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Estimation of synthetic dye erythrosine in food stuff and formulation and effect of dye on the protein binding of drug in BSA

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ABSTRACT

An efficient and accurate reverse phase – high performance liquid chromatographic method was developed and validated for the separation and determination of synthetic food colorant erythrosine E 127. This method was successfully applied for the estimation of erythrosine dye in certain food stuff (cream biscuits, cherry, gems, and candies) and in drug (Ibuprofen tablet - in which erythrosine present as a coating agent). Interaction study was carried out to find the effect of erythrosine on the protein binding of Ibuprofen in Bovine serum albumin. A Phenomenex C 18 Gemini column (150×4.6 mm), 5 µ particle size was used as stationary phase and Mobile phase contained a mixture of 10mM ammonium acetate buffer: Acetonitrile : Methanol in the ratio of (50:25:25v/v/v) at PH 8. The dye was successfully separated out at retention time of 5.6 min, by using isocratic elution technique. Photo diode array detector monitored the wavelength of erythrosine as 529nm and flow rate was selected as 1ml/min. The method was thoroughly validated. Detection limit for erythrosine was found to be 0.1ng/ml, where as limit of quantization of erythrosine was reported as 1ng/ ml. The intra-day precision and inter-day precision were determined as 1.33 % RSD and 0.98 % RSD respectively. The dye E127 was extracted and quantified in various food stuff such as (Cherry - 235µg/ml, Cream biscuits - 316µg/ml, Gems – 177µg/ml, Candies - 36µg/ml) and found that erythrosine content was more in cream biscuits compared to other food stuff. The amount of E127 present in ibuprofen tablet was found to be 5.6 µg/ tablet. A study on protein binding of Ibuprofen by UV spectroscopy and reverse phase high performance liquid chromatography were conducted. The effect of erythrosine on protein binding of Ibuprofen in BSA was carried out and monitored that protein binding of the drug was increased by the effect of dye. Unbound form of the drug was decreased by the effect of dye. Increase in the concentration of drug in BSA, the effect of dye on the drug was found to be more.

Keywords*:* High performance liquid chromatography, Ibuprofen, Photo diode-array detector, Protein binding.

INTRODUCTION

Colors are water soluble dyes and are extensively used in the pharmaceutical and food industries. The food and drug administration (FDA) is responsible for regulating all color additives used in United States. The permitted color additives are classified as ‘’ certifiable’’ or ‘’exempt from

certification. Whether a color is certifiable or exempt from certification it has no safety at all. Both types of color additives are subjected to rigorous standards of safety prior to their approval for use in foods (1-7).

Some of the synthetic colorants leads to a potential risks to human health; especially if they are excessively consumed. Due to this reason the acceptable daily intake have been especially determined and evaluated by food Agricultural Organization (FAO) and World Health Organization (WHO). According to Nutrition and Education Act of 1990, a certifiable color additive used in food must be listed in the ingredient statement by its common or usual name. All labels printed after July 1, 1991 must comply with this requirement (1-7).

Erythrosine FD&C Red No:3 : It is a Red color used in cherries ,canned fruit custard mix, sweets bakery, snack foods, can cause sensitivity to light ,can increase thyroid hormone levels and lead to hyperthyroidism[2] was shown to cause thyroid cancer in rats in a study in 1990,banned in January 1990;but not recalled by the US FDA ;banned in Norway(1-7).

Permissible level of color

The maximum permissible level of 200ppm of food color laid down in Rule 29 of the PFA Act was amended in 1997 to a level of 100ppm in the final food or beverage for consumption.

Permitted Colors in India and their Acceptable daily intake [ADI]

Color	Permitted Colors	ADI (mg/kg body weight)
Red	Erythrosine	0.1
	Carmosine	4.0
	Ponceau 4R	4.0
Yellow	Tartrazine	7.5
	Sunset yellow	2.5
Green	Fats Green FcF	2.5
Blue	Brilliant blue	12.5
	Indigo carmine	5.0

In a day a person may be exposed to synthetic food colors in the form of biscuits confectionary, soft drinks, sweet meals and deserts. A 20kg child between 5-7 years old who consumes 200gm of pink pastry with erythrosine (a bright pink permitted color) added in concentration of 100 ppm as it is permitted in India , will be consuming 10 times the ADI for Erythrosine[4]. Moreover synthetic colorant erythrosine was also found as an additive in formulations. *So* the maximum permitted intake of dye may be easily exceeded in any given day due to the wide spread usage of food colors in various food stuff and formulations. So the safety of permitted colors continues to be questioned (1-7).

