Results and Discussion

I- Spectrophotometric determination of cefotaxime (CEFO)

Preliminary investigations revealed that cefotaxime reacts directly with each of the reagents used [eosin bluish (EB), eosin yellowish (EY), bromocrysol purple (BCP) and orange G (OG),] to produce soluble ion-associate complexes. This was observed from the decrease in the absorption spectra of each reagent when scanned with the drug using buffer as a blank.

The optimum conditions favoring the formation of the ion – pair complexes were studies considering the following effects:

1- Effect of pH

Various aqueous buffers (acetate, borate, phosphate, and universal buffers) with different pH values were tested to establish the best buffer media. Universal buffer solutions at pH 2.04 -12.06 gave the best results. High and constant absorbance values were obtained at pH 3.30, 3.30,12.0 and 12.30 by using EB, EY, BCP and OG, respectively; therefore, all subsequent studies were carried out at these pH values at which the results were highly reproducible. Moreover, the optimum volume of the universal buffer solution was examined and found to be 3.0 ml in a total volume of 10 ml. Figures (1 - 4) show the effect of pH on the absorption spectra of cefatoxime with EB, EY, BCP and OG, respectively.

2- Determination of λ_{max} of complex species:

To determine the wavelength at which ion–pair complex species possesses maximum absorbance (λ_{max}), the following spectra were recorded:

A- Spectrum of pure reagent; 2.0 ml ($1x10^{-3}$ M) at the optimum pH value using the same buffer as a blank.

B- Spectrum of solution mixture of reagent (A) and drug (1.0 ml of $1x10^{-3}$ M) at the optimum pH value using the same buffer as a blank.

C- Spectrum of solution (B) against (A) as a blank.

The absorption spectra are shown in Fig.'s (5-8), from which the values of λ_{max} for each complex were determined and cited in Table (1). These optimal wavelengths are chosen for further investigation.

3- Effect of time and temperature:

The effect of time on complex formation was studied by measuring the absorbance of the complexes at optimum pH against a blank solution of the same pH at various time intervals. Also, the effect of temperature was studied for the same solution by incubating the sample and blank in water bath at different temperatures (25 – 50^{o}C). The absorbance was measured after cooling to room temperature.

The experiments showed that complexes are formed within few minutes (5 minutes) after mixing drug with reagent in the buffered media and remain stable for about 6 hours. It was found also that, increasing the temperature up to 50^{o}C has slight effect on the absorbance, while boiling destroys the complex.

4- Effect of sequence of addition:

The effect of sequence of addition on ion– pair complex formation was studied by measuring the absorbance of solutions prepared by different sequences of addition against a blank solution prepared in the same manner. Experiments showed that the sequence of reagent – buffer – drug is the best one. So, it seems that the buffer action must change the reagent to the anionic form [R^-] making it capable to interact with the drug in the cationic form [D^+] to form the ion – pair association complex [R^-][D^+].

5- Effect of reagent concentration:

To study the effect of reagent concentration on the complex formation between cefotaxime and different reagents under study, the concentration of the drug was kept constant (1.0 ml of $1x10^{-3}$ M) while that of the reagent was varied regularly (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0ml of $1x10^{-3}$ M). The resulted spectra showed that 2.0 ml of each reagent is sufficient for complete complexation.

6- Effect of buffer volume:

The effect of buffer volume on the reaction between the drug solution and the reagents was investigated by adding different buffer volumes of the selected pH (1.0, 2.0,…. 4.0 ml) to fixed concentrations of drug and reagent (1.0 ml of $1x10^{-3}$ M drug solution+ 2.0 ml of $1x10^{-3}$M reagent solution) and the volume was completed to 10.0 ml with bidistilled water. The absorbance of each sample solution was measured against a blank solution of reagent at the same pH. The optimum volume of buffer was found to be 3.0 ml chosen from the highest absorbance value. This volume is used for further studies.

