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HARMONIZED EXAMINATION OF MESALAMINE AND KAEMPFEROL: A SIMULTANEOUS ESTIMATION APPROACH IN PRE-FORMULATION STUDIES

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ABSTRACT

This study focuses on the pre-formulation assessment of Mesalamine, an established anti-inflammatory drug, and Kaempferol, a natural flavonoid with potential therapeutic benefits. The aim is to concurrently estimate the two compounds using advanced analytical techniques. The research encompasses a thorough investigation of the physicochemical properties, compatibility, and stability of Mesalamine and Kaempferol. The simultaneous estimation of these compounds is crucial for the development of combination formulations, where their synergistic effects can be harnessed for enhanced therapeutic outcomes. A novel, simple, sensitive and rapid spectrophotometric method has been developed for simultaneous estimation of Mesalamine and Kaempferol. The method involved solving simultaneous equations based on measurement of absorbance at two wavelengths, 303 nm and 365 nm, λ max of Mesalamine and Kaempferol respectively. Beer's law was obeyed in the concentration range of 5-35 µg/ml and 10-60 µg/ml for Mesalamine and Kaempferol respectively. The method was found to be precise, accurate, and specific. The proposed method was successfully applied for estimation of Mesalamine and Kaempferol combinely. This research contributes to the pharmaceutical field by providing valuable insights into the pre-formulation aspects and simultaneous estimation of Mesalamine and Kaempferol, paving the way for the development of pharmaceutical formulations and to serves as a foundation for optimizing drug combinations and potentially improving treatment efficacy

1. INTRODUCTION

After drug discovery, the drug must be formulated in a suitable form for administration, considering its physical, chemical, and derived powered properties. Pre-formulation studies are a crucial step in the drug development process, focusing on understanding the physical and chemical properties of a drug substance before it is formulated into a dosage form. These studies provide essential information for designing a stable and effective drug formulation **(Shargel et al., 1999; Patrick, 2023; Rowe et al., 2009)**. Preformulation is the process of applying biopharmaceutical concepts to the physical and chemical characteristics of a medication in order to create the best possible drug delivery system. A crucial stage in the preformulation stage of product development is characterizing the therapeutic molecule. Preformulation studies are therefore a crucial tool in the early stages of the creation of both API and pharmaceutical products **(Gopinath and Naidu, 2011).** Mesalamine, also known as 5-aminosalicylic acid (5-ASA), is a medication used to treat inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease. It belongs to the class of drugs known as aminosalicylates, which have anti-inflammatory properties. Mesalamine works by reducing inflammation in the colon and helping to maintain remission in patients with IBD **(Sandborn, 2000; Conrad et al., 2014).** Mesalamine's precise mode of action is unclear. The inflammatory response resulting from the cyclooxygenase and lipooxygenase pathways is thought to be modulated by it, hence reducing the synthesis of prostaglandins and leukotrienes. Moreover, the cellular activities of mucosal lymphocytes, macrophages, and natural killer cells, as well as the inhibition of tumor necrosis factor (TNF) and reduction of nuclear factor κB (NF-κB) activity, could potentially interfere with the generation of inflammatory cytokines **(Garud and Peppercorn, 2009; Meier and Sturm, 2011).** Beside, Kaempferol is a flavonoid, a type of polyphenolic compound found in various plants, fruits, and vegetables. It is known for its antioxidant and anti-inflammatory properties and has been studied for its potential health benefits**.** Kaempferol is naturally present in foods such as kale, broccoli, tea, apples, grapes, and tomatoes. It is also found in medicinal plants like Ginkgo biloba. Kaempferol has been investigated for its potential health-promoting effects, including antioxidant, anti-inflammatory, anticancer, and cardiovascular protective properties. It may also have neuroprotective and anti-diabetic effects, though more research is needed in these areas **(Calderon et al., 2011; Boesch et al., 2011).** Since no spectrophotometric method is reported for simultaneous estimation of Mesalamine and Kaempferol in combination therefore, in the present work, a successful attempt has been made to estimate both these drugs simultaneously by simple UVspectrophotometric methods (absorbance ratio method, Simultaneous equation method for analysis and Multicomponent mode method).