The growing evidence of thyroid toxicity of erythrosine has caused JECFA (joint expert FAO / WHO comity on food additives) to lower the ADI from time to time. The ADI of erythrosine in 1978 was 2.5 mg per kg body weight which was reduced to 1.25mg per kg body weight and then to 0.6mg per kg body weight in 1980. In 1990, evaluation the ADI was reduced to 0.1mg per kg body weight[5]. Binding of drugs to protein can affect the duration of action of the drug. Most drugs bind reversibly to plasma protein to some extent. The fraction of the drug bound can

change with plasma drug concentration and dose of the drug administered. If a patient has a low plasma protein concentration than for any given dose of drug, the concentration of free drug may be higher than anticipated.

Protein binding:
When a highly protein bound drug is displaced from binding by a second drug or agent , a sharp increase in the free drug concentration in the plasma may occur leading to toxicity. With drugs that are not highly bound to plasma protein, a small displacement from the protein causes a transient increase in the free drug concentration, which may cause a transient increase in pharmacological activity. A decrease in the protein binding result in freer drug concentration which allows more drug to penetrate or cross cell membrane and distribute in to all tissues. More drugs will be there for available to interact at a receptor site to produce more intense effects (8-12).

Free drug concentration in the plasma is responsible for the observed pharmacological effect or a therapeutic response. Plasma drug concentration are generally reported as the total, drug concentration in the plasma, including both a protein bound drug and an unbound drug concentrations. Interaction study carried out to determine the effect of dye on drug. The study indicates that the protein binding of ibuprofen was increased by the effect of dye. As a result unbound form of drug in plasma decreased. Hence if there is an Increase in the concentration of drug in BSA, the effect of dye on the drug was also found to be more (8-12).

Erythrosine E127 is used extensively as color additive in food, and drug. Depending on its long term and short term use, have potential carcinogic, mutagenic effects. Hence the objective of this research work, to estimate the amount of erythrosine in selected food stuff and formulation has its own significance. So that an efficient and accurate reverse phase – high performance liquid chromatographic method was developed to estimate synthetic food colorant erythrosine E 127 and this method was successfully applied for the determination of erythrosine dye in certain food stuff (cream biscuits, cherry, gems, and candies) and dye in formulation (Ibuprofen tablet - in which erythrosine present as a coating agent). An Interaction study was carried out to find the effect of erythrosine dye on the protein binding of Ibuprofen in BSA. The dye extracted from the food stuff by simple pre treatment like dilution or water extraction[6.]

MATERIALS AND METHODS

Drug and Dye Sample
The gift samples of pure drug were received from Ranbaxy Laboratories Limited, Mumbai, and Jenburkt pharmaceuticals Ltd, Gujarat and Abbots labs Goa. The dye was obtained from Bharath coats, Chennai.

Chemicals and solvents:
Methanol AR grade, HPLC grade (Qualigens Fine Chemicals, Mumbai.), Water HPLC grade (Merck Private Limited.), Acetonitrile HPLC grade (Merck Private Limited.), Hydrochloric Acid LR grade (Sd fine chem. limited, Mumbai.), Sulphuric Acid (Qualigens Fine Chemicals, Mumbai.), Sodium Hydroxide LR grade, Potassium dihydrogen phosphate LR grade,

Ammonium acetate LR grade, Triethylamine AR grade,Glacial acetic acid (sd fine chem, Mumbai.), Chloroform (Qualigens Fine Chemicals, Mumbai.), BSA (Loba chem)

Instruments used:

- Jasco V- 530 UV/VIS Spectrophotometer
- Shimadzu HPLC Class LC-10 AT VP system(Photodiode array detector)
- Pall Gelman Sciences, Vacuum pump
- Elico Pvt. Limited, India, pH meter – LI 127
- Shimadzu Digital Electronics Balance – BL220H
- Remi Centrifuge

RESULTS AND DISCUSSION

Erythrosine is a red color dye used in food stuff and formulation, reported that high levels of erythrosine intake are known to cause thyroid tumor and hyper thyroid condition.

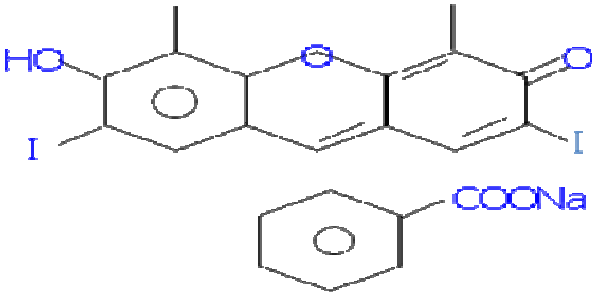

Fig.1. Erythrosine E127, CI 45430

Chemical structure, common name, E (European Community) and CI (Color Index) number of synthetic colorant studied(8-12).