7- Stoichiometry of complexes:

The molecular structure of the formed colored complex was determined by two spectrophotometric methods (mole ratio and continuous variation methods). The data obtained from these methods were used to calculate the stability constants of the colored products.

7-1 The continuous variation method

In the present work, the modification of Job's [56] continuous variation method performed by Vosburgh *et.al*.[57] was used to investigate the stoichiometry of the complex formed between drug and reagent. A series of solutions were prepared by mixing equimolar solution of the reagent and drug in different preparations keeping the total molar concentration constant ($2.0x10^{-3}$ M) in the presence of 3.0 ml of the selected buffer. A plot of the absorbance of the solution at the maximum wavelength against the mole fraction of the drug gives the molar ratio of the most stable formed complex. Experimental results revealed that the complexes formed have 1:1 stoichiometric ratio.

7-2 The mole ratio method

In the molar ratio method described by Yoe and Jones [55], the concentration of the drug was kept constant at (0.5 ml of $1x10^{-3}$ M) while that of the reagent was varied (0.2, …..2.4 ml of $1x10^{-3}$ M), 3.0 ml of the selected buffer solution is added and the volume is completed to 10.0ml with bidistilled water. The absorbance of the sample solution was measured against reagent blank at the maximum wavelength. The absorbance values were then plotted against the molar ratio [reagent/drug]. The inflection of the straight line obtained shows the molar ratio of 1:1 (drug : reagent) products. Results obtained from mole ratio and continuous variation methods are in agreement with each others.

8- Stability constants of the complexes:

The stability constants of the formed complex were calculated using the data obtained from the mole ratio and continuous variation methods applying the equation of Yeo and Jones [55] as modified by Issa *et al* [58].

$$K_n = \frac{(A/A_{max})}{[1-(A/A_{max})]^{n+1} C_R^n n^2}$$

where:

A : the absorbance at concentration C_R

A_{max} : the maximum absorbance value

n : the stoichiometric ratio of the complex

K_n : the stability constant

Log stability constants calculated from mole ratio and continuous variation methods are listed in Table (1).

9- Validity to Beer's law:

Under optimum conditions, mentioned above, different concentrations of cefotaxime (µg/ml) were transferred to 10.0 ml measuring flask containing 2.0 ml ($1x10^{-3}$ M) of reagent and 3.0 ml of buffer solution of the optimum pH. The volume was completed to the mark by bidistilled water and the content of the flask was mixed well. The absorbance was measured at optimum λ_{max}, then plotted against drug concentration [D] as shown in Fig.'s (9 - 12).

Limits of Beer's law, the molar absorptivity (ε; $lmol^{-1}cm^{-1}$) and Sandell sensitivity[59] values were calculated and listed in Table (1). Regression analysis for the results were as carried out using least square method. In all cases, Beer's law plots were linear with very small intercepts (-0.0084 – 0.0113) and good correlation coefficients (0.9884 - 0.9991).

For more accurate analysis, Ringbom [60] optimum concentration range was determined by calculating the percent transmittance (%T) from the following equation:

$$\%T = 10^{-A} x100$$

where A is the absorbance of the complex.

By plotting logarithm of drug concentration; log[D] in µg/ml against %T as in Fig.'s (13 -16), the linear portion of the sigmoid curve gave the accurate range of analysis. Results are listed in Table (1).

10- Accuracy and precision:

To determine the accuracy and precision of the proposed method; solutions of certain concentration (within the concentration range optained fom Beer's law and Ringbom methods) were prepared and analyzed in six replicates. The percentage relative standard deviation (% RSD) did not exceed 0.552 % indicating high accuracy and reproducibility of the proposed method (Table 2). The percentage recovery and the range of error (%) at 95% confidence level indicate the reasonable accuracy and precision. The results are considered as very satisfactory for the examined concentration levels.

