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Determination of Folic Acid in Pharmaceutical Dosage Forms: A Review

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1. INTRODUCTION

Folic acid (FA), chemically ispteroyl-L-glutamic acid or N- [4-[[(2-amino-1,4-dihydro-4-oxo-6-pteridinyl) methyl] amino] benzoyl]-L-glutamic acid, it is a member of the B vitamin group.The chemical formula of folic acid is $C_{19}H_{19}N_7O_6$[1]. The core of the molecule consists of heterocyclic pterin structure, with a methyl group in the sixth position bound to para-aminobenzoic and glutamic acids so that folic acid presents pteroylglutamic acid (Figure 1). Pterin is composed from pyrimidine and pyrazine rings («pteridine») with substituting keto- and amino groups in the second and fourth positions. Aromatic heterocyclic structures provide an ability for reversible electron-accepting [2,3]. "Folates" is a generic term encompassing folic acid and its derivatives-dihydro-, tetrahydro-, methyl-, formyl-compounds possessing metabolic activity. All folates are inherently conjugated with para-aminobenzoyl-glutamate as mono-, di-, tri-, and polyglutamates [4].

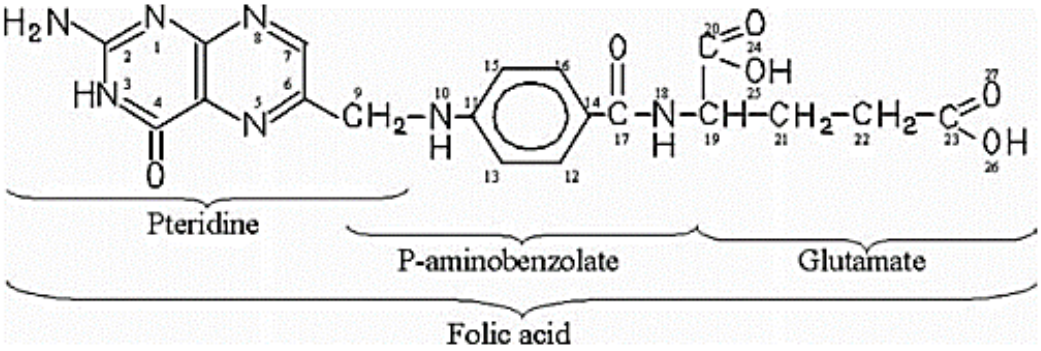

Figure 1. *The structural formula of folic acid (FA). The FAmolecule consists of three parts: pterine, p-aminobenzolate andglutamate.*

Folic acid as a vitamin which is not produced by natural metabolism; it is obtained from thediet. Most dietary folic acid loses its bioavailability during cooking and food processing. Yet folic acid is often recommended as a supplement when the amount of dietary folate is not sufficient [5].

Folic acid plays a crucial role in preventing major birth defects known as neural tube defects (NTDs). It is essential for cell replication, growth, and the synthesis of DNA and RNA. Folate helps prevent DNA alterations that can lead to cancer. Both adults and children need folate to produce normal red blood cells and prevent anemia. Additionally, supplementation with folic acid and vitamin B12 has been shown to improve vascular endothelial function in patients with coronary heart disease [6]. In vitro evidence shows that 5-methyltetrahydrofolate, the primary circulating metabolite of folic acid, can increase nitric oxide production and directly scavenge superoxide radicals, which may explain some of its cardiovascular benefits [7].

While most studies on folic acid have focused on heart health, recent findings suggest that folic acid may have antidepressant properties or act as an enhancing mediator for standard antidepressant

treatments. Evidence indicates that elderly individuals with depression have lower folate levels than their non-depressed peers. Supplementing with folate might reduce the incidence of depression in the elderly [8,9]. Folate deficiency is a common cause of anemia, with subtle signs such as diarrhea, loss of appetite, weight loss, weakness, a sore tongue, headaches, heart palpitations, and irritability. Additionally, a high risk of giving birth to children with NTDs is associated with women with type I diabetes, with epilepsy or who are under carbamazepine or antifolate medication [10].For the sake of reducing the risk of NTDs, supplementation of folic acid has been recommended before and during the first three months of pregnancy [11].