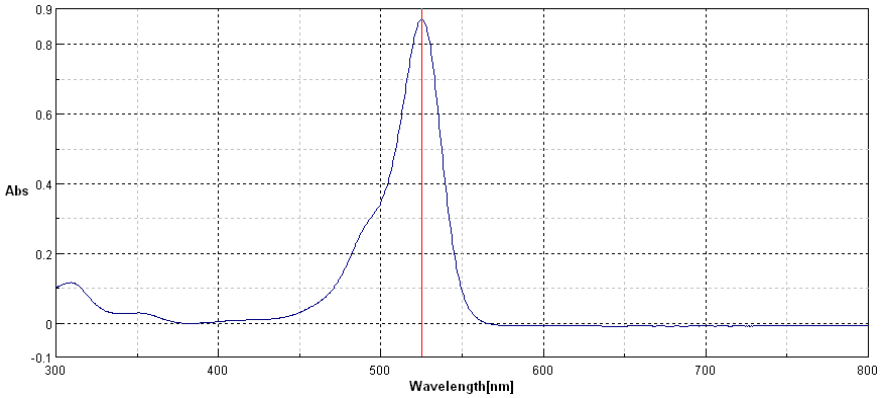

Fig.2. Standard UV spectrum of Erythrosine

Method development and validation of Erythrosine (E127) using RP-HPLC coupled with photo diode array detector:

The selection of HPLC method depends upon the selection of proper wavelength. An ideal wavelength is one that gives maximum absorbance and good response for the drug to be

detected. As UV spectrum of Erythrosine showed maximum absorbance at 529 nm, it was selected as the detection wavelength. (Fig.2)
(λmax = 525 nm)

The chromatographic conditions were for the estimation of erythrosine by RP-HPLC. Phenomenex C $_{18}$ Gemini column (150×4.6 mm), used as stationary phase and mixture of 10mM ammonium acetate buffer**:** Acetonitrile**:** Methanol (50**:**25**:**25v/v/v) selected as mobile phase. Isocratic elution mode was used and erythrosine gave good symmetric peak at pH8 (Fig.3). Photo diode array detector monitored the wave length as 529nm. Retention time recorded as 5.6min. Preparation of standard stock solution of erythrosine to obtain a concentration of 1000µg /ml. This solution was suitably diluted to get concentration of (0.2-1µ/ml) (Fig.4). (13-16)

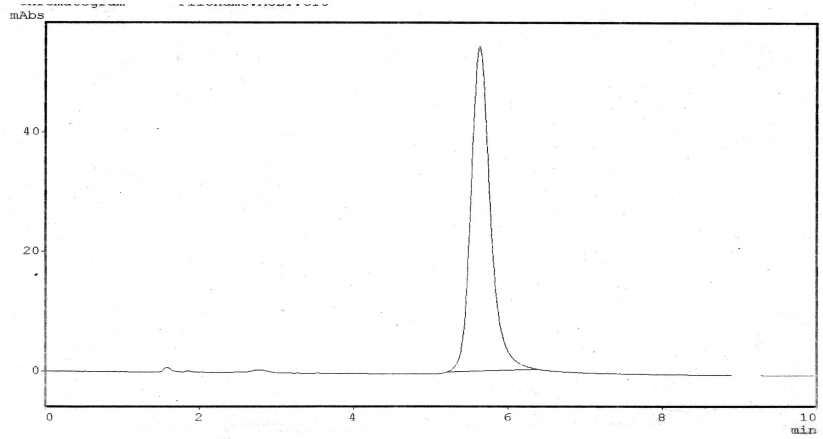

Fig.3. Chromatogram of Erythrosine at pH 8

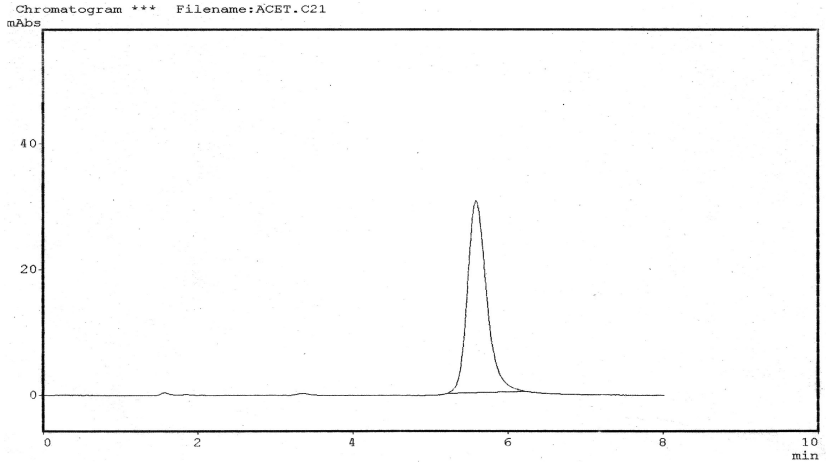

Fig.4.Chromatogram of standard erythrosine (0.6 µg /ml)

