CHAPTER - 4

SPECTROPHOTOMETRIC VALIDATION METHOD
UV-VISIBLE SPECTROPHOTOMETRY

The absorption of light by analytes (by raising an electron or electrons to a higher level, i.e. \( \pi^* \) or \( n^* \)) is due to the presence of chromophores in the molecules. The chromophores are specific portions of molecules that can absorb radiant energy in the visible region. This method of analysis is based on measuring the absorption of monochromatic light by colored compounds in the visible path of the spectrum (380-800 nm). If the analytes are colorless, they are converted into colored compounds by reaction with a suitable chromogenic reagent, which must be stable and have a constant composition and high color intensity. The photometric methods of analysis are based on the Bouger-Lambert-Beer's Law, which establishes, the absorbance by a solution is directly proportional to the concentration of the analyte.

Absorption Spectrum

The absorption spectrum is a graphical representation of the amount of light absorbed by a substance at definite wavelengths. To plot an absorption curve, the values of wavelength (\( \lambda \)) are laid off along the axis of abscissas and the values of the absorbance along the axis of ordinates. A characteristic of an absorption spectrum is a position of the peak (maxima) of light absorption, which is determined by the absorptivity at definite wavelength.

Plot of Beer’s Law

A standard specimen of the analyte is taken and the solutions of this specimen with known concentrations are prepared. The absorbance of all the solutions are measured at a definite wavelength (\( \lambda_{\text{max}} \)) and the calibration curve is plotted by lying off the known concentration along the axis of abscissas and the absorbance corresponding to them along the axis of ordinates. The calibration curve is used to determine the unknown concentration of the analyte in its solution.
The organic drugs, which are having functional groups in their molecules, i.e., reactive atoms or groups of atoms are determined by chemical reactions. The functional groups of these drugs determine the way of analyzing it because they are responsible for the properties of the compound [1-4].

CHROMOGENIC REAGENTS AND REACTIONS USED IN THE PRESENT INVESTIGATION

In the present investigation, [Di (4-amino N-acetyl) phenoxy] methyl ketone (DPMK), 4-acetamidophenyl N’-(sulphanilamide) acetate (APSA), Sodium [2-2, 6-dichlorophenyl [(4-amino N-acetyl) phenyl acetate]-amino] phenyl acetate] (DPAAPA) and 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-[4-[(4-amino N-acetyl) phenoxy carbonyl methyl]1-piperazinyl]-3-quinoline carboxylic acid (PPQC) are newly synthesized paracetamol derivatives and therefore no visible spectrophotometric methods have been reported for these synthesized compounds. The analytically useful, functional groups in the synthesized compounds are used to develop the new spectrophotometric method. Therefore, the synthesized compounds; DPMK, APSA, DPAAPA and PPQC has been selected in the present investigation.

Generally different type of the chemical reactions like nitrosation coupling reaction in the presence of sodium nitrite and hydrochloric acid in alkaline medium, condensation of aromatic amino compound with p-dimethylaminobenzaldehyde, oxidative coupling with potassium ferricyanide in alkaline medium and metal chelate complex formation with Fe (III) metal are used in developing visible spectrophotometric methods.

Nitrosation Reaction

In nitrosation, the nitrosonium electrophile is produced from HNO₂ by reaction of sodium nitrite and hydrochloric acid. The reaction pathway is given below:
Aromatic and alkyl amine, hydroxylamine, thiol compound, alcohol gives the nitrosation reaction. The aromatic amines produce stable nitroso compound as compared to alkyl amine. Primary and tertiary alkyl amine produces mostly unstable nitroso compound. Nitrosation compound produced by oxidation of hydroxylamine, nitroso alcohol and nitroso thiol [5] are prepared by reaction with nitrous acid, but nitroso alcohol compounds are less reactive than nitroso thiol compounds due to the fact that the oxygen atom is less nucleophilic than the sulfur atom. Substitution of a hydrogen atom in the aromatic nucleus by the NO group (nitroso) has been mostly limited to reactants that contain -OH, -OR, -NH₂ and -NHR groups, that is, groups that activate electrophilic aromatic substitution in the ortho and para position by their mesomeric effect.

The general nitrosation reagent is nitrous acid, generated from sodium nitrite and acid in water [6], while other nitrosating agents are N-haloamides and sodium nitrite under phase-transfer conditions [7], Fremy’s salt [8], bis(triphenylphosphine)nitrogen (+1) nitrite [9], Oxyhyponitrite [10], solid acids and sodium nitrite have been used [11]. The amines mostly give the nitrosation reaction [12]. The nitroso compounds produced by the nitrosation reaction absorb radiation in the visible region of the spectrum, but the wavelength and absorptivity depend on the structure of the substrate. The absorptivity is usually large enough to allow concentration of a few micrograms per milliliter to be measured. Nitrosation is used more widely in colorimetric analysis [13].

Several important pharmaceutical compounds are analyzed by nitrosation reaction. They include nitrite ion with acridine red [14], trace
Amounts of nitrite with N, N-bis(2-hydroxypropyl)aniline [15], acetaminophen and salicylamide [13], Oxyphenbutazone [16], chlorocresol [17], mesalamine [18] and paracetamol [19, 20].

**Condensation with Aromatic Aldehydes**

Certain amines condense with various aldehydes in acidic media to give products which are colored and oxidisable. Among the many aldehydes that have been shown to react are p-dimethylaminocinnamaldehyde, p-dimethylaminobenzaldehyde, benzaldehyde, piperonal, vanillin, formaldehyde, salicylaldehyde, paraldehyde, p-acetylaminobenzaldehyde, m- and p-nitrobenzaldehyde, m-aminobenzaldehyde and metaldehyde. The most common oxidant used is atmospheric oxygen, but the process has been hastened by the addition of hydrogen peroxide, nitrites, nitrates, ferric ion and several other metal ion catalysts. Apart from the all aldehydes, best results have been obtained with p-dimethylaminocinnamaldehyde and p-dimethylaminobenzaldehyde [21].