The body absorbs folic acid from supplements and fortified foods better than folate from naturally occurring foods. The recommend daily intake of dietary folate equivalents (DFEs) from food or vitamin sources is given in Table 1. The recommended daily dose of folic acid depends on the patient's history of birth abnormalities; the recommended preventative daily allowance of folic acid for women with no prior history of birth defects dose is 4.0 mg per day starting at least 30 days before conception and continuing throughout the first trimester of pregnancy [12].

Table 1. *The recommended daily intake of folic acid*

Age	Recommended amount
0–6 months	65 mcg dietary folate equivalent (DFE)
7–12 months	80 mcg DFE
1–3 years	150 mcg DFE
4–8 years	200 mcg DFE
9–13 years	300 mcg DFE
14–18 years	400 mcg DFE
19+ years	400 mcg DFE
During pregnancy	400–800 mcg DFE
During lactation	500 mcg DFE

Development of an appropriate and suitable methodfor quantitative pharmaceutical analysis is very significant. The subsequentmethod validation is obligatory to prove method reliability and it is carriedout as an integral part of quality control. The choice of proper instrumentalmethod depends on affordable equipment and the physicochemicalproperties of the analytes.

To the best of our knowledge, there are no published review articles on the analytical methods for determining folic acid in pharmaceutical dosage forms. This scientific literature review provides a comprehensive overview of the advancements in evaluating folic acid in pharmaceutical dosage forms from 2000 to 2024.This review categorizes the data according to the analytical methods used, facilitating a quick understanding of the different approaches employed in studying binary mixtures of azole antifungals and corticosteroids. By providing detailed insights from various published studies, this structured presentation enables pharmaceutical researchers and professionals to easily locate pertinent information about the analytical techniques relevant to their research or work.

2. Methods of Analysis

2.1. Official Pharmacoepial Methods

The British Pharmacopeia (BP) [13] monographs described high-performance liquid chromatography (HPLC) for the determination of folic acid in injections, tablets and oral solution.

The chromatographic method for the determination of the injections; involves using (C_{18}, 20 cm x 4.6 mm, 10 µm) column and 4 volumes of acetonitrile, 6 volumes of methanol and 90 volumes of 0.01 M sodium acetate, adjusted to pH 4.5 withacetic acid as mobile phase. The mobile phase is pumped at rate of 1.2 mL per minute and the analyteis monitored at 280nm.

The chromatographic conditions for oral liquid employs (C_{18}, 25 cm x 4.6 mm, 5µm) column and a two components mobile phase made of: mobile phase A: 10 volumes of methanol and 90 volumes of asolution containing 1.116 w/v%of potassium dihydrogenphosphate and 0.55% w/v of dipotassium hydrogen phosphate and mobile phase B methanol.The mobile phase is delivered in a gradient fashion; where 100% of mobile phase A was pumped for 45 minutes, then its composition was brought to 50% for within 2 minutes and kept as such for 14 minutes, then taken back to 100% A

within 1 minute and kept as such for 15 minutes.The mobile phase pumped at rate of 0.6 mL per minute and the analyteis monitored at 280nm.

The chromatographic method for the determination of the tablets form involves using (C_{18}, 20 cm x 4.6 mm, 10 µm) column and 7 volumes of acetonitrile and 93 volumes of 0.05M potassium dihydrogen orthophosphate adjusted to pH 6.0 with 5M sodiumhydroxide. The mobile phase is pumped at rate of 2 mL per minute and the analyteis monitored at 283nm.

According to the United States Pharmacopeia (USP)[14]; both the folic injection and tablet dosage are analyzed using the same chromatographic conditions, utilizing a (C_{18}, 25 cm x 4.6 mm, 5µm) and mobile phase prepared by transferring 35.1 g of sodium perchlorate and1.40 g of monobasic potassium phosphate to a 1-Lvolumetric flask followed by 7.0 mL of 1 N potassium hydroxideand 40 mL of methanol, the mixture is then diluted with water to volume, mixed well and adjusted to a pH of 7.2 with 1 N potassium hydroxide orphosphoric acid. The mobile phase pumped at rate of 1.0 mL per minute and the analyte was monitored at 254nm.

The USP method for oral liquid employs (C_{18}, 10 cm x 4.6 mm, 5µm) column maintained at 40 ° C and a two components mobile phase made of mobile phase A:50 mM monobasic potassium phosphateadjusted with phosphoric acid to a pH of 2.5and mobile phase B methanol.The mobile phase is delivered in a gradient fashion; the gradient program starts with 95% of mobile phase Akept as such for 2 minutes; followed by a gradient two steps, one from 95 to 90% over 8 minutes and another from 90% to 85% over 15 minutes, the two steps were followed by a small gradient segment from 85% to 65% over one minute and finally the composition was brought again to 95% mobile phase A.The mobile phase was pumped at rate of 1.0 mL per minute and the analyte was monitored at 280nm.

