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A Rapid and Precise Technique for Identifying 5-HIAA in Complex Biological Specimens

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ABSTRACT

Ehrlich reagent has been commonly used as colorimetric reagents for detection of indole group compound. The method is advancement for the colorimetric determination of 5-HIAA metabolite. The preparation of reaction mixture containing Ehrlich reagent (100 mg) in Conc HCl: Methanol (9:1) is simple, requires less time consuming. The sample is pre-treated with 0.1M per chloric acid for deproteinization of protein or interfering components is removed and the filtrate obtained after treatment of a small quantity of urine. The addition of Ehrlich's reagent mixture to this filtrate results in the immediate production of a stable green colour within 10sec of interaction of 5-HIAA present in urine sample. The determination is accurate, very sensitive and rapid, and reaction is completed within 1 min.

Keywords: 5-HIAA, Ehrlich reagent, Colorimetric detection.

INTRODUCTION

1. INTRODUCTION

For over a century, Ehrlich's reagent has been utilized in spot tests to detect indoles resulting from bacterial or intracellular enzyme action on tryptophan [1-3]. In these assays, the presence of indole triggers a color reaction between p-dimethylaminobenzaldhyde (DMAB, 1) and urine metabolites, aiding in the diagnosis of conditions such as liver diseases, hemolytic processes, common bile duct obstruction, and carcinoid syndrome [4]. Specific bacteria metabolize tryptophan, an amino acid, into various indolic compounds, including indole itself [1-3]. Ehrlich's indole test identifies the presence of such bacteria by producing a distinct red compound.

A novel method for determining urea levels, employing Ehrlich's reagent [5]. This reagent, as previously established by Ehrlich (1901) and others [6, 7], reacts chromatometrically not only with urea but also with various other substances like urobilinogen, indican, phenol, allantoin, tryptophan, and uric acid.

Their suggested approach involves determining urea in blood and urine after eliminating interfering substances. They achieve this by treating the deproteinized filtrate with activated carbon, ensuring that Ehrlich's reagent exclusively reacts with urea. Levine et al. demonstrated the specificity of this reaction by showing that the same filtrate, treated with urease, doesn't elicit a colorimetric response with Ehrlich's reagent. Additionally, they provided

evidence through identical spectral analyses of urea and blood filtrate complexes with Ehrlich's reagent.

Furthermore, [8] confirmed that detection of indoleacetic acid (IAA) and indolebutyric acid (IBA) in plant samples reacting with Ehrlich's reagent by producing blue and pink color in plant samples is described. The color change is based upon the reaction between the auxins and p-(dimethylamino)benzaldehyde (Ehrlich reagent) following the electrophilic substitution reaction mechanism at the indole ring.

The indole, in the bacterial product of tryptophan degradation, has a variety of important applications in the pharmaceutical industry and is a biomarker in biological and clinical specimens [9, 10]. Thus, indole in biological samples is often estimated using the simple and rapid Kovac's assay, which non-specifically detects a variety of commonly-occurring indole analogs.

MATERIALS & METHODS

The 5-HIAA standard solutions were prepared by diluting 1.0 mg Ml-1 solution of 5-hydroxy indole-3-acetic acid (5-HIAA) (Sigma Aldrich). A stock solution containing 100 mg/Ml p-(dimethylamino) benzaldehyde (PDAB, Ehrlich reagent, Himedia, India) in Conc HCl and methanol (9:1) was prepared. Perchloric acid (Merck India) were used in this study were of analytical grade. Double distilled water was used all through this work. All spectral measurements were performed on a Shimadzu UV-1900 UV-visible spectrophotometer (Shimadzu Corporation, assembled in China) in the wavelength range of 800-200 nm with 1 cm path length cell.

2.1 METHODOLOGY:

2.1 Development of chromophoric reaction in artificial biological fluid:

For development of chromophoric color detection of 5-HIAA in artificial biological fluids (as shown in table 1) the trials were carried out on the reported tests for functional group determination acid (KI-KIO_3 Test, Neutral ferric test), alcohol (Cerric ammonium nitrate, Lucas test, Chromic acid test), phenol (Libermann's nitroso test, Phthalein test, Azodye test) groups; the tests were selected based on the presence of specific functional group present in the metabolite structure. The 100 ug/ml of standard (5-HIAA) metabolite concentration were added in the artificial tear, saliva & urine fluid for chromophoric reaction [11, 12].

2.1.1 (a) Preparation of artificial biological fluids (tear, saliva, urine):

Table 1. Artificial tear, saliva, urine

Sr no	Artificial tear [13, 14]		Artificial Saliva [11]		Artificial Urine [9, 12]	
	Components	**Mmol/L**	**Components**	**g/Lit**	**Components**	**g/Lit**
1	Albumin	20.5	Calcium chloride	0.27	Ammonium chloride	1
2	Ammonium chloride	3	Magnesium chloride	0.1	Calcium chloride	0.651
3	Calcium chloride	16	Methyl Paraben	0.67	Creatinine	1.1
4	Citric acid	0.031	Methyl phydroxybenzoate	24	Formyl hydrazine	0.1500
5	Lactic acid	2.5	Potassium chloride	0.96	Magnesium chloride	0.651
6	Lysozyme	0.12	Potassium phosphate	1000	Potassium Chloride	1.6
7	Pyurvic acid	0.2	Propyl Paraben	0.96	Potassium hydrogen phosphate	2.8
8	Sodium bicarbonate	26	Sodium chloride	0.12	Sodium oxalate	0.02
9	Sodium chloride	100	Sorbitol	0.04	Sodium chloride	4.6
10	Urea	5	Water	0.01	Sodium sulfate	0.65
11	Vit C	0.008			Trisodium citrate dihydrate	0.65
12	y-globulins	0.53			Urea	25.0

2.1.2 Development of chromophoric color reaction for detection of 5-HIAA in rat serum/urine sample:
Exhaustive literature review was done wherein some common reagents were selected for chromophoric reaction and various trials were done individually and in combination of different by mixing them together.

2.1.3 Identification of specific reagent for chromophoric reaction of 5-HIAA:
Based on the structural moiety of 5-HIAA the commonly used reagents at laboratory used and some pilot trial experiments were carried out by reacting the metabolite with reagent. The trials were performed by using common laboratory reagents.

2.1.4 Identification of method for preparation and solvents for Ehrlich's reagent:
The initial trials were conducted based on the reported methods in literature and Indian Pharmacopeia 2018 considering the time of reaction to complete color development and stability of the color formed after chromophoric complex. The ratio of solvent combination was selected as follows 9:1, 8:2, 7:3, 6:4, 5:5, 4:6 3:7, 2:8, 1:9.