The reaction of p-dimethylaminobenzaldehyde with pyrroles has been used qualitatively and quantitatively [22]. The several spectrophotometric methods have been developed using p-dimethylaminobenzaldehyde, such as, hydralazine [23], metronidazole [24], isoproturon or metoxuron [25], ceftiofur hydrochloride [26], ganciclovir [27], rizatriptan benzoate [28] and Ceftriaxone [29]. In 1944 Werner investigated the reaction with nitrogen compounds and found that in a more dilute aqueous system aromatic compounds react in the presence of mineral acid, provided the –NH₂ group is directly attached to the benzene nucleus. No reaction occurs with (1) aliphatic amines and amino acids (2) N-substituted aromatic amines (3) heterocyclic amino compounds (4) amino derivatives of cycloparaffins.
Oxidation Followed by Complex Formation

Potassium ferricyanide plays a prominent role in the spectrophotometric determination of the organic compounds. Many tertiary amines [30], Morpholine (tetrahydro-1, 4-oxazine), piperidine (hexahydropyridine), piperazine (hexahydropyrazine) [31], double bond containing organic compound such indigo carmine [32] and flow injection-chemiluminescence reaction of terbutaline sulfate [33] are reacted with potassium ferricyanide in alkaline medium. An oxidative product was formed during the reaction with potassium ferricyanide in alkaline medium and converted Fe\(^{+3}\) ion into Fe\(^{+2}\) ion.

\[
\text{Substrate} + 2[\text{Fe(CN)}_6]^{3-} + 2\text{OH}^- \xrightarrow{\text{H}_2\text{O}} \text{Oxidative Product} + 2[\text{Fe(CN)}_6]^{4-}
\]

The reaction of six member ring, alicyclic and aliphatic tertiary amine with potassium ferricyanide in alkaline medium is form oxidative product. In oxidation reaction, alkaline solution was used as catalyst for the formation of oxidative product. During the reaction, carbanion and radical were formed and followed by the oxidation reaction and reduces ferricyanide to ferrocyanide. Here, alkaline solution of sodium hydroxide was used rather than other alkaline solution (KOH) because of the salvation number of sodium ion is higher than others ions (K\(^+\), NH\(_4^+\)). The potassium ferricyanide reagent is also capable to rupture the C=C double bonds present in indigo carmine in alkaline medium and ferricyanide reduces to ferrocyanide.

Chelate Formation by Ferric Sulphate

Ions or molecules bind with certain metal ions forms a chelate complex. During the chelate formation, polydentate (multiple bonded) ligand forms two or more co-ordination bonds with a metal which shows the affinity of chelating ligands towards metal ion. The chelating methods were often used for the spectrophotometric determination of drugs.
Ferric (III) ion is prominent metal ion used in the chelate formation of various drugs such as, Piroxicam [34], meconic acid as opium [35], acetohydroxamic acid [36] and Transferrin [37, 38]. Based on its chelate formation tendency, ferric (III) was suggested in the estimation of drugs by spectrophotometric method.

GENERAL METHODOLOGY AND METHOD VALIDATION
PARAMETERS FOR THE DEVELOPMENT OF NEW UV-VISIBLE SPECTROPHOTOMETRIC METHODS

DEVELOPMENT OF A METHOD

In developing a quantitative method for determining an unknown concentration of a given substance by absorption spectrophotometry, the first step will be the selection of analytical wavelength at which absorption measurements are made. The analytical wavelength can be chosen either from literature or experimentally by means of a scanning spectrum in the UV-Visible region. In order to enhance the sensitivity of the method and signal to noise ratio, the wavelength of maximum absorbance is chosen as analytical wavelength.

After selection of the analytical wavelength, the color developing reagent and the absorbing product must be stable for a considerable period of time. Always the preparation of standards and unknowns should be on a definite time schedule.

OPTIMIZATION OF ANALYTICAL METHOD [39]

In the present investigation, the basis of the spectrophotometric method are (a) diazotization and coupling (b) condensation with p-dimethylaminobenzaldehyde (c) oxidative complex formation with potassium ferricyanide (d) chelate complex formation with ferric sulphate. In each method, the absorbance of the colored species is measured. The sensitivity of the method, rate of color formation and stability of colored species are affected by the concentration of the
reagent in the solution. The nature of the solvent, the temperature, the pH of the medium, order of addition of reactants and reaction time also affect the above parameters. For simple system having no interaction between variables, the one variable at a time (OVAT) strategy appears to be simple, efficient and effective to establish the optimum conditions. The OVAT approach requires all variables, but one to be held constant while a univariate search is carried out on the variable of interest. The details of fixing optimum conditions in different procedures used in the present investigations are furnished in subsequent chapters.

**METHOD VALIDATION**

Typical validation parameters are:

- Linearity
- Range
- Limit of detection (LOD)
- Limit of quantification (LOQ)
- Precision (repeatability and reproducibility)
- Accuracy
- Recovery
- Specificity
- Robustness
- Stability

The validation of analytical procedures (methodology) is followed according to International Conference on Harmonisation (ICH) guidelines (ICH Topic Q2B, 1996) [40]. The validation of analytical methods must meet the following parameters:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity</td>
<td>$r$ should be between 0.995 and 1.</td>
</tr>
<tr>
<td>Precision</td>
<td>RSD should not be more than 2%</td>
</tr>
<tr>
<td>Accuracy</td>
<td>% recovery should be between 95 to 105%</td>
</tr>
<tr>
<td>Specificity</td>
<td>No interference with the placebo</td>
</tr>
</tbody>
</table>

Where, RSD is relative standard deviation, $r$ is correlation coefficient.
Calibration

Calibration is one of the most important steps in drug analysis. The calibration function can be obtained by fitting an adequate mathematical model through the experimental data. The most convenient calibration function is linear; passing through the origin and is applicable over a wide dynamic range. For the majority of analytical techniques the analyst used the calibration equation.

\[ Y = a + bX \]  \hspace{1cm} (4.1)

In calibration, univariate regression is applied, which means that all observations are dependent upon a single variable “X”.