11- Analytical applications:

The validity of the proposed procedure was tested for determination of cefotaxime in pharmaceutical preparations manufactured in local companies such as cefotaxime and claforan ampoules (containing 1.0 and 0.5 mg respectively, of cefotaxime per 2 ml). The standard additions method was used, in which variable amounts of the pure drug were added to the previously analyzed portion of the pharmaceutical formulations. The data, *c.f*. Table (3), showed that the proposed method is highly sensitive; therefore, it could be used easily for routine determination of CEFO in its pure form and in its pharmaceutical formulations.

The performance of the proposed method was judged further by the Student's t-test for accuracy and F-test for precision. At 95% confidence level, the calculated t- and F-values did not exceed the tabulated values (t = 2.57 and F = 5.05) suggesting that the method is accurate and precise as the reference method.

II- Spectrophotometric determination of ceftazidime

Preliminary investigations revealed that fourtum reacts readily with each of the reagents used [eosin bluish (EB), orange G (OG), bromocrysol purple (BCP) and arsenazo I (ARZ I),] to produce soluble ion-associate complexes. The importance of utility of such reagents stems from several points, namely, high selectivity of the reactions, high solubility of the colored complexes, exact stoichiometric composition and stability of the colored complexes.

To investigate the optimum conditions favoring the formation of the colored complexes, the following points were extensively studied:

1- Effect of pH

2- Selection of the suitable wavelength at which complex species maximally absorb.

3- Effect of time and temperature.

4- Effect of sequence of addition.

5- Effect of reagent concentration.

6- Effect of buffer volume.

1- The effect of pH on the ion – pair complex formation was studied by recording the absorption spectra of series of solutions containing 2.0 ml ($1.0x10^{-3}$ M) of reagent, 3.0 ml universal buffer solution of the pH range 2.60 – 11.62 and 1.0 ml ($1.0x10^{-3}$ M) of the drug against blank solutions prepared in the same way without drug at the same pH. The absorption spectra are shown in Fig.'s (17 - 20). Inspection of the data gathered from these figures shows that the optimum pH values giving maximum absorbance are 3.35, 7.81, 12.0 and 12.0 for EB, OG, BCP and ARZ I respectively. These values are recommended for subsequent studies.

2- The wavelength at which ion – pair complex species possesses maximum absorbance (λ_{max}), was determined by recording the following spectra:

(A) Spectrum of pure reagent (2.0 ml of $1x10^{-3}$ M) at the optimum pH using the same buffer as a blank.

(B) Spectrum of solution mixture of reagent (A) and drug (1.0 ml of $1x10^{-3}$ M) at the optimum pH value using the same buffer as a blank.

(C) Spectrum of solution (B) against (A) as a blank.

The absorption spectra are shown in Fig.'s (21 - 24), from which the values of λ_{max} for each complex were determined and cited in Table (4). These optimal wavelengths are chosen for further investigation.

3- Experiment on the effect of time and temperature on complex formation showed that complexes are formed within few minutes (5 minutes) after mixing drug with reagent in the buffered media and remain stable for about 6 hours. It also showed that, increasing the temperature up to $50^{o}C$ has slight effect on the absorbance, while boiling destroys the complex.

4- The effect of sequence of addition on ion – pair complex formation was studied as previously discussed where it was found that the best sequence is reagent–buffer–drug. So, it is clear that the buffer action must change the reagent to the anionic form $[R^{-}]$ making it capable to interact with the drug in the cationic form $[D^{+}]$ to form the ion – pair association complex $[R^{-}][D^{+}]$.

5- The effect of reagent concentration on the complex formation was studied by recording the absorption spectra of series of solutions containing different reagent concentration and constant drug concentration. The resulted spectra showed that 2.0 ml ($1x10^{-3}$ M) of each reagent is sufficient for developing complete complexation.

6- The effect of buffer volume on the reaction between the drug solution and the reagents is investigated as mentioned early. The optimum volume of buffer is found to be 3.0 ml, chosen from the highest absorbance value, and was used for further studies.

Stoichiometryand stability constants of complexes

The molecular structure of the formed colored complex is determined by both mole ratio and continuous variation methods. Investigation of molecular structure of EB, OG, BCP and ARZ I complexes with ceftazidime in the light of the results obtained by the two methods reveals the formation of 1:1 complexes.

