Analytical Method Development

2.1. Introduction

Artemether is official in International Pharmacopoeia and the reported method of analysis is 'High Pressure Liquid Chromatography' while Mefloquine hydrochloride and primaquine phosphate are official in British Pharmacopoeia, and the reported method of analysis is potentiometric titration. The present study reveals a simple, ultraviolet spectrophotometric method for the determination of all three drugs. These developed methods were further used in evaluation of taste masked formulations.

2.2. Artemether (ARM)

Various methods have been reported for artemether determination out of which high-pressure liquid chromatography (International Pharmacopoeia, 2005; Sandrenan et al., 1997; Thomas and Ward, 1992), reductive electrochemical method (Acton et al., 1985; Melendez et al., 1991) and capillary electrophoresis (Gabriels et al., 1999) are some important methods.

The chemical structure of ARM displays an endoperoxide bridge and lacks ultraviolet (UV) chromophore appropriate for their routine measurement using UV detection, shown in Figure 2.1a. This problem of UV detection of ARM has been tackled by acid treatment, inducing the production of a UV detectable product (Figure 2.1b).

In addition, an ultraviolet spectroscopic method has not so far been suggested as a method of analysis. In present study, a simple, ultraviolet spectrophotometric method for the determination of ARM in methanol with analytical parameters has been reported. This developed method was further used in analyzing taste masked formulations.

(a) **(b)**

Figure 2.1 Structure of (a) artemether and (b) UV detectable compound

2.2.1. Experimental

2.2.1.1. Apparatus

Shimatzu UV-1700 UV visible spectrophotometer with quartz cells of 10-mm path length; Bath sonicator (Model 120W, Vibronics Pvt. Ltd, Mumbai), 589 Blue ribbon S & S filter paper circles of diameter 125 mm (Schleider and Schuell GmbH, Germany).

2.2.1.2. Materials

Artemether (ARM) was kindly gifted by Ajanta Pharma Ltd., Mumbai Methanol and hydrochloric acid of analytical grade were purchased from S.D. Fine Chem Ltd, Boisar. Stock solution of ARM (100 mcg/ml) was prepared by dissolving 10 mg in 100 ml of methanol.

2.2.1.3. Preparation of calibration curve

Suitable aliquots (0.6–2.2 ml) of the stock solution were pipetted into 10 ml volumetric flasks. Five milliliter of 5M hydrochloride solutions was added to respective flasks. The solution was heated at 50-60 ^{0}C for 5 min and allowed to cool at room temperature. The volume was made up with methanol, shaken well for proper mixing and the absorbance was measured at 256 nm.

2.2.1.4. Stability

Stability of the solutions of ARM, used for preparing the calibration curves, was ascertained by observing for changes in the absorbance at their respective analytical wavelengths over a period of 48 hr.

2.2.1.5. Selectivity

The selectivity of these methods for the estimation of the drug in presence of various tablet excipients such as starch, lactose, microcrystalline cellulose, sodium starch glycolate, talc and magnesium stearate was investigated.

A placebo comprising 63% w/w lactose, 15% w/w starch, 15% w/w microcrystalline cellulose, 4% w/w sodium starch glycolate, 2% w/w talc and 1% w/w magnesium stearate was prepared. A 1:1 blend of drug and placebo was prepared. Drug was then extracted from this blend using methanol. This methanol extract was filtered through 589 Blue ribbon S & S filter paper circles of diameter 125 mm (Schliecher and Schuell, GmbH, Germany) and absorbance of the filtrates, appropriately diluted, was measured at 256 nm.

2.2.1.6. Precision and accuracy

In order to determine precision and accuracy of the methods, solutions containing known amounts of pure drug were prepared and analyzed in three replicates. Drug was also extracted from a blend of placebo and known amount of drug were analyzed in three replicates.

2.2.2. Results and discussion

ARM in methanol after acid treatment yields a characteristic curve when scanned in the ultraviolet wavelength range between 200 and 400 nm. The scan shows absorption maxima at 205 and 256 nm, as shown in Figure 2.2.

Though the absorptivity at 205 nm was high it was not selected as analytical wavelength due to its proximity to the lower limit of the UV transparent region of methanol. The optical characteristics for the solution of ARM are given in Table 2.1. The absorptivity at 256 nm was found to be 10,234.43 l mol−1 cm−1 and hence was selected for further investigations. Correlation coefficient was found to be 0.9954, signifying that a linear relation existed between absorbance and concentration of the drug.

