceftriaxone and Cefuroxime mixture at their respective concentration levels of 200%

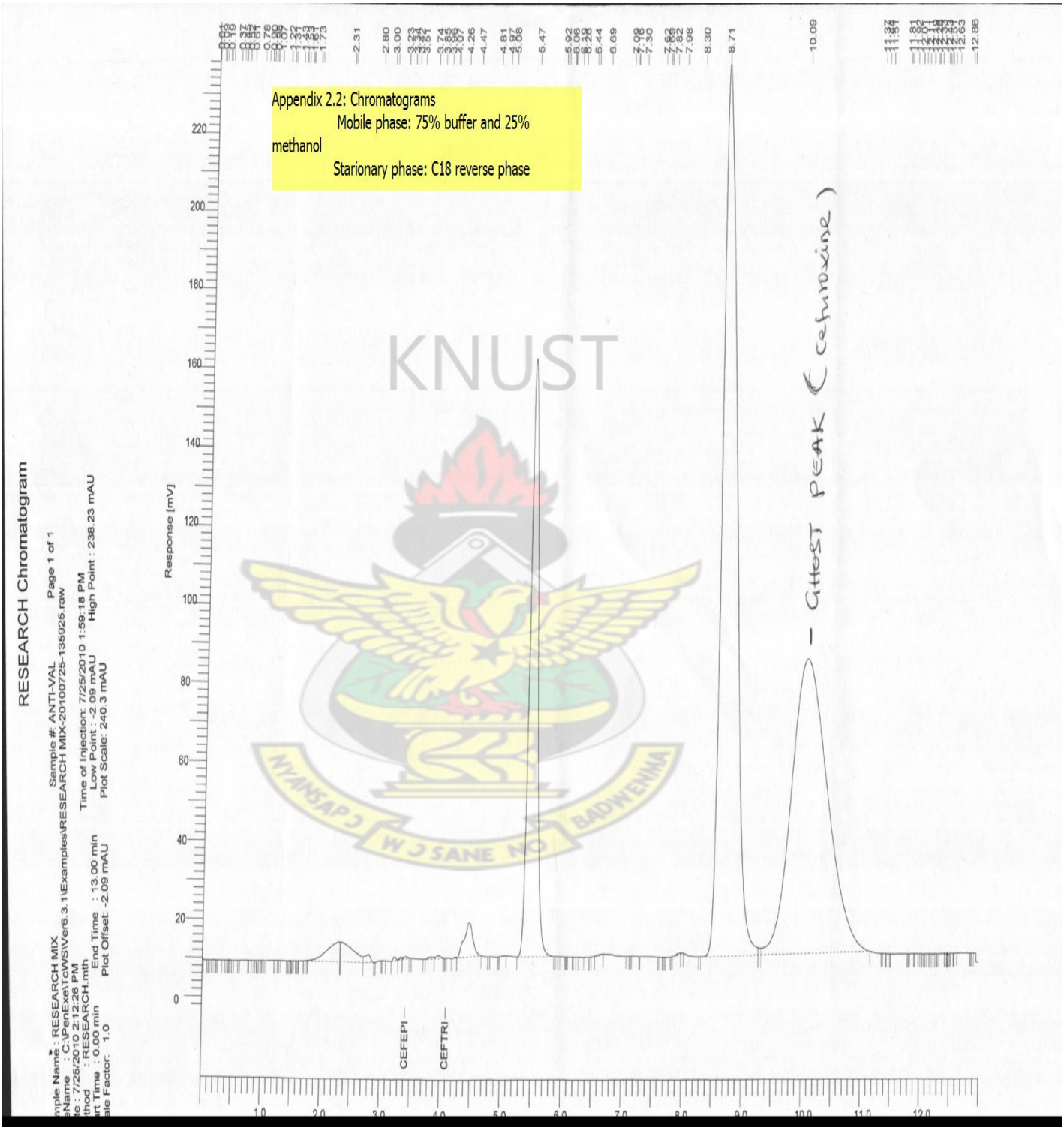

Fig 7.8 Chromatograms of Cefepime, Ceftriaxone and Cefuroxime mixture

7.1 Preparation of Solutions

Preparation of 0.2Molar NaOH:

Percentage purity is 98%^{w}/v.

40g NaOH≡1000ml of 1MNaOH

8g NaOH≡1000ml of 0.2NaOH

2g NaOH≡250ml of 0.2NaOH

But purity is only 98%. Therefore finding appropriate mass is =

(100/ 98)* 2.00g = 2.041g

Preparation of the 100ml standard solution sulphamic acid;

97.09gH_2NSO_3H≡1000ml of 1MNaOH

19.418gH_2NSO_3H≡1000ml of 0.2MNaOH

1.9418gH_2NSO_3H≡100ml of 0.2MNaOH

0.19418gH_2NSO_3H≡10ml of 0.2MNaOH

0.019418gH_2NSO_3H≡1ml of 02MNaOH

Standardizing 0.2 M sodium hydroxide:

Average mass of sulphamic acid weighed in to the conical flask = 0.4849g

From the Milliequivalent the expected titer volume is = 24.97ml

Actual average titer volume = 24.85 ml

Factor of 0.2 M sodium hydroxide (F_1) = 24.97 / 24.85

F_1= 1.005

Preparation of 0.2M KH_2PO_4 acid (USP Phosphate buffers)

27.22g KH_2PO_4 ≡1000ml of 0.2M KH_2PO_4

6.805g KH_2PO_4 ≡250ml of 0.2M KH_2PO_4

7.2 Abbreviations

B.P. – British Pharmacopoeia

USP – United States Pharmacopoeia

Rt – Retention time

AUC – Area under the curve

ODS – Octadecylsilylsilane

C_{18} - Octadecylsilylsilane

C_8 - Octasilylsilane

ICH - International Conference on Harmonization

HPLC - High-performance liquid chromatography

FSQ - Full spectrum quantification

KH_2PO_4 - Potassium dihydrogen phosphate

NaOH – Sodium Hydroxide

UV – VIS – Ultra violet- visible spectrum

LOQ – Limit of Quantification

LOD – Limit of Detection

cGMP – Current Good Manufacturing Practice

% RSD – Percentage Relative Standard Deviation