2.2. Spectroscopic Methods

UV-Vis spectroscopy is an analytical method for qualitative and quantitative determination of compounds containing chromophores that absorb characteristic wavelengths of ultraviolet (UV) or visible light. According to the Beer–Lambert law, absorption is related to the concentration of the sample. In addition to its simplicity, it is characterized by simple sample preparation, low cost, and relatively short analysis time.

Analytical chemists encounter significant challenges when analyzing and ensuring the quality of analytes that lack a chromophore system, are present in very low concentrations, or exist within complex matrices using direct spectrophotometry. Consequently, they turn to alternative approaches such as chemical derivatization, derivative spectroscopy, or chemometric techniques to increase the selectivity or sensitivity of the method.

2.2.1. Direct UV Methods

The content of folic acid in tablets was determined by direct measurement of the absorbance of its solution in 0.01 M NaOH at 255 nm using 0.01 M NaOH as a blank. The method was validated and proved to be have good accuracy, precision and low limir of quantitation (LOQ). The linear range of the method was 10-50µg/mL [15].

Another method was reported for direct spectrophotometric determination of folic acid in tablets. The method based on measuring the absorbance of the folic acid solution in phosphate buffer pH 9.0 at 282.5 nm. The method was confirmed to be accurate and precise, the linear range of the method was 1.00 and 17.5 µg/mL [5].

Folic acid in tablets wasalso determined in the presence of pteroic acid, glutamic acid, aminobenzoic acid, and other products of degradation of folic acid by measurement of its solution in0.1 M sodium carbonate at 285 nm.The linear range of the method was 1-25 µg/mL [16].

Direct spectrophotometric determination of folic acid in tablets was carried out by measuring the absorbance of the aqueous solution at 250 nm against water as a blank. The method shown to be accurate, precise and linear over the range of 5-30 µg/mL [17].

2.2.2. Derivative Spectrophotometric Methods

First order derivative spectrophotometric method was used for the determination of folic acid in different pharmaceutical formulations, using 0.1 MNaOH as solvent, spectrophotometric

measurements were made at the zero crossing point wavelength 393 nm. The method is shown to be simple, selective, and robust. Good linearity was achieved over the range of 1.80 - 2.85 µg/mL [18].

Three derivative based spectrophotometric methods were reported for the determination of folic acid, para-aminobenzoic acid and, pteroic acid [19]. The first method is a ratio difference spectro photometric method (RDSM) which depends on measuring the difference value in the ratio spectrum, where the difference between 291 and 313 nm was used for the determination of folic acid, while the difference between 305 and 319 nm was selected for the estimation of para-aminobenzoic acid; on the other hand, pteroic acid can be determined using the first derivative of ratio spectra spectro photometric method at 262 nm. The second method was a double-divisor spectrophotometric method (DDSM); this method is based on using the ratio spectrum obtained by the division of the spectrum of ternary mixture (folic acid and para-aminobenzoic acid and, pteroic acid) by the spectrum of binary mixture containing two of the three mentioned components, and in this method, folic acid, para-aminobenzoic acid, and pteroic acid were measured at 242, 313, and 258 nm, respectively. The third method was a mean-centering of ratio spectra spectrophotometric method (MCR); in this method, folic acid, para-aminobenzoic acid, and pteroic acid can be measured using the mean-centered second ratio spectra amplitudes at 317–318 (peak to peak), 264–265 (peak to peak), and 232 nm, respectively.

2.2.3. Colorimetric Methods

Two colorimetric method were proposed for determination of Folic acid in pharmaceutical formulations and bulkdosage forms.The first methodwas based on the formation of colored species on binding of folic acidwith sodium nitroprusside and ammonia reagent to produce a dark yellow colored chromogen was measured at390 nm,while in the second method the complex is formed between MBTH and ferric chloride and the green colored chromogen was measured at 690 nm.The two methods were linear over the range 10-50 µg/mL [20].