Precise and sensitive RP-HPLC method developed and validated for the estimation of erythrosine. The standard stock solution containing erythrosine was prepared at different ranges 0.2-1µg/ml,1-5µg/ml,5-25µg/ml in distilled water .Among these ranges ,0.2-1 µg/ml was found to be more linear .Calibration graphs were plotted using peak area of standard erythrosine .The slope , intercept and correlation co-efficient was found to be 0.0044, 0.00757 and

0.99957(Fig.5.). LOD was determined by injecting progressively lower concentration of erythrosine. The limit of detection and was found to be 0.1ng/ml. The limit of quantization and was found to be 1ng/ml (Fig.6, and Fig.7.)

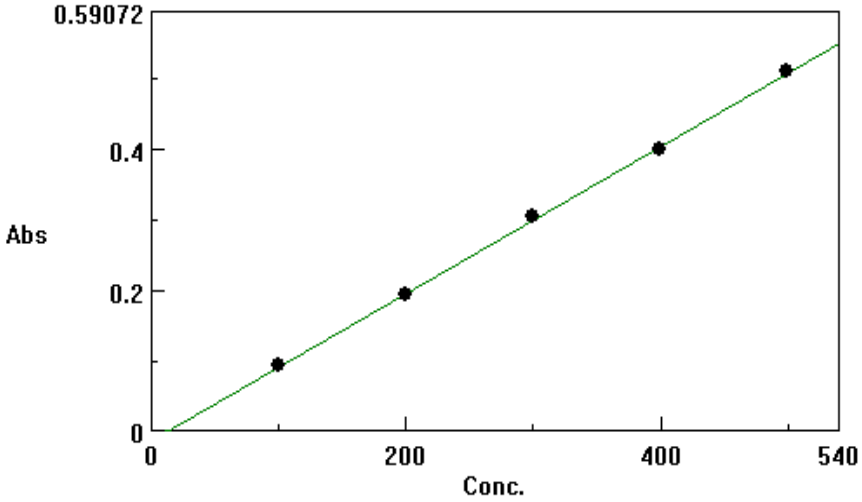

Fig.5. Calibration graph of erythrosine (0.2-1µg/ml)

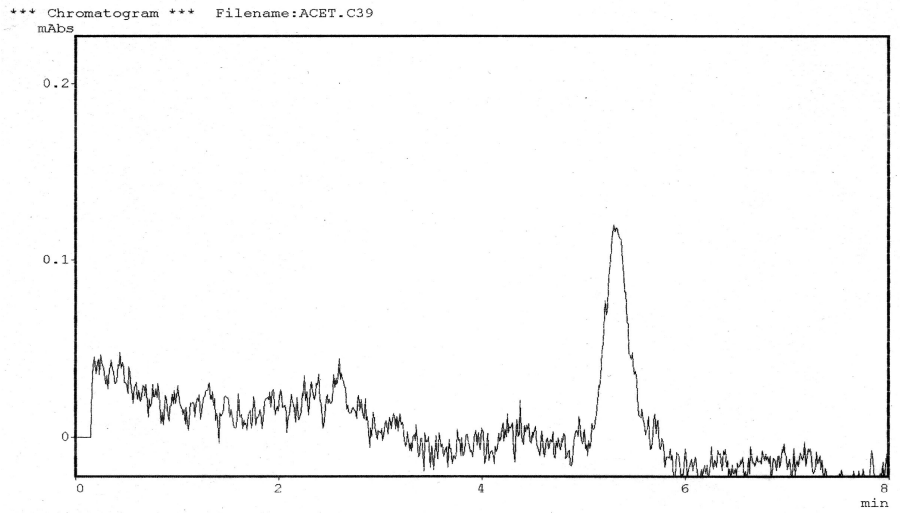

Fig.6. Chromatogram of Erythrosine limit of detection (LOD) (0.1ng/ml)

Estimation of erythrosine in various food stuff using RP-HPLC

Extraction of erythrosine from various water soluble food stuff such as cherry, cream biscuits, gems, and candies with simple pre treatment of water (suitable dilution or water extraction, filtration) (13-16) and estimated by using RP-HPLC (Fig.8,9,10 and 11).A 25ml of sample of drink was diluted with mobile phase in a volumetric flask; the sample was degassed by strong string. The solid sample was homogenized. A portion of 25mg cream biscuits, gems and candies accurately weighed and dissolved in 25ml of water. Keep aside the sample solution for complete extraction of the colorants. Solution was filtered through What Mann filter paper; the filtrate was collected and injected after filtering through 0.45µm disposable syringe filter. The RP-HPLC

method developed and determined the synthetic dye erythrosine in various water soluble food stuff (Table.1) (Fig.12). (17-20)