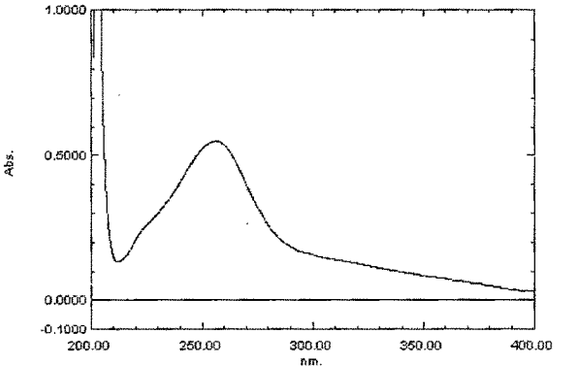

Figure 2.2 Wavelengths scan for ARM (10 mcg/ml) in methanol

Table 2.1. Optical characteristics for ARM in methanol

Characteristic	Value in methanol
Absorption maxima (nm)	205, 256[a]
Beer's law limits (mcg/mL)[b]	6-22 mcg/ml
Apparent molar Absorptivity[b] (l mol-1 cm-1)	10,234.43

[a] Analytical wavelength for proposed method. [b] At analytical wavelength

Beer's law was found to be obeyed between 6 and 22 mcg/ml. Regression analysis was performed on the experimental data. The raw data along with the results of regression analysis (method of least squares) is shown in Table 2.2. Regression equation for ARM was $y=0.0345x+ 0.0081$.

Table 2.2 Mean absorbance values, regressed values and statistical data of the calibration curve for the estimation of ARM in methanol using 5M hydrochloric acid.

Concentration (mcg/mL)	Mean ABS[a] (±S.E.)	Regressed values[b]
6	0.212 ± 0.011	0.215
8	0.277 ± 0.014	0.284
10	0.343 ± 0.025	0.353
12	0.438 ± 0.017	0.422
14	0.519 ± 0.029	0.491
16	0.554 ± 0.023	0.560
18	0.620 ± 0.029	0.629
20	0.694 ± 0.030	0.698
22	0.768 ± 0.022	0.767
24	0.834 ± 0.020	0.836

Regression equation statistical data. Intercept (a) = $0.81x10^{-2}$; slope (b) = $3.45x10^{-2}$; correlation coefficient = 0.9954. [a] Mean of three values. [b] Using regression equation

The stability of ARM in methanol with different molar hydrochloric acid (1M-5M) pretreatment was monitored over a period of 48 hr. Absorbance of ARM showed variation with different molar hydrochloric acid. The variation decreases with increase in molarity of the hydrochloric acid. This has been confirmed by calculating SD and %RSD (Table 2.3). This may be because of the incomplete conversion of ARM to UV detectable compound. ANOVA studies of the mean absorbance values of the solutions of different concentrations at pre-selected time intervals indicated that no significant difference existed between the readings with 5M hydrochloric acid. Thus, ARM

is stable over a period of 48 hr in methanol when pretreated with 5M hydrochloric acid.

Table 2.3 Effect of different molar concentration of hydrochloric acid on standard deviation and % RSD

	1M	2M	3M	4M	5M
SD	0.100	0.199	0.145	0.057	0.004
% RSD	93.62	56.20	24.50	7.74	0.50

In order to determine the precision and accuracy of the methods, known amount of pure drug were subjected to recovery studies in triplicate. Table 2.4 summarizes the results of these investigations. Accuracy of each method was ascertained by using the '*t*' test at each level.

The computed '*t*' values at 10, 15 and 20 mg are 2.532, 0.473 and 2.645, respectively. These values are lower than the tabulated 't' value of 4.30 ($P <$ 0.05) indicating no significant difference between the added and the estimated quantity.

Estimation of ARM was carried out in the presence of various commonly used tablet excipients at the levels they are normally used. From Table 2.4, it can be seen that there is no significant difference between the amount added to the placebo and the amount recovered (computed 't'=1.447, calculated t=4.30 at two degrees of freedom) $P < 0.05$.

In order to determine the precision and accuracy of the methods, known amounts of pure drug were subjected to recovery studies in triplicate. Table 2.4 summarizes the results of these investigations. Accuracy of each method was ascertained by using the '*t*' test at each level.

