

Chapter 4: Verification of compendial methods

Introduction

In order to ensure accurate and reliable test results, the quality control laboratory (QCL) needs to use analytical methods (and accompanying specifications) that are validated, justified and suitable to test the specific quality parameters of the FPP. It is not always the case that the QCL that developed the method be the one to perform the tests (as is the case of pharmacopoeial monographs which are developed by one entity but used throughout the world). Therefore all QCL's must be able to prove that the method they use will perform as it was intended (USP, 2013). It is not necessary to revalidate a method that has already been validated (WHO, 2010; Graham, 2011; ICH, 2005). Compendial methods that have already been validated need only be verified by the QCL. Method verification ensures that the laboratory is capable of carrying out the method and produce accurate and reliable results as it is intended to (WHO, 2010). The purpose of this chapter is to briefly explain the terminology associated with method validation and method verification, and to present the verification results applicable to this study.

4.1 Method validation

Methods and procedures that are used to assess the quality of pharmaceuticals must meet certain standards of accuracy and reliability (USP, 2013). The validation of a method entails the execution of certain tests to prove the accuracy, reliability and suitability of the method.

The International Conference on Harmonization (ICH) published the Q2 (R1) guideline which summarises the principles and practices for analytical method validation (ICH, 2005). The process of validation entails the execution of a set of analytical tests and calculations to prove that the particular method is fit for its purpose. Typical questions that should be answered during method validation include:

- Do the experimental obtained values correspond to the expected values? (accuracy);
- Is the method repeatable? (precision);
- Can the analyte be detected in the presence of other substances? (specificity);
- What is the lowest amount of analyte that can be accurately quantified? (limit of quantitation - LOQ);
- What is the lowest amount of analyte that can be detected? (limit of detection - LOD);

- In what concentration range is the detection linear? (linearity and range);
- To what extent is the accuracy of the method influenced when small changes are made to the experimental conditions ? (robustness).

The decision as to which validation parameters should be evaluated is mainly determined by the objective of an analytical procedure or the justification thereof and is shown in Table 4-1 (ICH, 2005).

Table 4-1: Validation parameters considered during analytical method validation (ICH, 2005)

Validation Parameters	Type of analytical procedure			
	Identification	Testing for impurities		Assay Dissolution Content/potency
		quantitative	Limit	
Accuracy	-	+	-	+
Precision:				
<i>Repeatability</i>	-	+	-	+
<i>Intermediate Precision</i>	-	+(1)	-	+(1)
<i>Reproducibility</i>	-	+	-	+
Specificity	+	+	+	+
Detection limit	-	-(3)	+	-
Quantitation limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

- signifies that this characteristic is not normally evaluated.

+ signifies that this characteristic is normally evaluated.

1 in cases where reproducibility has been performed, intermediate precision is not needed.

2 lack of specificity of one analytical procedure could be compensated by other supporting analytical procedures.

3 may be needed in some cases.

4.1.1 Terminology: validation parameters

The following section provide a short overview on terminology associated with analytical method validation.

4.1.1.1 Accuracy

An analytical method is considered accurate when the experimental values correspond (within reasonable limits) to the actual true/known values, also referred to as the closeness of agreement (USP, 2013; Graham, 2011). The ICH Q2(R1) document (ICH, 2005) recommends covering the specified concentration range with a minimum of three concentrations with at least nine determinations e.g. three different concentrations and then replicating the reading of each concentration three times. Accuracy is reported as the percentage of recovery from the experimental values and theoretical values (USP, 2013).

4.1.1.2 Precision

Precision is defined as the measurement of scatter or as the agreement between replicate measurements of a homogeneous sample (USP, 2013; Graham, 2011). Precision could be reported as the relative standard deviation (%RSD) of replicate measurements. A sufficient number of replicate measurements must be made to be able to calculate a statistically significant relative standard deviation value (USP, 2013). The ICH Q2(R1) document (ICH, 2005) recommends covering the specified concentration range with a minimum of three concentrations with at least nine determinations e.g. three different concentrations and then replicating the reading of each concentration three times or a minimum of 6 determinations of the 100% test concentration.

Precision could be divided into three levels: repeatability, intermediate precision and reproducibility (Graham, 2011):

- Repeatability: represents precision under the same operating conditions over a short interval of time (ICH, 2005);
- Intermediate precision: precision represented as within laboratory variations e.g. different days, different analysts, different equipment etc;
- Reproducibility: represents precision between laboratories and are usually applied when standardising methodology.

4.1.1.3 Specificity

An analytical method may be considered specific when it can selectively distinguish between the analyte of interest in the presence of other components (impurities, degradation products and matrix) especially components with closely related structures (ICH, 1999).

In the case of chromatographic procedures representative chromatograms should clearly indicate separation and identification of peaks (USP, 2013).

4.1.1.4 Limit of detection

The detection limit is defined as the lowest amount of analyte in a sample that can be detected. It is not necessary to quantify the limit of detection into an exact value and a signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit (ICH, 2005).

The detection limit may also be calculated by using the standard deviation of the response and the slope of the calibration curve (ICH, 2005):

$$\text{Detection limit} = \frac{3.3 \sigma}{\text{slope}} \quad \text{Equation 4.1}$$

Where:

σ = the standard deviation of the response

slope = the slope of the calibration curve

4.1.1.5 Quantitation limit

The quantitation limit is the lowest amount of analyte that can be determined with acceptable precision and accuracy under the stated experimental conditions (USP, 2013). The quantitation limit could be calculated by means of a signal-to-noise ratio with a ratio of 10:1 being acceptable (Graham, 2011; ICH, 2005).

The quantitation limit may also be calculated by using the standard deviation of the response and the slope of the calibration curve (ICH, 2005):

$$\text{Quantitation limit} = \frac{10 \sigma}{\text{slope}} \quad \text{Equation 4.2}$$

Where:

σ = the standard deviation of the response

slope = the slope of the calibration curve

4.1.1.6 Linearity and range

“The linearity of an analytical procedure is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range” (USP, 2013).

The range of an analytical procedure is the interval between the upper and lower concentrations for which suitable level of precision, accuracy and linearity exists. The ICH suggests that a minimum of five concentrations over the intended concentration range are used to establish linearity (ICH, 2005). The response vs. concentration is plotted and analysed using linear regression. This type of graph is termed a calibration curve (Graham, 2011).

4.1.1.7 Robustness

The capacity of an analytical procedure to remain unaffected by small, but deliberate variations in method parameters is referred to as its robustness. This is an indication of the reliability of the analytical procedure during normal usage (ICH, 2005). When an analytical method is transferred from one site to another, robustness plays an important role during the method transfer process. During method development small deliberate changes such as changes in pH, reagent concentration, temperature etc. could be applied to investigate the robustness of the method (Graham, 2011).

4.2 Verification of analytical methods

The USP defines verification of compendial test procedures (i.e. method verification) as “the assessment of whether the procedure can be used for its intended purpose, under the actual conditions of use for specified drug substances or drug product matrix” (USP, 2013). The quinine sulfate tablet monographs used in this study were obtained from the USP, BP and *Ph.Int.* and are considered to be validated. It was not the objective of this study to revalidate the methods in these monographs, but rather to perform method verifications to ensure the methods perform as they were intended by the laboratory that developed and validated the methods.

Method verification evaluates parameters similar to that evaluated for method validation but are not as elaborate as method development e.g. to prove linearity during validation a minimum of five concentrations over the intended range needs to be measured, however when linearity is established during verification a minimum of three concentrations over the intended range is considered sufficient (FDA, 2013).

Method verifications were performed in order to prove that the methods were suitable for their intended purpose in the laboratory where the studies were performed (Research Institute for Industrial Pharmacy[®] incorporating CENQAM[®]). When performing method verification one will evaluate and confirm specificity, precision, recovery and linearity, keeping in mind which validation parameters are affected and applicable to the objective of the analytical procedure or method (USP, 2013).

4.3 Verification of the quinine sulfate tablet monograph methods

Where applicable (when required for the type of test), the analytical procedures were verified. Method verification is not required for qualitative compendial tests (e.g. the various wet chemistry procedures such as pH measurements) but quantitative compendial tests (like dissolution, assay testing and related substances) requires method verification (USP, 2013). Parameters evaluated for quantitative tests method verification are listed in Table 4-2.

Table 4-2: Parameters that were considered during method verification of the quantitative tests for this study

Verification Parameters	Type of analytical procedure			
	Identification	Testing for impurities		Assay Dissolution Content/potency
		quantitative	limit	
Accuracy	-	+	-	+
Precision:				
<i>Repeatability</i>	-	+	-	+
<i>Intermediate Precision</i>	-	-	-	-
<i>Reproducibility</i>	-	-	-	-
Specificity	-	+	-	+
Detection limit	-	-	-	-
Quantitation limit	-	-	-	-
Linearity	-	+	-	+
Range	-	+	-	+

- signifies that this characteristic is not normally evaluated

+ signifies that this characteristic is normally evaluated

Table 4-3 provides a summary of all the compendial methods which will be verified for the purpose of this study.

Table 4-3: Compendial methods which were verified for the purpose of this study

Compendium	Description of method
<i>Ph.Int.</i>	Dissolution method for quinine sulfate tablets Testing for related cinchona alkaloids using HPLC for analysis
USP	Dissolution method for quinine sulfate tablets Assay method using HPLC for analysis
BP	Dissolution method for quinine sulfate tablets Assay method using non-aqueous titration for analysis
Developmental method	Dissolution method for quinine sulfate tablets

4.3.1 Verification of the dissolution method specified in the International Pharmacopoeia for quinine sulfate tablets

For determining the amount of quinine sulfate that was released and dissolved during the dissolution testing of quinine sulfate tablets, UV-Vis spectroscopy was used. The validation parameters evaluated for the method verification of the quinine sulfate tablet dissolution method are listed in Table 4-2 and discussed in the following sections.

4.3.1.1 Specificity

4.3.1.1.1 Procedure

The specificity of this method was investigated by recording the UV spectra of a reference standard solution and sample solutions dissolved in dissolution medium over the wavelength range 200 nm to 800 nm. The absorbance spectra duly produced were then compared to ensure that the matrix present in the sample solution does not interfere with the UV detection of quinine sulfate dissolved in the dissolution medium.

4.3.1.1.2 Acceptance criteria

The absorbance spectra of the sample solutions and reference standard solution should exhibit absorption maxima at corresponding wavelengths (ICH, 2005).

4.3.1.1.3 Results

As seen in Figure 4-1 the UV absorbance spectra of the reference standard solution and sample solutions were comparable.

The UV-absorbance spectra of the reference standard solution and the sample solutions showed absorbance maxima at the same wavelengths. The criteria for specificity were thus met and the specificity of the method could be considered suitable.

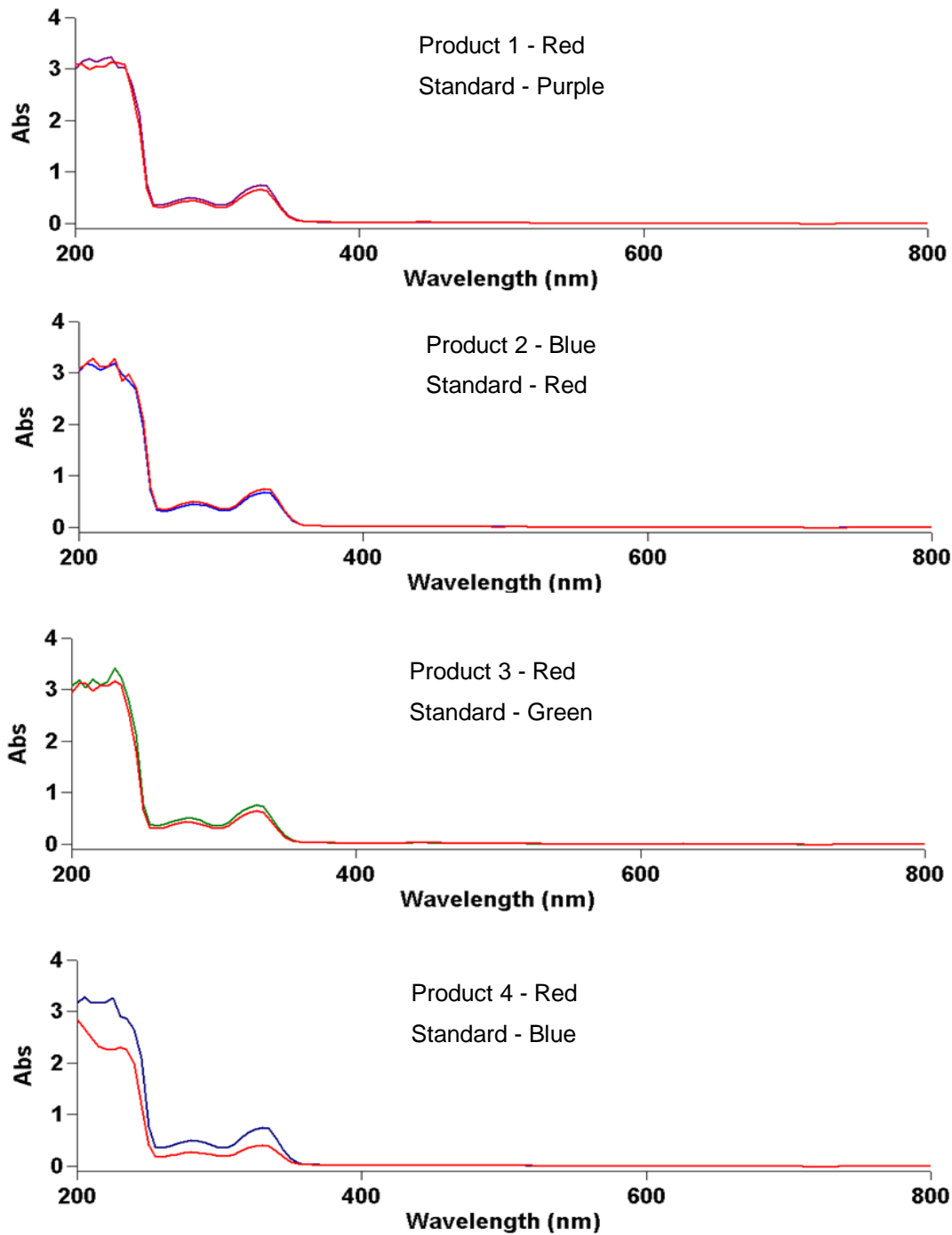


Figure 4-1: The UV absorbance spectra of quinine sulfate reference standard solution and each quinine sulfate sample solution (prepared using Products 1 - 4) phosphate buffer, pH 6.8 as solvent in accordance with the *Ph.Int.*

4.3.1.2 Linearity and range

4.3.1.2.1 Procedure

The dissolution method of the *Ph.Int.* employs phosphate buffer, pH 6.8 as dissolution medium. A tablet with a label claim of 300 mg quinine sulfate per tablet is tested in 500 ml of dissolution medium. Therefore the theoretical final concentration, assuming complete dissolution, will be 600 µg/ml. 5 ml of the withdrawn sample is then diluted to 50 ml with dissolution medium, resulting in a solution with a final theoretical concentration of 60 µg/ml. 60 µg/ml was considered as the target concentration (also referred to as the 100% concentration), across which a linear range was established.

A reference standard stock solution was prepared by transferring approximately 60 mg of quinine sulfate reference standard (RS) into a 100 ml volumetric flask and diluted to volume with dissolution medium (theoretical concentration = 600 µg/ml). From the reference standard stock solution, several diluted solutions were prepared using different dilutions as described in Table 4-4.

Table 4-4: Preparation of the different concentrations of quinine sulfate solution used for the linear regression analysis of the method verification of the dissolution method specified in the Ph.Int

Volume of stock solution used	Diluted to volume	Theoretical concentration of final solution	Range %
2 ml	100 ml	12 µg/ml	20%
5 ml	100 ml	30 µg/ml	50%
10 ml	100 ml	60 µg/ml	100%
12 ml	100 ml	72 µg/ml	120%
15 ml	100 ml	90 µg/ml	150%

The UV spectrophotometer was set to 330 nm (wavelength specified by the monograph), and blanked using the dissolution medium solution (phosphate buffer, pH 6.8). The different standard solutions (ranging from 20% - 150% of the intended theoretical concentration) were subsequently analysed at the specified wavelength. The absorbance values of the respective reference standard solutions were plotted as a function of the theoretical concentrations. A linear regression analysis was performed using STATISTICA 11 (2012) software (StatSoft, Inc., USA) and the correlation coefficient, y-intercept and slope of the regression line determined.

4.3.1.2.2 Acceptance criteria

The acceptance criteria relevant to the linearity parameter specify a correlation coefficient of ≥ 0.99 (Lobrutto and Patel, 2007:461). The y-intercept of the linear regression equation should not be significantly different from zero.

4.3.1.2.3 Results

The results obtained are summarised in Table 4-5. The linear regression analysis reported r^2 value of 1.000. A linear graph was evident over the concentration range of 12.11 – 90.83 $\mu\text{g/ml}$ (as seen in Figure 4-2). The intercept of the regression line was calculated as 0.0024, which did not differ significantly from zero. It can thus be concluded that this method is linear over the concentration range of 12.11 – 90.83 $\mu\text{g/ml}$.