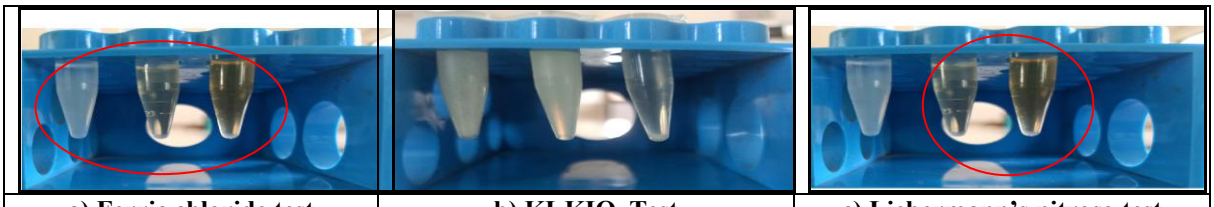
2.1.5 Optimization of solvent mixture for Ehrlich's reagent:
The series of trials were conducted by using selected Ehrlich's reagent that were mixed with solvent combination of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6 3:7, 2:8, 1:9 for a chromophoric reaction with 5-HIAA, the best suited combination of solvent mixture were finalized and optimized.

2.1.6 Optimization of Ehrlich's reagent 1 concentration & sample volume:
Various trials were performed by increasing volume of reagent concentration and sample volume in graded manner and the volume was optimized based on the visible colour obtained after chromophoric reaction between the reagent concentration and sample volume was selected.

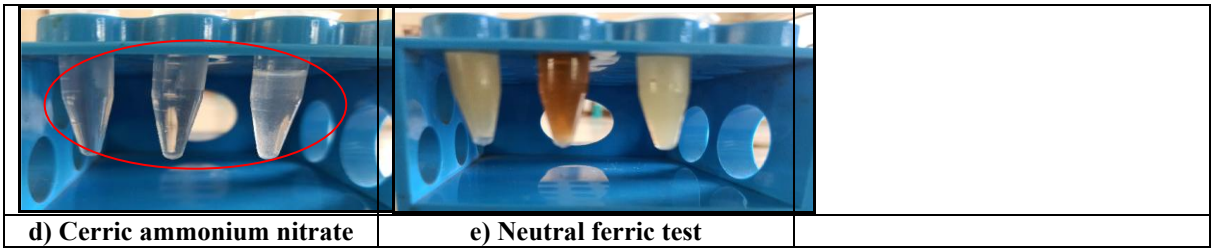
2.1.7 Determining the lowest conc. Of Ehrlich reagent and lowest conc. Of metabolite (5-HIAA) to be detectable:
The optimized reaction mixture conc HCL: methanol (9:1, 8:2) for Ehrlich reagent concentration (100, 10 mg/ml) was reacted with different concentrations of 5-HIAA 100, 50, 10, 1 µg/ml to determine the lowest concentration of 5-HIAA to be detectable by chromophoric reaction.

3. RESULT

3.1 Development of chromophoric reaction in artificial biological fluid:
The chromophoric reaction was established by performing different trials of 5-HIAA, HVA, VMA by adding 100 µg/ml of each in artificial tear, saliva, urine sample to find out the suitable biological fluid. Various functional tests were carried out to identify the specific reagent for each 5-HIAA, HVA, VMA where it was found that chromophoric reaction for these metabolites was achieved (fig 1). Based on the trials conducted it was observed that as observed in (fig no 1) test no a, c, d reaction of metabolite with reagent chemical produced clear solution after reaction, but in test b, e it produces turbid & precipitated solution. From the observation Ferric chloride was selected as one of reagent.



a) Ferric chloride test	b) KI-KIO_3 Test	c) Liebermann's nitroso test



d) Cerric ammonium nitrate	e) Neutral ferric test	

Fig 1: Different trials carried out in artificial biological fluids

3.2 Development of chromophoric color reaction for detection of 5-HIAA, HVA in Wistar rats serum/urine Sample:

The detection of 5-HIAA, HVA is possible by existing analytical techniques like HPLC, LCMS, ELISA method in serum, plasma, urine samples for research purpose [11]. These methods are time consuming, requires several steps for sample preparation, and not used at laboratory practice for clinical diagnosis. Thus these methods are not routinely performed at laboratory scale for diagnosis. Thus a simple qualitative & quantitative method based on chromophoric approach can be developed.

3.2.1 Identification of specific reagent for chromophoric reaction of 5-HIAA:

Different trials were conducted for identification of specific reagent for 5-HIAA metabolite. Various reagents were used for chromophoric color reaction with 5-HIAA in different combinations of this Ferrous sulphate, Ammonium iron 327ilkins327 hexahydrate, Ammonium chloride, Barium sulphate, DTNB, Potassium Ferric cyanide, Ellaman's reagent, Bromocresol green, Bromothymol blue, Ninhydrin, Sodium nitrite, Potassium hydroxide, Ehrlich reagent. The results obtained from trials conducted showed that Ehrlich reagent showed chromophoric color change by specifically forming complex with indole present in 5-HIAA structure (fig 2). Further trials were conducted to identify solvent for Ehrlich reagent solubility as shown in table 2, where reaction mixture contains: 1 gm of Ehrlich reagent in following below solvents and for achieving chromophoric reaction the 200 µl of sample + 800 µl of Ehrlich reagent reaction mixture was reacted.