2. MATERIAL AND METHOD

2.1 Chemical and Reagents

All the chemicals used in this study were obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem Chem. Ltd. (Mumbai, India). All the chemicals and solvent used in this study were of analytical grade.

2.2 Pre-formulation studies

2.2.1 Organoleptic Properties

Organoleptic properties were observed by visual observation. The Organoleptic studies of Kaempferol and Mesalamine such as appearance, color, odor, state etc. were observed.

2.2.2 Solubility study

Qualitative solubility of Kaempferol and Mesalamine in different solvents was determined according to **USP NF, 2007**. Drug (1 mg) was accurately weighed and transferred into a 10 ml test tube; then, it was dissolved in the different methanol, ethanol, DMSO, water, chloroform, Acetone, and n-hexane.

2.2.3 Melting Point

Melting point was analyzed by open Capillary method using Thiele's tube. Few quantity of the drugs (separately) was placed in a thin walled capillary tube 10-15 mm long, about 1mm inside diameter, and closed at one end. Liquid paraffin oil was filled in the thieles tube and placed in the contact of flame. The capillary was suspended into the thiele's tube and heat the sample slowly; thermometer was attached to check the temperature. The temperature at which the sample starts to melt was taken as the melting point of the sample **(Arslan and Tirnaksiz, 2010).**

2.2.4 pH

pH was determine using digital pH meter (EI). A 1-2 mg drug was dissolved in 10 ml distilled water and pH was measure by digital pH meter **(Chandel et al., 2011).**

2.1.5 Selection of common solvent

After the solubility study of both drugs in different solvents, water: methanol was confirmed as a common solvent for developing spectral characteristic.

2.3 UV estimation For Mesalamine and Kaempferol

Preparation of standard stock solution:

According to Indian Pharmacopoeia standard stock solution for API were prepared for that 10 mg of Mesalamine was dissolve in 10 ml of methanol (1000 µg/mL). Out of this stock 0.25-1.75 ml was pipetted and diluted up to 5 ml by solvent water: methanol (5-30 µg/mL) and examined between 200-800 nm and 10 mg of Kaempferol was dissolve in 10 ml of methanol (1000 µg/mL). **(AK Dash et al., 2013)** Out of this stock 0.1 ml was pipetted and diluted up to 10 ml by methanol (10-60 µg/mL) and examined between 200-800 nm. The maximum absorbance was determined using UV-Vis Spectrophotometer (UV- 1700, Shimadzu, Japan) to confirm the λ max of the drugs **(Joseph et al., 2015; Bhagav et al., 2010)**.

2.3.1 Drug: drug interference study

Standard stock solution (100 µg/ml) of Mesalamine and Kaempferol were prepared separately in methanol by serial dilution technique. The absorbance values for Mesalamine and Kaempferol were recorded at 303 nm and 365 nm respectively, using methanol (50:50) as a blank. Absorptivity values a (1%. 1 cm) were calculated for both wavelengths from absorbance values **(Dahshan et al., 2019; Patel and Kothari, 2020)**

2.3.2 Simultaneous Equation Method

From the standard stock solutions both drugs solutions were taken and made it to final concentration of 5-35 µg/ml and 10-60 µg/ml. Absorbance was measured at both the wavelengths by using solvent as blank. The reading was taken in triplicate. Absorbance maxima of both the drugs were recorded at both the wavelengths. The concentration was determined by using simultaneous equation method **(Gondalia et al., 2010; Dadhania et al., 2011).**

2.3.3 Simultaneous equation method formula

By using the below equations the concentrations in the samples were obtained **(Panchale et al., 2020)**

CX= A1ay2 – A2ay1 / ax1ay2 – ax2ay1 Eq. 1

CY = A1ax2 – A2ax1 / ay1ax2 – ay2ax1 Eq. 2

Where A1 and A2 are absorbances of mixture at 303 nm and 365 nm respectively, ax1 and ax2 are absorptivities of Mesalamine at λ1 and λ2 respectively, ay1 and ay2 are absorptivities of Kaempferol at λ1 and λ2 respectively, Cx and Cy are concentrations of Mesalamine and Kaempferol respectively.