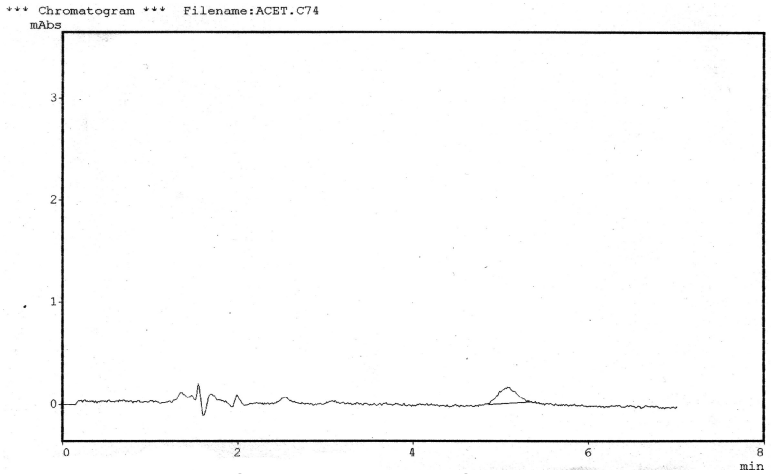

Fig.7.Chromatogram of Erythrosine limit of quantization (LOQ) (1ng/ml)