The Method of Least Squares

Least squares regression analysis [41] is used to describe the relationship between signal and concentration. All models describing the relationship between response (Y) and concentration (X) can be represented by the general function.

\[ Y = f (X, a_1, b_1, ............b_m) \]

Where, \( a_1, b_1, ............b_m \) are the parameters of the function.

We adopt the convention that the “X” values related to the controlled or independent variable and the “Y” values related to the dependent variable. It means that “X” values have no error. The values of the unknown parameter \( a_1, b_1, ............b_m \) must be estimated in such a way that the model fits the experimental data as far as possible. The true relationship between X and Y is considered to be given by a straight line. The relationship between each observation pair \( (X_i, Y_i) \) can be represented as

\[ Y_i = a + \beta X_i + e_i \]

The signal \( Y_i \) is composed of a deterministic component predicted by linear model and a random component \( e_i \). One must now find the estimates ‘a’ and ‘b’ of the true values and \( a \) and \( \beta \) which are constants. This is done by calculating values of ‘a’ and ‘b’ for which \( \sum e_i^2 \) is minimal. The component \( e_i \) represent the differences between the observed \( Y_i \) and
values and predicted $Y_i$ value by the model. The $e_i$ is called the residuals, ‘a’ and ‘b’ is the intercept and slope respectively. The equations given for the slope and intercept of the line are as follows.

\[
\text{Slope (b)} = \frac{n\sum X_i Y_i - \sum X_i \sum Y_i}{n\sum X_i^2 - [\sum X_i]^2} \tag{4.2}
\]

\[
\text{Intercept (a)} = \frac{\sum Y_i \sum X_i^2 - \sum X_i \sum X_i Y_i}{n\sum X_i^2 - [\sum X_i]^2} \tag{4.3}
\]

**Correlation Coefficient (r)**

The correlation coefficient $r \ (x, y)$ is more useful to express the relationship of the chosen scale. To obtain a correlation coefficient, the covariance is divided by the product of the standard deviation of $X$ and $Y$.

\[
\begin{align*}
    r &= \frac{\sum_{i=1}^{n} (X_i - \bar{X})(Y_i - \bar{Y})}{(n - 1) \left[ \sum_{i=1}^{n} (X_i - \bar{X})^2 \right]^{1/2} \left[ \sum_{i=1}^{n} (Y_i - \bar{Y})^2 \right]^{1/2}} \\
    &= \frac{\sum_{i=1}^{n} (X_i - \bar{X})(Y_i - \bar{Y})}{(n - 1) \left[ \sum_{i=1}^{n} (X_i - \bar{X})^2 \sum_{i=1}^{n} (Y_i - \bar{Y})^2 \right]^{1/2}} \tag{4.4}
\end{align*}
\]

Where, sigma $X$ and $Y$ are the arithmetic means of $X$ and $Y$ respectively.

**Linearity and Sensitivity of the Method**

Knowledge of the sensitivity of the color is important and the following terms are commonly employed for expressing the sensitivity. According to the Beer’s law

\[
A = \log \frac{\text{Intensity of incident light}}{\text{Intensity of transmitted light}} \tag{4.5}
\]

\[
A = \varepsilon \ C \ t \tag{4.6}
\]

The absorbance ($A$) is proportional to the concentration ($C$) of the absorbing species if absorptivity ($\varepsilon$) and thickness of the medium ($t$) are constant. When ‘$C$’ is a concentration in mole per liter and the constant is called the molar absorptivity. Beer’s law limits and $\varepsilon_{\text{max}}$ values are
expressed as $\mu g \text{ mL}^{-1}$ and mole cm$^{-1}$ respectively. Sandell’s sensitivity [42] refers to the number of $\mu g$ of the drug determined, converted to the colored product, which in a column of cross section 1 cm$^2$ and shows an absorbance of 0.001 (expressed as $\mu g \text{ cm}^{-2}$).

The Limit of Detection (LOD) and the Limit of Quantification (LOQ)

The limit of detection (LOD) and Limit of quantification (LOQ) were computed from the calibration equation and using the formula, [43]

$$\text{LOD} = 3.3 \times \frac{SD}{b}$$

$$\text{LOQ} = 10 \times \frac{SD}{b}$$

Where, SD is the standard deviation of calibration curve and $b$ is the slope of the calibration curve.

Precision

Precision [44] refers to the reproducibility of measurement within a set that is to scatter or dispersion of a set from its central value. The term ‘set’ is defined as referring to a number (n) of independent replicate measurements of some property.

One of the most common statistical terms employed is the standard deviation of a population of observation. Standard deviation is the square of the sum of squares of deviations of individual result from the mean, divided by one less than the number of results in the set. The standard deviation ‘SD’ is given by

$$SD = \left[ \frac{1}{(n - 1)} - \frac{n}{\sum_{i=1}^{n} (X_i - \bar{X})^2} \right]^{1/2}$$

(4.9)

Standard deviation has the same units as the property being measured. The square of the standard deviation is called the variance ($SD^2$). Relative standard deviation (RSD) is the standard deviation,
expressed as a fraction of the mean. It is sometimes multiplied by 100 and expressed as a percent relative standard deviation [Coefficient of variance (%CV)]. This is a more accurate measure of the precision.

\[
%CV = \frac{SD \times 100}{X}
\]  

(4.10)

Where, SD = Standard deviation, X = Mean, %CV = Coefficient of variation

**Intra-Assay Precision**

This is also known as repeatability i.e. the ability to repeat the same procedure with the same analyst, using the same reagent and equipment in a short interval of time, e.g. within a day and obtaining similar results.