A1 = ax1Cp + ay1Cs (At 365 nm)

A2 = ax2Cp + ay2Cs (At 303 nm)

A1 = ax1Cp + ay1Cs (At 365 nm)

A2 = ax2Cp + ay2Cs (At 303 nm)

A1 = absorbance value of the sample solution at 365 nm

A2 = absorbance value of the sample solution at 303 nm

ax1 = absorptivity of Mesalamine at 365 nm

ax 2 = absorptivity of Mesalamine at 303 nm

ay1 = absorptivity of Kaempferol at 303 nm

ay2 = absorptivity of Kaempferol at 365 nm

Cy = concentration of the Kaempferol in µg/ml

Cx = concentration of the Mesalamine in µg/ml

2.3.4 Study of Beer's Lambert Law

The solutions having concentrations in range 5-35 µg/ml and 10-60µg/ml for both Mesalamine and Kaempferol were prepared in methanol using working standard solution. The absorbances of resulting solutions were measured at 303 nm and 365 nm. Calibration curves were plotted at these wavelengths. Both the drugs obeyed linearity individually and combination within the concentration range of 5-35 µg/ml and 10-60µg/ml for both Mesalamine and Kaempferol **(Mehta et al., 2011).**

2.3.5 Precision Study (validation for method during analysis)

Both Inter- day precision and Intra-day precision were carried out as per the statistical requirement to support reproducibility of the method **(ICH, 2005; Balan *et al.*, 2011).**

2.3.5.1 Intra Day Assay (validation for method during analysis)

The assay procedure was carried out in the same day in the duration of 2 hours to 3 hours at fixed concentration and the results were compared.

2.3.5.2 Inter Day Assay (validation for method during analysis)
The assay procedure was carried out in the after day in the duration of 24 hours at fixed concentration and the results were compared.

2.3.6 Ruggedness study (validation for method during analysis)
The ruggedness of the method was determined by carrying out the analysis using two different analysts and the respective absorbance was noted. Ruggedness of the methods was assessed by carrying out assay 6 reading with different analyst by using same equipment **(ICH, 2005)**.

2.3.7 Robustness study (validation for method during analysis)
To determine the robustness, the same procedure was carried out by changing the temperature and the result is compared with the same previous procedure **(Swartz and Krull, 2006).**

2.4 Fourier transmission Infra-Red Spectroscopy
FT-IR spectrum of Drug and excipient combination was recorded over the range of 4000 to 400 cm−1 by KBr pellet method using a FT-IR spectrophotometer. The KBr disc was prepared using 1 mg of each drug and drug + polymers in 100 mg of spectroscopic grade KBr which has been dried using IR lamp. Both KBr and drug was mixed and subjected to hydraulic pressure to form disc. This disc was placed in FT-IR chamber. Infrared spectrum was recorded in the 4000 - 400 cm-1 region **(Luciene *et al.*, 2008).**

2.5 DSC
Thermal behaviour of samples was assessed using differential scanning calorimetry (DSC) analysis. Samples were sealed in an aluminium crimp cell and heated at a speed of 10°C/min from 30 to 300°C under nitrogen atmosphere (60 mL/min). A control empty pan was subjected to the same conditions **(Ghaderi et al., 2016).**

3. RESULTS

3.1 Organoleptic properties

Table 1 : Organoleptic properties of Kaempferol and Mesalamine

Organoleptic properties	**Observation (Kaempferol)**	**Observation (Mesalamine)**
Color	yellow powder	Off white color
Odour	Characteristic	Aromatic
Appearance	Powder	Powder
State	Solid powder	Solid powder

3.2 Solubility study

Table 2: Solubility study of Kaempferol and Mesalamine

Solvents	**Observation/Inference (Kaemferol)**	**Observation/Inference (Mesalamine)**
Water	soluble	Slightly soluble
Methanol	Soluble	Freely soluble
Ethanol	Insoluble	Freely soluble
DMSO	Freely soluble	soluble
Acetone	Insoluble	Sparingly soluble
Chloroform	Insoluble	Soluble