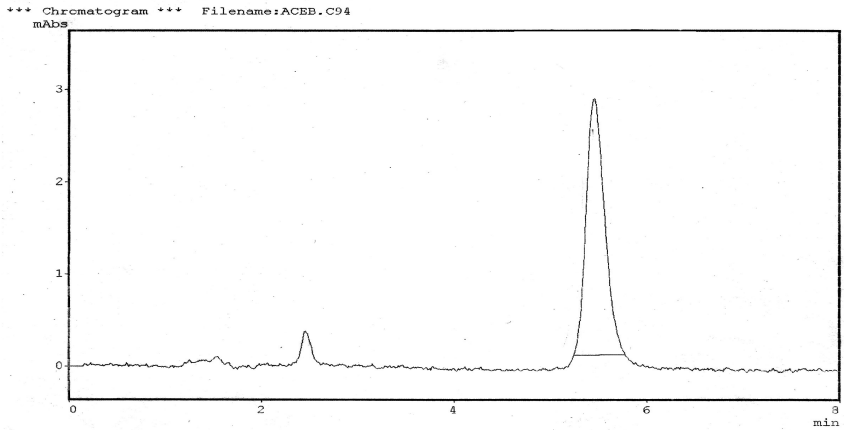

Fig.8.Chromatogram of erythrosine in BISCUITS

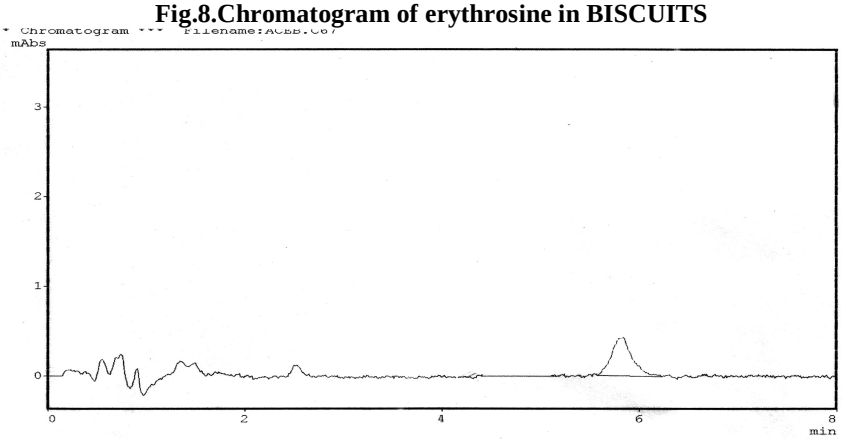

Fig.9.Chromatogram of erythrosine in CHERRY

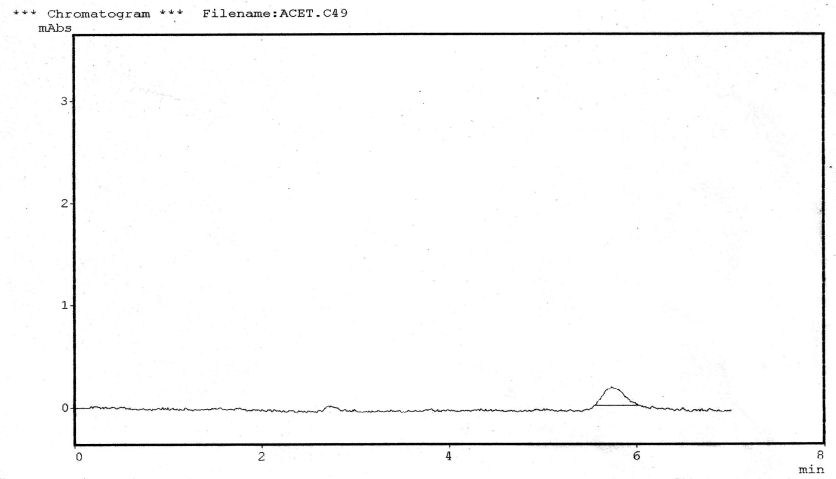

Fig .10.Chromatogram of erythrosine from POPPINS

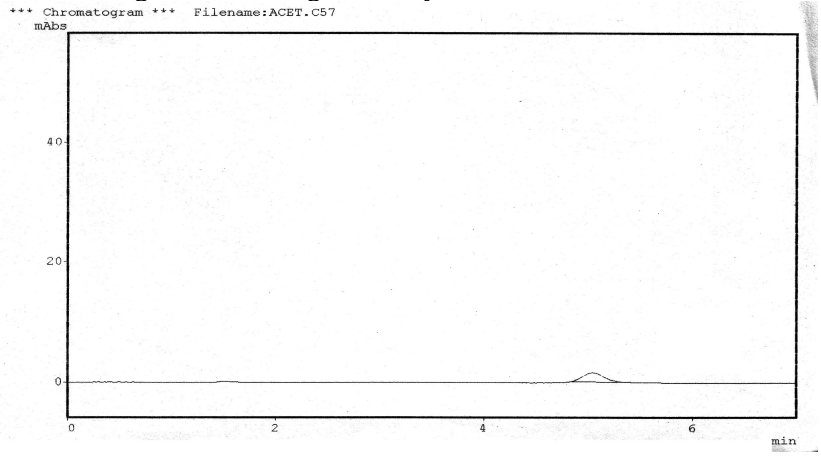

Fig.11.Chromatogram of erythrosine from GEMS

Table.1.Amount of erythrosine present in different food stuff

Food stuff	Erythrosine µg/ml
Cherry	235
Cream biscuits	316.65
Gems	177.8
Candies	36.74

The amount of erythrosine content was found to be high in cream biscuits when compared to other food stuffs

Analysis of erythrosine E127 from formulation

UV spectra of the drug were recorded and the wavelength of ibuprofen was selected as 265nm as it gave good absorbance and good detection. The erythrosine dye coat was peeled out from the ibuprofen tablet and extracted with water, and mixed with mobile phase, filtered out and injected. (Fig: 13). Accurate and precise RP-HPLC method developed by using Shimadzu HPLC system to estimate erythrosine which is present as a coating agent in formulation. (13-22)

The amount of dye in ibuprofen tablet was found 5.6 as µg/ tablets (Table: 2).