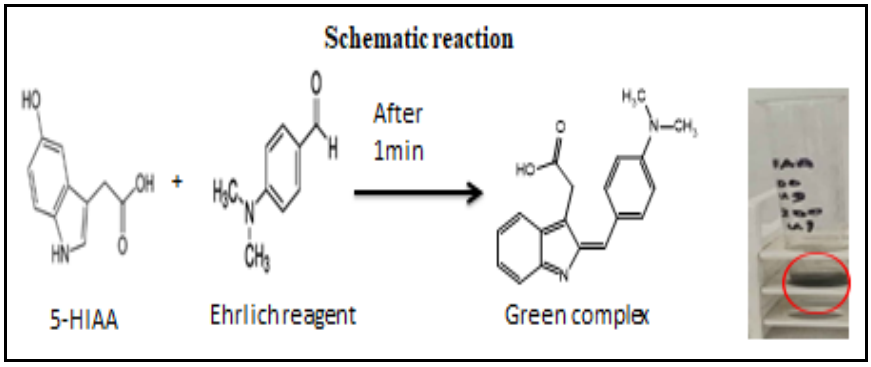


Fig 2: Schematic reaction for 5-HIAA with Ehrlich reagent

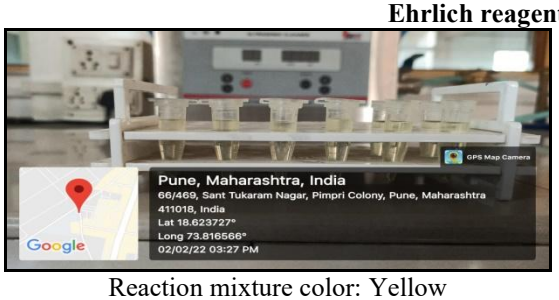
The observations obtained after dissolving Ehrlich reagent with different solvents where it was observed that the Ehrlich reagent was completely soluble in methanol (color of reaction mixture was light purple) but when treated with 5-HIAA it did not show any color change Thus next trials were conducted by selecting methanol as one of the components of reaction mixture.

Table 2: Identification of solvent for Ehrlich reagent solublity

Solvents	Ehrlich reagent	Observations		
		HVA	VMA	5-HIAA
Diethyl ether	soluble	No reaction	No reaction	Dark brown
Chloroform	soluble	Layer separation		
Acetic acid	soluble	No reaction	No reaction	Light blue
Methanol	soluble	No reaction	No reaction	Light Purple

Additional trials were conducted on method 1, for finding an alternative to as Conc HCl is highly acidic. Various combinations of solvents were tried to replace Conc HCl by using weak acids (acetic acid, o-phosphoric acid) in fig 3 to fig 9, summarized in table 3.

Fig 3: Trial no 1: Conc HCL: Methanol
Reaction vol: 1ml
HIAA Conc: 1mg in 10 ml
Ehrlich reagent Conc: 100mg/ml



Reaction mixture color: Yellow

After reaction, Reaction mixture color change: Blue to light green

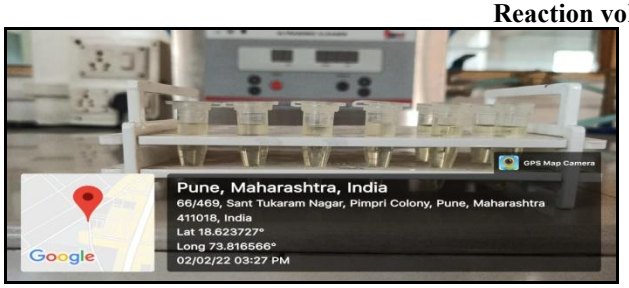
Solvents	Dilution ratio								
Conc HCL (µl)	9	8	7	6	5	4	3	2	1
Methanol (µl)	1	2	3	4	5	6	7	8	9

Fig 4: Trial no 2: Ethyl acetate: Acetic acid
Reaction vol: 1ml

Reaction mixture color: Dark brown

After reaction, Reaction mixture color change: Dark brown

Fig 5: Trial no 3: Conc HCL: 2-propanol
Reaction vol: 1ml



Reaction mixture color: Yellow

After reaction, Reaction mixture color change: Variation in color observed no specific color

Solvents	Dilution ratio								
Conc HCL (µl)	9	8	7	6	5	4	3	2	1
2-propanol (µl)	1	2	3	4	5	6	7	8	9

Fig 6: Trial no 4: Ethanol: 1-butanol: Conc HCl
Reaction vol: 1ml

Reaction mixture color: Yellow

After reaction, Reaction mixture color change: Dark blue, Time-consuming reaction

Solvents	**Dilution ratio**					
Ethanol (µl)	100	75	50	125	50	100
1-butanol (µl)	600	450	300	700	900	800
Conc HCl (µl)	100	75	50	125	50	100

Fig 7: Trial no 5: Ethyl acetate: Conc HCl
Reaction vol: 1ml

Reaction mixture color: Yellow

After reaction, Reaction mixture color change: Variation in color observed no specific color

Solvents	**Dilution ratio**								
Ethyl acetate (µl)	9	8	7	6	5	4	3	2	1
Conc HCl (µl)	1	2	3	4	5	6	7	8	9

Fig 8: Trial no 6: Methanol: Acetic acid
Reaction vol: 1ml

Reaction mixture color: Yellow

After reaction, Reaction mixture color change: Yellow

Solvents	**Dilution ratio**								
Methanol (µl)	9	8	7	6	5	4	3	2	1
Acetic acid (µl)	1	2	3	4	5	6	7	8	9

Fig 9: Trial no 7: Methanol: O-phosphoric acid
Reaction vol: 1ml

Reaction mixture color: Orange

After reaction, Reaction mixture color change: Orange

Solvents	**Dilution ratio**								
Methanol (µl)	9	8	7	6	5	4	3	2	1
O-phosphoric acid (µl)	1	2	3	4	5	6	7	8	9

Table 3: Summarize results for trials conducted to finalize reagent for 5-HIAA metabolite

Trial no	**Chemicals composition**	**Observation**	**Inference**
1	Di methyl amino benzaldehyde (100 mg) + Conc HCl+ Methanol	Blue to light green	positive
2	Di methyl amino benzaldehyde (100 mg) + Ethyl acetate + Acetic acid	No color change	Negative
3	Di methyl amino benzaldehyde (100 mg) + 2-propanol + Conc HCl	No color change	Negative
4	Di methyl amino benzaldehyde (100 mg) + ethanol + 1-butanol + Conc HCl	No color change	Negative
5	Di methyl amino benzaldehyde (100 mg) + Methanol+ Acetic acid	No color change	Negative
6	Di methyl amino benzaldehyde (100 mg) + Methanol + O-Phosphoric acid	No color change	Negative
7	Di methyl amino benzaldehyde (100 mg) + Ethyl acetate + Conc HCl	No color change	Negative

From the trials conducted above weak acids were not able to form chromophoric complex, thus the trial 1 (fig 3) where the Ehrlich reagent was found to form chromophoric complex with 5-HIAA by changing colour from yellow to blue to light green was finally chosen for further study. Reproducible results were observed by dissolving Ehrlich reagent (100 mg) in Conc HCl: Methanol (900:100µl and 800:200µl). Thus Ehrlich reagent was selected as specific reagent for 5-HIAA.