3.3 Determination of melting point and pH

Table 3: Melting Point and pH

S. No.	Drug	Observation (Melting point)	Observation (pH)
1.	Mesalamine	280°C	6.8±0.051
2.	Kaemferol	275°C	6.15±0.05

3.4 Lambda max of Kaempferol and Mesalamine

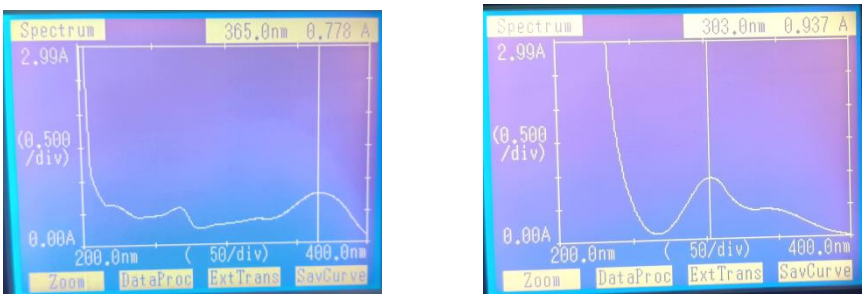

Figure 1: Lambda max of Kaempferol and Mesalamine

Table 4: Lambda max

S. No	Drug	UV absorption maxima (Lambda max)
1.	**Kaemferol**	365.0 nm
2.	**Mesalamineee**	303.0 nm

3.5 Standard curve of Kaempferol and Mesalamine

Table 5: Calibration curve of Kaempferol and Mesalamine

Concentration (µg/ml) of Kaempferol	Absorbance (365 nm) of Kaempferol	Concentration (µg/ml) of Mesalamine	Absorbance (303 nm) of Mesalamine
10	0.251	5	0.132
20	0.446	10	0.275
30	0.646	15	0.467
40	0.837	20	0.632
50	1.02	25	0.796
60	1.212	30	0.974
Mean	0.735333	**Mean**	0.546
SD	0.359125	**SD**	0.266616
%RSD	47.6	**%RSD**	48.8

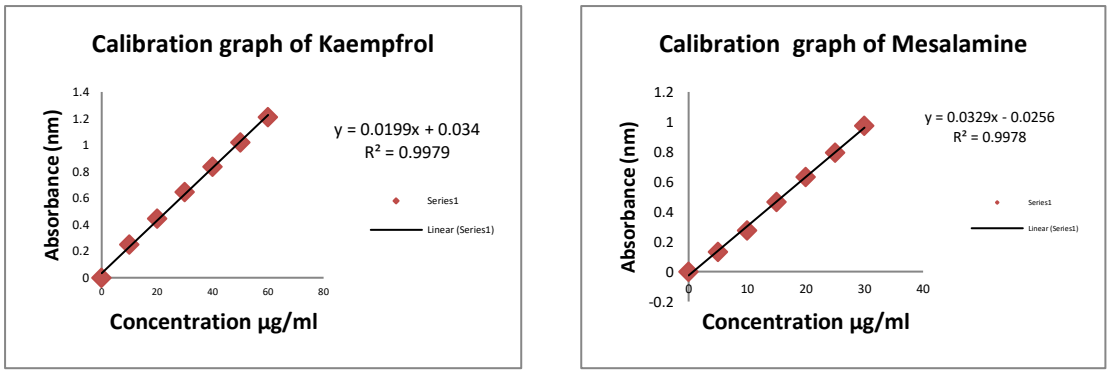

Figure 2: Calibration curves of Kaempferol and Mesalamine

3.6 Precision study

Table 6: Precision of Kaempferol and Mesalamine

Precision	**Concentration (µg/ml)**	**Mean**	**SD**	**% RSD**
Kaempferol	20	0.4415	0.00501	1.134
Intraday		0.4420	0.00337	0.748
Inter day		0.4366	0.00195	0.441
Mesalamine	20	0.62983	0.00264	0.419
Intraday		0.62827	0.00415	0.656
Inter day		0.62717	0.00347	0.55