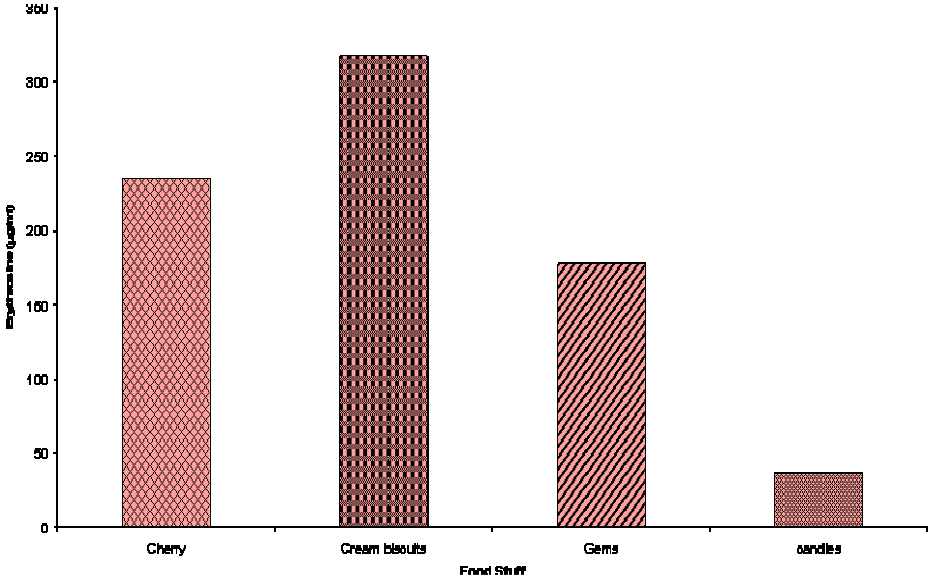

Fig.12. Graphical representation of erythrosine in various food stuffs

Table2. Amount of Erythrosine in ibuprofen tablet as coating material

Dye	Amount of dye in ibuprofen tab µg/ tabs
Erythrosine	5.6 µg/ tabs

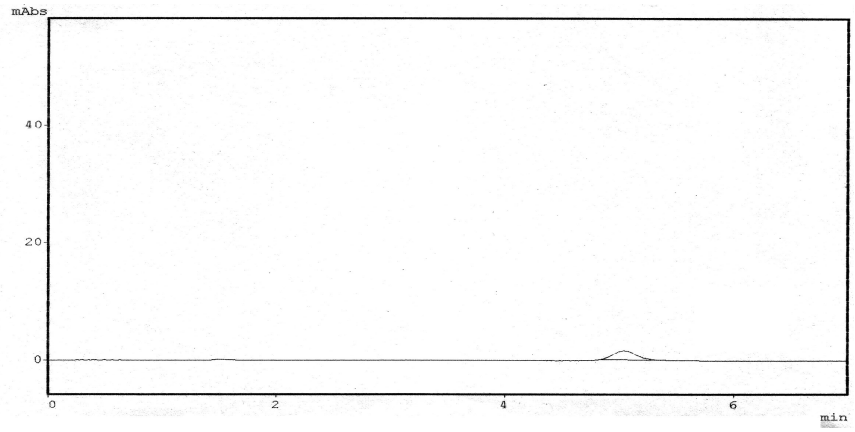

Fig.13.Chromatogram of dye erythrosine from ibuprofen

Interaction Study

A study on protein binding of drug by RP-HPLC and UV spectrophotometric method (Table .4)

Preparation of standard stock solution: 100Mg of drug ibuprofen dissolved in 2ml of 0.1N NaOH and made up to 1ml with distilled water to get 1000mcg/ml. This was used as primary stock solution.

Preparation of pH 7.2 buffer solution: 50ml of 0.2N Potassium hydrogen phosphate is added to 34.7 ml of 0.2N NaOH solution and made up to 200ml with distilled water.

Preparation of 2.8×10^{-4} m solution of egg albumin: 0.315g of egg albumin flakes is dissolved in distilled water. It is shaken well (till flakes are completely dissolved) and was kept aside.

Preparation of buffer solution of ibuprofen: Dissolve 10 mg of ibuprofen in 10 ml mobile phase to get 1000µgm /ml. From this 3.36 ml transferred into a 100 ml standard flask and made up to the volume with buffer 7.21 ml of the above solution should contain 0.336 mcg of ibuprofen. This 25 ml taken into a beaker and used for the study.

A boiling tube open on both sides was taken and a semi permeable membrane is tied onto the neck of the boiling tube. The egg albumin solution 10 ml was taken inside the semi permeable membrane. The boiling tube was then immersed into the beaker containing the drug ibuprofen 1.63×10^{-4} M. Immediately at zero time 1 ml of the drug solution is pipette out from the beaker and was replaced with 1 ml of water and injected to RP-HPLC column. Readings were taken at 0, 10, 30,45,1 hr, 1.15, 1.30, 1.45, 2hrs (Table.3, Table.4)

*Once equilibrium is reached there will be no further change in absorbance, hence the constant value of absorbance was noted. So there will not be any further change in peak area for particular drug, and constant value of peak area was noted for each drug. Ibuprofen absorbed from GIT, and peak plasma concentration occurs about 1-2hrs, after an injection 90-99% bound to plasma proteins and has a plasma half life of 2 hrs.

Table.3) Protein binding of drugs by – RPHPLC method

Time	Absorbance of 1.63×10^{-4} M Ibuprofen
0 min	46272
10min	45487
30min	44985
45min	43940
1.00hr	43368
1.15hr	43187
1.30hr	42216*
1.45hr	42521*
2.00hr	42773*

Table.4.) Protein binding of drugs - by UV Spectrophotometric method

Time in min	Absorbance of 1.63×10^{-4} M Ibuprofen at 265 nm
0	0.0666
10	0.0713
20	0.0660
40	0.0686*
60	0.0639*
80	0.0616*

Table 5: Report for protein binding of drugs

DRUG	% OF PROTEIN BINDING	PEAK PLASMA CONCENTRATION (hrs)	HALF LIFE (hrs)	UV-RESULTS (hrs)	HPLC RESULTS (hrs)
Ibuprofen	90-99	1-2	2	60-80	1.30

Effect of erythrosine on protein binding of drug

Solution1: Ibuprofen was accurately weighed and diluted to get a concentration of 10mcg/ml. From this stock solution, concentration range of 0.2-1mcg/ml was prepared.