Folic acid in tablet and injection dosages was determined colorimetrically by its reduction to 2,4,5-triamino-6-hydroxypyrimidine (TAHP) using zinc dust in an acidic environment (4N HCl) with heating for 45 minutes at 35°C. The reduction product was then treated with ninhydrin to form a stable purple complex. This complex has an absorbance maximum at 555 nm and follows Beer's law within the concentration range of 4.5- 45 µg/mL [21].

Three spectrophotometric methods were developed for the determination of folic acid in its pure form or pharmaceutical preparations [22]. These methods are based on the probable diazotization of p-aminobenzoylglutamic acid, which is obtained after the reductive cleavage of folic acid using zinc and concentrated hydrochloric acid. This is followed by coupling with different reagents to produce measurable colored products:

- Coupling with iminodibenzyl(IDB) yields a violet product, measurable at 580 nm.

The reaction carried by adding 2 ml of 5 M hydrochloricacid, followed by the addition of 1 mlof 2% $NaNO_2$. To this solution, 1.5 ml of2% sulfamic acid was added along with 2 ml of 0.5%IDB and 3 ml of alcoholto the sample in a 25 mL volumetric flask.The solution was heated in aboiling water bath for 5 min, cooled and diluted to themark with 1:1 (v/v) H_2SO_4.

- Coupling with 3-aminophenol (AMP) produces an orange-yellow product, measured at 460 nm.

The reaction carried by adding 2 ml of 5 M hydrochloric acid, followed by the addition of 2 ml of 1% $NaNO_2$ and 2 ml of 2% sulfamic acid to the sample in a 25 mL volumetric flask.The solution was mixed with 2 mlof 1% 3-aminophenol, heated in a boiling water bath for5 min, cooled, followed by the addition of 3 ml of 5 MHCl, swirled, and diluted with water to the mark.

- Coupling with sodium molybdate and pyrocatechol results in a pale red product, measured at 490 nm.

After diazotization as mentioned above,2 ml of 8% sodium molybdate was used along with1.5 ml of 0.4% pyrocatechol. The solution was swirledwell, heated in a boiling water bath for 5 min, andcooled, the solution was diluted and with 1:1 (v/v)H_2SO_4 up to the markin a 25 mL volumetric flask.

Two kinetic spectrophotometric methods (rate data and fixed time method) have been developed for the determination of folic acid (FA) in bulk and pharmaceutical Formulations [23]. The methods

based on the oxidation of FA by Fe(III) in sulfuric acid medium. Fe(III) subsequently reduces to Fe(II) which is coupled with potassium ferricyanide to form Prussian blue.

The rate method involves addition of ammonium ferric sulfate solution (3.0×10-3 M) followed by 1.5 mL of potassium ferricyanide solution (2.5×10-3 M) to aliquot volumes of the drug solution in different 10 mL and then diluted to the volume with double distilled water at 25°C. The content of mixture of each flask was mixed well and the increase in absorbance at 725 nm was recorded as a function of time over 5–20 min against reagent blank treated similarly. The rate data of the reaction at different concentrations was obtained from the slope of the tangent to absorbance time curves. The calibration graphs were constructed by plotting the logarithm of the rate data of the reaction versus logarithm of molar concentration of FA.

The rate method involves addition of ammonium ferric sulfate solution (3.0×10-3 M) followed by 1.5 mL of potassium ferricyanide solution (2.5×10-3 M) to aliquot volumes of the drug solution in different 10 mL and then diluted to the volume with double distilled water at 25°C. The content of mixture of each flask, was mixed well and the absorbance of each sample solution at preselected fixed time (15 min) was accurately measured and plotted against the final concentration of the FA.

A spectrophotometric method has been described for estimating folic acid (vitamin B9) in pharmaceutical formulations. This method involves the reaction of folic acid with 1,2-naphthoquinone-4-sulphonate (NQS) in an alkaline medium ($NaOH/NaHCO_3$buffer, pH 11), producing a deep yellow compound. The method follows Beer's law for folic acid concentrations ranging from 0.75 - 10.5 µg/mL at 436 nm [24].

A spectrophotometric method for determining folic acid (FA) in pharmaceutical preparations. The method involves the initial oxidation of folic acid by aqueous potassium permanganate in an alkaline medium, followed by the addition of 4-nitro aniline. After diluting to the mark with water, the solution's absorbance was measured at 374 nm. The method exhibited linearity over the concentration range of 1-21 µg/mL [25].