The stability constants of the formed complex were calculated using the data obtained from the molar ratio and continuous variation methods. The data listed in Table (4) indicate high stability of the formed complexes.

Validity to Beer's law

The use of EB, OG, BCP and ARZ I as chromophoric reagents for the spectrophotometric determination of ceftazidime is checked by the validity of Beer's law. Series of solutions in which the concentration of each reagent is kept constant (2.0 ml of $1x10^{-3}$ M) while that of the drug is regularly varied, were prepared at the recommended pH. The absorbance was then measured at the corresponding wavelength for each complex and plotted vs concentration of the drug [D; µg/ml], (*c.f.* Fig.'s 25 - 28).

Limits of Beer's law, molar absorptivity (ε=3.43–$6.31x10^{4}$ $lmol^{1}cm^{-1}$) and Sandell sensitivity (0.036 – 0.072 $\mu g/cm^{2}$) values were calculated and listed in Table (4). Regression analysis for the results was also carried out using least square method. In all cases, Beer's law plots were linear with very small intercepts (-0.017 - 0.063) and good correlation coefficients (0.9984 -0.9993).

For more accurate analysis, Ringbom optimum concentration range was determined by plotting logarithm of drug concentration, log[D], in µg/ml against %T as in Fig.'s (29 - 32). The linear portion of the sigmoid curve gave the accurate range of analysis. Results are listed in Table (4).

Accuracy and precision

To determine the accuracy and precision of the proposed method; solutions of certain concentration (within the concentration range optained fom Beer's law and Ringbom methods) were prepared and analyzed in six replicates. The percentage relative standard deviation (% RSD) did not exceed 0.132 % indicating high accuracy and reproducibility of the proposed method (Table 5). The percentage recovery and the range of error (%) at 95 % confidence level indicate the reasonable accuracy and precision. The results are considered as very satisfactory for the examined concentration levels.

Analytical applications:

The validity of the proposed procedure was tested for determination of ceftazidime in two of its pharmaceutical formulations (fourtum and fortaz, containing 1.0 and 1.0 mg of ceftazidime per ampoule). The standard additions method was used, in which variable amounts of the pure drug were added to the previously analyzed portion of the pharmaceutical formulations. The data, *c.f*. Table (6), showed that the proposed method is highly sensitive; therefore, it could be used easily for routine determination of ceftazidime in its pure form and in its pharmaceutical formulations.

The performance of the proposed method was judged further by the Student's t-test for accuracy and F-test for precision. At 95% confidence level, the calculated t- and F-values did not exceed the tabulated values (t = 2.57 and F = 5.05) suggesting that the method is accurate and precise as the reference method.

III- Spectrophotometric determination of cefepime

Preliminary investigations showed that cefepime reacts directly with each of the reagents used [eosin yellowish (EY), eosin bluish (EB), orange G (OG) and arsenazo I (ARZ I)] to produce soluble ion-associate complexes. This was acertained from styding the absorption spectra of each reagent (in ethanol as a solvent) compaired with that of the reagent and cefepime in the same solvent. The decrease in the maximum absorbance in the later case is taken as an evidence for complex formation.

The optimum conditions favoring the formation of the ion – pair complexes between cefepime and the reagents under study were extensively studied taking into consideration the following effects:

1- Effect of pH

The effect of pH on the ion – associate complex formation between cefepime and the four reagents under investigation was studied in universal buffer solutions within the pH range 2.60 – 11.62 as previously mentioned, illustrative spectra are shown in Fig.'s (33 - 36). Careful investigation of these spectra shows that the formed ion – associate complexes absorb maximally at the pH values 3.35, 4.52, 12.30 and 10.21 for EY, EB, OG and ARZ I respectively. These values are recommended for subsequent studies.