The computed '*t*' values at 10, 15 and 20 mg are 2.532, 0.473 and 2.645, respectively. These values are lower than the tabulated 't' value of 4.30 ($P <$ 0.05) indicating no significant difference between the added and the estimated quantity.

Estimation of ARM was carried out in the presence of various commonly used tablet excipients at the levels they are normally used. From Table 2.4, it can

be seen that there is no significant difference between the amount added to the placebo and the amount recovered (computed 't'=1.447, calculated t=4.30 at two degrees of freedom) $P < 0.05$.

Table 2.4 Evaluation of accuracy and precision of the proposed method

Exact amount of drug added (mg)	Individual amounts of drug found (mg); mean (S.D.)[a]	Coefficient of variation (CV)	Standard Error (SE)	Confidence limits
(A) Pure drug				
10.17	9.94	0.533	0.030	9.92 ± 0.132
10.24	9.86			
10.03	9.96			
	9.92 (0.052)			
15.06	15.04	1.552	0.135	15.11 ± 0.583
14.92	15.38			
15.10	14.93			
	15.11 (0.234)			
20.01	19.87	1.281	0.144	19.59 ± 0.624
20.30	19.53			
19.94	19.38			
	19.59 (0.251)			
(B) Drug with placebo (1:1)				
11.00	11.67	4.530	0.290	11.09 ± 1.249
10.60	10.81			
10.80	10.79			
	11.09 (0.509)			

[a] *n=3.* [b] *confidence limits at P=0.95 and two degree of freedom.*

Thus, excipients like starch, lactose, microcrystalline cellulose, sodium starch glycolate, talc and magnesium stearate did not interfere with the estimation.

Also, the filtration medium did not absorb the drug to any extent. Thus the present method has the advantages of simplicity, stability, sensitivity, reproducibility and accuracy.

The non-interference of commonly used excipients makes the method suitable for the estimation of the ARM in formulations and hence further used for the routine quality control of ARM formulations.

2.3. Mefloquine hydrochloride (MFL)

Various methods have been reported for MFL determination, out of which high pressure liquid chromatography (Bergqvist et al., 1993; Bergqvist et al., 1994; Wallen et al., 1994), gas liquid chromatography (Nakagawa et al., 1979; Schwartz and Ranalder, 1981), gas chromatography (Dadgar et al., 1985; Heizmann and Geschke, 1984; Neal et al., 1994), thin layer chromatography (Betschart et al., 1991), fluorimetry (Fidanza and Aaron, 1989) and capillary zone electrophoresis (Fanali and Camera, 1996) are some important methods. Even though an ultraviolet spectroscopy method (Lim, 1985) has been suggested as a method of analysis, no other analytical parameter except absorptivity has so far been reported.

In present study, a simple ultraviolet spectroscopic method for the determination of MFL in methanol has been reported. Analytical parameters have also been established.

2.3.1. Experimental

2.3.1.1. Apparatus

Apparatus was used as mentioned in 2.2.1.1.

2.3.1.2. Materials

Mefloquine hydrochloride (MFL) was kindly gifted by Ajanta Pharma Ltd., Mumbai. Methanol was purchased from SD Fine chemicals, Bhoisar and used as received. Stock solution of MFL (100 mcg/ml) was prepared by dissolving 10 mg of MFL in 100 ml of methanol.

2.3.1.3. Preparation of calibration curve

Suitable aliquots (1.5-5.5 mL) of the stock solution were pipetted into 10 ml volumetric flasks. The volume was made up with methanol, shaken well for proper mixing and the absorbance was measured at 284 nm.

2.3.1.4. Stability

Stability study was carried out as mentioned in 2.2.1.4.

2.3.1.5. Selectivity

Selectivity study was carried out as mentioned in 2.2.1.5.

2.3.1.6. Precision and accuracy

Precision and accuracy studies were carried out as mentioned in 2.2.1.6.

2.3.2. Result and discussion

MFL in methanol yields a characteristic curve when scanned in the ultraviolet wavelength range between 200 and 400 nm. MFL in methanol yields a characteristic curve with absorption maxima at 222, 284 and 317 nm, as shown in Figure 2.3.