3.7 Ruggedness

Table 7: Result of ruggedness of Kaempferol

Analyst-1		**Analyst-2**	
Concentration (µg/ml)	**Absorbance**	**Concentration (µg/ml)**	**Absorbance**
20	0.446	20	0.446
20	0.441	20	0.449
20	0.447	20	0.445
20	0.448	20	0.456
20	0.450	20	0.457
20	0.451	20	0.459
Mean	0.447167	**Mean**	0.452
SD	0.003545	**SD**	0.006066
% RSD	0.793	**% RSD**	1.34

3.8 Ruggedness

Table 2: Result of ruggedness of Mesalamine

Analyst-1		**Analyst-2**	
Concentration (µg/ml)	**Absorbance**	**Concentration (µg/ml)**	**Absorbance**
20	0.632	20	0.631
20	0.634	20	0.630
20	0.639	20	0.641
20	0.642	20	0.655
20	0.643	20	0.656
20	0.652	20	0.657
Mean	0.640333	**Mean**	0.645
SD	0.007174	**SD**	0.012665
% RSD	1.12	**% RSD**	1.96

3.9Robustness

Table 3: Results showing robustness of Kaempferol

Temperature 25^0C		**Temp 30^0C**	
Concentration (µg/ml)	**Absorbance**	**Concentration (µg/ml)**	**Absorbance**
20	0.446	20	0.441

20	0.447	20	0.449
20	0.449	20	0.442
20	0.448	20	0.456
20	0.450	20	0.457
20	0.452	20	0.452
Mean	0.448667	**Mean**	0.4495
SD	0.00216	**SD**	0.008042
% RSD	0.48	**% RSD**	1.78

3.10 Robustness

Table 10: Results showing robustness of Mesalamine

Temperature 25^{0}C		**Temperature 30^{0}C**	
Concentration (µg/ml)	**Absorbance**	**Concentration (µg/ml)**	**Absorbance**
20	0.632	20	0.631
20	0.630	20	0.629
20	0.636	20	0.628
20	0.641	20	0.626
20	0.648	20	0.630
20	0.649	20	0.629
Mean	0.639333	**Mean**	0.628833
SD	0.008042	**SD**	0.001722
% RSD	1.25	**% RSD**	0.274

3.11 Determination of Iso-bestic point and selection of suitable Wavelength

An Iso-bestic λ point (a wavelength of equal absorptivity of the two components) was determined by taking overlain spectrum of the solutions Mesalamine and Kaempferol (20 µg/ml each) in methanol in UV range against the solvent blank.