2- Determination of λ_{max} of complex species

The maximum wavelength (λ_{max}) at which each ion – pair complex species absorbs was determined, as previously mention, by recording the following spectra:

A- Spectrum of pure reagent; 2.0 ml ($1x10^{-3}$ M) at the optimum pH value using the same buffer as a blank.

B- Spectrum of solution mixture of reagent (A) and drug (1.0 ml of $1x10^{-3}$ M) at the optimum pH value using the same buffer as a blank.

C- Spectrum of solution (B) against (A) as a blank.

The absorption spectra are shown in Fig.'s (37 - 40), from which the values of λ_{max} for each complex were determined and cited in Table (7). These optimal wavelengths are chosen for further investigation.

3- Effect of time and temperature

By measuring the absorbance of the complexes at optimum pH against a blank solution of the same pH at various time intervals, it was found that complexes are formed within few minutes (5 minutes) after mixing drug with reagent in the buffered media and remain stable for about 6 hours.

Also, studying the effect of temperature on complex formation, showed that increasing the temperature up to $50^{o}C$ has slight effect on the absorbance, while boiling destroys the complex.

4- Effect of sequence of addition

Experiments on the effect of sequence of addition showed that the sequence of reagent – buffer – drug is the best one indicating that the buffer solution changes the reagent to the anionic form $[R^{-}]$ making it capable to interact with the drug in the cationic form $[D^{+}]$ to form the ion– pair association complex $[R^{-}][D^{+}]$.

5- Effect of reagent concentration

Studying the effect of reagent concentration on the complex formation between cefepime and reagents under study, showed that 2.0 ml of each reagent is sufficient for complete complexation.

6- Effect of buffer volume

Experiments on the effect of buffer volume on the complex formation, performed as previously mentioned, showed that the optimum volume of buffer is 3.0 ml. This volume is used for further studies.

7- Stoichiometry and stability constant of complexes

The molecular structure of the formed colored complex was determined by two spectrophotometric methods (mole ratio and continuous variation methods). The data obtained from these methods are used to calculate the stability constants of the colored products. The experimental data showed the formation of (1:1) (drug : reagent) ion – pair complex.

The stability constants of the formed complex are calculated using the data obtained from the molar ratio and continuous variation methods. Log stability constants are listed in Table (7). The values obtained revealed that the complexes formed are fairly stable.

8- Validity to Beer's law

Under optimum conditions mentioned in the preceding discussion, different concentrations of cefepime (µg/ml) were transferred into 10.0ml measuring flask containing 2.0 ml ($1x10^{-3}$ M) of reagent and 3.0 ml of buffer solution of the optimum pH. The volume was completed to the mark by bidistilled water and the content of the flask was mixed well. The absorbance was measured at optimum λ_{max}, then plotted against drug concentration [D] as shown in Fig.'s (41 -44)

Limits of Beer's law, the molar absorptivity (ε; $lmol^{-1}cm^{-1}$) and Sandell sensitivity, ($\mu g/cm^2$) values were calculated and listed in Table (7). Regression analysis for the results were as carried out using least square method. In all cases, Beer's law plots were linear with very small intercepts (-0.011 - 0.018) and good correlation coefficients (0.9978 - 0.9998).

For more accurate analysis, Ringbom optimum concentration range was determined by calculating the percent transmittance (%T) from the following equation:

$$\%T = 10^{-A} x100$$

where A is the absorbance of the complex.

By plotting logarithm of drug concentration, log[D] in µg/ml against %T as in Fig.'s (45 - 48), the linear portion of the sigmoid curve gave an accurate range of analysis. Results are listed in Table (7).

9- Accuracy and precision

To determine the accuracy and precision of the proposed method; solutions of certain concentration (within the concentration range obtained from Beer's law and Ringbom methods) were prepared and analyzed in six replicates. The percentage relative standard deviation (% RSD) did not exceed 0.247% indicating high accuracy and reproducibility of the proposed method (Table 8). The percentage recovery and the range of error (%) at 95% confidence level indicate the reasonable accuracy and precision. The results are considered as very satisfactory for the examined concentration levels.