Table 4-5: Solution concentrations and range used during the linear regression analysis and analytical results obtained (*Ph.Int.* dissolution method verification)

Concentration ($\mu\text{g/ml}$)	Range %	Absorbance values	Average for absorbance values	%RSD for absorbance values
12.11	20.2	0.154 0.154 0.154 0.154	0.154	0.08
30.27	50.5	0.384 0.385 0.384 0.384 0.384	0.384	0.05
60.55 (1)	100.9	0.758 0.758 0.758 0.758	0.758	0.04
60.33 (2)*	100.6	0.758 0.758 0.758 0.757 0.758	0.758	0.03
72.65	121.1	0.913 0.913 0.914 0.913 0.914	0.913	0.05
90.83	151.4	1.143 1.142 1.145 1.142 1.143	1.143	0.11

* Duplicate standard solution prepared for accuracy or recovery evaluation (section 4.3.1.4).

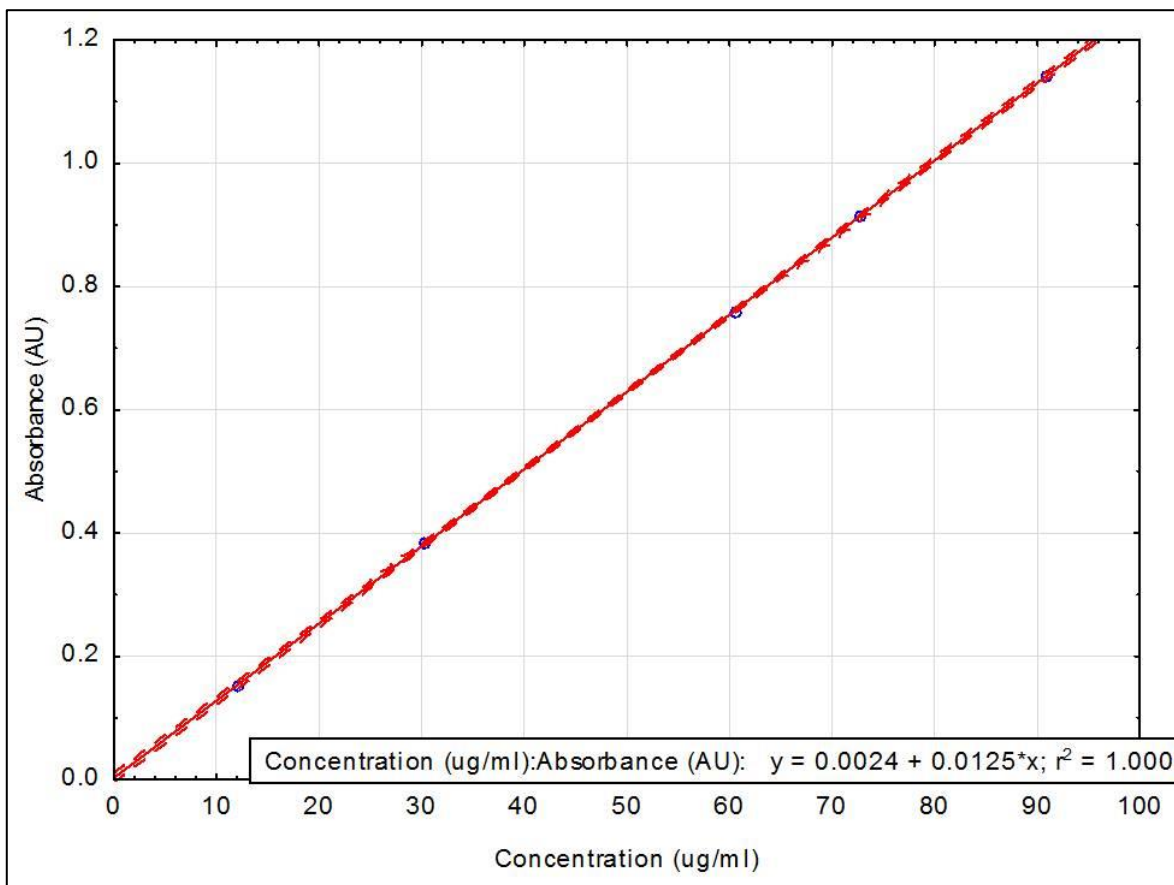


Figure 4-2: Linear regression analysis for the *Ph.Int.* monograph for dissolution testing of quinine sulfate tablet method verification. The dashed line illustrates the calculated 95 % confidence interval for the linear regression.

4.3.1.3 Precision and repeatability

4.3.1.3.1 Procedure

Repeatability was evaluated by performing replicate measurements of the reference standard solutions (prepared as described in Table 4-4) and calculating the %RSD of the replicate measurements (Table 4-5).

4.3.1.3.2 Acceptance criteria

A %RSD of replicate analytical values less than 2% is considered acceptable to prove repeatability (Lobrutto and Patel, 2007:461).

4.3.1.3.3 Results

The calculated %RSD values at each concentration showed acceptable repeatability (%RSD < 2%) - see Table 4-5.

4.3.1.4 Accuracy/recovery

4.3.1.4.1 Procedure

To evaluate the accuracy/recovery of the analytical method, a duplicate reference standard solution with a known concentration of approximately 60 µg/ml (see Table 4-5) was prepared.

The absorbance values obtained were used to calculate the experimental concentration of the duplicate reference standard solution by means of the equation obtained from the linear regression analysis (Figure 4-2). The percentage recovery was calculated by means of the following equation:

$$\% \text{ Recovery} = \frac{\text{Experimental concentration}}{\text{Theoretical concentration}} \times 100 \quad \text{Equation 4.3}$$

4.3.1.4.2 Acceptance criteria

A percentage recovery ranging between 95.0% - 105.0% is generally considered acceptable for dissolution method verification (Lobrutto and Patel, 2007:461).

4.3.1.4.3 Results

The experimental concentration was calculated to be 60.45 µg/ml and the theoretical concentration was 60.33 µg/ml.

The percentage recovery was calculated to be 100.2% and deemed acceptable (95.0 % < 100.2% < 105.0%).

4.3.2 Method verification of the dissolution method specified in the United States Pharmacopoeia for quinine sulfate tablets

For determining the amount of quinine sulfate that was released and dissolved during the dissolution testing of quinine sulfate tablets, UV-Vis spectroscopy was used.

The validation parameters evaluated for the method verification of the quinine sulfate tablet dissolution method are listed in Table 4-2 and the same as those evaluated for the quinine sulfate tablet dissolution method of the *Ph.Int.*

4.3.2.1 Specificity

4.3.2.1.1 Procedure

The specificity of this method was investigated by recording UV spectra of a reference standard solution and sample solutions dissolved in dissolution medium over the wavelength range 200 nm to 800 nm. The absorbance spectra duly produced were then compared to ensure that the matrix present in the sample solution does not interfere with the UV detection of quinine sulfate dissolved in the dissolution medium.

4.3.2.1.2 Acceptance criteria

The absorbance spectra of the sample solutions and the reference standard solution should exhibit absorption maxima at corresponding wavelengths (ICH, 2005).

4.3.2.1.3 Results

As seen in Figure 4-3 the UV absorbance spectra of the reference standard solutions and sample solutions were comparable. The UV-absorbance spectra of the reference standard solution and the sample solutions showed absorbance maxima at the same wavelengths. The criteria for specificity were thus met and the specificity of the method considered suitable.

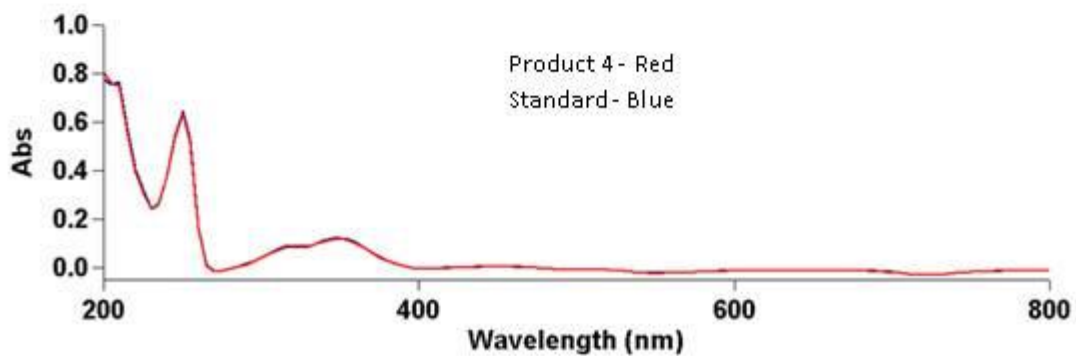
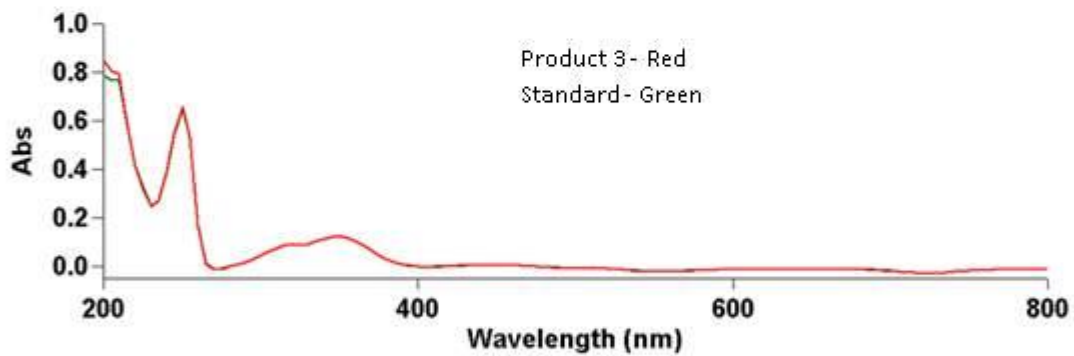
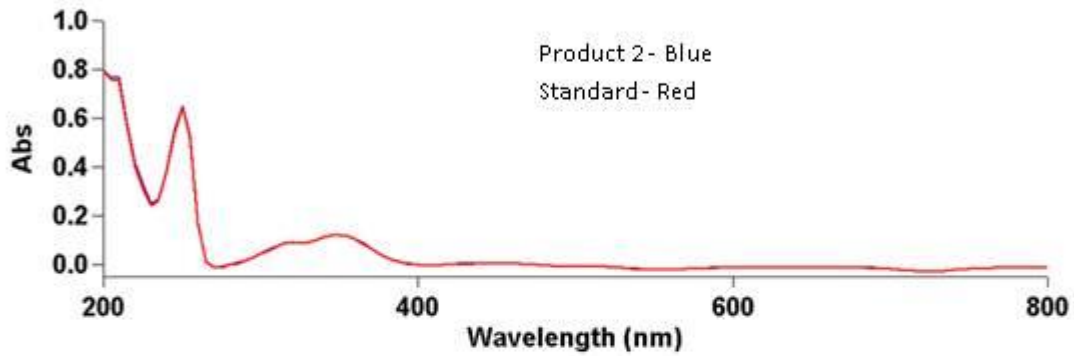
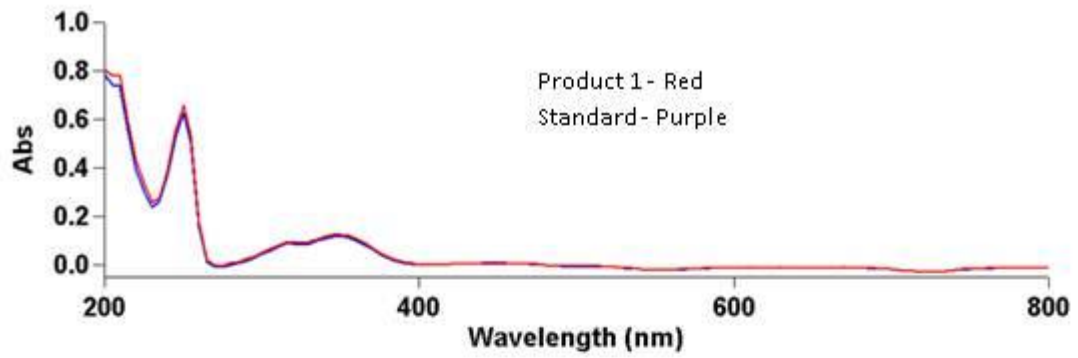


Figure 4-3: The UV absorbance spectra of quinine sulfate reference standard solution and each quinine sulfate sample solution (prepared using Products 1 -4) prepared using 0.01 M hydrochloric acid as solvent in accordance with the USP.

4.3.2.2 Linearity and range

4.3.2.2.1 Procedure

The dissolution method of the USP employs 0.01 M hydrochloric acid as dissolution medium. A tablet with a label claim of 300 mg quinine sulfate per tablet is tested in 900 ml of dissolution medium. Therefore the theoretical final concentration, assuming complete dissolution, will be 333.33 µg/ml. 3 ml of the withdrawn sample is then diluted to 100 ml with dissolution medium, resulting in a solution with a final theoretical concentration of 10 µg/ml. 10 µg/ml was considered as the target concentration (also referred to as the 100% concentration), across which a linear range was established.

A reference standard stock solution was prepared by transferring approximately 18 mg of quinine sulfate reference standard (RS) into 200 ml volumetric flask and diluted to volume with dissolution medium (theoretical concentration = 90 µg/ml). From the reference standard stock solution, several diluted solutions were prepared using different dilutions as seen in Table 4-6.

Table 4-6: Preparation of the different concentrations of quinine sulfate solution used for the linear regression analysis of the method verification of the dissolution method specified in the USP

Volume of stock solution used:	Diluted to volume:	Concentration of final solution:	Range %
4 ml	100 ml	3.6 µg/ml	40%
5 ml	100 ml	4.5 µg/ml	50%
7 ml	100 ml	6.3 µg/ml	70%
10 ml	100 ml	9.0 µg/ml	100%
15 ml	100 ml	13.5 µg/ml	150%

The UV spectrophotometer was set to 248 nm (wavelength specified by the monograph), and blanked using the dissolution medium solution (0.01 M hydrochloric acid). The different reference standard solutions (ranging from 40.0% - 150.0% of the intended theoretical concentration) were subsequently analysed at the same wavelength. The absorbance values of the respective reference standard solutions were plotted as a function of the theoretical concentrations. A linear regression analysis was performed using STATISTICA 11 (2013) software (StatSoft, Inc., USA) and the correlation coefficient, y-intercept and slope of the regression line determined.

4.3.2.2.2 Acceptance criteria

The acceptance criteria relevant to the linearity, comprise a correlation coefficient of ≥ 0.99 (Lobrutto and Patel, 2007:461). The y-intercept of the linear regression equation should not be significantly different from zero.

4.3.2.2.3 Results

The results obtained are summarised in Table 4-7. The linear regression analysis reported r^2 value of 0.9999. A linear graph was evident over the concentration range of 3.78 – 14.15 $\mu\text{g/ml}$ (as seen in Figure 4-4). The intercept of the regression line was calculated as 0.0032, which did not differ significantly from zero. It can thus be concluded that this method is linear over the concentration range of 3.78 – 14.15 $\mu\text{g/ml}$.

Table 4-7: Solution concentrations and range used during the linear regression analysis and analytical results obtained (USP dissolution method verification)

Concentration ($\mu\text{g/ml}$)	Range %	Absorbance values	Average for absorbance values	%RSD for absorbance values
3.78	42.0	0.268 0.268 0.268 0.268 0.268	0.268	0.01
4.72	52.5	0.332 0.332 0.332 0.332 0.332	0.332	0.03
6.60	73.5	0.470 0.470 0.470 0.470 0.470	0.470	0.03
9.44 (1)	105.0	0.670 0.670 0.670 0.670 0.670	0.670	0.04
9.42 (2)*	104.7	0.663 0.663 0.663 0.664 0.663	0.663	0.02
14.15	157.4	0.996 0.996 0.996 0.997 0.996	0.996	0.02

* Duplicate standard solution prepared for accuracy or recovery evaluation (section 4.3.2.4)

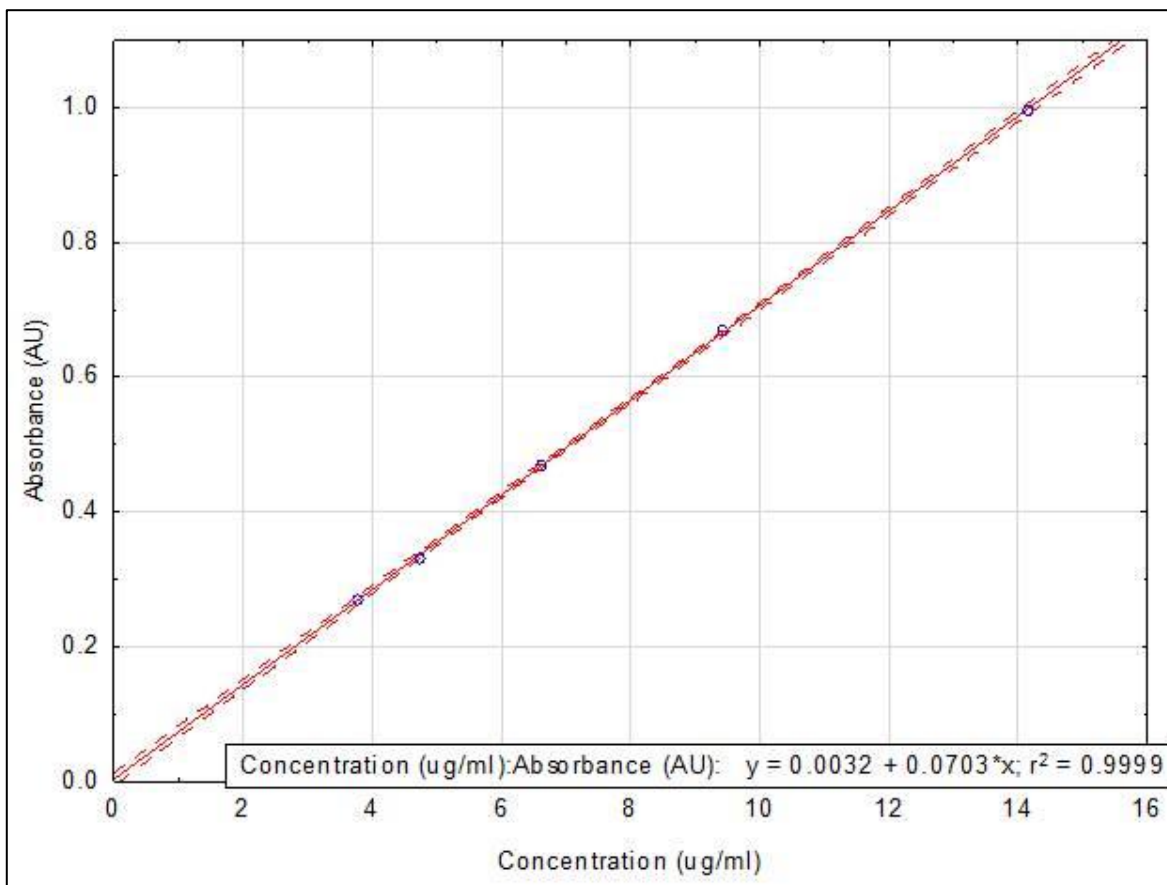


Figure 4-4: Linear regression analysis for the USP monograph for dissolution testing of quinine sulfate tablets method verification. The dashed line illustrates the calculated 95% confidence interval for the linear regression.