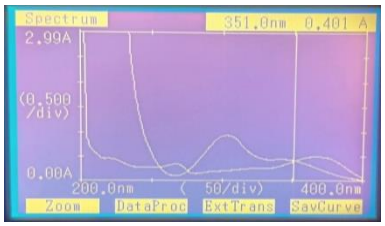

Figure 3: Overlay spectra of Mesalamine and Kaempferol with Iso absorptive point

3.12 Compatibility study by FTIR

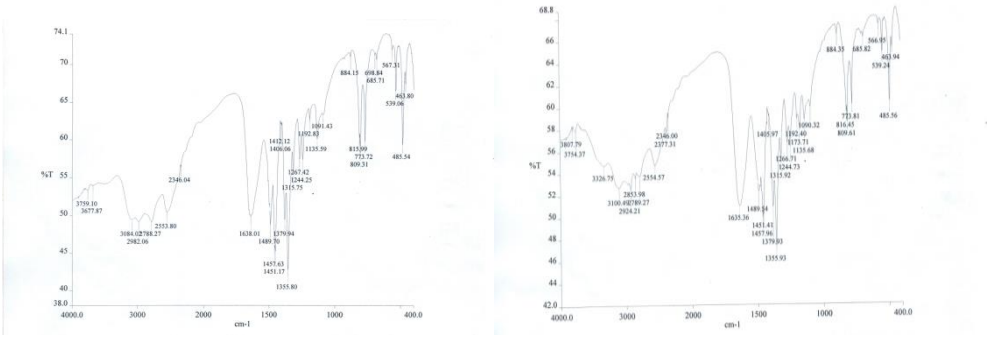

Figure 4: FTIR of Mesalamine and Mesalamine + Kaempferol

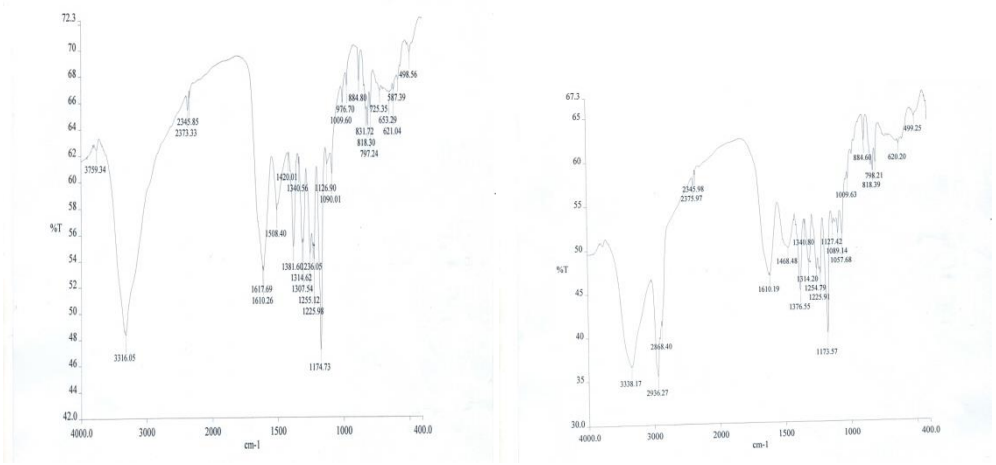

Figure 5: FTIR of Kaempferol and Mesalamine + Kaempferol+ Exceipients (cholestrol)

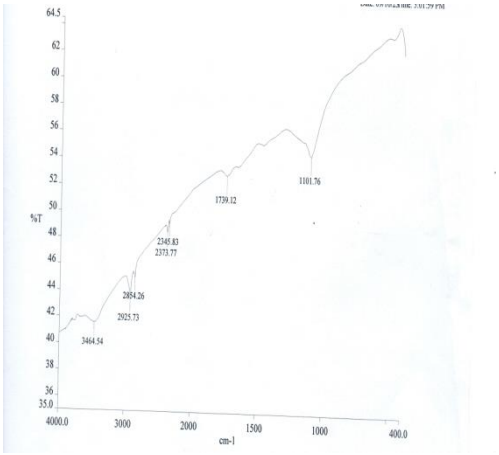

Figure 6: FTIR of Mesalamine + Kaempferol+ Exceipients (soyalecithin)

4. DISCUSSION

An evaluation of the API's organoleptic qualities, including Appearance, color, odor, and state, was conducted. Kaempferol was discovered to have a yellow powder color to it when tested and Mesalamine was discovered to have an off white color to it when tested. Kaempferol has a characteristic odor and has a solid state powder form along Mesalamine exhibited the same appearance, color, odor and state as the I.P. requirements for these characteristics. Result shown in **Table 1.** The solubility of Kaempferol and Mesalamine was determined in various non-volatile or volatile liquid vehicles such as water, methanol, ethanol, DMSO, acetone and chloroform shown in **Table 2**. From the results, it was observed that the Kaempferol was soluble in water and methanol along Mesalamine was freely soluble in Methanol, ethanol and DMSO, Soluble in chloroform and slightly soluble in water. The capillary method is used to determine the melting point of a substance. The melting point of the Kaempferol and Mesalamine was found to be 280°C ºC and 275^{0}C, which is well within the limits of the drug specification range. The digital pH meter used to determination the pH of the Mesalamine and Kaempferol. These were found to be 6.8 ± 0.05 and 6.15±0.051. These were well within the limits of the drug specification range. UV- visible spectrophotometer (1700- Shimadzu) is used to determine the lambda max (absorption maxima) of a substance. The lambda max of the Kaempferol and Mesalamine was found to be 365nm and 303 nm respectively. These values