A spectrophotometric technique for measuring folic acid in both pure and pharmaceutical forms has been outlined. This method involves the oxidation of pyrocatechol with iron (III) in an acidic environment, which then reacts with folic acid (FA) to form a stable, water-soluble orange compound. This compound has a maximum absorption at 350 nm when compared to the blank reagent. The charge transfer complex was analyzed under optimal conditions, and the titration graph demonstrated linearity within the range of 0.5-25 µg/mL [26].

2.3. Separation Methods

2.3.1. High-Performance Liquid Chromatographic Methods (HPLC)

HPLC is particularly well-suited for assessing the purity and quality of pharmaceutical preparations, especially when gas-liquid chromatography (GLC) is unsuitable due to the insufficient thermal stability or low volatility of the components. As a result, HPLC is favored over GLC for product quality control in most pharmaceutical companies and is included in most international pharmacopeias. The development of highly selective adsorbents and advancements in the sensitivity of flow-through spectrophotometric, fluorimetric, and electrochemical detectors have further boosted the use of HPLC in pharmaceutical analysis.

Reversed-phase high-performance liquid chromatography (RP-HPLC) has been widely utilized for quantifying folic acid in different matrices. Most of the reported methods employed isocratic elution using a reversed-phase column (C_8 or C_{18}) and mobile phases composed of organic solvent and buffers mixtures adjusted to specific pH levels.

Although all the reported methods have been validated, none have been systematically developed to justify the experimental conditions used. Moreover, the full potential of HPLC techniques has not been utilized in these methods to develop a stability-indicating method. This is crucial given the nature of folic acid, which degrades readily and is highly sensitive to environmental factors such as heat, UV light, and oxygen[27]. Description of the reported HPLC methods is given in Table 2.

Table 2: *HPLC methods used for the analysis folic acid*

No	Matrix	Column	Mobile phase	Determination range(µg/mL)	ƛ(nm)	(Ref)
1	folic acid in bulk and tablet dosage form	C_{18} (250 x 4.6 mm, 5 µm)	0.05M potassium dihydrogen orthophosphate buffer: methanol (70:30v/v). The flow rate was maintained at 0.6mL/min	10-50	212	[28]
2	folic acid and its related substances in tablets	C_8 (250 x 4.6 mm, 5 µm) 30°C	methanol and phosphate buffer (11.16 g/L KH_2PO_4 and 5.5 g/L dipotassium hydrogen phosphate with pH adjusted to pH=6.4 with dilute orthophosphoric acid) (12:88, v/v %). The flow rate was maintained at 0.7 mL/min	0.01 - 5.91	280	[29]
3	folic acid in multivitamin-mineral preparations	C_{18} (300 x 4.0 mm, 10µm)	To 13 mL of 10% aqueous tetrabutylammoniumhydroxide is added, 840 mL of water, 50mg of ethylenediaminetetraacetic acid disodium salt (EDTA), and160 mL of acetonitrile. The solution is adjusted by the additionof potassium dihydrogen phosphate to pH 7.6	0-5	280	[30]
4	folic acid in multivitamin and minerals preparations	C_{18} (300 x 3.9 mm, 20µm)	Acetonitrile:0.014M tetrabutylammonium hydroxide pH 7.2 16:84 v/v	5-20	283	[31]
5	folic acid in tablets	C_{18} (300 x 4.6 mm, 5 µm) 30°C	0.1% v/v trifluoroacetic acid and acetonitrile at ratio (80:20 v/v)	1-5	290	[32]
6	folic acid in pharmaceutical preparations	C_{18} (150 mm × 3 mm, 5 µm)	50 ml of a solution of 4.3 g sodium hexane sulfonate in 100 ml acetonitril/water 50/50, adjusted to pH 2.65 with glacial acetic acid in 1000 ml of a mixture of 23.4/76.6 methanol/water.	0.6–12	PDA detector	[33]
7	folic acid in tablets	C_{18} (150x4.6 mm, 3.5 µm)	acetate buffer pH 5.2 (50:50 v:v), at flow rate 1.0 mL/min	3.22-12.86	240	[34]
8	folic acid in tablets	C_{18} (250 x 4.6 mm, 5 µm) 40°C	trifluoroacetic acid and acetonitrile (90:10) The flow rate of 1.0 mL/min.	5-200	290	[35]
9	folic acid in tablets	C_8 (100 x 2.1 mm, 1.7µm)	Acetonitrile :0.005 M hexane sulfonic acid salt made pH 2.5 with phosphoric acid in the ratio of 10:90 v/v. The Flow rate was 0.4 ml/min	20-140	210	[36]

2.3.2 Thin-layer Chromatographic Methods

In the last two decades' thin layer chromatography (TLC) has become one of the most useful chromatographic methods, especially for qualitative analysis and preparative separations. Quantitative determination by TLC has never been as popular as gas chromatography and high-performance liquid chromatography, due to problems with sample applications, development and evaluation. However, due to the recent developments in instrumentation for TLC will which lead to a general improvement in accuracy and precision, TLC now is increasingly being used for the quantitative determination of drugs determination in tablets, capsules, solutions, ointments, and many other formulation types [37].