4.3.2.3 Precision and Repeatability

4.3.2.3.1 Procedure

Repeatability was evaluated by performing replicate measurements of the reference standard solutions and calculating the %RSD of the replicate measurements (Table 4-7).

4.3.2.3.2 Acceptance criteria

A %RSD of replicate analytical values less than 2% is considered acceptable to prove repeatability (Lobrutto and Patel, 2007:461).

4.3.2.3.3 Results

The calculated %RSD values at each concentration showed acceptable repeatability (%RSD < 2%) – Table 4-7.

4.3.2.4 Accuracy/recovery

4.3.2.4.1 Procedure

To evaluate accuracy/recovery of the analytical method, a duplicate reference standard solution with a known concentration of approximately 9 µg/ml was prepared.

The absorbance values obtained were used to calculate the experimental concentration of the duplicate reference standard solution by means of the equation obtained from the linear regression analysis (Figure 4-4). The percentage recovery was calculated by means of equation 4.3.

4.3.2.4.2 Acceptance criteria

A percentage recovery between 95.0% - 105.0% is generally considered acceptable for dissolution verification (Lobrutto and Patel, 2007:461).

4.3.2.4.3 Results

The experimental concentration was calculated to be 9.39 µg/ml and the theoretical concentration was 9.42 µg/ml. The percentage recovery was calculated to be 99.7% and deemed acceptable (95 % < 99.7% < 105%).

4.3.3 Method verification of the dissolution method specified in the British Pharmacopoeia for quinine sulfate tablets

For determining the amount of quinine sulfate that was released and dissolved during the dissolution testing of quinine sulfate tablets, UV-Vis spectroscopy was used. The validation parameters evaluated for the method verification of the quinine sulfate tablet dissolution method are listed in Table 4-2 and the same as those evaluated for the quinine sulfate tablet dissolution method of the *Ph.Int.*

4.3.3.1 Specificity

4.3.3.1.1 Procedure

The specificity of this method was investigated by recording UV spectra of a reference standard solution and sample solutions dissolved in dissolution medium over the wavelength range 200 nm to 800 nm. The absorbance spectra duly produced were then compared to ensure that the matrix present in the sample solution does not interfere with the UV detection of quinine sulfate dissolved in the dissolution medium.

4.3.3.1.2 Acceptance criteria

The absorbance spectra of the sample solutions and reference solution should exhibit absorption maxima at corresponding wavelengths (ICH, 2005).

4.3.3.1.3 Results

As seen in Figure 4-5 the UV absorbance spectra of the reference standard solution and sample solutions were comparable. The UV absorbance spectra of the reference standard solution and the sample solutions showed absorbance maxima at the same wavelengths. The criteria for specificity were thus met and the specificity of the method considered suitable.

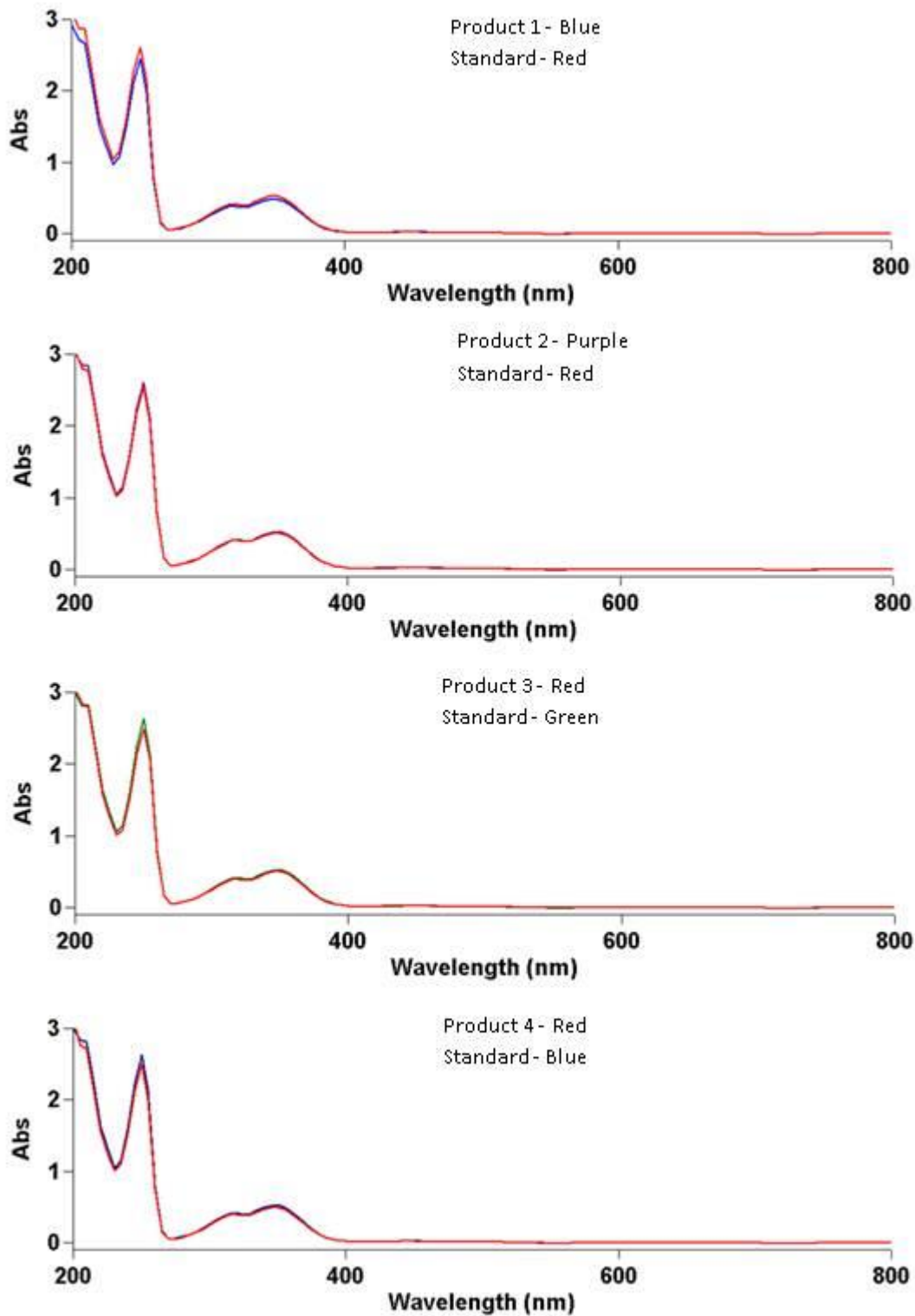


Figure 4-5: The UV-absorbance spectra of quinine sulfate reference standard solution and each sample solution prepared (prepared using Products 1 - 4) using 0.1 M hydrochloric acid as solvent in accordance with the BP.

4.3.3.2 Linearity and range

4.3.3.2.1 Procedure

The dissolution method of the BP employs 0.1 M hydrochloric acid as dissolution medium. A tablet with a label claim of 300 mg quinine sulfate per tablet is tested in 900 ml of dissolution medium. Therefore the theoretical final concentration, assuming complete dissolution, will be 333.33 µg/ml. 5 ml of the withdrawn sample is then diluted to 50 ml with dissolution medium, resulting in a solution with a final theoretical concentration of 33.33 µg/ml. 33.33 µg/ml was considered as the target concentration (also referred as the 100% concentration), across which a linear range was established.

A reference standard stock solution was prepared by transferring approximately 36 mg of quinine sulfate RS into a 100 ml volumetric flask and diluted to volume with dissolution medium (theoretical concentration = 360 µg/ml). From the reference standard stock solution several diluted solutions were prepared using different dilutions described in Table 4-8.

Table 4-8: Preparation of the different concentrations of quinine sulfate solution used for the linear regression analysis of the method verification of the dissolution method specified in the BP

Volume of stock solution used:	Diluted to volume:	Concentration of final solution:	Range %
5 ml	100 ml	18.0 µg/ml	50%
7 ml	100 ml	25.2 µg/ml	70%
10 ml	100 ml	36.0 µg/ml	100%
12 ml	100 ml	43.2 µg/ml	120%
15 ml	100 ml	54.0 µg/ml	150%

The UV spectrophotometer was set to 348 nm (wavelength specified by the monograph), and blanked using the dissolution medium solution (0.1 M hydrochloric acid). The different reference standard solutions (ranging from 50.0% - 150.0% of the intended theoretical concentration) were subsequently analysed at the same wavelength. The absorbance values of the respective standard solutions were plotted as a function of the theoretical concentrations. A linear regression analysis was performed using STATISTICA 11 (2013) software (StatSoft, Inc., USA) and the correlation coefficient, y-intercept and slope of the regression line determined.

4.3.3.2.2 Acceptance criteria

The acceptance criteria relevant to the linearity parameter specify a correlation coefficient of ≥ 0.99 (Lobrutto and Patel, 2007:461). The y-intercept of the linear regression equation should not be significantly different from zero.

4.3.3.2.3 Results

The results obtained are summarised in Table 4-9. The linear regression analysis reported r^2 value of 0.9998. A linear graph was evident over the concentration range of 18.38 – 55.13 $\mu\text{g/ml}$ (as seen in Figure 4-6). The intercept of the regression line was calculated as 0.004, which did not differ significantly from zero. It can thus be concluded that this method is linear over the concentration range of 18.38 – 55.13 $\mu\text{g/ml}$.

Table 4-9: Solution concentrations and range used during the linear regression analysis and analytical results obtained (BP dissolution method verification)

Concentration ($\mu\text{g/ml}$)	Range %	Absorbance values	Average for absorbance values	%RSD for absorbance values
18.38	51.05	0.259 0.259 0.259 0.260 0.258	0.259	0.31
25.73	71.46	0.362 0.363 0.362 0.362 0.362	0.362	0.09
36.75 (1)	102.09	0.511 0.511 0.511 0.512 0.511	0.511	0.06
36.44 (2)*	101.22	0.513 0.513 0.513 0.513 0.513	0.513	0.04
44.10	122.51	0.612 0.612 0.612 0.612 0.612	0.612	0.04
55.13	153.14	0.771 0.771 0.771 0.771 0.770	0.770	0.06

* Duplicate standard solution prepared for accuracy or recovery evaluation (section 4.3.3.4)

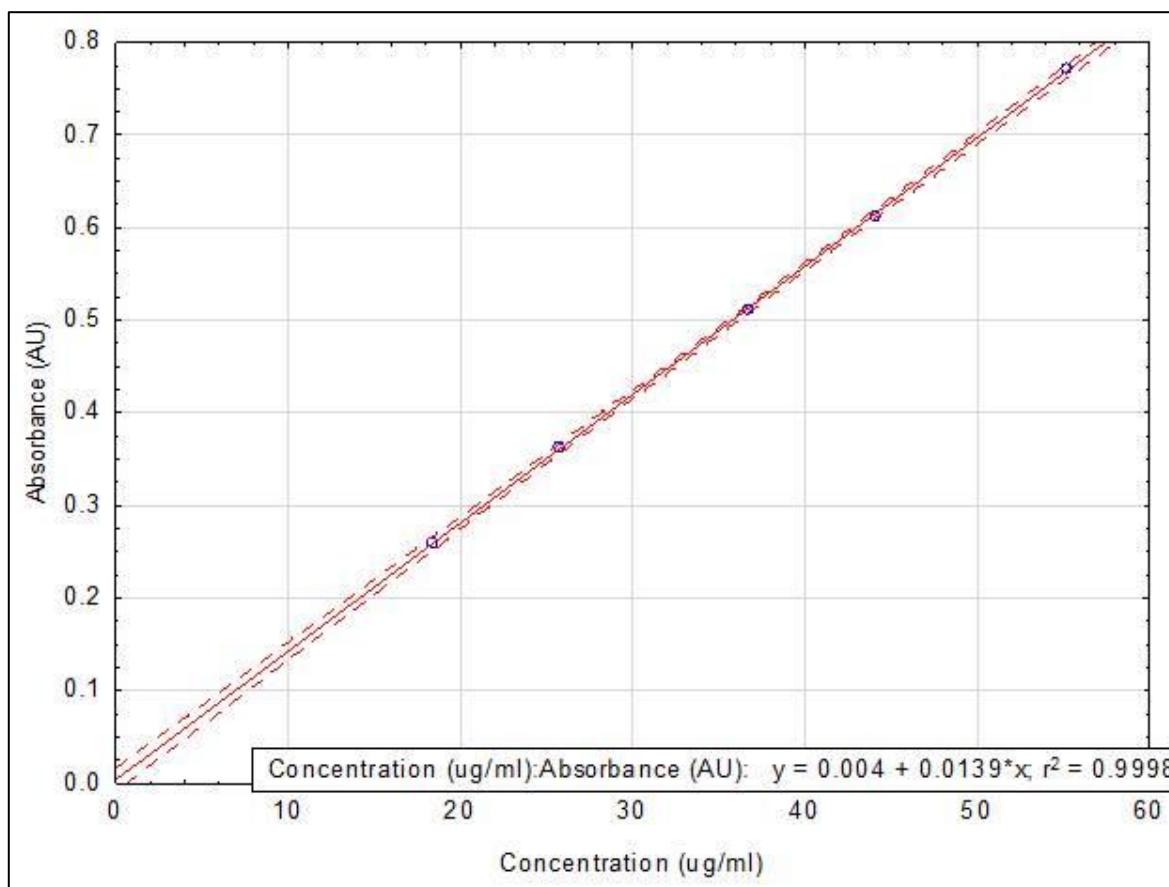


Figure 4-6: Linear regression analysis for the BP monograph for dissolution testing of quinine sulfate tablets method verification. The dashed line illustrates the calculated 95% confidence interval for the linear regression.

4.3.3.3 Precision and repeatability

4.3.3.3.1 Procedure

Repeatability was evaluated by performing replicate measurements of the reference standard solutions and calculating the %RSD of the replicate measurements.

4.3.3.3.2 Acceptance criteria

A %RSD of replicate analytical values less than 2% is considered acceptable to prove repeatability (Lobrutto and Patel, 2007:461).

4.3.3.3.3 Results

The calculated %RSD values at each concentration showed acceptable repeatability (%RSD < 2%) see Table 4-9.

4.3.3.4 Accuracy/recovery

4.3.3.4.1 Procedure

To evaluate accuracy/recovery of the analytical method, a duplicate reference standard solution with a known concentration of approximately 36 µg/ml (see Table 4-9) was prepared. The absorbance values were used to calculate the experimental concentration of the duplicate reference standard solution by means of the equation obtained from the linear regression analysis (Figure 4-6). The percentage recovery was calculated by means of equation 4.3.

4.3.3.4.2 Acceptance criteria

A percentage recovery between 95.0% - 105.0% is generally acceptable for dissolution verification studies (Lobrutto and Patel, 2007:461).

4.3.3.4.3 Results

The experimental concentration was calculated to be 36.62 µg/ml and the theoretical concentration was 36.44 µg/ml. The recovery was calculated to be 100.5% and deemed acceptable ($95.0\% < 100.5\% < 105.0\%$)

From the linear equation (Figure 4-6) the experimental $A_{1\text{cm}}^{1\%}$ value was calculated to be 139, which is correlation of 102.2% with that specified by the monograph ($A_{1\text{cm}}^{1\%} = 136$). This also falls within the acceptance criteria for recovery, as specified in 4.3.3.4.2 above.

4.3.4 Method validation of the dissolution method using acetate buffer with a pH of 4.5 as dissolution medium

Acetate buffer, pH 4.5 was considered as an alternative dissolution medium (for reasons to be discussed in Chapter 6). Therefore it was required to perform a preliminary method validation to ensure at least the minimum analytical criteria (as for method verification) are met prior to performing any dissolution studies using acetate buffer, pH 4.5 as dissolution medium. The validation parameters that were investigated included:

- Linearity and range
- Repeatability
- Specificity
- Accuracy

For determining the amount of quinine sulfate that was released and dissolved during the dissolution testing of quinine sulfate tablets, UV-Vis spectroscopy was used.

4.3.4.1 Specificity

4.3.4.1.1 Procedure

The specificity of this method was investigated by recording UV spectra of a reference standard solution and sample solutions dissolved in dissolution medium over the wavelength range 200 nm to 800 nm. The absorbance spectra duly produced were then compared to ensure that the matrix present in the sample solution does not interfere with the UV detection of quinine sulfate dissolved in the dissolution medium.