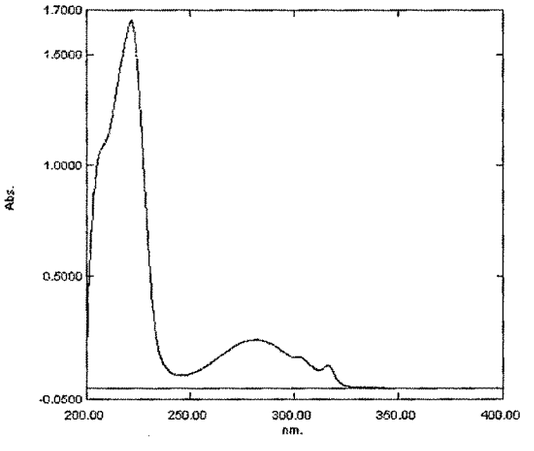

Figure 2.3 Wavelengths scan for MFL (15 mcg/ml) in methanol

Though the absorptivity at 205 nm was high, it was not selected as analytical wavelength, due to its proximity to the lower limit of the UV transparent region of methanol. The optical characteristics for the solution of MFL are given in Table 2.5.

Table 2.5 Optical characteristics for MFL in methanol

Characteristic	Value in methanol
Absorption maxima (nm)	222, 284[a], 317
Beer's law limits (mcg/mL)[b]	15-55 mcg/ml
Apparent molar Absorptivity[b] (l mol-1 cm-1)	5890.16

[a] Analytical wavelength for proposed method. [b] At analytical wavelength

The absorptivity at 284 nm was found to be 5890.16 l mol−1 cm−1 more suitable and hence was selected for further investigations. Correlation coefficient was found to be 0.9994, signifying that a linear relation existed between absorbance and concentration of the drug.

Beer's law was found to be obeyed between 15 and 55 mcg/mL. Regression analysis was performed on the experimental data. The raw data along with the results of regression analysis (method of least squares) is shown in Tables 2.6. Regression equation for MFL was y=0.0148x- 0.0153.

Table 2.6 Mean absorbance values, regressed values and statistical data of the calibration curve for the estimation of MFL in methanol

Concentration (mcg/mL)	Mean ABS[a] (±S.E.)	Regressed values[b]
15	0.207 ± 0.023	0.207
20	0.284 ± 0.017	0.281
25	0.353 ± 0.019	0.355
30	0.434 ± 0.027	0.429
35	0.478 ± 0.014	0.503
40	0.545 ± 0.021	0.577
45	0.657 ± 0.015	0.651
50	0.731 ± 0.024	0.725
55	0.807 ± 0.017	0.799

Regression equation statistical data. Intercept (a) = 1.48x10^{-2}; slope (b) = -1.53x10^{-2}; correlation coefficient = 0.9994. [a] Mean of three values. [b] Using regression equation

The stability of MFL in methanol was monitored over a period of 48 hr. ANOVA studies of the mean absorbance values of the solutions of different concentrations at pre-selected time intervals indicated that no significant difference existed between the readings. Thus, MFL is stable over a period of 48 hr in methanol.

Table 2.7 Evaluation of accuracy and precision of the proposed method

Exact amount of drug added (mg)	Individual amounts of drug found (mg); mean (S.D.)[a]	Coefficient of variation (CV)	Standard Error (SE)	Confidence limits
(A) Pure drug				
20.10	19.97	0.738	0.086	20.10 ± 0.369
20.02	20.06			
20.40	20.26			
	20.10 (0.148)			
25.17	25.09	0.179	0.026	25.13 ± 0.112
25.08	25.18			
25.22	25.13			
	25.13 (0.045)			
30.12	30.17	0.595	0.104	30.36 ± 0.450
30.68	30.53			
30.40	30.38			
	30.36 (0.180)			
(B) Drug with placebo (1:1)				
10.05	9.63	6.741	0.401	10.31 ± 1.727
10.11	10.29			
11.26	11.02			
	10.31 (0.695)			

[a] n=3. [b] confidence limits at P=0.95 and two degree of freedom.

In order to determine the precision and accuracy of the methods, known amount of pure drug were subjected to recovery studies in triplicate. Table 2.7 summarizes the results of these investigations. Accuracy of each method was ascertained by using the '*t*' test at each level. The computed '*t*' values at 20, 25 and 30 mg are 1.312, 0.377 and 0.682, respectively.

These values are lower than the tabulated 't' value of 4.30 ($P < 0.05$) indicating no significant difference between the added and the estimated quantity.