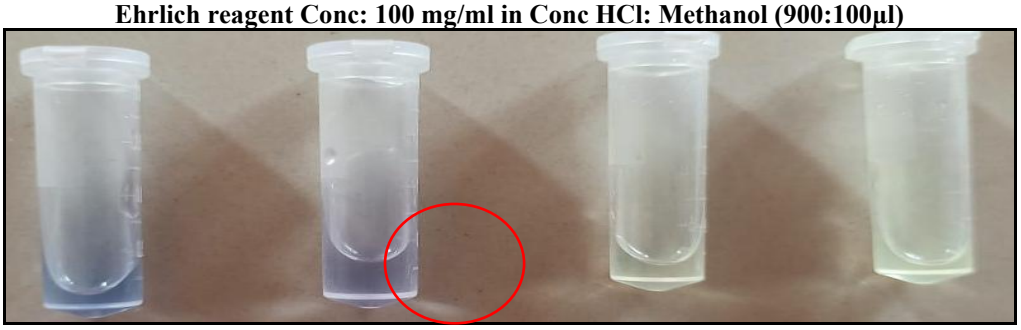
3.2.2 Optimization of solvent mixture for Ehrlich reagent

The series of trials were conducted by using Ehrlich reagent that were mixed with different solvent combination of Conc HCl: Methanol in ratio of 900:100µl, 800:200µl, 7:3, 6:4, 5:5, 4:6 3:7, 2:8, 1:9 for a chromophoric reaction with 5-HIAA. The combination of conc HCl: methanol in 900:100µl, 800:200µl was found to form chromophoric complex with 5-HIAA, based on observations it was observed that as the concentration of conc HCl decreases the time required for colour development is more, intensity of colour formed was less as the complex of Ehrlich reagent with 5-HIAA was formed only in acidic medium because of slightly acidic Ph of reaction mixture i.e. 0.62 which is highly acidic due to conc HCl. Thus, Ph of reaction mixture should be below 3 while carrying out reaction.

3.2.3 Optimization of Ehrlich reagent concentration & 5-HIAA metabolite concentration

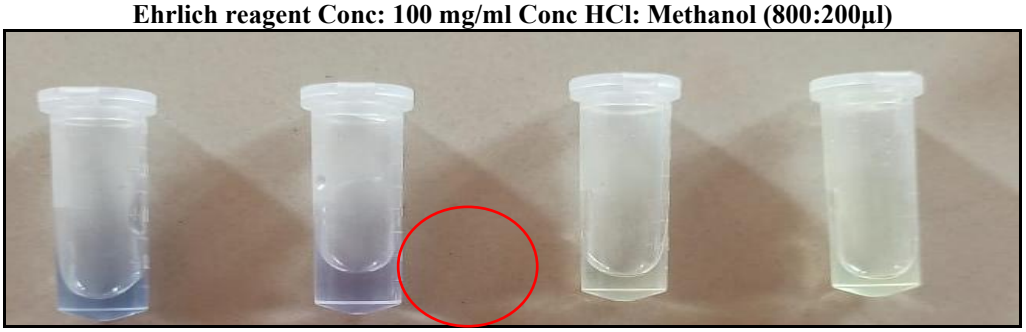
The concentration of Ehrlich reagent was reduced to identify its lowest concentration that forms chromophoric color reaction with 5-HIAA. Series of trials were conducted where trial no 1 & 2 (fig 10, 11) Ehrlich reagent Conc: 100 mg/ml in Conc HCl: Methanol (900:100µl; 800:200µl) was able to form complex with 5-HIAA (50 µg/ml) in artificial urine, but when the concentration of Ehrlich reagent was reduced of Ehrlich reagent Conc: 10 mg/ml in Conc HCl: Methanol (900:100µl; 800:200µl) the complex was formed only at 5-HIAA (100 µg/ml; 50 µg/ml) in artificial urine trial no 3 & 4 (fig 12, 13) that was observed by naked eye. Thus, the best results were observed at concentration of 100 mg/ml of Ehrlich reagent in Conc HCl: Methanol (900:100µl).

Fig 10: Trial no 1
Ehrlich reagent Conc: 100 mg/ml in Conc HCl: Methanol (900:100µl)



5-HIAA Conc µg/ml	100 µg	50 µg	10 µg	1 µg

Fig 11: Trial no 2
Ehrlich reagent Conc: 100 mg/ml Conc HCl: Methanol (800:200µl)

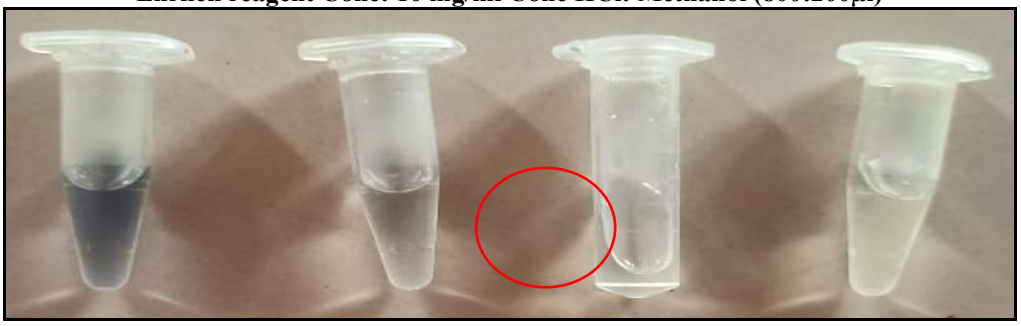


5-HIAA Conc µg/ml	100 µg	50 µg	10 µg	1 µg

Fig 12: Trial no 3
Ehrlich reagent Conc: 10 mg/mlConc HCl: Methanol (900:100µl)

5-HIAA Conc µg/ml	100 µg	50 µg	10 µg	1 µg

Fig 13: Trial no 4
Ehrlich reagent Conc: 10 mg/ml Conc HCl: Methanol (800:200µl)



5-HIAA µg/ml	100 µg	50 µg	10 µg	1 µg

Table 4: Summary of reaction mixture for 5-HIAA

Sr no	Parameters	
1	Ehrlich reagent conc	100 mg/ml
2	Reaction mix. Combination	Conc HCl: Methanol (900:100µl)
3	Reaction mixture vol	1 ml
4	Sample Volume	200 µl
5	Metabolite conc (visible color was produced)	50 µg/ml

3.2.4 Development of UV method for detection of 5-HIAA by Ehrlich reagent in urine sample

The development UV method was carried out considering parameters while UV method development such as λ_{max}, time of reaction, interference of other components on colour reaction, effect of change in Ph of urine sol. on colour reaction, complex confirmation by IR & LCMS method.