**Inter-Assay Precision**

The ability to repeat the same method under different conditions, e.g. change of analyst, reagent, or equipment; or on subsequent occasions, and over several weeks or months, is covered by the between batch precision or reproducibility, also known as inter-assay precision.

The reproducibility of a method is of prime interest to the analyst since this will give a better representation of the precision during routine use as it includes the variability from a greater number of sources [45]. A minimum of three concentrations in the range of expected concentrations is recommended. The %CV determined at each concentration level, should not exceed 15 % except for the LOQ, where it should not exceed 20 % [46].

**Accuracy [47, 48]**

The accuracy of an analytical method is a measure of the systematic error or bias and is defined as the agreement between the measured value and the true value. Accuracy is best reported as percentage bias that is calculated from the expression:
Bias = \frac{\text{Measured value} - \text{true value}}{\text{true value}} \times 100 \quad (4.11)

Some of the possible error sources causing biased measurement are: error in sampling, sample preparation, preparation of calibration line and sample analysis. The method accuracy can be studied by comparing the results of a method with results obtained, by analysis of certified reference material (CRM) or standard reference material (SRM). Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The mean value should be within 15 % of the actual value except at LOQ, where it should not deviate by more than 20 % [46].

**Recovery experiments**

Absolute recovery of an analytical method is the measured response of a processed spiked matrix standard expressed as a percentage of the response of a pure standard, which has not been subjected to sample pre-treatment and indicates whether the method provides a response for the entire amount of the analyte that is present in the sample [49].

% Recovery = \frac{\text{Response of spiked plasma (Processed)}}{\text{Response of standard solution (Unprocessed)}} \times 100 \quad (4.12)

The matrix effect can also be studied by comparing the response of extracted samples spiked before extraction with the response of the extracted blank matrix sample to which analyte has been added at the same nominal concentration just before injection [45]. Good precision and accuracy can be obtained from methods with moderate recoveries, provided they have adequate sensitivity. Indeed it may be desirable to intentionally sacrifice high recovery in order to achieve better selectivity with some sample extraction procedure. Solvents such as ethyl acetate normally give rise to high recovery of analyte, however these solvents...
simultaneously extract many interfering compounds. Therefore, provided that an adequate sensitive detection limit is attained with good precision and accuracy, the extent of recovery should not be considered an issue in analytical method development and validation [50].

Robustness and Ruggedness

Robustness

ICH [40] defines the robustness of an analytical procedure as a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. It is provided as an indication of the procedure’s reliability during normal usage.

For the determination of a method’s robustness, a number of parameters, such as pH, reaction time, working temperature and the quantitative influence of the variables are determined. If the influence of the parameters is within a previously specified tolerance, the parameter is said to be within the method’s robustness range.

Ruggedness

Ruggedness has been replaced by reproducibility which has the same meaning. Ruggedness is not addressed in the ICH documents, but it is defined by the USP [47] as the degree of reproducibility of results obtained under a variety of conditions, such as different laboratories, analysts, instruments, environmental conditions, operators and materials. Ruggedness is a measure of the reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst. Ruggedness is determined by the analysis of aliquots from homogenous lots in different laboratories.

Stability

The stability of the analyte is often critical in biological samples, even over a short period of time. Degradation is not unusual, even when all precautions are taken to avoid specific known stability problems of
the analyte (e.g. light sensitive). It is therefore important to verify that there is no sample degradation during the time of collection of the sample and their analysis. Stability evaluation is done to show that the concentration of analyte at the time of analysis corresponds to the concentration of the analyte at the time of sampling [51].

An essential aspect of method validation is to demonstrate that analyte (s) is (are) stable in the biological matrix and in all solvents encountered during the sample work-up process, under the conditions to which study samples will be subjected [50].

According to the recommendations of the Washington conference report by Shah et al., (1992), [52] the stability of the analyte in the matrix at ambient temperature should be evaluated over a time that encompasses the duration of typical sample preparation, sample handling and analytical run time. Similarly, Dagar & Brunett (1995) [50] gave the following details to be investigated.

**Bench top stability**

This is evaluated following the storage under laboratory conditions (at room temperature) used for the sample work-up for a period of 6-24 hr. The data are compared with freshly prepared same samples and analyzed without delay.

**Long term stability**

This is done to assess stability of the analyte in the plasma matrix under the sample storage conditions (-20 ºC) for the time period required for the samples generated in a clinical study to be analyzed.

**Standard stock solution stability**

The stability test for the standard stock solution must be done at the same temperature, container and solvent as that to be used for the study. The time period should be at least 6 hrs.
Acceptable stability is a 2% change in standard solution or sample solution response relative to a freshly prepared standard. Acceptable stability at the LOQ (Limit of quantification) for standard solution and sample solution is a 20% change in response to a freshly prepared sample [53].

**Specificity and Selectivity**

ICH [40] defines specificity as “the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically this might include impurities, degradants, matrix etc.” Selectivity and specificity are important metrological performance characteristics of analytical methods, especially in connection with validation procedures. The concept of selectivity and specificity are used interchangeably and synonymously. Analysts are in agreement that selectivity of an analytical method refers to the extent to which particular analytes can be determined in real samples (mixtures or matrices, simple or complex) under given conditions without interferences by other components.

The selectivity can determine simultaneously several components independently from each other. The specificity can be determined independently from all the other components which give no analytical signal in this case. The selectivity and specificity are only meaningful with respect to a completely described analytical procedure the course of which is prescribed in all details, including the type of samples that have to be analyzed. Selectivity studies should also assess interferences that may be caused by the matrix, such as urine, blood, soil, water or food. Optimized sample preparation can eliminate most of the matrix components.