Estimation of MFL was carried out in the presence of various commonly used tablet excipients at the levels they are normally used. From Table 2.7, it can be seen that there is no significant difference between the amount added to the placebo and the amount recovered (computed 't'=0.9, calculated t=4.30 at two degrees of freedom) $P < 0.05$.

Thus, excipients like starch, lactose, microcrystalline cellulose, sodium starch glycollate, talc and magnesium stearate did not interfere with the estimation. Also, the filtration medium did not absorb the drug to any extent.

Thus the present method has the advantages of simplicity, stability, sensitivity, reproducibility and accuracy. The non-interference of commonly used excipients makes the method suitable for the estimation of the MFL in formulations and hence further used for the routine quality control of MFL formulations.

2.4. Primaquine Phosphate (PRM)

PRM is official in British Pharmacopoeia 2007 and the official method of estimation is potentiometric titration (British Pharmacopoeia, 2007). Many methods for estimation of PRM are reported, including a HPLC method for the separation and identification of the oxidation products of PRM as well as determination of PRM in plasma and blood cells (Dua et al., 1996; Dua et al., 1998; Guentert et al., 1979; Stensrud et al., 2000). Dean et al. have used electrochemical detection for PRM (Dean et al., 1994) and Endoh et al. have determined primaquine in calf plasma by EC detection (Endoh et al., 1992). Others have developed HPLC methods for PRM using various detection techniques (Clark et al., 1984; Nora et al., 1984; Parkhurst et al., 1984;

Ward et al., 1984). High performance thin layer chromatographic (HPTLC) and HPLC methods for the analysis of PRM have been described (Ni et al., 1992).

In present study, a simple, sensitive and reproducible ultraviolet spectrophotometric method for the determination of PRM in distilled water has been reported. Analytical parameters for the method have also been established.

2.4.1. Experimental

2.4.1.1. Apparatus

Apparatus was used as mentioned in 2.2.1.1.

2.4.1.2. Materials

Primaquine phosphate (PRM) was kindly gifted by Unicure Remedies Pvt. Ltd.; Baroda. Stock solution of PRM (100 mcg/ml) was prepared by dissolving 10 mg of PRM in 100 ml of distilled water.

2.4.1.3. Preparation of calibration curve

Suitable aliquots (0.4-1.4 mL) of the stock solution were pipetted into 10 ml volumetric flasks. The volume was made up with distilled water, shaken well for proper mixing and the absorbance was measured at 259 nm.

2.4.1.4. Stability

Stability study was carried out as mentioned in 2.2.1.4.

2.4.1.5. Selectivity

Selectivity study was carried out as mentioned in 2.2.1.5.

2.4.1.6. Precision and accuracy

Precision and accuracy studies were carried out as mentioned in 2.2.1.6.

2.4.2. Results and discussion

PRM in distilled water yields a characteristic curve when scanned in the ultraviolet wavelength range between 200 and 400 nm. PRM in distilled water yields a characteristic curve with absorption maxima at 206, 259 and 357 nm, as shown in Figure 2.4. The optical characteristics for the solution of PRM are given in Table 2.8. The absorptivity at 259 nm was found to be 25,815.51 l

mol−1 cm−1 and hence was selected for further investigations. Correlation coefficient was found to be 0.9987, signifying that a linear relation existed between absorbance and concentration of the drug.

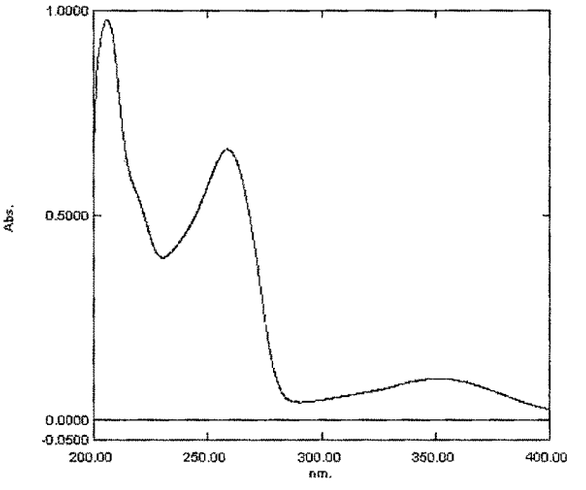

Figure 2.4 Wavelengths scan for PRM (12mcg/ml) in distilled water

Table 2.8. Optical characteristics for PRM in distilled water

Characteristic	Value in distilled water
Absorption maxima (nm)	206, 259 [a], 351
Beer's law limits (mcg/mL)[b]	4-14 mcg/ml
Apparent molar Absorptivity[b] (l mol-1 cm-1)	25,815.51