3.2.4 (a) Identification of λ_{max}

The λ_{max} of chromophoric complex of 5-HIAA with Ehrlich reagent was identified by establishing reaction of 200 µl of 5-HIAA (100 µg/ml) reacted with 1 ml of 100 mg/ml of Ehrlich reagent and the spectra was recorded from 400 to 800 nm (in UV visible range) as the color is visible by naked eye and the λ_{max} of complex was found to be 441 nm (fig 14). Also, whether solvents used are interfering in color formation or not, thus the spectra of methanol (Fig 15), conc HCl (Fig 16), blank reaction mixture (Fig 17).

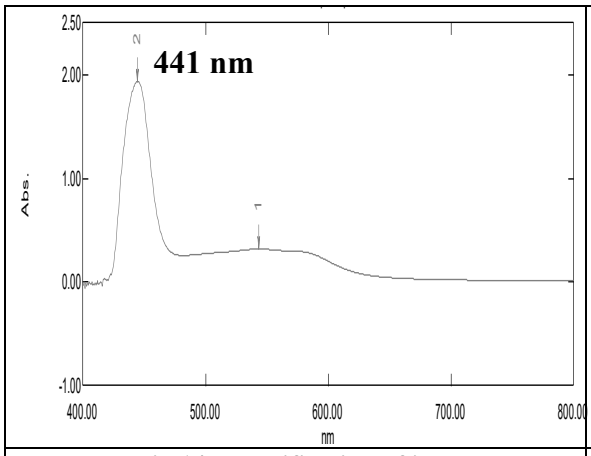


Fig 14: Identification of λ_{max}

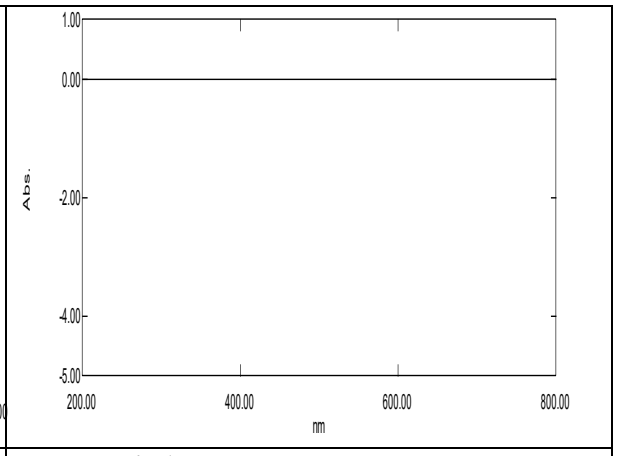


Fig 15: Methanol blank spectrum

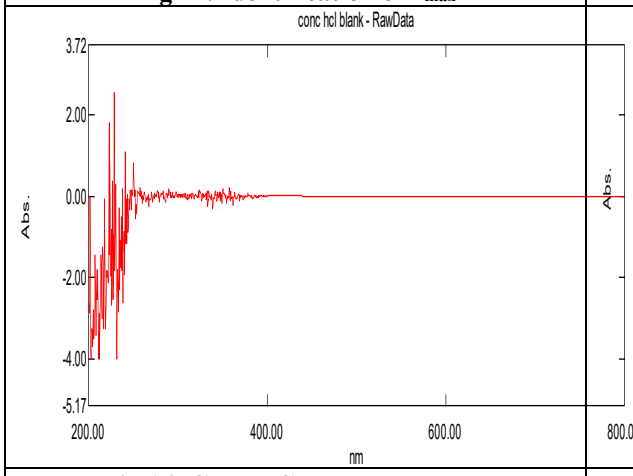


Fig 16: Conc HCl blank spectrum

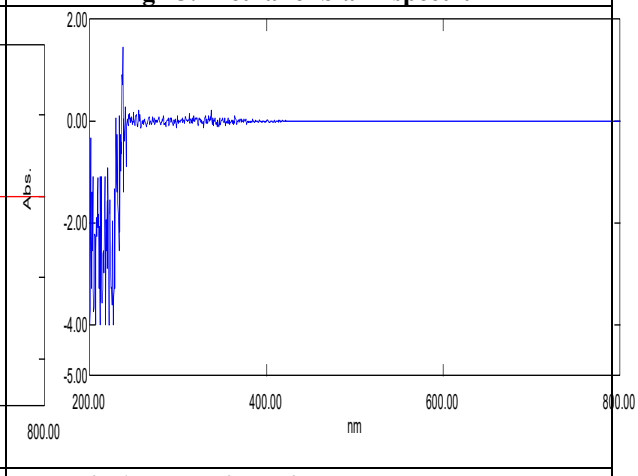


Fig 17: Reaction mixture blank spectrum

3.2.4 (b) Identification of Time of reaction

The time of reaction was identified by reacting 200 µl of 5-HIAA (100 µg/ml)

solution with 1 ml of 100 mg/ml of Ehrlich reagent reaction mixture & the spectra was recorded after 1 min on color development and at 5 min to determine the stability of complex formed. The reaction was completed within 1 min (red line) and even the complex was stable after 5min (blue line) (fig 18).

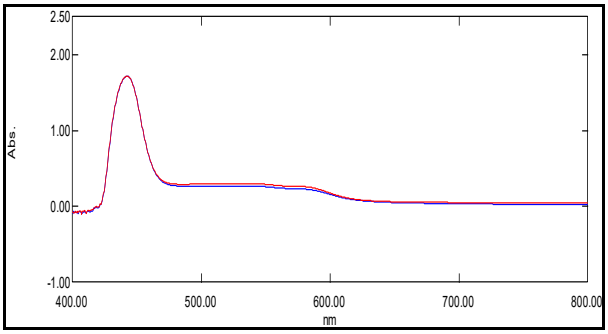
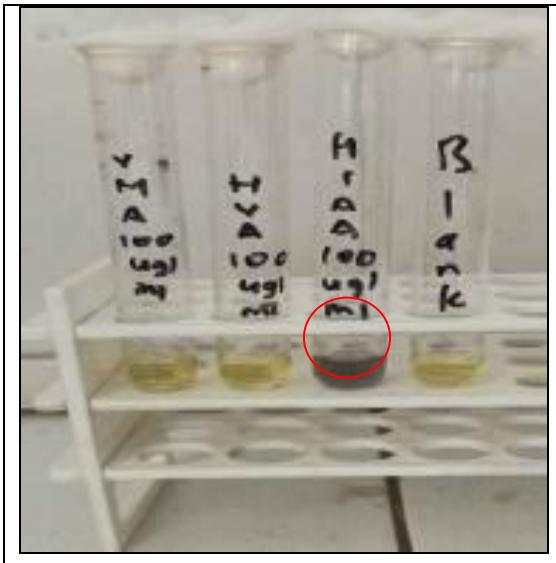
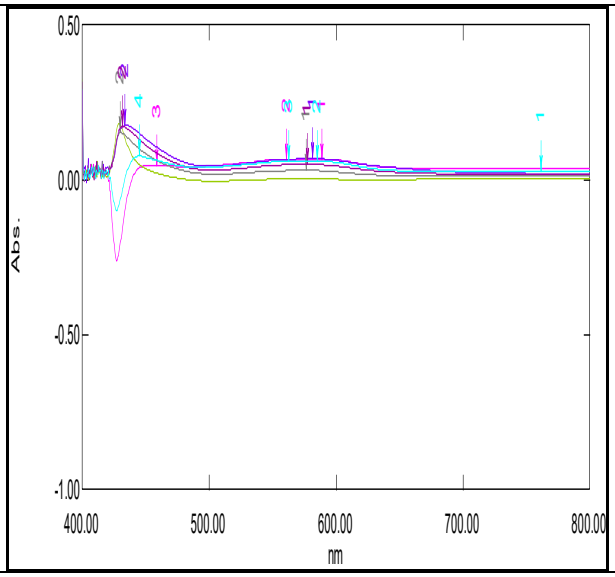