4.3.4.1.2 Acceptance criteria

The absorbance spectra of the sample solutions and reference standard solution should exhibit absorption maxima at corresponding wavelengths (ICH, 2005).

4.3.4.1.3 Results

As seen in Figure 4-7 the UV absorbance spectra of the reference standard solution and sample solutions were comparable. The UV absorbance spectra of the reference standard solution and the sample solutions showed absorbance maxima at the same wavelengths.

The criteria for specificity were thus met and the specificity of the method could be considered suitable. The absorbance spectra was also used to identify the single wavelength at which further analysis was performed at the wavelength maximum.

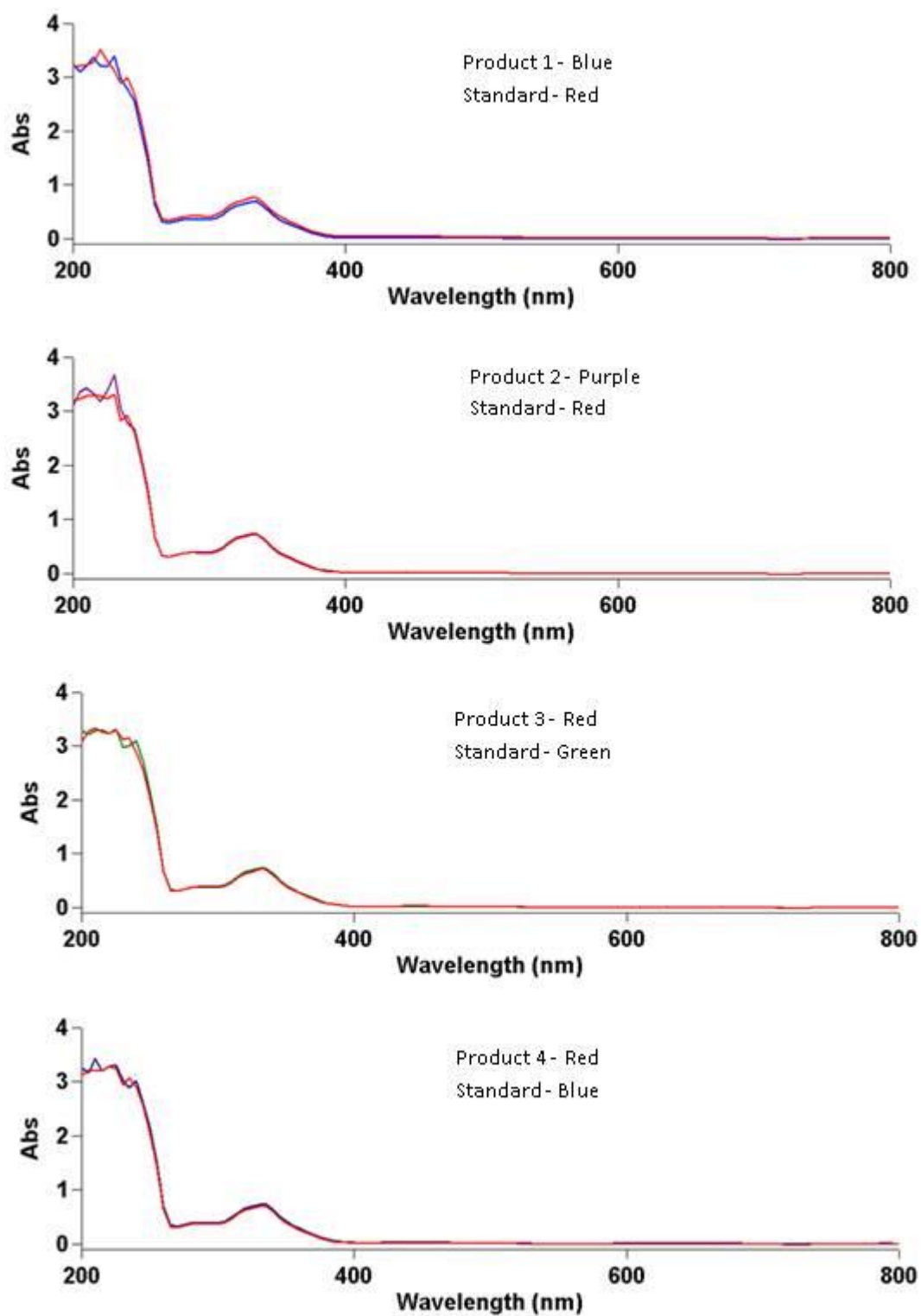


Figure 4-7: The UV absorbance spectra of quinine sulfate reference standard solution and each quinine sulfate sample solution (prepared using Products 1 - 4) prepared using acetate buffer, pH 4.5 as solvent.

4.3.4.2 Linearity and range

4.3.4.2.1 Procedure

Standard solutions of quinine sulfate in acetate buffer (pH 4.5) were prepared in varying concentrations. The theoretical 100% final concentration was set to be 60 µg/ml, across which a linear range was established.

A reference standard stock solution was prepared by transferring approximately 12.50 mg of quinine sulfate reference standard (RS) into a 100 ml volumetric flask and diluted to volume with dissolution medium (theoretical concentration = 125 µg/ml). From the reference standard stock solution several diluted solutions were prepared using different dilutions as described in Table 4-10.

Table 4-10: Preparation of the different concentrations of quinine sulfate solution used for the linear regression analysis of the preliminary method validation of the dissolution method considered with acetate buffer as dissolution medium

Volume of stock solution used:	Diluted to volume:	Concentration of final solution:	Range %
6 ml	25 ml	30 µg/ml	50.0%
8 ml	25 ml	40 µg/ml	60.7%
10 ml	25 ml	50 µg/ml	83.4%
12 ml	25 ml	60 µg/ml	100.1%
15 ml	25 ml	75 µg/ml	125.1%

The wavelength of maximum absorbance for quinine sulfate in acetate buffer, pH 4.5 was detected at 333 nm (Figure 4-7). The UV spectrophotometer was set to 333 nm, and blanked using the dissolution medium solution (acetate buffer, pH 4.5). The different standard solutions (ranging from 50% to 125% of the intended theoretical concentration) were subsequently analysed at the specific wavelength. The absorbance values of the respective reference standard solutions were plotted as a function of the theoretical concentrations. A linear regression analysis was performed using STATISTICA 11 (2013) software (StatSoft, Inc., USA) and the correlation coefficient, y-intercept and slope of the regression line determined.

4.3.4.2.2 Acceptance criteria

The acceptance criteria relevant to the linearity parameter specify a correlation coefficient of ≥ 0.99 (Lobrutto and Patel, 2007:461). The y-intercept of the linear regression equation should not significantly differ from zero.

4.3.4.2.3 Results

The results obtained are summarised in Table 4-11. The linear regression analysis reported r^2 value of 1.000. A linear graph was evident over the concentration range of 29.99 – 74.98 $\mu\text{g/ml}$ (as seen in Figure 4-8). The intercept of the regression line was calculated as - 0.0022, which did not differ significantly from zero. It can thus be concluded that this method is linear over the concentration range of 29.99 – 74.98 $\mu\text{g/ml}$.

Table 4-11: Solution concentrations and range used during the linear regression analysis and analytical results obtained (Acetate buffer, pH 4.5 dissolution method preliminary validation)

Concentration ($\mu\text{g/ml}$)	Range %	Absorbance values	Average for absorbance values	%RSD for absorbance values
29.99	49.98	0.365 0.365 0.365 0.364 0.364	0.365	0.03
39.99	66.65	0.490 0.490 0.490 0.490 0.490	0.490	0.05
49.99	83.32	0.610 0.610 0.611 0.610 0.611	0.610	0.01
59.99 (1)	99.98	0.734 0.735 0.735 0.735 0.735	0.735	0.04
59.80 (2)*	99.67	0.718 0.718 0.718 0.718 0.719	0.718	0.03
74.98	125.00	0.917 0.916 0.917 0.916 0.917	0.917	0.03

* Duplicate standard solution prepared for accuracy or recovery evaluation (section 4.3.4.4)

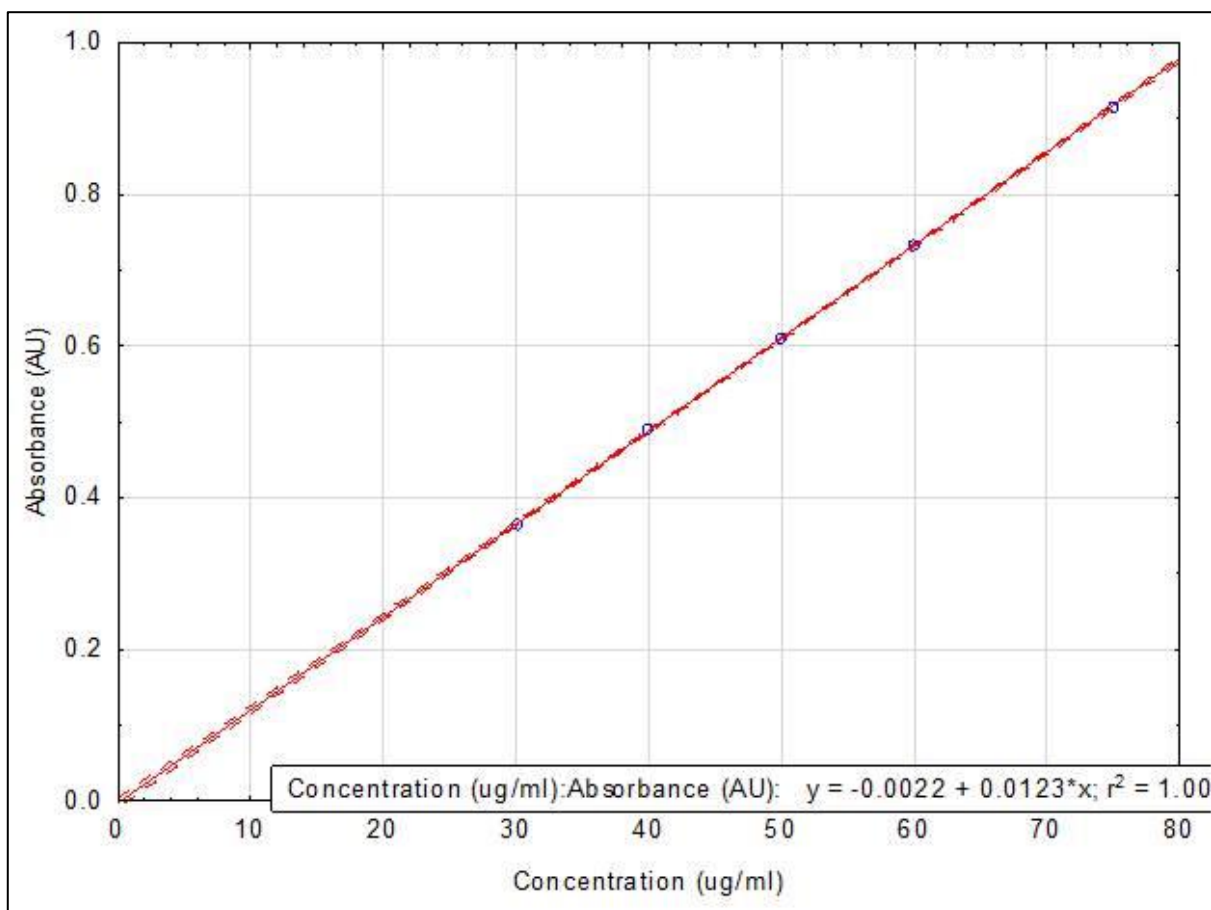


Figure 4-8: Linear regression analysis for the developmental study using acetate buffer, pH 4.5 for dissolution testing of quinine sulfate tablets preliminary method validation.

4.3.4.3 Precision and repeatability

4.3.4.3.1 Procedure

Repeatability was determined by performing replicate measurements of the reference standard solutions and calculating the %RSD of the replicate measurements (Table 4-11).

4.3.4.3.2 Acceptance criteria

A %RSD of replicate analytical values less than 2% is considered acceptable to prove repeatability (Lobrutto and Patel, 2007:461).

4.3.4.3.3 Results

The calculated %RSD values at each concentration showed acceptable repeatability (%RSD < 2%) (Table 4-11).

4.3.4.4 Accuracy/recovery

4.3.4.4.1 Procedure

To evaluate accuracy/recovery of the analytical method, a duplicate reference standard solution with a known concentration of approximately 60 µg/ml (see Table 4-11) was prepared. The absorbance values obtained were used to calculate the experimental concentration of the duplicate reference standard solution by means of the equation obtained from the linear regression analysis (Figure 4-8). The percentage recovery was calculated by means of equation 4.3.

4.3.4.4.2 Acceptance criteria

A percentage recovery between 95.0% - 105.0% is generally acceptable (Lobrutto and Patel, 2007:461).

4.3.4.4.3 Results

The experimental concentration was calculated to be 58.55 µg/ml and the theoretical concentration was 59.80 µg/ml. The recovery was calculated to be 97.9% and deemed acceptable (95 % < 97.9% < 105%).

For reasons discussed in Chapter 6, the investigational dissolution studies using acetate buffer pH 4.5 did not deem it as a suitable candidate as an alternative dissolution medium. For this reason, the preliminary method development for acetate buffer pH 4.5 was not further elaborated upon.

4.3.5 Method verification of the non-aqueous titration assay method as specified by the International Pharmacopoeia and British Pharmacopoeia for quinine sulfate tablets

The *Ph.Int.* and BP quinine sulfate tablet monographs both specify non-aqueous titration methods for assay determination. A comparison between the methods are given in Table 4-12.

Table 4-12: Comparison between non-aqueous titration methods of the BP and *Ph.Int.* for quinine sulfate tablets

	BP	Ph.Int.
Sample preparation:	Weigh tablet powder equivalent to 0.4 g quinine sulfate and dissolve as completely as possible, using heat, in 40 ml acetic anhydride. (Sample concentration, 10 mg/ml)	Weigh tablet powder equivalent to 0.2 g quinine sulfate and dilute in 40 ml acetic anhydride and gently stir for 15 minutes to dissolve. (Sample concentration, 5 mg/ml)
Titrant:	0.1 M perchloric acid	0.1 M perchloric acid
Equivalence:	1 ml titrant = 26.10 mg quinine sulfate	1 ml titrant = 26.10 mg quinine sulfate
Detection method:	Crystal violet indicator	Potentiometric
Specifications:	95% - 105% of label claim	90% - 110% of label claim

From Table 4-12 three differences can be observed, namely sample concentration (10 mg/ml vs. 5 mg/ml), means of detection (indicator vs. potentiometric) and the specifications (95-105% vs. 90-110%). The same titrant (in the same concentration) and solvent for sample preparation are used, ultimately providing with the same equivalency per ml for the determination of the sample concentration. It was decided that for this study only to execute the titration method according to the BP method, since the two methods were deemed comparable (would provide with the same results).

As explained in Chapter 3 (section 3.1.5), the reaction stoichiometry for quinine sulfate with perchloric acid is 1:3 (resulting in 1 ml of 0.1 M perchloric acid to be equivalent to 26.10 mg quinine sulfate). This is a fixed ratio (chemical reaction stoichiometry) which cannot be changed. That is the reason why the BP and *Ph.Int.* provide the same chemical equivalence for the assay determination, as both use the same titrant (and concentration thereof). Even though the sample concentrations which are specified by the monographs are different, the reaction stoichiometry is fixed and apply to any analyte concentration. The titrant volume for a sample prepared in a concentration of 10 mg/ml should be double that expected for the 5 mg/ml solution. As seen in section 4.3.5.2, Figure 4-10 (linearity and range), a linear response was obtained, with a r^2 value of 1.000 and a y-intercept of - 0.0134, indicating that the response of titrant was linear to that of quinine sulfate concentration. The repeatability/accuracy at low concentrations were also evident (Table 4-13).

The BP assay method for quinine sulfate tablets (utilising crystal violet indicator) which was selected to represent assay by means of titration presented with method verification parameters that complied with the acceptance criteria, showing that the indicator was indeed fit for use. The results obtained using the chosen method may therefore be considered as accurate and true representations of assay by means of titration, which was compared with the outcome from HPLC (USP) – Chapter 5 section 5.3.

Although it is expected that the two titration methods (BP vs. *Ph.Int.*) will provide comparable results, it should be noted that the specifications are different between the monographs. It is therefore possible that even though the methods may provide comparable results, that the outcome be different between the two monographs. The most common specifications applied for assay for tablets by monographs is 90-110%, seeing that these methods may be employed for both release and stability testing. The USP and BP monographs assay specifications for quinine sulfate tablets is set at 95 – 105% and 90 – 110%, and it is therefore proposed that the *Ph.Int.* quinine sulfate tablet assay specification be amended to 90-110% to harmonise the requirement between the *Ph.Int.* and BP pharmacopoeia.

Standardisation is the determination of the concentration of a volumetric solution. For this study, 0.1 M perchloric acid volumetric solution was standardised with a potassium hydrogen phthalate solution (Figure 4-9). The molar concentration of the titrant is dependent on the mass of substance that is weighed for preparation and also the exact volume of the final preparation. For this reason one cannot calculate and proceed with a theoretical molar concentration of the titrant, as it may influence the accuracy of the results. For this reason, the potassium hydrogen phthalate solution was titrated with the 0.1 M perchloric acid titrant to standardise the titrant (to experimentally obtain the concentration of the titrant).

The reaction stoichiometry between potassium hydrogen phthalate and perchloric acid culminate to 1 ml of 0.1 M perchloric titrant being equal to 20.42 mg of potassium dihydrogen phthalate.