**EXPERIMENTAL**

**Preparation of Stock Solutions**

*Di (4-Amino N-Acetyl) Phenoxy] Methyl Ketone (DPMK)*

Aqueous stock solutions (2.0 M) of HCl (Hydrochloric acid), NaNO₂ (Sodium nitrite), NaOH (Sodium hydroxide), NH₄OH (Ammonium hydroxide) and KOH (Potassium hydroxide) were prepared. The various concentrations of each solution were prepared by dilution method. A stock solution of sodium nitrite (1.0 mg mL⁻¹) was prepared by dissolving 100.0 mg in 100.0 mL distilled water. An aqueous stock solution (1.0 mg mL⁻¹) and stock solution of DPMK (1.0 mg mL⁻¹) was prepared by dissolving 100.0 mg of DPMK in 100.0 mL of distilled water and 100.0 mL of methanol respectively. A working solution of 100.0 µg mL⁻¹ was thereafter made from the stock solution-1 and 2 in respective solvents. Spike DPMK in serum and urine standard working solutions (100.0 µg mL⁻¹) were prepared by using a blank serum and urine respectively. Stock solutions were stored at 4 ºC.

*4-Acetamidophenyl N’-(Sulphanilamide) Acetate (APSA)*

A stock solution of 2.0 M H₂SO₄ (sulphuric acid) was prepared by diluting 11.2 mL sulphuric acid up to 100.0 mL with double distilled water. 2.0% p-Dimethylaminobenzaldehyde (DMAB) stock solutions were prepared by dissolving 2.0 g DMAB in 100.0 mL of 0.1 to 2.0 M H₂SO₄ solutions respectively. 1.0 mg mL⁻¹ stock solution of APSA was prepared by dissolving 100.0 mg of APSA in 100.0 mL methanol. Serum and urine standard working solutions (100.0 µg mL⁻¹) were prepared by mixing of 1000.0 µL diluted stock solution of APSA (100.0 µg mL⁻¹) with a 1000.0 µL blank serum and urine, respectively. Solutions of lower concentrations were prepared by appropriate dilution of the stock solutions with suitable solvents. The prepared stock solutions were stored at 4 ºC till the analyses are performed.
Sodium [2-[2, 6-Dichlorophenyl [(4-Amino N-Acetyl) Phenyl Acetate]-Amino] Phenyl Acetate] (DPAAPA)

An aqueous stock solution of 3.0 M of NaOH and 2.0 mM of K₃[Fe(CN)₆] (Potassium ferricyanide) was prepared by dissolving 12.0 g of NaOH and 0.065 g of K₃[Fe(CN)₆] in 100 mL volumetric flasks and diluting up to the mark with distilled water respectively. An aqueous standard stock solution and a standard solution of DPAAPA (1.0 mg mL⁻¹) was prepared by dissolving 100.0 mg of DPAAPA in 100.0 mL of distilled water and 100.0 mL of methanol, respectively. Working solutions were prepared by appropriate dilution of the standard solution. Spike stock solutions of 100.0 µg mL⁻¹ of DPAAPA were prepared in blank serum and urine by properly mixing of 100.0 µL of 100.0 µg mL⁻¹ stock solution of DPAAPA with 100.0 µL blank serum and urine, respectively.

1-Ethyl-6-Fluoro-1, 4-Dihydro-4-Oxo-7-[4-[(4-Amino N-Acetyl) Phenoxy Carbonyl Methyl]-1-Piperazinyl]-3-Quinoline Carboxylic Acid (PPQC)

An aqueous stock solution of 2.0 M H₂SO₄ was prepared by 11.2 mL of H₂SO₄ transferred in 100 mL volumetric flasks and diluted up to the mark with distilled water. An accurately weighed (0.19 g) of Fe₂(SO₄)₃ (Ferric sulphate) was transferred into 100 mL volumetric flasks to get the final concentration of 5.0 mM in 0.5 M H₂SO₄. Stock solutions of PPQC were prepared separately in water (stock solution-1) and methanol (stock solution-2) to reach a concentration of 1.0 mg mL⁻¹ respectively. Further, working solutions were prepared by simple dilution method. Spiked serum and urine samples were prepared by using stock solution-2 of PPQC. Calibration curves were obtained from these solutions.

Stock Solutions of Interfering Radicals

The appropriate amounts of possible interfering substances such as Ca²⁺, Na⁺, K⁺, Mg²⁺, Zn²⁺, Fe²⁺, L-Alanine, Glycine, Tyrosine, glucose,

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uric acid, paracetamol and L-ascorbic acid were dissolved in distilled water. After further dilution with the same solvent, the final concentrations for interference study were obtained 100.0 µg mL⁻¹. The appropriate concentrations were prepared during the study by using dilution method.

**Determination of Wavelength (λmax)**

**[Di (4-Amino N-Acetyl) Phenoxy] Methyl Ketone (DPMK)**

A 1.0 mL stock solution of DPMK was taken into 10 mL volumetric flask. Then after 1.0 mL of each NaNO₂ and HCl stock solutions were added respectively. The resulting solution was made up to 10.0 mL with 2.0 M NaOH stock solution. The reddish-yellow colored complex of nitroso-DPMK was formed, which was allowed to stay at room temperature for 10 minutes. A similar set was prepared without DPMK to act as blank. The absorption spectrum of nitroso-DPMK complex was determined against the blank. The wavelength at which maximum absorption was observed is reported as λmax.

**4-Acetamidophenyl N’-(Sulphanilamide) Acetate (APSA)**

An aliquot of working solution (100.0 µg mL⁻¹) of APSA was taken in 10 mL calibrated flask, with 0.5 mL, 2.0% of DMAB solution prepared in 2.0 M sulphuric acid and diluted up to the mark with methanol solvent. The contents of the flask were mixed well and kept at room temperature till most intensely colored visualized. This sample was scanned on UV-Visible spectrophotometer in 400-800 nm in range and determined λmax corresponding to maximum absorbance at room temperature.