Fig 18: Time of reaction (1 min & 5 min)

3.2.4 © Interference of other components on colour reaction

To determine the specificity of reagent selected the interference of other metabolites (HVA, VMA) that excreted through urine. The stock solution of 100 µg/ml of HVA, VMA, 5-HIAA solutions were prepared and each 200 µl + 1ml Ehrlich reagent reaction mixture was individually reacted with reagent where other two metabolites (HVA (blue line), VMA (pink line)) did not show any color change (fig 19 a, b) thus it can concluded that Ehrlich reagent will form complex with 5-HIAA only in biological fluid which was confirmed in experiments.





Tube 1: VMA std stock sol (1 mg in 10ml)
Tube 2: HVA std stock sol (1 mg in 10ml)
Tube 3: 5-HIAA std stock sol (1 mg in 10ml)
Tube 4: Blank reaction mixture

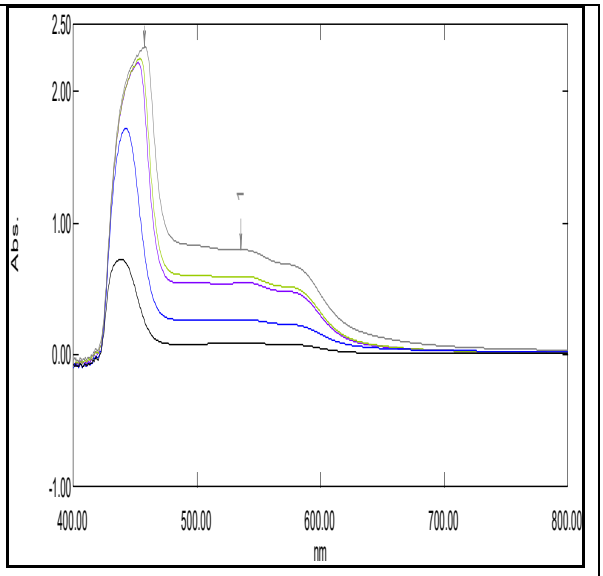
Fig 19 a: Interference of other components on colour reaction

Fig 19 b: UV spectra of interference of other components on colour reaction

3.2.4 (d) Effect of change in Ph of urine sol. on colour reaction

Another factor that was taken into consideration was difference in Ph of urine as urine is biological fluid their chances the Ph of urine may vary from person to person, thus different solution of 5-HIAA were prepared with varying Ph where it was

observed that as the Ph of 5-HIAA solution increases the intensity of colour formed was less (fig 20), that does not mean that the concentration of metabolite will be less thus need to be taken into consideration while carrying out reaction.



Tube 1: Sol. Ph 4, Tube 2: Sol. Ph 5, Tube 3: Sol. Ph 6, Tube 4: Sol. Ph 7, Tube 5: Sol. Ph 8

Fig 20 a: Effect of change in Ph of urine sol. on colour reaction

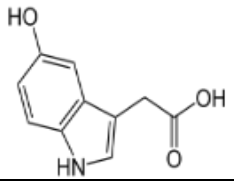
Fig 20 b: UV spectra of effect of change in Ph of urine sol. on colour reaction

3.2.5 Confirmation of functional group of 5-HIAA complex by IR technique

Functional group determination was performed by recording IR spectra for complex formed. The results of FTIR analysis for standard 5-HIAA, blank reaction mixture of 5-HIAA, reaction mixture complex of 5-HIAA. The FTIR spectrum is given in Figure 21, 22, 23 and Table 5, 6, 7.

Fig 21: IR spectra of Std 5-HIAA

Structure:



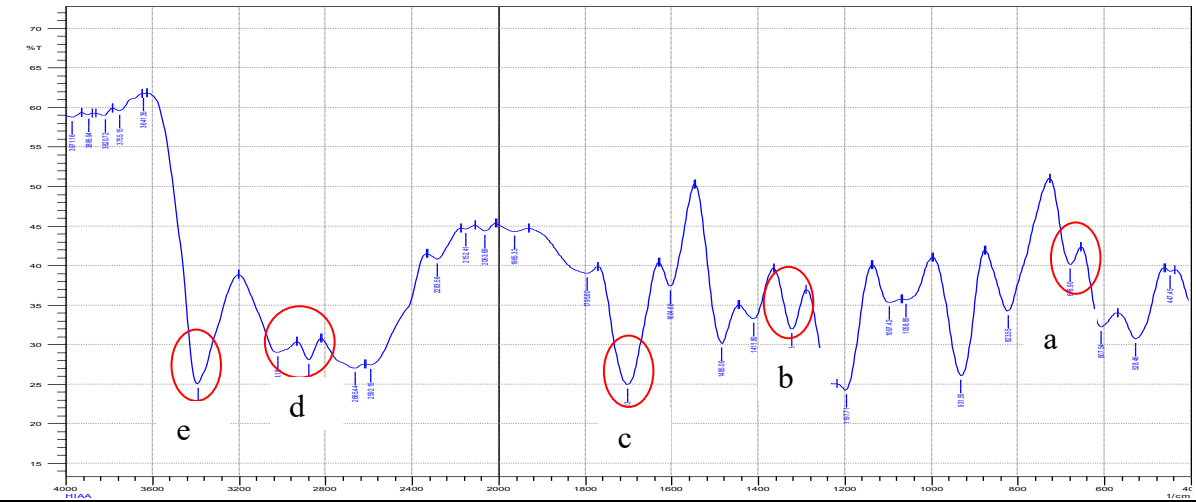


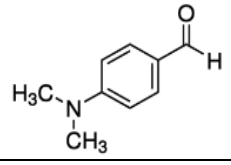
Table 5: The functional groups retrieved from FTIR spectrum for 5-HIAA