The 0.1 M perchloric acid (titrant) was prepared and standardised and the final concentration was established to be 0.112 M. The BP (and *Ph.Int.*) specify that 1 ml of 0.1 M titrant is equivalent to 26.10 mg of quinine sulfate (refer to Chapter 3, section 3.1.5 for stoichiometric ratio calculations). Adjusted to apply to the experimental titrant concentration (0.112 M), it was calculated that each 1 ml of 0.112 M titrant is equivalent to 29.232 mg of quinine sulfate.

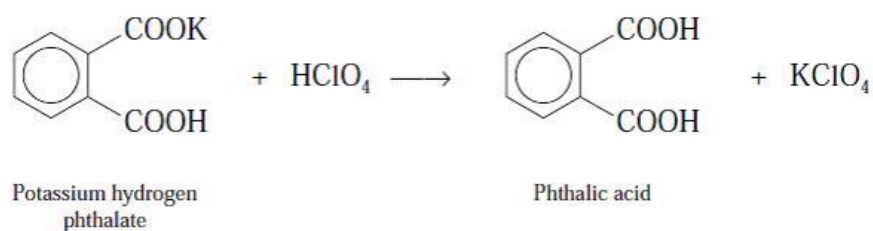


Figure 4-9: Standardisation of 0.1 N perchloric acid showing the reaction between the potassium hydrogen phthalate and the perchloric acid (Kar, 2005:109).

4.3.5.1 Specificity

Titration is a non-specific method (Watson, 2005:58), however the stoichiometry of the reaction between quinine sulfate and the perchloric acid is fixed - refer to Chapter 3 (section 3.1.5).

4.3.5.2 Linearity and range

4.3.5.2.1 Procedure

The BP quinine sulfate tablet monograph specifies that an amount of tablet powder equivalent to 400 mg of API should be weighed for assay test samples. For this reason, 400 mg quinine sulfate in 40 ml acetic anhydride (10.0 mg/ml) was considered as the 100% concentration. The linearity was determined by measuring the response (i.e. titre volume) of standard solutions (three concentrations) with a concentration range of $\pm 50 - 150$ % of the target concentration (10.0 mg/ml) – Table 4-13. The analytical values (i.e. titre volume) of the respective standard solutions were plotted as a function of the theoretical masses. A linear regression analysis was performed using STATISTICA 11 (2013) software (StatSoft, Inc., USA) and the correlation coefficient, y-intercept and slope of the regression line determined.

4.3.5.2.2 Acceptance criteria

The acceptance criteria relevant to the linearity parameter specify a correlation coefficient of ≥ 0.99 (Lobrutto and Patel, 2007:461). The y-intercept of the linear regression equation should not be significantly different from zero.

4.3.5.2.3 Results

The results obtained are summarised in Table 4-13. The linear regression analysis reported r^2 value of 1.0000. A linear graph was evident over the concentration range of 5 - 15 mg/ml (as

seen in Figure 4-10). The y-intercept of the regression line was calculated as -0.0134, which did not differ significantly from zero. It can thus be concluded that this method is linear over the concentration range of 5 - 15 mg/ml.

Table 4-13: Solution concentrations and range used during the linear regression analysis and analytical results obtained (non-aqueous titration method verification) of quinine sulfate

Standard solution number	Mass (mg)	Range (%)	Titre volume(ml)	Mass obtained experimentally (mg)	% Recovery
1	200.53	50.13	6.80	198.78	99.13
2	200.77	50.19	6.90	201.70	100.46
3	200.15	50.04	6.75	197.32	98.58
Average			6.82	199.27	99.39
%RSD			1.12	1.12	0.97
4	400.65	100.16	13.80	403.40	100.69
5	400.49	100.12	13.80	403.40	100.73
6	400.39	100.10	13.90	406.32	101.48
7	400.34	100.09	13.60	397.56	99.30
8	400.45	100.11	13.70	400.48	100.00
Average			13.76	402.23	100.44
%RSD			0.83	0.83	0.82
9	600.30	150.08	20.60	602.18	100.31
10	600.40	150.10	20.30	593.41	98.84
11	600.40	150.10	20.50	599.26	99.81
Average			20.47	598.28	99.65
%RSD			0.75	0.75	0.75

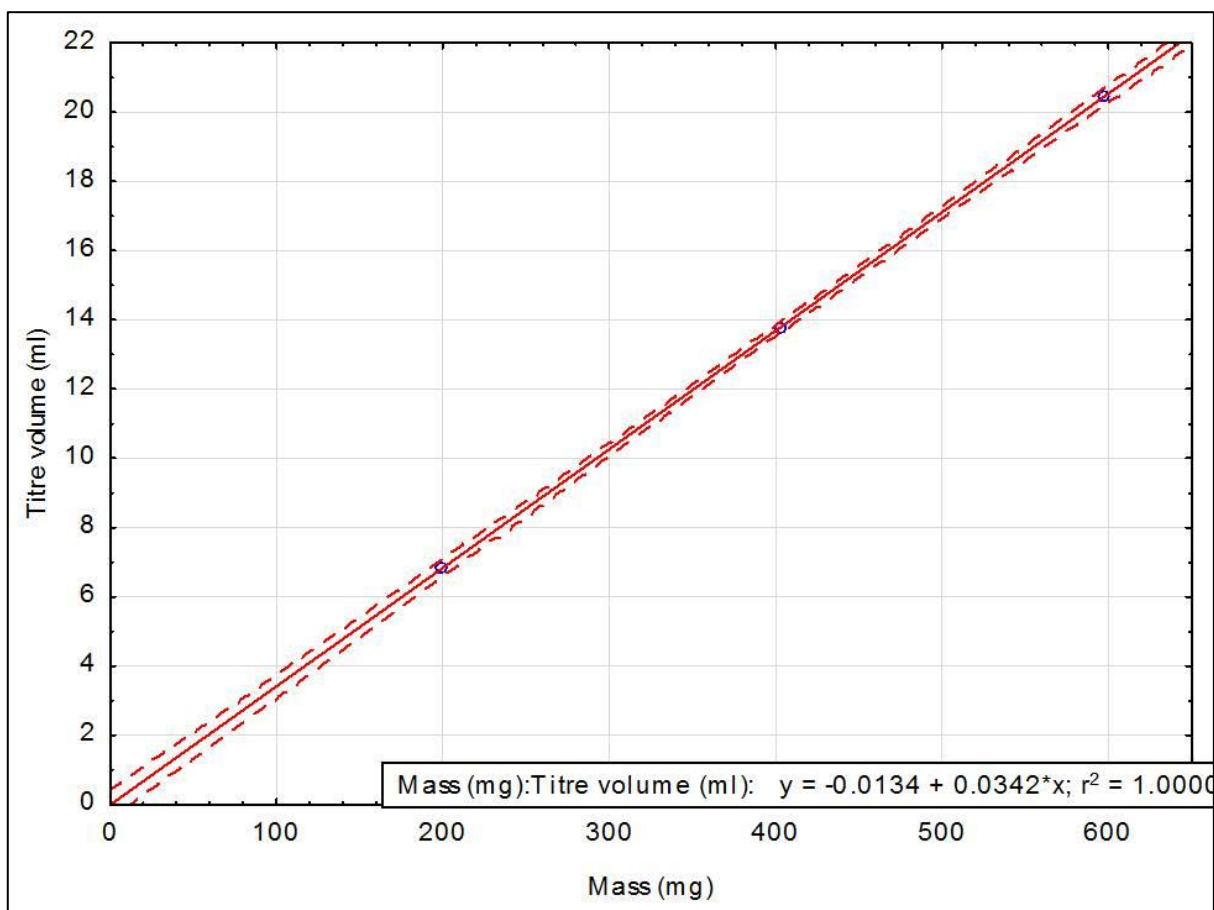


Figure 4-10: Linear regression analysis for the BP monograph for the assay of quinine sulfate tablets method verification. The dashed line illustrates the calculated 95% confidence interval for the linear regression.

4.3.5.3 Precision and repeatability

4.3.5.3.1 Procedure

Repeatability was evaluated by performing replicate titrations of the reference standard solutions and calculating the %RSD of the replicate analysis (Table 4-13).

4.3.5.3.2 Acceptance criteria

A %RSD of replicate analytical values less than 2% is considered acceptable to prove repeatability (Lobrutto and Patel, 2007:461).

4.3.5.3.3 Results

The calculated %RSD values at each concentration showed acceptable repeatability (%RSD < 2%) - see Table 4-13.

4.3.5.4 Accuracy/recovery

4.3.5.4.1 Procedure

To evaluate accuracy/recovery of the analytical method, the masses obtained experimentally were divided by the actual mass weighed and multiplied by a 100 as indicated by equation 4.4.

$$\% \text{ recovery} = \frac{\text{Experimental value obtained} \times 100}{\text{Actual mass weighed}} \quad \text{Equation 4.4}$$

4.3.5.4.2 Acceptance criteria

A percentage recovery between 98.0% - 102.0% is generally acceptable for assay validation purposes (Lobrutto and Patel, 2007:461).

4.3.5.4.3 Results

The calculated recovery values were found to be between 98.0% and 102.0% and deemed acceptable (Table 4-13).

4.3.6 Method verification of the HPLC assay method as specified by the United States Pharmacopoeia

For determining the amount of quinine sulfate that is present in the dosage units/tablets using a pooled sample, the USP specifies that the assay of quinine sulfate tablets be determined by means of HPLC. The validation parameters evaluated for the method verification of the HPLC assay are listed in Table 4-2.

4.3.6.1 Specificity

4.3.6.1.1 Procedure

The specified conditions for the assay of quinine sulfate tablets by means of HPLC are as follows:

Column: 300 x 3.9 mm, containing L1 packing - C 18, 10 µm (µBondapak, Waters)

Mobile phase: Acetonitrile, methanesulfonic acid solution (solution A), diethylamine solution (solution B) and water in the ratio of 100:20:20:860. Adjust with solution B to pH 2.6 if required.

Methanesulfonic acid solution: Add 35.0 ml methanesulfonic acid to 20.0 ml of glacial acetic acid and dilute to 500 ml with water.

Diethylamine solution: Dissolve 10.0 ml of diethylamine in water to obtain a 100 ml solution.

Injection volume: 50 µl

Flow rate: 1.0 ml/min

Detection wavelength: 235 nm

Standard preparation: Transfer about 20 mg of quinine sulfate RS to a 100 ml volumetric flask and dissolve and dilute to volume with mobile phase.

System suitability solution: 0.2 mg/ml of quinine sulfate and 0.2 mg/ml dihydroquinine dissolved in 5% of the final volume with methanol and dilute to volume with mobile phase.

Assay sample preparation: Accurately weigh and powder 20 tablets. Transfer a weighed portion of the powder equivalent to 160 mg of quinine sulfate to a 100 ml volumetric flask. Add 80 ml methanol and shake by mechanical means for 30 minutes. Dilute to volume with methanol. Filter and discard the first 10 ml of filtrate. Transfer 3 ml of the filtrate to a 25 ml volumetric flask and make up to volume with mobile phase.

Equation 4.5 is the calculation specified by the USP monograph for the quinine sulfate tablets assay:

$$\text{quinine sulfate (mg)/portion of tablets} = \frac{2500 \times C \times (r_{b,U} + r_{d,U})}{3 \times (r_{b,S} + r_{d,S})} \quad \text{Equation 4.5}$$

Where:

C = the concentration (mg/ml) of USP quinine sulfate RS in the standard preparation

$r_{b,U}$ = The peak area responses of quinine sulfate obtained from the standard solution

$r_{b,S}$ = The peak area response of quinine sulfate obtained from the assay solution

$r_{d,U}$ = The peak area response of dihydroquinine obtained from the standard solution

$r_{d,S}$ = The peak area response of dihydroquinine obtained from the assay solution

Since equation 4.4 is stipulated for assay determination, the method verification results in Table 4-15 present AUC_t , which is the sum of the peak area response of quinine sulfate (AUC_{qs}) and the peak area response of dihydroquinine (AUC_{dhq}).

4.3.6.1.2 Acceptance criteria

Acceptable specificity outcome for a chromatographic method may be verified by conformance with the specified system suitability requirements (USP, 2013). The method requires that the resolution between the quinine sulfate peak (approximately 23 minutes retention time) and dihydroquinine peak (approximately 33 minutes retention time) be at least 1.2, to ensure acceptable specificity between the two compounds. The monograph specifies that the relative retention times (relative to quinine sulfate) of quinine sulfate and dihydroquinine sulfate should be 1.0 and 1.5 respectively.

Therefore, for an acceptable specificity outcome all the peaks that present in a chromatogram should be separated (Lobrutto and Patel, 2007:461).

4.3.6.1.3 Results

As seen in Figure 4-11, the mobile phase peaks presented at approximately 3.8 minutes. The peaks due to the mobile phase did not interfere with the peaks of interest - quinine sulfate (approximately 23 minutes) and dihydroquinine (approximately 33 minutes).

Figure 4-12 is representative of the system suitability solution, Figure 4-13 of the standard solution and Figure 4-14 of a sample solution. The resolution between the quinine sulfate and dihydroquinine peaks was found to be 7.3 and therefore within the limits for system suitability.

The peaks from the system suitability solution (Figure 4-12) that eluted during the analysis were identified as quinine at ± 23 minutes (1.0×23 min) and dihydroquinine at ± 34 minutes ($\pm 1.5 \times 23$ min) and therefore corresponded with that specified by the monograph. It is a general requirement for HPLC chromatography to be acceptable, that the tailing factor of a peak be 2 or less (USP, 2013). None of the peaks displayed tailing factors above 2, rendering the peaks acceptable for integration (peak area).

Since no interferences were present, and all other requirements for system suitability (specified by the monograph) were met, the analysis continued.

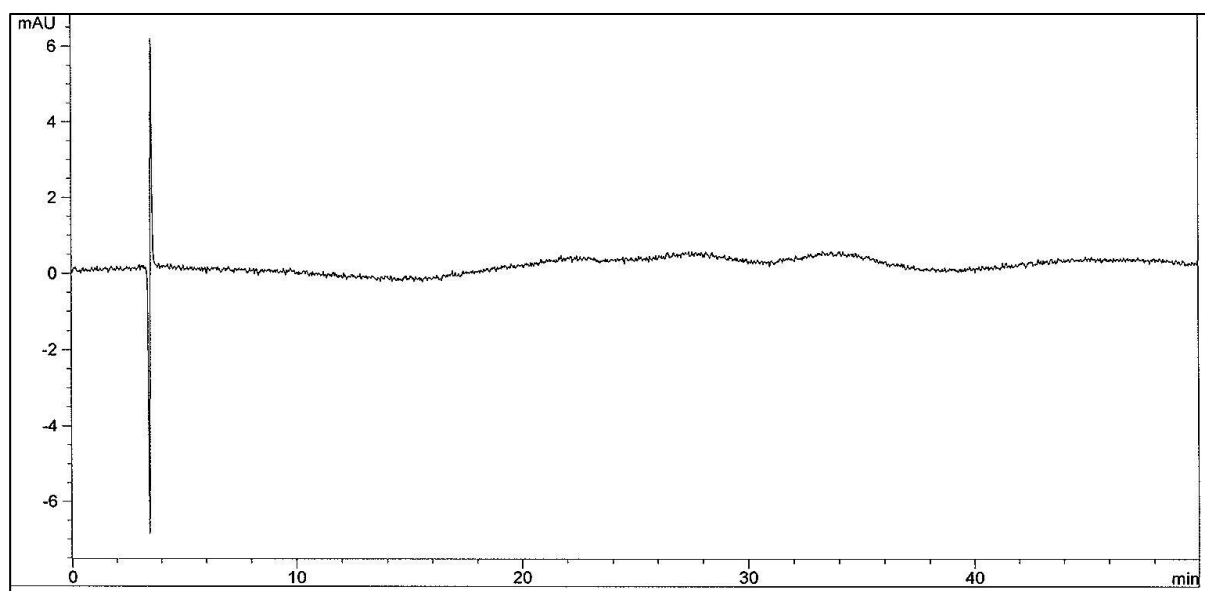


Figure 4-11: Example of a chromatogram of the mobile phase.

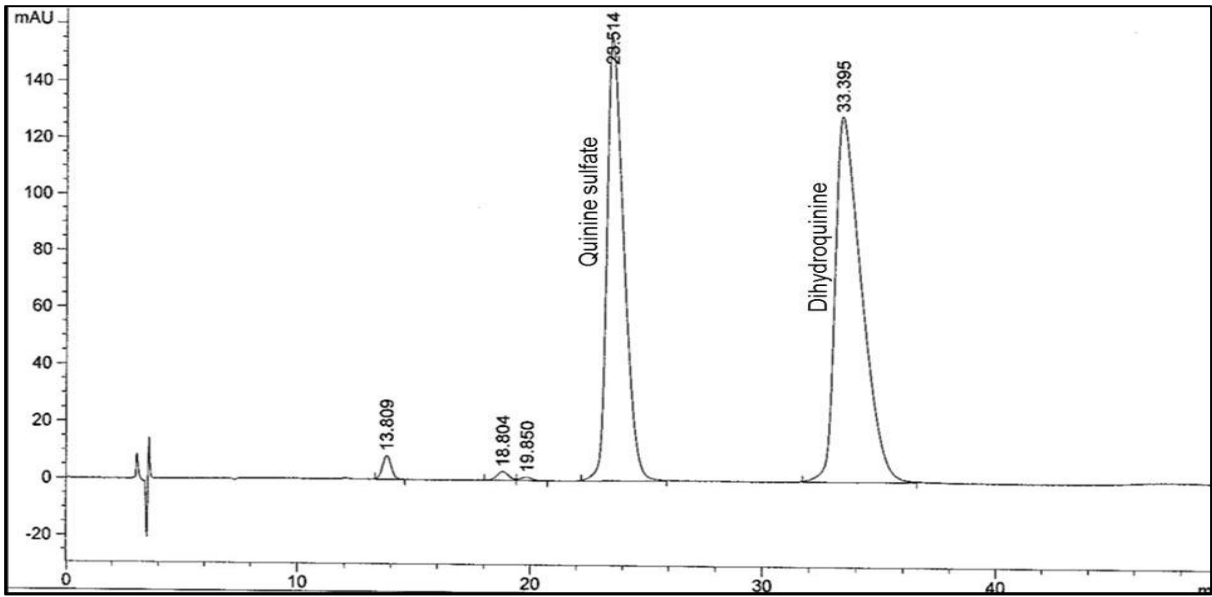


Figure 4-12: Example of a chromatogram of the system suitability solution.