A TLC-densitometric method was developed for the separation and quantification of folic acid in the presence of its two impurities (photodegradation products), pteroic acid and para-aminobenzoic acid, on TLC silica gel 60 F_{254} plates. The developing system used was methanol:isopropanol:water: acetic acid (9:0.5:0.5:0.2, v/v). The separated bands were then measured densitometrically at 280 nm. The

method exhibited linearity over the ranges of 0.4-4 µg/mL for folic acid, 0.1-1.5 µg/mL for para-aminobenzoic acid, and 0.2-1.8 µg/mL for pteroic acid [19].

A high-performance thin layer chromatographic (HPTLC) method has been developed for identification andquantification of folic acid. The chromatography was performed on 20 cm × 10 cm silica gel $60F_{254}$ HPTLC plates. Plates weredeveloped to a distance of 90 mm in a 20 cm × 10 cm verticalchamber containing 40 mL mobile phase (isopropyl alcohol–ethylacetate–water–aqueous ammonia, 4 + 2 + 2 + 1 v/v/v/v). The spots detection was carried at 289 nm. The method was linear over the range of 25 -70 µg/mL [38].

A High Performance Thin Layer Chromatography (HPTLC) method was developed for analyzing folic acid in tablets. The separation was performed on pre-coated HPTLC silica gel 60 F254 glass plates using a mobile phase of ethyl acetate: methanol mixture (15:15:0.5 v/v/v). Detection was carried out at a wavelength of 280 nm, and the method exhibited a linear range of 317.19–761.25 ng/spot [39].

3. Conclusion

This study provides a thorough overview of commonly employed methods for analyzing folic acid. It encompasses a wide range of methods described for these compounds in various bulk or pharmaceutical dosage forms. The review spans from 1981 to the present day, offering a comprehensive analysis of the literature on this topic. The primary distinction among existing methods lies between chromatographic and non-chromatographic techniques. As depicted in Figure 2, chromatographic methods are predominant, constituting 40% of the utilized techniques, followed by colorimetric methods at 36% with direct UV and thin-layer chromatographic methods (TLC) at 12% each.

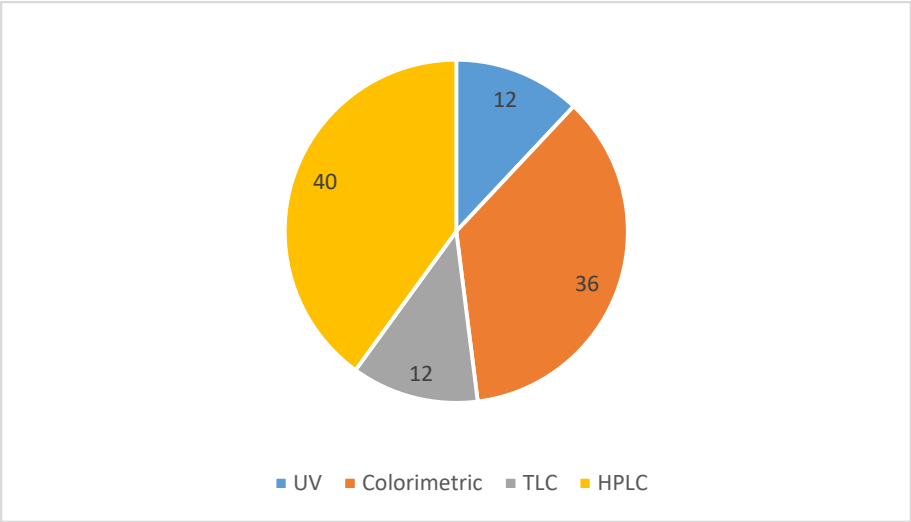

Figure 2. *Pie chart indicating the % ratio of the analytical methods used for the determination of folic acid*

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