**Sodium [2-[2, 6-Dichlorophenyl [(4-Amino N-Acetyl) Phenyl Acetate]-Amino] Phenyl Acetate] (DPAAPA)**

1.0 mL of stock solution-1 of DPAAPA was taken in 10 mL of volumetric flask. Then after, 2.0 mL of stock solutions of NaOH and
**Chapter-4 Spectrophotometric Validation Method**

K$_3$[Fe(CN)$_6$] was added in the volumetric flask and mixed properly. The resulting solution was diluted up to the mark with distilled water and scanned on UV-Visible spectrophotometer in the range between 400-800 nm and determined the maximum wavelength against the blank.

**1-Ethyl-6-Fluoro-1,4-Dihydro-4-Oxo-7-[4-[(4-Amino N-Acetyl)Phenoxy Carbonyl Methyl]-1-Piperazinyl]-3-Quinoline Carboxylic Acid (PPQC)**

A standard solution-1 (1.0 mg mL$^{-1}$) of PPQC in a 10 mL of volumetric flask was mixed with 1.0 mL of Fe$_2$(SO$_4$)$_3$ stock solutions. The resulting solution was properly mixed and diluted up to the mark with distilled water. After intensive color formation, the solution was scanned in the range of 400-650 nm on UV-Visible spectrophotometer and note down the maximum wavelength correspond to maximum absorbance.

**OPTIMIZATION STUDIES**

**The Effect of Diluting Solvent**

**[Di (4-Amino N-Acetyl) Phenoxy] Methyl Ketone (DPMK)**

The effect of the diluting media of DPMK was investigated by using sodium hydroxide, ammonium hydroxide and potassium hydroxide respectively. In each case, the reaction mixture was made up to 10.0 mL with different media of various concentrations of 0.05, 0.1, 0.5, 1.0 and 2.0 M. The effective media and it’s optimal concentration was taken as one which gave the highest absorbance reading at $\lambda_{max}$ against blank at optimal condition.

**4-Acetamidophenyl N’-(Sulphanilamide) Acetate (APSA)**

The effects of diluting solvents of APSA were examined by using water, methanol, ethanol, propanol, butanol and acetonitrile solvent respectively. In each case, after mixing of 1.0 mL of APSA (100.0 µg mL$^{-1}$) and 0.5 mL of DMAB solution, the resulting reaction mixture was made up to 10.0 mL with respective solvents. The absorbance was noted on spectrophotometer against blank solvent. The diluting solvent that
showed maximum absorbance was selected as the best diluting solvent for the spectrophotometric method.

**Sodium [2-[2, 6-Dichlorophenyl [(4-Amino N-Acetyl) Phenyl Acetate]-Amino] Phenyl Acetate] (DPAAPA)**

The effect of diluting solvent was examined using acetonitrile, ethanol, methanol, water, propanol and butanol solvents. A 10.0 µg mL\(^{-1}\) of DPAAPA solution was used to determine the effect of diluting solvent. An aliquot of DPAAPA was taken in 10 mL volumetric flask and properly mixed after addition of 2.0 mL of stock solutions of NaOH and K\(_3\)[Fe(CN)\(_6\)]. The finally, resulting solution was diluted up to the mark with acetonitrile, ethanol, methanol, water, propanol and butanol solvents respectively. The absorbance was measured at \(\lambda_{\text{max}}\) against respective blank solvents.

**1-Ethyl-6-Fluoro-1, 4-Dihydro-4-Oxo-7-[4-[(4-Amino N-Acetyl) Phenoxy Carbonyl Methyl]-1-Piperazinyl]-3-Quinoline Carboxylic Acid (PPQC)**

The effect of diluting solvent was examined using acetonitrile, methanol, ethanol, propanol, butanol and water. After addition of certain aliquots of PPQC (10.0 µg mL\(^{-1}\)) and 1.0 mL of Fe\(_2\)(SO\(_4\))\(_3\) stock solutions in 10 mL volumetric flasks were diluted up to the mark with each solvent. The absorbance was measured at \(\lambda_{\text{max}}\) against blank solvent.

**The Effect of Reagent Concentration**

**[Di (4-Amino N-Acetyl) Phenoxy] Methyl Ketone (DPMK)**

The effect of varying reagent concentrations of NaNO\(_2\) and HCl was carried out using different concentrations 0.05, 0.1, 0.5, 1.0, and 2.0 M. To each set of NaNO\(_2\) solution, 1.0 mL of stock solution-1 of DPMK and 1.0 mL stock solution of HCl were added and diluted to 10.0 mL with 0.5 M NaOH. Similarly, to each set of HCl solution, 1.0 mL of stock solution-1 of DPMK and 1.0 mL stock solution of NaNO\(_2\) and diluted to 10.0 mL
with 0.5 M NaOH. The absorbance readings of the nitroso-DPMK complex formed were measured at λmax on UV-Visible spectrophotometer against the blank at optimal condition. The maximum absorbance reading was considered as an effective reagent concentration.

4-Acetamidophenyl N’-(Sulphanilamide) Acetate (APSA)

The effect of DMAB concentration on the absorbance value was investigated in the concentration range of 0.1-2.0% w/v in 2.0 M sulphuric acid. Also the effect of sulphuric acid concentration on the absorbance value was examined in the concentration range of 0.1-2.0 M using 2.0% w/v DMAB. The effect of reagent concentration was studied and noted the best reagent concentration that was suitable for the formation of condensation product.