Peak no	Absorption(cm-1)	Functional groups	Compound class

a	678.90	C=C bending	alkene
b	1323.08	-OH bending	phenolic
c	1703.03	C=O	carbonyl carbon
d	2665.44 to 3024.18	OH- stretching	H bonded OH
e	3392.55	-NH secondary amine	Indole NH Band

Fig 22: IR spectra of reaction mixture blank of 5-HIAA

Structure:



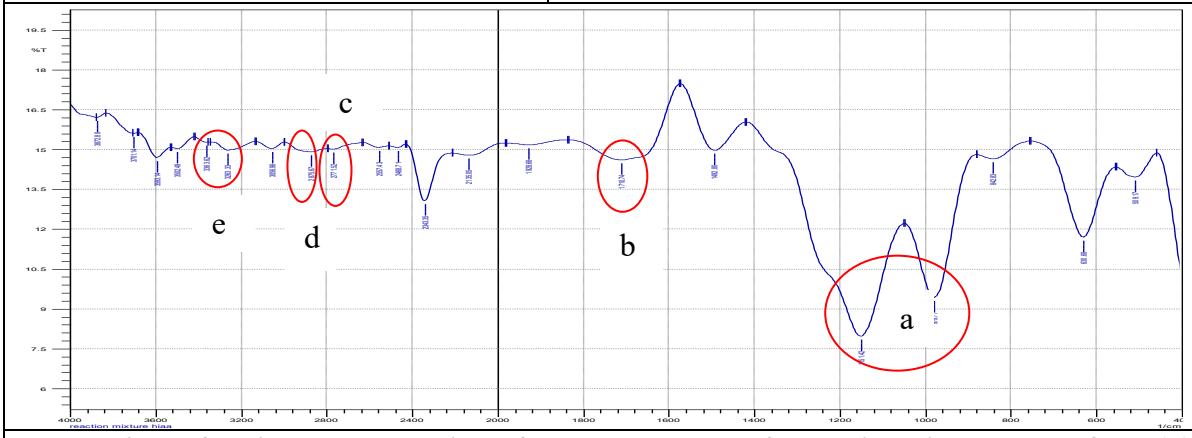
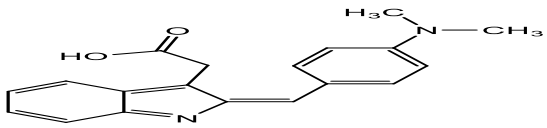


Table 6: The functional groups retrieved from FTIR spectrum for reaction mixture blank of 5-HIAA

Peak no	Absorption(cm-1)	Functional groups	Compound class
a	979.77- 1151.42	C-N	stretching amine
b	1710.74	C=O	aldehyde
c	2771.52	O-H stretching	alcohol intramolecular bonded
d	2875.67- 3056.96	C-H stretching	alkane
e	3263.33-3363.62	N-H	Aliphatic amine, tertiary amine

Fig 23: IR spectra of reaction mixture complex with 5-HIAA

Structure:



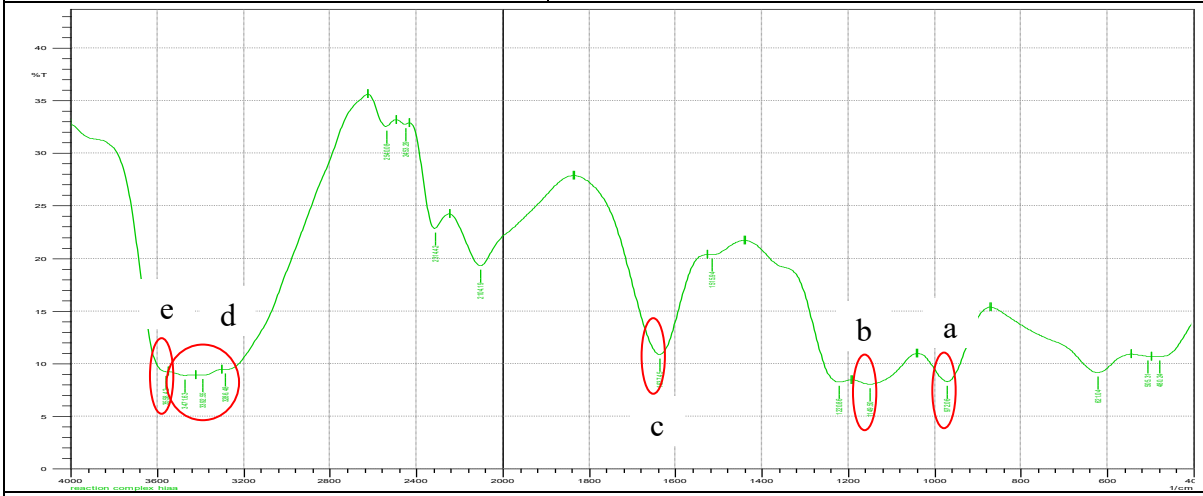


Table 7: The functional groups retrieved from FTIR spectrum for reaction mixture complex with 5-HIAA

Peak no	Absorption(cm-1)	Functional groups	Compound class

a	972.06	C=C bending	Alkene, mono substituted
b	1149.50	C-O stretching	alcholic
c	1637.45	C=C stretching	Alkene, mono substituted
d	3286.48- 3471.63	O-H stretching	Alcohol, intermolecµlar bonded
e	3558.42	N-H	amine

3.2.6 Confirmation of complex by LCMS method

The developed LCMS spectra for reaction mixture blank Ehrlich reagent and complex were identified based on mass to charge ratio (m/z) as shown in fig 24, fig 25 the theoretical molecular weight calculated for blank reaction mixture Ehrlich reagent is 149 gm and m/z ratio 149.19 g/mol, 150 (m+1), 151 (m+2), for reaction mixture Ehrlich reagent (Conc HCl: methanol 900:100µl) + 5-HIAA theoretical molecular weight 338 gm and m/z ratio 337 g/mol, 338.34 (m+1).