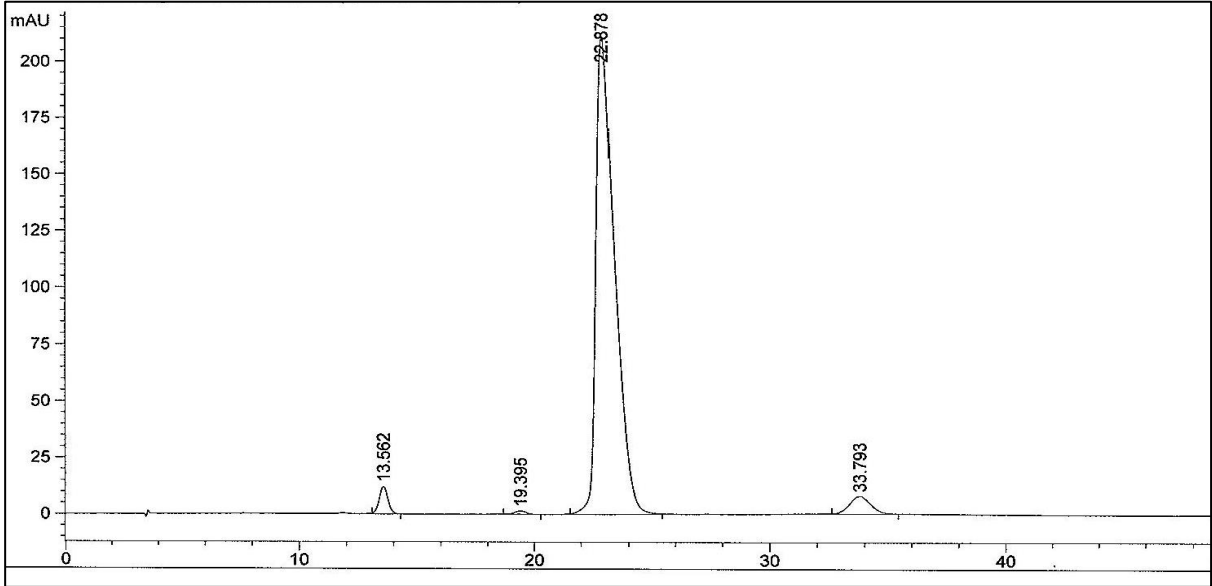


Figure 4-13: Example of a chromatogram of the standard solution.

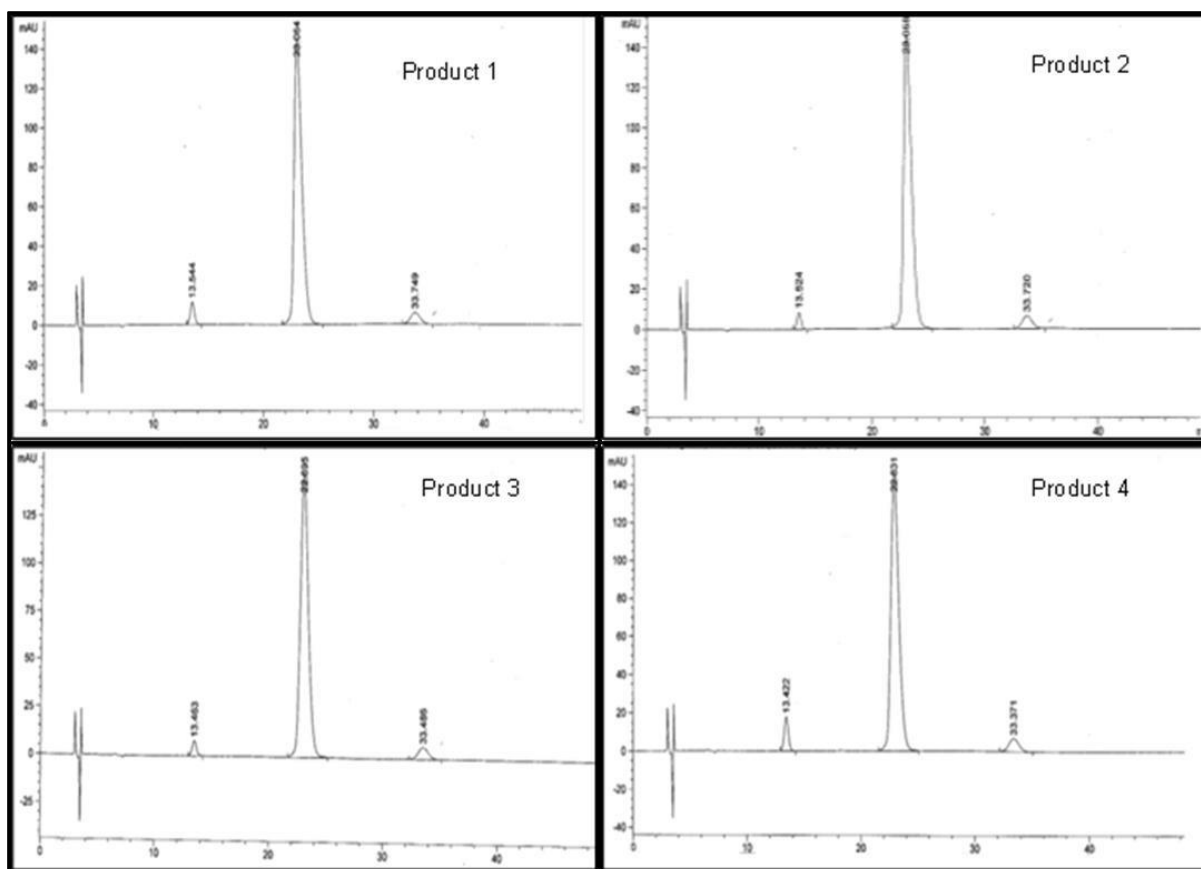


Figure 4-14: Example of the chromatograms obtained from the sample solutions of Product 1, 2, 3 and 4.

4.3.6.2 Linearity and range

4.3.6.2.1 Procedure

The USP specifies the 100% standard solution concentration to be 200 µg/ml (with reference to quinine sulfate). For this reason, 200 µg/ml quinine sulfate was considered as the 100% concentration, across which a linear range needed to be established. A 100% reference standard solution was prepared by transferring approximately 20 mg of quinine sulfate RS into a 100 ml volumetric flask and diluted to volume with mobile phase (theoretical concentration = 200 µg/ml). From the 100% reference standard solution a 50% reference standard solution was prepared using a dilution factor of 2:

25 ml → 50 ml, diluted to volume (50% equivalent to 100 µg/ml)

150 % reference standard solution was prepared by transferring approximately 30 mg of quinine sulfate RS into a 100 ml volumetric flask and diluted to volume with mobile phase (theoretical concentration = 300 µg/ml)

From the 150% reference standard solution a 120% reference standard solution was prepared using a dilution factor of 1.25:

20 ml → 25 ml, diluted to volume (120% equivalent to 240 µg/ml)

Table 4-14: Summary of the preparation of the different concentrations of quinine sulfate solutions used for the linear regression analysis of the assay method verification

Preparation	Concentration of solution:	Range %
25 ml of 100% concentration solution in 50 ml volumetric flask, up to volume with mobile phase	100 µg/ml	50 %
20 mg into 100 ml volumetric flask, up to volume with mobile phase	200 µg/ml	100 %
20 ml of 150% concentration solution in 25 ml volumetric flask, up to volume with mobile phase	240 µg/ml	120 %
30 mg into 100 ml volumetric flask, up to volume with mobile phase	300 µg/ml	150 %

The sum of the quinine sulfate and dihydroquinine peak areas of the respective standard solutions were plotted as a function of the theoretical concentrations. A linear regression analysis was performed using STATISTICA 11 (2013) software (StatSoft, Inc., USA) and the correlation coefficient, y-intercept and slope of the regression line determined.

4.3.6.2.2 Acceptance criteria

The acceptance criteria relevant to the linearity parameter specify a correlation coefficient of ≥ 0.99 (Lobrutto and Patel, 2007:461). The y-intercept of the linear regression equation should not be significantly different from zero.

4.3.6.2.3 Results

The results obtained are summarised in Table 4-15. The linear regression analysis reported r^2 value of 1.0000. A linear graph was evident over the concentration range of 103.95 µg/ml - 302.50 µg/ml (as seen in Figure 4-15). The intercept of the regression line was calculated as -

89.074, which did not differ significantly from zero taking into consideration that the target concentration is approximately 8152 mAu. It can thus be concluded that this method is linear over the concentration range of 103.95 µg/ml - 302.50 µg/ml.

Table 4-15: Concentrations of solutions and range of the linear regression analysis (USP assay method verification)

Concentration (µg/ml)	Range (%)	Sum of peak areas (rb,s + rd,s)	Average for peak areas	% RSD for peak areas
103.95	52.0	4045.191 4038.024 4040.765	4041.329	0.09
207.89 (1)	103.9	8145.530 8266.673 8118.917 8124.365 8139.579 8108.610	8151.629	0.80
205.09 (2)*	102.5	8071.941 8060.471 8087.571	8073.328	0.17
242.00	121.0	9516.005 9524.302 9527.417	9522.574	0.06
302.50	151.3	11966.474 11899.106 11899.470	11921.683	0.33

* Duplicate standard solution prepared for accuracy or recovery evaluation (section 4.3.6.4)

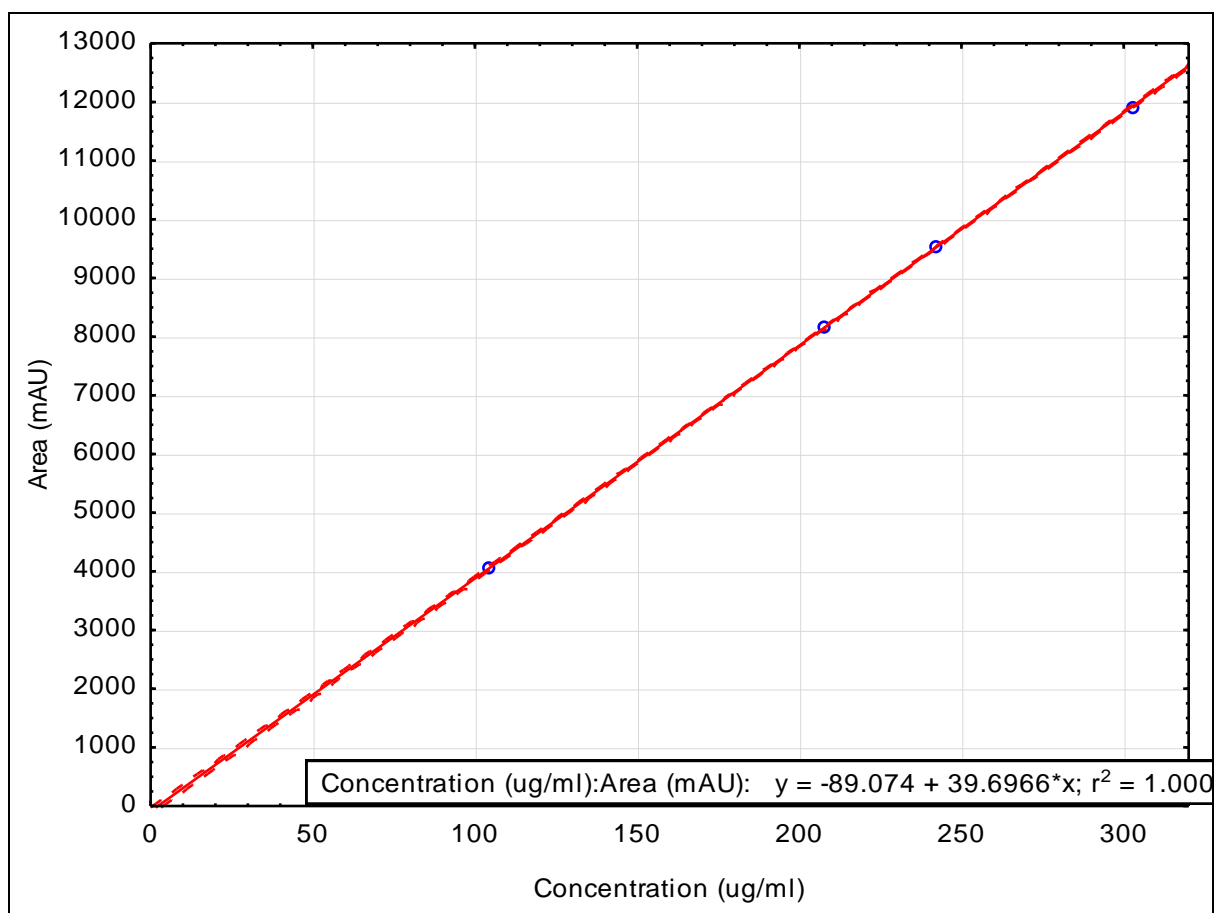


Figure 4-15: Linear regression analysis for the USP monograph for assay testing of quinine sulfate tablet method verification. The dashed line illustrates the calculated 95% confidence interval for the linear regression.

4.3.6.3 Precision and repeatability

4.3.6.3.1 Procedure

Repeatability was evaluated by performing replicate measurements of the reference standard solution and calculating the %RSD of the replicate measurements (Table 4-15).

4.3.6.3.2 Acceptance criteria

A %RSD of replicate analytical values less than 2% is considered acceptable to prove repeatability (Lobrutto and Patel, 2007:461).

4.3.6.3.3 Results

The calculated %RSD values at each concentration showed acceptable repeatability (%RSD < 2%) - see Table 4-15.

4.3.6.4 Accuracy/recovery

4.3.6.4.1 Procedure

To evaluate accuracy/recovery of the analytical method, a duplicate reference standard solution with a concentration of approximately 200 µg/ml (refer to Table 4-15) was prepared. The analytical values obtained were used to calculate the experimental concentration of the reference standard solution by means of the equation obtained from the linear regression analysis (Figure 4-15). The percentage recovery was calculated by means of equation 4.3.

4.3.6.4.2 Acceptance criteria

A percentage recovery between 98% - 102% is generally acceptable for assay verification purposes (Lobrutto and Patel, 2007:461).

4.3.6.4.3 Results

The experimental concentration was calculated to be 205.62 µg/ml and the theoretical concentration was 205.09 µg/ml. The percentage recovery was calculated to be 100.3% and this was deemed acceptable (98.0 % < 100.3% < 102.0%).

4.3.7 Verification of the method testing related cinchona alkaloids/other cinchona alkaloids as specified by the International Pharmacopoeia and British Pharmacopoeia

For determining the presence and the concentration of the related cinchona alkaloids in quinine sulfate tablets, the BP and *Ph.Int.* specify testing by means of HPLC. The validation parameters evaluated for the method verification of the HPLC procedure are listed in Table 4-2.

The HPLC procedures concerning related cinchona alkaloids (*Ph.Int.*) and the other cinchona alkaloids (BP) were found to be comparable. Table 4-16 summarises the main conditions of the HPLC procedures specified for the related cinchona alkaloids (*Ph.Int.*) and the other cinchona alkaloids (BP). For ease of reference, the differences between the methods (Table 4-16) are presented in bold.

Table 4-16: The main conditions of the related/other cinchona alkaloid methods of the BP and *Ph.Int*

	BP	<i>Ph.Int</i>
Flow rate:	1.5 ml/min	1.3 ml/min
Mobile phase:	6.8 g potassium dihydrogen orthophosphate and 3.0 g hexylamine in 700 ml water adjusted to pH 2.8 , adding 60 ml acetonitrile thereafter and diluting to 1000 ml with water	6.8 g potassium dihydrogen orthophosphate and 3.0 g hexylamine in 900 ml water adjusted to pH 3.0 and diluted to 1000 ml with water (Solution 1) Mobile phase is 920:80 (Solution 1: acetonitrile)
Detection wavelength:	316 nm (for quinine)	316 nm
Column type:	5 µm, 250mm×4.6mm , C 18	5 µm, 150 mm×4.6 mm , C 18
Injection volume:	10 µl	10 µl
Specification:	Dihydroquinine not more than 10% Cinchonidine note more than 5% Any other related substance not more than 2.5% The sum of the related substances is not more than 15%	Dihydroquinine not more than 10% Cinchonidine note more than 5% (elutes before quinine – Figure 4-19) Any other related substance not more than 2.5% No specification for total related substances

From Table 4-16 it can be seen that the:

- flow rate between the BP monograph and the *Ph.Int.* monograph differs by 15%,
- the pH of the mobile phase of the BP monograph and the *Ph.Int.* monograph differs by 0.2 units,
- the acetonitrile in the mobile phase differs by 33% between the BP monograph and the *Ph.Int.* monograph and
- length of the column required by the BP monograph differ 66% from the *Ph.Int.* monograph.