Sodium [2-[2, 6-Dichlorophenyl [(4-Amino N-Acetyl) Phenyl Acetate]-Amino] Phenyl Acetate] (DPAAPA)

The effect of NaOH and K$_3$[Fe(CN)$_6$] concentration on absorbance were investigated in the range of 0.1-3.0 M and mM respectively. Aliquots of DPAAPA were taken in 10 mL volumetric flasks and 2.0 mL of various concentrations of NaOH and 2.0 mL of stock solution of K$_3$[Fe(CN)$_6$] in each flask were added. The resulting solutions were diluted up to the mark with water. Similarly, a fixed amount of NaOH and various concentrations of K$_3$[Fe(CN)$_6$] was studied. The maximum absorbance was noted at 451 nm λmax against the blank selected as an optimum concentration of the reagents.

1-Ethyl-6-Fluoro-1, 4-Dihydro-4-Oxo-7-[4-[(4-Amino N-Acetyl) Phenoxy Carbonyl Methyl]-1-Piperazinyl]-3-Quinoline Carboxylic Acid (PPQC)

The effect of reagent concentration was studied using various concentrations of 0.1-2.0 M H$_2$SO$_4$ and 0.1-5.0 mM of Fe$_2$(SO$_4$)$_3$. The
effect was examined for each reagent concentration on the fixed concentration of PPQC (10.0 µg mL\(^{-1}\)). The maximum absorbance at a definite concentration was considered as the optimal reagent concentration for the proposed method.

**The Effect of Reaction Temperature**

The temperature was optimized using the method of steepest ascent \[46\]. The optimization of temperature studies for each synthesized compound was carried out by using 10.0 µg mL\(^{-1}\) solutions of each synthesized compound. After reagent addition, as per the spectrophotometric method of each synthesized compound, the resulting mixture was mixed in a vortex mixer followed by incubating at various temperatures in the range of 5-80 °C. The absorbance of the resulting solution was measured for each temperature at λmax on visible spectrophotometer. Each determination was done in triplicate.

**The Effect of Reaction Time**

The steepest ascent method was used for optimization of reaction time \[46\]. The optimal reaction time of each synthesized compound was carried out as similar to the effect of temperature. The absorbance of the resulting solution was measured at various time intervals in the range of 0-60 min. The optimal reaction time at an optimized temperature was taken as the time corresponding to the maximal absorbance of the sample.

**Stoichiometric Ratio Determination**

The Job’s method was used to check the stoichiometric reaction between synthesized compound and reagents \[54\].

**[Di (4-Amino N-Acetyl) Phenoxy] Methyl Ketone (DPMK)**

Equimolar solutions (1.0 mg mL\(^{-1}\)) of sodium nitrite and DPMK were prepared in double distilled water. The various volumes of 0.00,
0.25, 0.33, 0.50, 0.67, 0.75 and 1.00 mL of sodium nitrite solution were taken 10 mL volumetric flasks and made up to 1.0 mL with DPMK solution. Subsequently, the mixtures were made up to 10.0 mL with 0.5 M NaOH. The blank solutions were similarly prepared by the above method without addition of DPMK. The absorbance was noted at λ_{max} on visible spectrophotometer in optimal condition against each blank solution.

4-Acetamidophenyl N’-(Sulphanilamide) Acetate (APSA)

Equimolar solutions (1.0 mg mL\(^{-1}\)) of the DMAB (in 0.9 M H\(_2\)SO\(_4\)) and APSA (in methanol) were used to determine the stoichiometry ratio. In seven different volumes of 0.00, 0.25, 0.33, 0.50, 0.67, 0.75 and 1.00 mL of DMAB were taken in volumetric flasks and made up to 1.0 mL with APSA solution. Subsequently, diluted to 10.0 mL with methanol and the absorbance were noted at 446 nm on spectrophotometer under optimum conditions against blank reagents.

Sodium [2-[2, 6-Dichlorophenyl [(4-Amino N-Acetyl) Phenyl Acetate]-Amino] Phenyl Acetate] (DPAAPA)

The stoichiometric ratio was determined by using equimolar of solutions of the K\(_3\)[Fe(CN)\(_6\)] and DPAAPA (1.0 mg each). Seven different volumes of 0.00, 0.4, 0.66, 1.00, 1.34, 1.60 and 2.00 of K\(_3\)[Fe(CN)\(_6\)] were taken in 10 mL volumetric flasks and diluted up to 2.0 mL with DPAAPA solution. Finally, the resulting solutions were diluted up to 10.0 mL with water. The absorbance was measured at 451 nm against the blank, prepared similar way except DPAAPA.

1-Ethyl-6-Fluoro-1,4-Dihydro-4-Oxo-7-[4-[(4-Amino N-Acetyl)Phenoxy Carbonyl Methyl]-1-Piperazinyl]-3-Quinoline Carboxylic Acid (PPQC)

The standard equimolar solutions (1.0 mg mL\(^{-1}\)) of PPQC and Fe\(_2\)(SO\(_4\))\(_3\) were used to determine the stoichiometry ratio. A series of 1.0 mL portions of the standard solutions of PPQC with the reagent
Fe$_2$(SO$_4$)$_3$ were made up of different complementary proportions (0.00:1.00, 0.25:0.75, 0.33:0.67, 0.50:0.50, 0.67:0.33, 0.75:0.25 and 1.00:0.00 mL) in 10 mL volumetric flasks. Then, each flask was made up to the mark with distilled water. The reactions were performed at optimal conditions. The absorbance of the resulting solutions was measured at 418 nm against reagent blanks treated similarly.

**VALIDATION STUDIES [40, 46]**

A series of standard solutions of synthesized compounds were prepared in a suitable range in µg mL$^{-1}$. Absorbance was plotted as a function of the concentration of synthesized compounds. A linear relationship between the absorbance (A) and the concentration (c) of synthesized compounds was obtained. The 95% confidence limit for the slope and intercept was computed using origin software. The correlation coefficient (r) and molar absorptivity was obtained from standard equation 4.4 and 4.6 respectively. The limit of detection (LOD) and limit of quantification (LOQ) for developing method was calculated using the standard equation 4.7 and 4.8. Sandell’s sensitivity was determined by the concentration that gives an absorbance reading of 0.001.