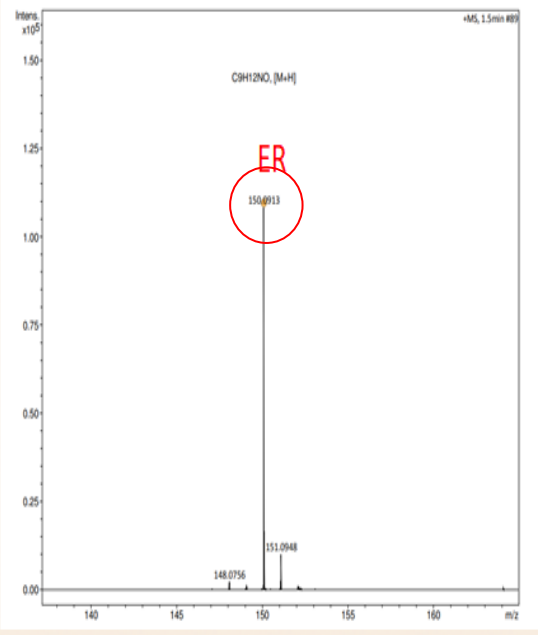


Fig 24: Mass spectra of reaction mixture blank Ehrlich reagent (Conc HCl: methanol 900:100µl)
Mol.Wt 149 gm
MS m/z : 149.19 g/mol, 150 (m+1), 151 (m+2)

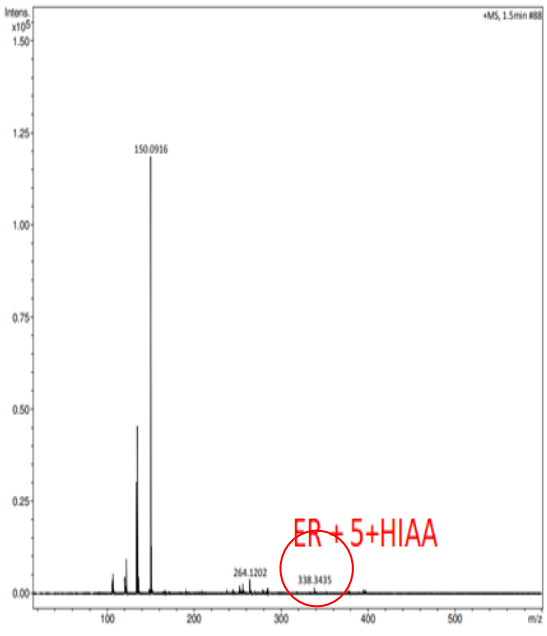


Fig 25: Mass spectra of Reaction mixture Ehrlich reagent (Conc HCl: methanol 900:100µl) + 5-HIAA
Mol. Wt: 338 gm
MS m/z : 337 g/mol, 338.34 (m+1)

DISCUSSION

The aging global population where individuals are at risk, increasing the societal burden of these diseases. Advances in research have identified potential disease-modifying therapies, but these are most effective when initiated early in the disease progression. Furthermore, early diagnosis allows for timely lifestyle adjustments, better management of symptoms, and more informed decision-making for patients and their families, enhancing their quality of life. The development of less invasive and more accessible diagnostic tools also makes early detection more feasible and practical in contemporary healthcare settings. The development of a UV method for detecting 5-HIAA using Ehrlich reagent was carried out by considering several key parameters. These parameters included the determination of λ_{max}, reaction time, interference from other components, the effect of pH changes in the urine solution, and complex confirmation using IR and LCMS methods. The λ_{max} of the chromophoric complex formed between 5-HIAA and Ehrlich reagent was identified to be 441 nm. This was achieved by reacting

200 µl of 5-HIAA with 1 ml of Ehrlich reagent (100 mg/ml) and recording the spectra from 400 to 800 nm. The selection of this range was based on the visible color change observed by the naked eye. The spectra of methanol, concentrated HCl, and a blank reaction mixture were also recorded to ensure that the solvents did not interfere with color formation.

The reaction time was determined by reacting 200 µl of 5-HIAA solution with 1 ml of Ehrlich reagent, and the spectra were recorded at 1 minute and 5 minutes after color development. The reaction was found to be complete within 1 minute, and the complex remained stable for at least 5 minutes. To ensure the specificity of the Ehrlich reagent, the interference of other metabolites excreted in urine was evaluated. When HVA and VMA solutions (100 µg/ml) were individually reacted with the Ehrlich reagent, no color change was observed. This indicated that the Ehrlich reagent selectively forms a complex with 5-HIAA in biological fluids, which was further confirmed through experiments.

The effect of varying the pH of the 5-HIAA solution was also investigated. It was observed that as the pH of the 5-HIAA solution increased, the intensity of the color formed decreased. This suggests that the pH of the urine sample needs to be considered when performing the reaction, as changes in pH can affect the color intensity and, consequently, the accuracy of the 5-HIAA quantification. The functional groups of the complex formed were confirmed using IR spectroscopy. IR spectra were recorded for standard 5-HIAA, a blank reaction mixture, and the reaction mixture complex. The functional groups identified in each spectrum were detailed in tables, providing information on the molecular structure and bonding characteristics of the complex. The complex was further confirmed using the LCMS method. The mass spectra for the blank reaction mixture and the complex were identified based on their mass-to-charge ratio (m/z). The theoretical molecular weight for the blank reaction mixture was calculated to be 149 gm with an m/z ratio of 149.19 g/mol, while the theoretical molecular weight for the Ehrlich reagent + 5-HIAA complex was 338 gm with an m/z ratio of 337 g/mol.

In clinical practice, methods like HPLC with electrochemical detection or mass spectrometry are often preferred for their high sensitivity and ability to quantify multiple metabolites simultaneously. However, the UV method could be valuable in settings where advanced equipment is not available, or as a screening tool before more detailed analyses are performed. In summary, the developed UV method offers a practical alternative for 5-HIAA detection, particularly in resource-limited settings, due to its simplicity, specificity, and rapid reaction time.

CONCLUSION

The findings showcased the practicality of the colorimetric method for detecting and selectively identifying 5-HIAA. When compared to other techniques such as HPLC, immunoassay, and capillary electrophoresis, this colorimetric method stands out as it doesn't require any complicated instruments. The process of determining 5-HIAA aligns well with the sample preparation, resulting in a streamlined experimental procedure. The affordability and simplicity of this method make it an appealing choice for regular testing of 5-HIAA in urine samples.

Declaration by Authors

Ethical Approval: Not required.

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Conflict of Interest: Nil.

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