According to the general chapter <621> Chromatography of the USP (USP, 2013):

- the length of a HPLC column can be adjusted from that specified by as much as 70%;
- the flow rate can be adjusted from that specified by as much as 50%;
- the injection volume can be reduced as long as the precision remains acceptable;

- the pH of mobile phase may differ by maximum 0.2 pH units; and
- the ratio of minor mobile phase components (50% or less of the mobile phase) can be adjusted by approximately 30%.

The allowed adjustments as specified above have been established as a part of comprehensive robustness testing during method development. Since published monographs are considered to be fully validated, it implies that it should be robust enough to withstand small adjustments. Since similar techniques, procedures and specifications are applied by the BP and *Ph.Int.*, it was decided to perform the *Ph.Int.* method only, since the differences in the methods are within the tolerated variances as described above.

Quinine is extracted from the bark of the cinchona tree and a small percentage of related cinchona alkaloids will always be present even in a purified reference standard. There are more than 20 cinchona alkaloids that are structurally related (Vinetz *et al.*, 2011). The related cinchona alkaloids that are specified in the *Ph.Int.* monograph for quinine sulfate tablets are cinchonidine, quinindine sulfate, quinine sulfate, dihydroquinidine and dihydroquinine (Figure 4-16).

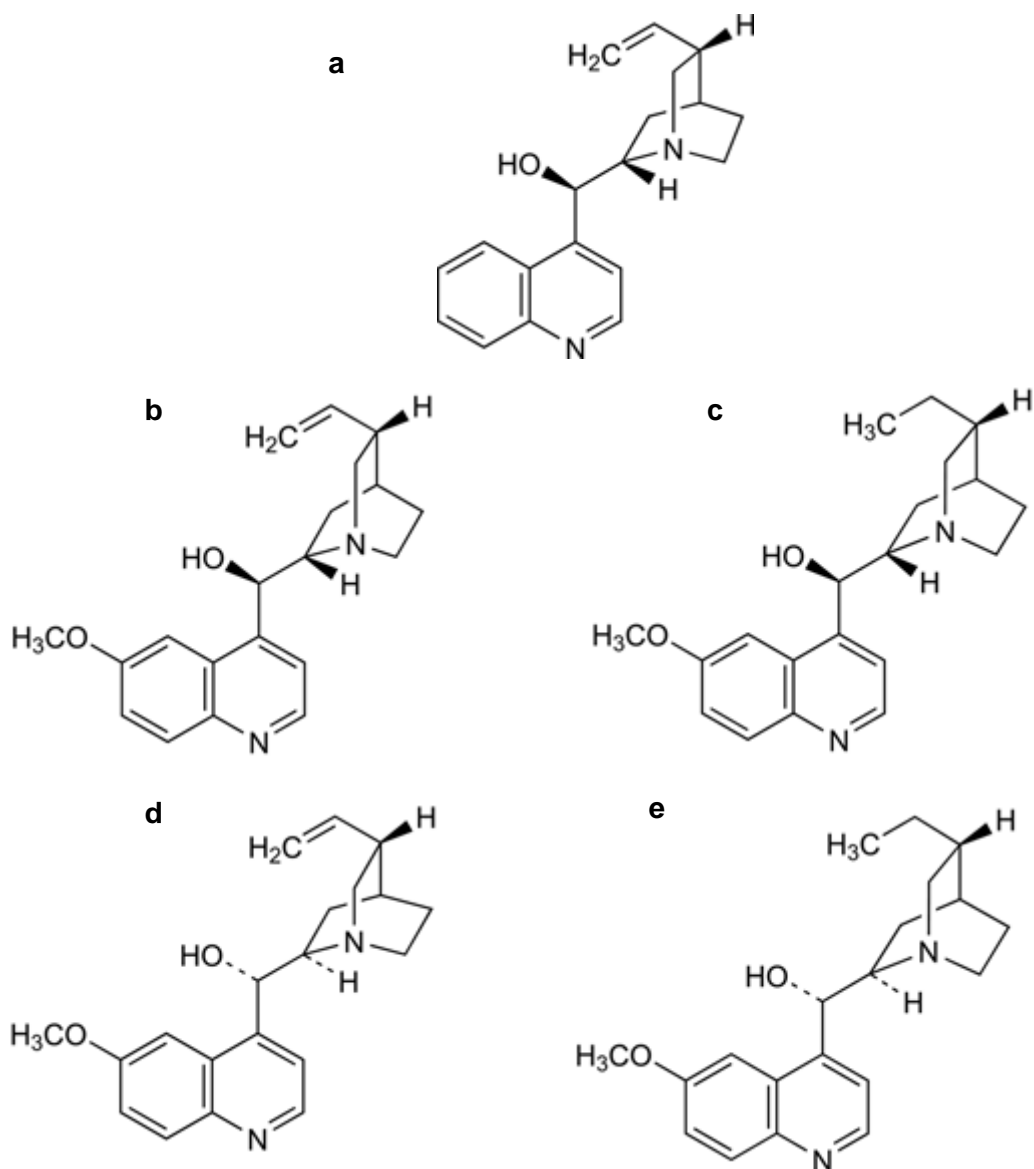


Figure 4-16: Cinchona alkaloids specified in the *Ph.Int.* monograph for quinine sulfate tablets include: (a) cinchonidine, (b) quinine, (c) dihydroquinine, (d) quinidine and (e) dihydroquinidine (Moffat *et al.*, 2011).

The *Ph.Int.* specifies that the related cinchona alkaloids to be quantified by means of peak normalization. The *Ph.Int.* specifies the use of two reference standards: Quinine sulfate RS and Quinidine sulfate RS. The monograph reports the relative retention times (with reference to quinine) for the following alkaloids: cinchonidine about 0.6; quinidine about 0.8; dihydroquinidine about 1.2 and dihydroquinine about 1.8 (*Ph.Int.*, 2013).

The quinine sulfate reference standard solution is prepared as described in section 4.3.7.1.1 (solution 2). From solution 2 one would expect to see at least one peak (quinine sulfate),

however the chromatogram of solution 2 (Figure 4-20) illustrates three peaks. The principal peak at ± 8.8 minutes was identified as quinine sulfate, and the peaks at ± 5.0 minutes and ± 12.9 minutes were identified as cinchonidine and dihydroquinine respectively.

The resolution solution (containing quinidine sulfate) is prepared as described in section 4.3.7.1.1 (solution 3). From solution 3, one would expect to see at least two peaks (quinine sulfate and quinidine sulfate). The chromatogram of solution 3 (Figure 4-19) displayed five peaks, three of which from solution 2 (all those in Figure 4-20), indicating that the quinidine reference material contained quinidine (peak at ± 7.1 minutes), but also dihydroquinidine (± 10.4 minutes). It is thus clear that the official quinine pharmacopoeial standards consist of complex mixtures. The *Ph.Int.* (2013) states that peak normalization is generally used for the determination of the component composition of complex mixtures, especially when the relative response factors of the individual components are similar. The similarity of the relative response factors of quinine sulfate and the related cinchona alkaloids have been proved during the extensive method validation by the *Ph.Int.* and the BP thus it need not be re-established or illustrated in this study.

At the time of this study no individual, pharmacopoeial standards were available for the related cinchona alkaloids, thus individual specificity, linearity, range and accuracy analyses for each of the related cinchona alkaloids could not be performed as part of the method verification. Taking into consideration that the related cinchona alkaloids display similar relative response factors, method verification criteria based on the cumulative responses of the quinine sulfate, cinchonidine and dihydroquinine were used to assess the method verification parameters (in conjunction with normal system suitability criteria).

4.3.7.1 Specificity

4.3.7.1.1 Procedure

The specified HPLC conditions for analysis for the related cinchona alkaloids present in quinine sulfate tablets are as follows:

Column type:	Phenomenex Luna C 18, 150 mm \times 4.6 mm, 5 μ m
Mobile phase:	6.8 g potassium dihydrogen orthophosphate and 3.0 g of hexylamine in 900 ml water, adjusting the pH to 3.0 with orthophosphoric acid and dilute to 1000 ml with water. Mix 920 ml of this solution with 80 ml of acetonitrile.
Flow rate:	1.3 ml/min

Injection volume:	10 µl
Detection wavelength:	316 nm
Solvent:	Prepared using 80 volumes of water, 20 volumes acetonitrile and 0.1 volume of orthophosphoric acid.
Solution 1:	Transfer powdered tablet containing equivalent 60 mg of quinine sulfate into a 20 ml volumetric flask. Add 15 ml of solvent and sonicate for 5 minutes. Allow to cool to room temperature and dilute to volume with solvent. Filter using a 0.45 µm Millipore filter.
Solution 2:	Transfer 30 mg of quinine sulfate reference standard to a 10 ml volumetric flask. Dilute to volume. Filter using a 0.45 µm Millipore filter.
Solution 3:	Transfer 15 mg of quinidine sulfate reference standard to a 5 ml volumetric flask. Dilute to volume using solution 2 as solvent. Filter using a 0.45 µm Millipore filter.

4.3.7.1.2 Acceptance criteria

Acceptable specificity outcome for a chromatographic method may be verified by conformance with the specified system suitability requirements (USP, 2013). In addition to the normal system suitability requirements the *Ph.Int.* method specifies the following relative retention times for the related substances with reference to quinine (retention time about 10 minutes):

- quinidine about 0.8;
- dihydroquinidine about 1.2;
- dihydroquinine about 1.5; and
- cinchonidine about 0.6.

The method stipulates that the resolution between the quinidine and quinine peak be at least 1.5 and the resolution between the quinine and dihydroquinidine peak be at least 1.5 to ensure acceptable specificity between the related substances.

4.3.7.1.3 Results

As seen in Figure 4-17 and Figure 4-18, the mobile phase and solvent peaks presented between 2 and 3 minutes. The peaks due to the mobile phase did not interfere with the peaks of interest – cinchonidine (approximately 5 minutes), quinidine sulfate (approximately 7 minutes), quinine sulfate (approximately 9 minutes), dihydroquinidine (approximately 10 minutes) and dihydroquinine (approximately 13 minutes).

Figure 4-19 is representative of the resolution solution. The resolution between the quinidine and quinine was found to be 4.2 and between the quinine and dihydroquinidine was 3.3, therefore within the limits for system suitability.

It is a general inherent requirement for acceptable chromatography that the tailing factor of a peak be 2 or less (USP, 2013). None of the peaks displayed tailing factors above 2, rendering the peaks acceptable for integration (peak area).

From the sample and standard solution chromatograms it can be seen that the retention times of the quinine sulfate and related substances correspond, which implies a positive identification. The chromatograms of the sample solution and standard solution showed that there was no interference at the retention times of interest (5.0 minutes, 7.1 minutes, 8.9 minutes, 10.4 minutes and 12.9 minutes). The relative retention times specified by the monograph corresponded with the relative retention times obtained experimentally as seen in Table 4-17. Since no interferences were present (also see chromatograms of the four products, Figure 4-21), and all other requirements for system suitability (specified by the monograph) were met, the analysis specificity was demonstrated.

Table 4-17: Relative retention times specified by the *Ph.Int.* monograph compared to the experimental relative retention times obtained

	Relative retention time specified by monograph	Experimental relative retention time
Cinchonidine	0.6	0.6
Quinidine sulfate	0.8	0.8
Quinine sulfate	1.0	1.0
Dihydroquinidine	1.2	1.2
Dihydroquinine	1.5	1.5

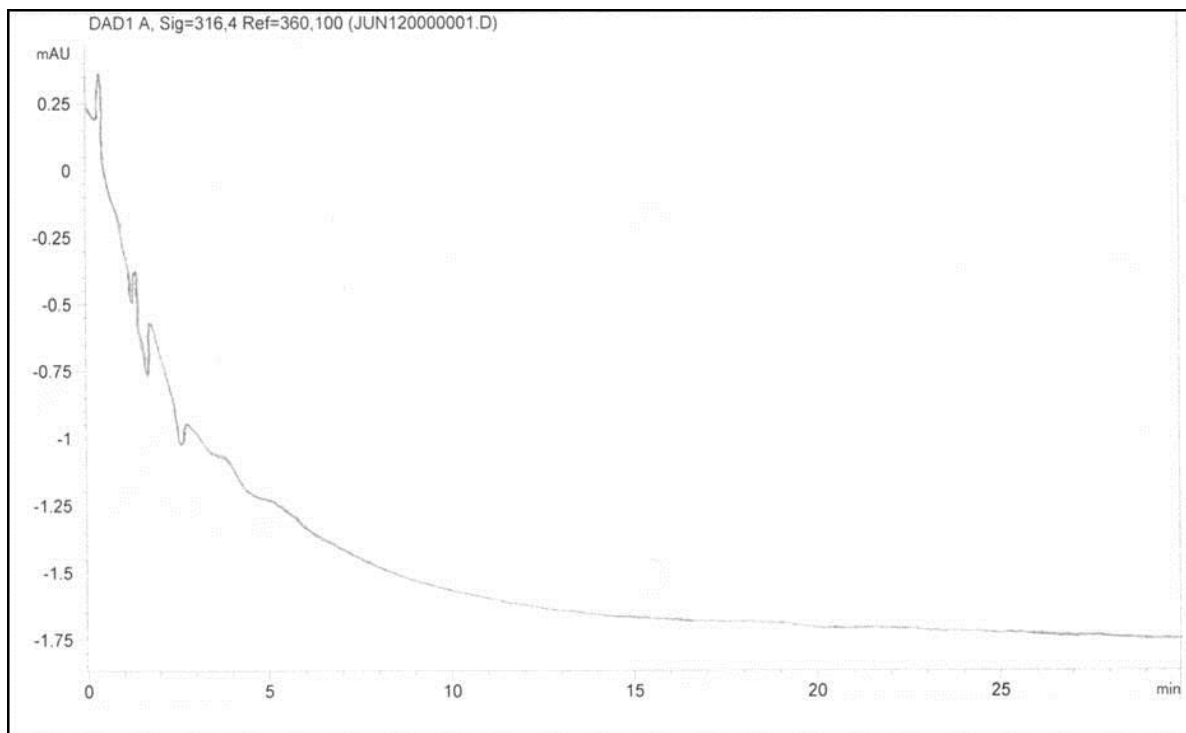


Figure 4-17: Example of a chromatogram of the mobile phase.

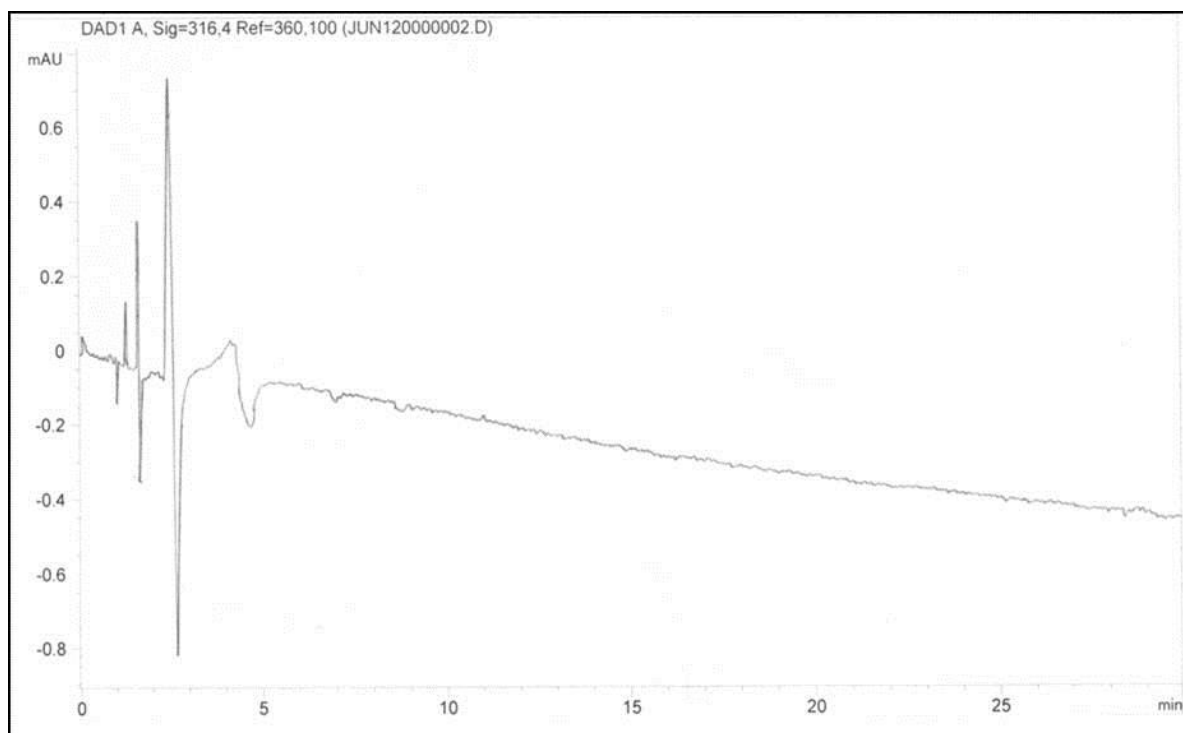


Figure 4-18: Example of a chromatogram of the solvent.