[a] *Analytical wavelength for proposed method.* [b] *At analytical wavelength*

Beer's law was found to be obeyed between 4 and 14 mcg/mL. Regression analysis was performed on the experimental data. The raw data along with the results of regression analysis (method of least squares) is shown in Tables 2.9. Regression equation for PRM was $y=0.0547x+ 0.0101$.

The stability of PRM in distilled water was monitored over a period of 48 hr. ANOVA studies of the mean absorbance values of the solutions of different concentrations at pre-selected time intervals indicated that no significant difference existed between the readings. Thus, PRM is stable over a period of 48 hr in distilled water.

In order to determine the precision and accuracy of the methods, known amount of pure drug were subjected to recovery studies in triplicate. Table 2.10 summarizes the results of these investigations.

Table 2.9. Mean absorbance values, regressed values and statistical data of the calibration curve for the estimation of PRM in distilled water.

Concentration (mcg/mL)	Mean ABS[a] (±S.E.)	Regressed values[b]
4	0.220 ± 0.016	0.229
6	0.342 ± 0.014	0.338
8	0.452 ± 0.029	0.448
10	0.567 ± 0.018	0.557
12	0.661 ± 0.021	0.667
14	0.771 ± 0.011	0.776

Regression equation statistical data. Intercept (a) = $5.47x10^{-2}$; slope (b) = $1.01x10^{-2}$; correlation coefficient = 0.9987. [a] Mean of three values. [b] Using regression equation

Accuracy of each method was ascertained by using the '*t*' test at each level. The computed '*t*' values at 5, 7.5 and 10 mg are 0.458, 1.357 and 2.523, respectively. These values are lower than the tabulated 't' value of 4.30 ($P < 0.05$) indicating no significant difference between the added and the estimated quantity.

Estimation of PRM was carried out in the presence of various commonly used tablet excipients at the levels they are normally used. From Table 2.10, it can be seen that there is no significant difference between the amount added to the placebo and the amount recovered (computed 't'=1.058, calculated t=4.30 at two degrees of freedom) $P < 0.05$. Thus, excipients like starch, lactose, microcrystalline cellulose, sodium starch glycolate, talc and magnesium stearate did not interfere with the estimation. Also, the filtration medium did not absorb the drug to any extent.

Table 2.10 Evaluation of accuracy and precision of the proposed method

Exact amount of drug added (mg)	Individual amounts of drug found (mg); mean (S.D.)[a]	Coefficient of variation (CV)	Standard Error (SE)	Confidence limits
(A) Pure drug				
5.10	5.14	0.815	0.024	5.10 ± 0.103
4.94	5.06			
5.20	5.12			
	5.10 (0.041)			
7.58	7.43	0.962	0.041	7.49 ± 0.180
7.64	7.57			
7.45	7.47			
	7.49 (0.072)			
10.20	10.21	0.995	0.058	10.15 ± 0.251
9.98	10.04			
10.18	10.22			
	10.15 (0.101)			
(B) Drug with placebo (1:1)				
10.07	9.93	5.484	0.328	10.38 ± 1.415
10.11	10.19			
11.26	11.02			
	10.38 (0.569)			

[a] *n=3.* [b] *confidence limits at P=0.95 and two degree of freedom.*

Thus the present method has the advantages of simplicity, stability, sensitivity, reproducibility and accuracy. The non-interference of commonly used excipients makes the method suitable for the estimation of the PRM in formulations and hence further used for the routine quality control of PRM formulations.

2.5. Summary

Characteristics	ARM	MFL	PRM
Absorption maxima (nm)	205, 256[a]	222, 284[a], 317	206, 259[a], 351
Beer's law limits (mcg/mL)[b]	6-22 mcg/ml	15-55 mcg/ml	4-14 mcg/ml
Apparent molar Absorptivity[b] (l mol-1 cm-1)	10,234.43	5890.16	25,815.51

[a] *Analytical wavelength for proposed method.* [b] *At analytical wavelength*

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