Solution 2: Erythrosine dye was prepared in a concentration range 0.2-1mcg/ml.

Stock solution of ibuprofen:

5ml of the solution 1 was evaporated to dryness using nitrogen gas at room temperature in a flask along with 5ml BSA and vortexes for 60 seconds, to that 5ml of methanol was added and centrifuged at 2000 rpm for 5 minutes. From this organic layer, 1ml was taken evaporated under nitrogen gas and finally the residue was reconstituted in mobile phase and injected to RPHPLC column and analyzed and noted the readings.

Preparation of stock solution of Ibuprofen in BSA with Erythrosine

5ml of solution 1 and 5ml of BSA were added. These solutions were mixed thoroughly and kept aside for 30 min. Centrifuged and extracted by using the above mentioned extraction procedure and volume was made up with mobile phase. To that solution2 was added and then mixed well and injected to analyze for Ibuprofen content by using HPLC. The interaction study of erythrosine with ibuprofen in BSA was done using RP-HPLC and noted the effect of dye on drug. (Fig 14, Table 5). The concentration of the drug was found to be altered, due to the effect of dye (8-12).

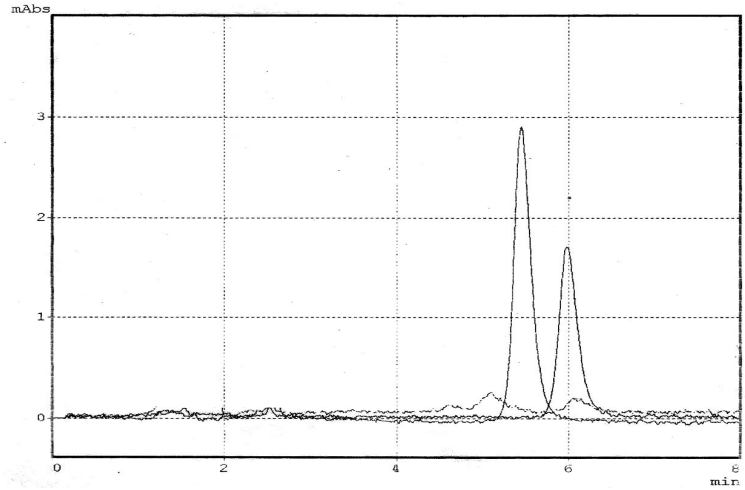

Fig 14: Chromatogram of standard ibuprofen in BSA with erythrosine (0.4µg/ml)

Interaction study of Erythrosine E127 on protein binding of drug Ibuprofen indicates that

- Protein binding of the drug is increased by the effect of dye.
- Unbound form of the drug is decreased by the effect of dye.

- Increase in the concentration of drug in BSA, the effect of dye on the drug was found to be more

Table.6.) Peak area of standard ibuprofen in BSA Vs Peak area of ibuprofen in BSA along with erythrosine

Concentration (µg/ml)	PEAK AREA OF IBUPROFEN IN BSA	PEAK AREA OF IBUPROFEN IN BSA - ERYTHROSINE
0.2	1066	0915
0.4	1226	1131
0.6	1466	1433
0.8	1523	3617
1.0	2003	36920

CONCLUSION

1. Accurate and precise RP-HPLC method was developed and validated for the estimation of erythrosine E127.
2. Estimated amount of Erythrosine in certain food stuff

- Erythrosine in Cherry - 235µg/ml
- Erythrosine in Cream biscuits - 316µg/ml
- Erythrosine in Gems – 177µg/ml
- Erythrosine in Candies - 36µg/ml

Erythrosine content found to be more in cream biscuit compared to other food stuff

3. Estimated amount of Erythrosine in ibuprofen tablet
   Erythrosine in Ibuprofen – 5.6µg/ml

4. Interaction study

The dye have specific effect on the drug in BSA such as

- Protein binding of the drug is increase by the effect of dye
- Unbound form of the drug is decreased by the effect of dye
- Is there is an Increase in the concentration of drug in BSA, the effect of dye on the drug was also found to be more.

So there is sufficient evidence for that even in the permissible limit of this dye may lead to be carcinogenic. Use of this permitted colors in permissible levels also not safe and there is a need to minimize the indiscriminate use of colors especially erythrosine in foods and pharmaceuticals were the consumption could be more**.**

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