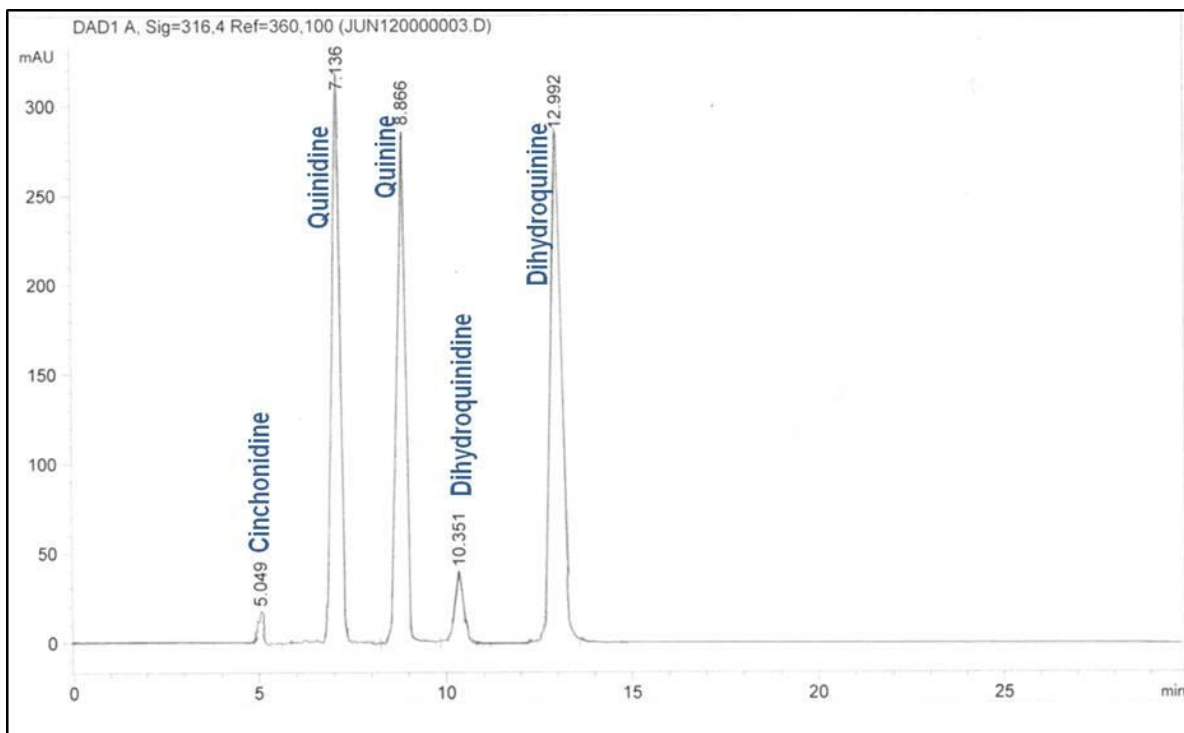


Figure 4-19: Example of a chromatogram of the resolution solution (solution 3).

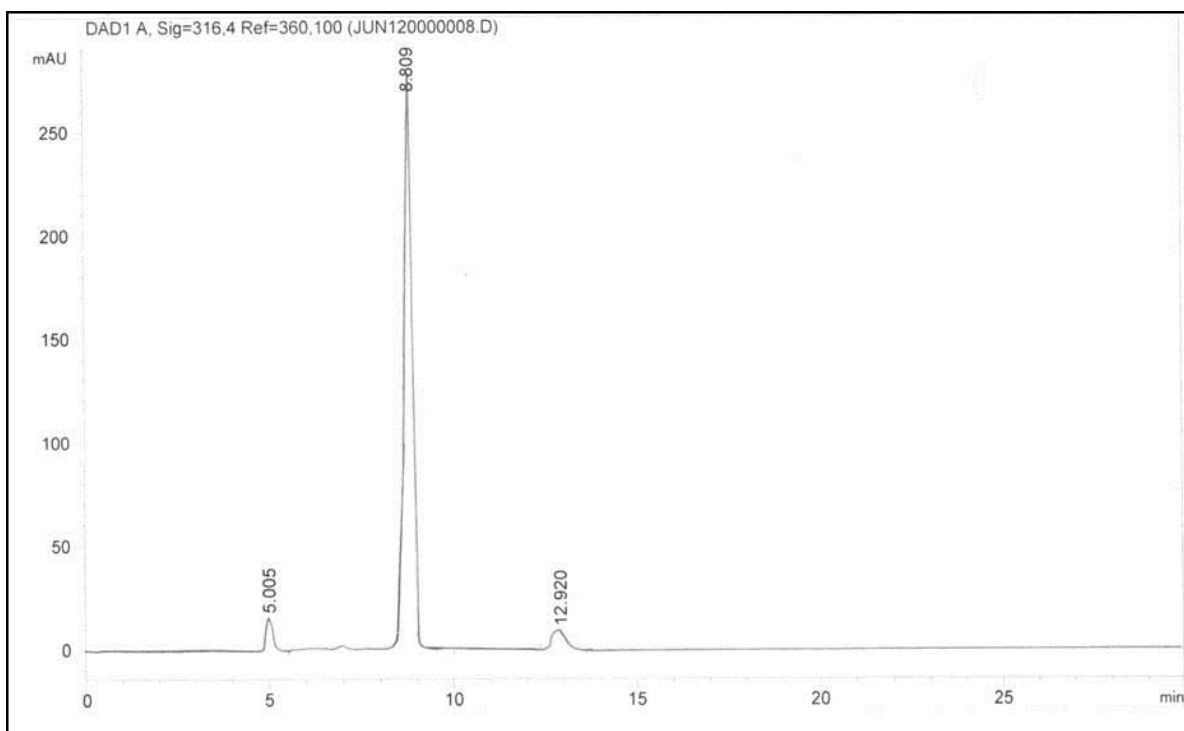


Figure 4-20: Example of a chromatogram of the 100% standard (solution 2).

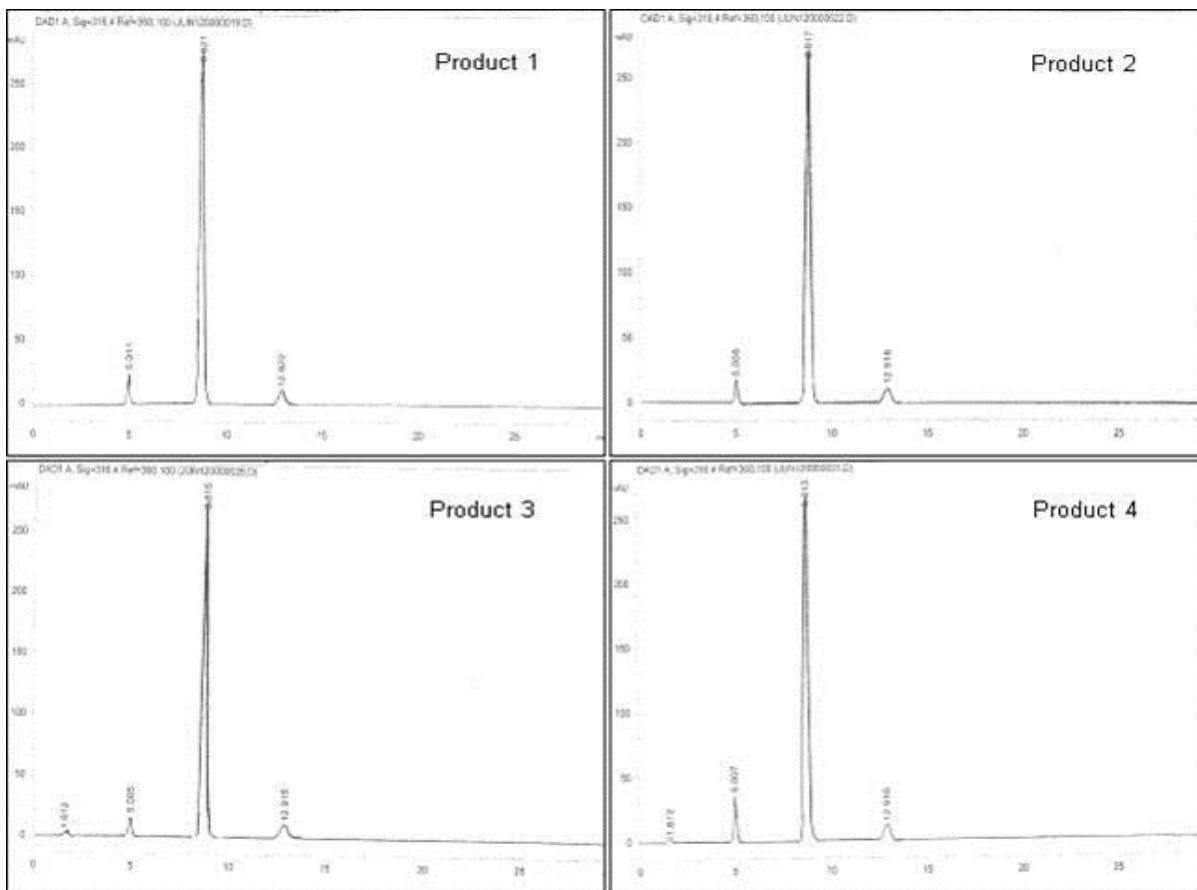


Figure 4-21: Sample chromatograms of each of the four products (solution 1).

4.3.7.2 Linearity and range

4.3.7.2.1 Procedure

The *Ph.Int.* specifies that the 100% standard solution concentration to be 3000 µg/ml (quinine sulfate). For this reason, 3000 µg/ml was considered as the 100% concentration, across which a linear range needed to be established. A 100% reference standard solution was prepared transferring 30 mg of quinine sulfate RS into a 10 ml volumetric flask and diluted to volume with solvent (theoretical concentration = 3000 µg/ml) - refer to Table 4-18.

A 50% reference standard solution was prepared transferring 15 mg of quinine sulfate RS into a 10 ml volumetric flask and diluted to volume with solvent (theoretical concentration = 1500 µg/ml) - refer to Table 4-18.

A 150% reference standard solution was prepared transferring 45 mg of quinine sulfate RS into a 10 ml volumetric flask and diluted to volume with solvent (theoretical concentration = 4500 µg/ml) - refer to Table 4-18.

Table 4-18: Summary of the preparation of the different concentrations of quinine sulfate solutions used for the linear regression analysis of the HPLC procedure for related cinchona alkaloids method verification

Preparation	Concentration of solution:	Range %
15 mg into 10 ml volumetric flask, up to volume with solvent	1500 µg/ml	50%
30 mg into 10 ml volumetric flask, up to volume with solvent	3000 µg/ml	100%
45 mg into 10 ml volumetric flask, up to volume with solvent	4500 µg/ml	150%

The analytical values/peak areas of the sum of the cinchonidine, quinine sulfate and dihydroquinine were plotted as a function of the theoretical quinine sulfate reference standard solution concentrations. A linear regression analysis was performed using STATISTICA 11 (2013) software (StatSoft, Inc., USA) and the correlation coefficient, y-intercept and slope of the regression line determined.

4.3.7.2.2 Acceptance criteria

The acceptance criteria relevant to the linearity specify a correlation coefficient of ≥ 0.99 (Lobrutto and Patel, 2007:461). The y-intercept of the linear regression equation should not be significantly different from zero.

4.3.7.2.3 Results

The results obtained are summarised in Table 4-19. The linear regression analysis reported a r^2 - value of 0.9999. A linear graph was evident over the concentration range of 1509.49 – 4563.43 µg/ml (as seen in Figure 4-21). The intercept of the regression line was calculated as 4.8437, this is relatively close to zero taking into consideration that the target concentration is approximately 4950 mAu.. It can thus be concluded that this method is linear over the concentration range of 1509.49 – 4563.43 µg/ml.

Table 4-19: Concentrations and range of the linear regression analysis (*Ph.Int.* test for cinchona alkaloids method verification)

Concentration (µg/ml)	Range (%)	Cumulative Peak areas	Average for peak areas	% RSD for peak areas
1509.49	50.3	2464.142 2448.117	2456.129	0.46
3010.99 (1)	100.4	4945.287 4946.314 4943.974 4951.894 4957.805 4956.597	4950.312	0.12
3012.98 (2)*	100.4	4924.989 4924.106	4924.548	0.01
4563.43	152.1	7441.166 7451.059	7446.113	0.09

* Duplicate standard solution prepared for accuracy or recovery evaluation (section 4.3.7.4)

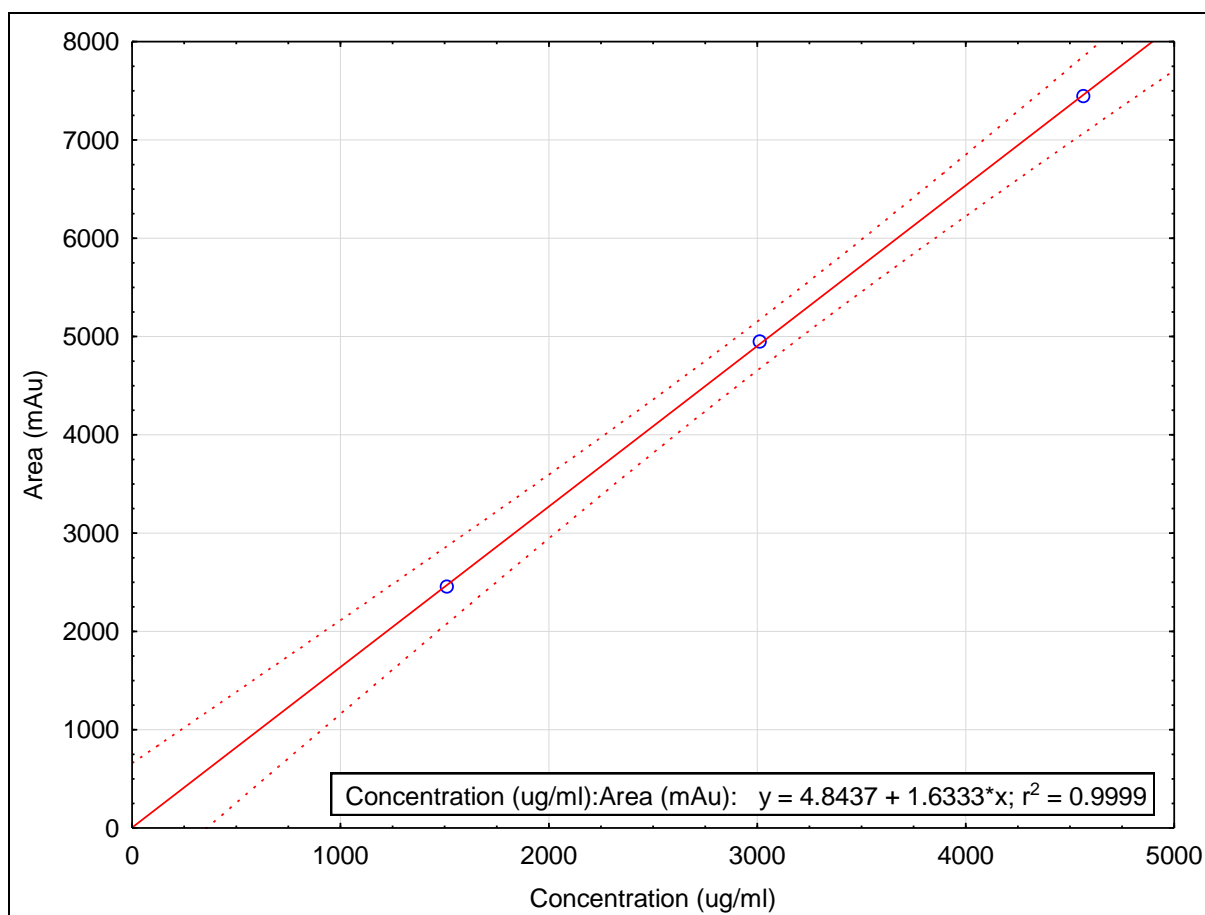


Figure 4-22: Linear regression analysis for the *Ph.Int.* monograph for testing of related cinchona alkaloids method verification. The dashed line illustrates the calculated 95% confidence interval for the linear regression

4.3.7.3 Precision and repeatability

4.3.7.3.1 Procedure

Repeatability was determined by performing replicate measurements of the reference standard solutions and calculating the %RSD of the replicate measurements (Table 4-19).

4.3.7.3.2 Acceptance criteria

A %RSD of replicate analytical values less than 2% is considered acceptable to prove repeatability (Lobrutto and Patel, 2007:461).

4.3.7.3.3 Results

The calculated %RSD values at each concentration showed acceptable repeatability (%RSD < 2%) -see Table 4-19.

4.3.7.4 Accuracy/recovery

4.3.7.4.1 Procedure

To evaluate accuracy/recovery of the analytical method, a duplicate reference standard solution (quinine sulfate, cinchonidine and dihydroquinine) with a known concentration of approximately 3000 µg/ml (refer to Table 4-19) was prepared.

The analytical values obtained were used to calculate the concentration of the reference standard solution by means of the equation obtained from the linear regression analysis (Figure 4-22). The percentage recovery was calculated by means of equation 4.3.

4.3.7.4.2 Acceptance criteria

A percentage recovery between 90% - 110% is generally acceptable for related substances verification purposes (Lobrutto and Patel, 2007:461).

4.3.7.4.3 Results

The experimental concentration of quinine sulfate cinchonidine and dihydroquinine was calculated to be 3012.13 µg/ml and the theoretical concentration was 3012.98 µg/ml. The recovery was calculated to be 99.9% and deemed acceptable (95 % < 99.9% < 105%).

Conclusion

It is required to perform method verification on established methods to ensure that the methods perform as they were intended under alternate conditions and using alternate equipment. Method verifications were performed on all the quantitative tests of the quinine sulfate tablet monographs (dissolution, assay and impurities/related substances).

The parameters that were evaluated included specificity, repeatability, linearity and recovery. The criteria for method verification were met for all these tests in all instances, which confirmed the successful transfer of these methods to the laboratory .