**Determination of Synthesized Compounds in Biological Fluids (Blood and Urine Sample) [55]**

Blank blood (0.5 mL) sample was collected from Adult Wistar albino rat, through retro-orbital sinus puncture [56] using 0.1 mL capillary tubes into 2 mL polyethylene centrifuge tube. 1.0 mL ethyl acetate was previously added into 2 mL polyethylene centrifuge tube. A 0.5 mL blood was spiked with a 0.5 mL stock solution of synthesized compounds prepared in methanol (1.0 mg mL$^{-1}$). The mixture was hand shaken for about 5 minutes until the contents were properly mixed. The mixture of methanol and ethyl acetate (1:2) was found to be an ideal solvent for blood extraction because of blood proteins were not precipitated by using only methanol or ethyl acetate solvent. Then the
tubes were shaken for 5 minutes on a vortex mixture and centrifuged at 2000 rpm for 5 minutes at 30 °C temperature. The clear supernatant was obtained which did not require further clean up. The convenient volumes of the supernatants were collected in 10 mL volumetric flasks and analyzed by developed method. The final concentrations of each synthesized compound in blood supernatants were 1.25, 2.50 and 3.75 µg mL\(^{-1}\) respectively. The absorbance was measured at λ\(_{\text{max}}\) against the blank reagent which were prepared in the same way except the addition of synthesized compounds. The calibration curve of synthesized compounds was plotted for serum sample in the appropriate range in µg mL\(^{-1}\).

The blank urine sample was collected from Wistar albino rats. Urine sample (1.0 mL) was diluted up to 100 mL with distilled water. Diluted urine sample (1.0 mL) was spiked with convenient amounts of each synthesized compound (1.0 mg mL\(^{-1}\)) and diluted up to 10.0 mL with distilled water. The spiked urine samples were withdrawn in convenient aliquots in 10 mL volumetric flasks and then followed by the assay method according to developed for each synthesized compound. The final concentrations of each synthesized compound in urine samples were 10.0, 15.0 and 20.0 µg mL\(^{-1}\), respectively. Blank was prepared in the diluted urine sample except the addition of synthesized compounds and measured the absorbance at λ\(_{\text{max}}\). The calibration curve was prepared for spiked urine samples in the appropriate concentration range in µg mL\(^{-1}\).

**Accuracy and Precision [45, 46]**

The accuracy and precision of the developed methods were determined from intra-day and inter-day analysis of each synthesized compound spiked at three different concentrations levels in blood (1.25, 2.50 and 3.75 µg mL\(^{-1}\)) and urine (10.0, 15.0 and 20.0 µg mL\(^{-1}\)) samples. The precision of the developed methods was described as the percentage relative standard deviation (%RSD). The accuracy was described as a
percentage of measured concentration of each synthesized compound. Intra-day precision and accuracy of the developed method for blood and urine samples were evaluated by analyzing each sample three times on the same day, while inter-day precision and accuracy of the developed method for blood and urine sample were evaluated by analyzing each sample in six consecutive days.

**Recovery Study**

The recoveries study was carried out using three different spiked concentrations of each synthesized compound in blood (1.25, 2.50 and 3.75 µg mL\(^{-1}\)) and urine (10.0, 15.0 and 20.0 µg mL\(^{-1}\)) samples. The recovery study was described as a percentage of measured concentration of each synthesized compound.

**Robustness**

The robustness was examined by evaluating the influence of small variation in the method. In these experiments, one parameter was changed, whereas the others were kept unchanged, and it was calculated in terms of standard deviation and %RSD. The robustness of the proposed method was assessed by changes in the analytical wavelength (±1 nm) at two different concentrations (5.0 and 10.0 µg mL\(^{-1}\)) of analytes and changes in working temperatures (±2 °C) and reaction time (±3 min) at 10.0 µg mL\(^{-1}\) concentration levels of analytes.

**Ruggedness**

Ruggedness was tested by applying the proposed method to the assay of synthesized compounds using the same operational conditions, when performed by two different analysts and day-to-day variations in the analysis. The ruggedness of the proposed methods was checked by using two different analysts at two different concentrations (5.0 and 10.0 µg mL\(^{-1}\)) of analytes and also examined day-to-day reproducibility in blood (2.5 µg mL\(^{-1}\)) and urine (10.0 µg mL\(^{-1}\)) samples for six consecutive days.
days. Ruggedness was calculated in terms of standard deviation and % RSD.

**Stability Study**

The stability of each synthesized compound in blood and urine samples was measured for typical storage and handling conditions. At three different concentration levels of Spiked blood (1.25, 2.50 and 3.75 µg mL\(^{-1}\)) and urine (10.0, 15.0 and 20.0 µg mL\(^{-1}\)) samples were stored at bench top (at room temperature for 6-24 hr) and long-term (at -20 ºC for a week) conditions. The bench-top and long-term stability were examined by analyzing the stored sample using developed assay method for synthesized compounds and compared with freshly prepared intra-day blood and urine samples.

**Specificity and Selectivity**

The specificity of the method was investigated by possible interferences caused through other potential substances on the determination of synthesized compounds. Under the developed experimental conditions for each synthesized compound, the effect of interfering species was evaluated by addition of various concentrations of interfering species (e.g. Ca\(^{2+}\), Na\(^{+}\), K\(^{+}\), Mg\(^{2+}\), Zn\(^{2+}\), Fe\(^{2+}\), L-Alanine, Glycine, Tyrosine, glucose, uric acid, paracetamol and L-ascorbic acid) to spiked blood and urine samples containing a fixed amount of each synthesized compound (10.0 µg mL\(^{-1}\)). The tolerance of the various interferents and selectivity of the proposed method was examined.
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