were well within the limits of the drug specification. The linearity of the proposed method was established by least squares linear regression analysis of the calibration curve. The regression equation of Kaempferol and Mesalamine was obtained by plotting absorbance versus concentration of Kaempferol in the range of 10-60 µg/mL and Mesalamine in the range of 5-30µg/mL. The linearity of an analytical procedure was its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Six points calibration curve were obtained in a concentration range from 10-60 µg/mL for Kaempferol and seven points calibration curve were obtained in a concentration range from 5-30µg/mL for Mesalamine. The response of Kaempferol was found to be linear in the investigation concentration range and the linear regression equation was $y = 0.019x + 0.034$ with correlation coefficient $R^2 = 0.997$. For Mesalamine the regression equation was $y = 0.032x - 0.025$ and the regression coefficient ($R^2 = 0.997$) was calculated. The precision of the developed method was expressed in terms of % relative standard deviation (% RSD). The intermediate precision was assessed by comparing the results obtained on three different days. The experimental values (%RSD) obtained for repeatability (intra-day precision) and intermediate precision (inter-day precision) were found to be 89.6, 58.3 and 0.656, 0.55 respectively for Kaempferol and Mesalamine. Ruggedness of the proposed method was evaluated by comparison of the absorbance of both drugs that have been measured by two different analysts in the same laboratory. Ruggedness was carried out at 20 µg/ml concentration. The results are expressed as standard deviation and relative standard deviation and it was found 0.447167 ± 0.003545 for analyst-1 and 0.452± 0.006066 for analyst-2 whereas the value of % RSD was recorded 0.793 and 1.34 respectively for Kaempferol, for Mesalamine it was found 0.640333 ± 0.007174 for analyst-1 and 0.645 ± 0.012665 for analyst-2 whereas the value of % RSD was recorded 1.12 and 1.96 respectively. The results are presented in **Table 7 and 8.** The robustness of the proposed method was assessed with changes in the analytical temperature. Robustness was carried out at concentration 20 µg/ml at temperature 25^0C and 30^0C. The results are expressed as mean with standard deviation and relative standard deviation and were recorded 0.448667 ± 0.00216 and 0.4495 ± 0.008042 whereas % RSD was found 0.48 and 1.78 for Kaempferol and 0.639333 ± 0.008042 and 0.628833 ± 0.001722 whereas % RSD was found 1.25 and 0.274 for Mesalamine respectively. The results are compiled in **Table 9 and 10.** From the overlain spectra of the two drugs, it was found that Mesalamine showed λ max at 303 nm and Kaempferol showed λ max at 280 nm. Iso-absorptive point was found out at 365 nm, as Iso-absorptive point was selected for estimation of Drug simultaneously shows in **fig: 3**. The individual concentration range for beer-lambert was found 5-35 µg/ml for both Mesalamine and Kaempferol at 303 nm and 365 nm with correlation coefficient 0.997 and 0.997. UV scan of 5-30 µg/ml and solution of Mesalamine and aloin combination showed the absorption maxima at 303 nm, 365 nm and 351 nm. The simultaneous estimation was done to check the interference between both the drugs at the λ max of one another. By substituting absorbance and absorptivity values in simultaneous equation, C1 and C2 were calculated, Cx: 5.3 µg/ml and C2: 7.38 µg/ml. The percentage of Mesalamine and Kaempferol recovered after the combination was found to be 88.9 % and 78.6 % respectively indicating no interference between both the drugs. The Linearity was observed by the linear regression equation method for Mesalamine and Kaempferol in different concentration range. The correlation coefficient of these drugs was found to be close to 1.00, indicating good linearity. Hence proposed method

can be used for routine analysis of these two drugs in combined dosage form. It was validated as per ICH guidelines.

5. CONCLUSION

The proposed UV spectrophotometric method was found very simple, rapid and economical. However, the most important outcome of the simultaneous estimation was that can formulate and analyse both the drugs in combination for any suitable dosage form in a very safe and effective way. The method was validated in compliance with ICH guidelines was suitable for simultaneous estimation of Mesalamine and Kaempferol with excellent recovery, precision and linearity.

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