AAPS PharmSciTech, Vol. 16, No. 3, June 2015 (© 2014)
DOI: 10.1208/s12249-014-0228-2

Research Article

Simultaneous Determination of Acetaminophen and Synthetic Color(s) by Derivative Spectroscopy in Syrup Formulations and Validation by HPLC: Exposure Risk of Colors to Children

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Received 6 May 2014; accepted 18 September 2014; published online 6 November 2014

Abstract. Color additives are used in pediatric syrup formulations as an excipient; though not prerequisite, but pediatric syrup formulations are normally colored. An attempt has been made to measure simultaneously the single drug, acetaminophen (AT), along with the colors, carmoisine (CA), erythrosine (ET), and sunset yellow FCF (SSY) added in it by three derivative spectroscopy methods namely, 1st order, ratio, and differential derivative methods. Moreover, evaluation has been made for the exposure assessment of the colors added as excipient because some colors have been reported to cause allergic reactions and hypersensitivity in children. The present methods provide simple, accurate, and reproducible quantitative determination of the drug, AT, along with the color in synthetic mixtures and commercial drug formulations without any interference. The limit of detection varied from 0.0001–0.31 μg/ml while limit of quantification ranged from 0.002–1.04 μg/ml in all the three methods. The calibration curve of all the three derivative methods exhibited good linear relationship with excellent regression coefficients (0.9986–1.000). Both intra-day and inter-day precisions showed %RSD value less than 2% while the percentage recovery was found between 96.8–103.8%. The sensitivity of the proposed methods is almost comparable to HPLC and thus, can be used for determination of drug AT, and color simultaneously in pharmaceutical formulation on routine basis. The present methods also showed that colors like SSY and ET are saturating more than 50% of acceptable daily intake (ADI) value which is alarming and needs to be considered for modification by regulatory authorities to safeguard the health of children.

KEY WORDS: 1st-order derivative; acetaminophen; colors; differential derivative; exposure assessment; ratio derivative.

INTRODUCTION

Color additives are used in pediatric syrup formulations as an excipient that impart the preferred color and match the flavor of the formulation, e.g., green with mint-flavored solutions and red for strawberry-flavored formulations (1). Although the inclusion of colors is not a prerequisite for all pharmaceutical solutions, pediatric syrup formulations are normally colored (2).

Color additives used in pediatric syrup formulations may be natural or synthetic although the former is substituted due to better color, uniformity, stability, and easily blending property of synthetic dyes (3,4). But both natural and synthetic dyes are known to pose risk to health. Carmine red, a natural dye is known to cause occupational asthma and food allergy mediated by IgE (5,6). Synthetic dyes, like amaranth, erythrosine, carmoisine, ponceau 4R, indigo carmine, tartrazine, and sunset yellow FCF are used in pediatric formulations (5). Colors such as SSY, tartrazine and ponceau 4R are reported to provoke allergic reactions including urticaria, dermatitis, angioedema, and exacerbation of asthma in sensitive individuals (7). Tartrazine and SSY have also been implicated to cause irritability, restlessness, sleep disturbance, and hyperactivity in children (8–14). The enhanced health risk due to the use of colors has subjected the regulatory authorities to increase legislative control, restricting them to selective use (15–17). However, no regulatory authority has imposed any restriction over the specified levels of these colors.

Literature survey reveals various analytical techniques, with chromatographic methods playing a significant role in pharmaceutical analysis (18–29). However, these methods require sophisticated and expensive equipment, provision for use and disposal of solvents, labor-intensive sample preparation procedures, and personal skills.

Derivative spectroscopy is an alternate approach that has been proved advantageous in the determination of mixtures with two or more components having overlapping spectra and in eliminating interference from formulation matrix by using

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the zero-crossing techniques (21,30–40). Though absorption spectrophotometry have been reported for the estimation of individual drugs or in case of mixtures where absorption maxima are distinctly apart (41–49), however, no method is available for the simultaneous estimation of the drug and color additive. Also, there is a need to evaluate the exposure assessment of the colors added to syrup formulations to safeguard the health of vulnerable population including children. Hence, an attempt has been made to measure simultaneously the drug along with the color added in it by derivative spectroscopy and the validation of the proposed method with HPLC technique along with intake profile of color additives.

MATERIALS AND METHODS

Experimental

A double-beam spectrophotometer (Perkin-Elmer Lambda Bio 20; Perkin-Elmer Instruments, Schwerzenbach, Switzerland) was used for absorption and derivative spectrophotometric measurements with a quartz cell of 10 mm path length.

Chromatographic analysis was carried out with a Waters LC module (Waters Associates, Vienna, Austria) equipped with a dual pump (Model 510), Rheodyne injector with a 20-μL loop and a tunable absorbance detector (Model 2489). The chromatograms were recorded and processed by Waters Empower software version.

Reagents

Analytical reagent grade sodium hydroxide and concentrated hydrochloric acid were procured from Qualigens, Mumbai, India. Methanol and acetonitrile (HPLC grade) were purchased from Merck Limited, Mumbai, India. The standards of synthetic dyes viz carmoisine, erythrosine, and sunset yellow FCF were obtained from Bush Boake Allen, Chennai (India). Standard acetaminophen was a gift from Dr Surrendra Reddy, CSIR-Central Drug Research Institute, Lucknow. All the other chemicals used were of highest purity available commercially.

Collection of Acetaminophen Syrup Formulations

A total of nine branded samples of acetaminophen were procured from the market. These samples were of Cipla (4), GlaxoSmith Kline (2), East India Pharmaceutical Ltd (1), and Micro Lab Ltd (2) companies. Out of these nine samples, three samples contained ET, three having CA while the remaining three had SSY.

Procedures

Derivative Methods

1st Derivative. Individual solution of the four standards namely, acetaminophen (AT), carmoisine (CA), erythrosine (ET) and sunset yellow FCF (SSY) were prepared at a concentration of 1 mg/ml in methanol: milliQ water (50:50, v/v) in a 25 ml volumetric flask and diluted to the mark. The solutions were further diluted (10 times) with milliQ water to obtain working standards of 100 μg/ml. Different concentration ranging from 2.5 to 20 μg/ml of all the four standards was prepared from the respective working standard solutions of 100 μg/ml. The absorption spectra of the standard solutions were recorded between 200 to 700 nm with a scan rate of 480 nm per min, against a blank of Milli-Q

Table I. Statistical Parameters of Calibration Graph for Each Component

Equation	Regression coefficient	Linearity (μg/ml)	Slope	Intercept	LOD (μg/ml)	LOQ (μg/ml)
1st derivative						
$^{1}D_{302}$AT	0.9993	2.5–12.5	5.56×10^{-3}	-6.80×10^{-4}	0.12	0.41
$^{1}D_{570}$CA	0.9999	5.0–25.0	9.54×10^{-3}	-1.07×10^{-3}	0.31	1.04
$^{1}D_{536}$ET	0.9997	5.0–25.0	3.66×10^{-2}	-6.68×10^{-3}	0.011	0.04
$^{1}D_{520}$SSY	0.9993	5.0–25.0	1.45×10^{-2}	3.49×10^{-3}	0.026	0.09
Ratio spectra derivative						
CA as divisor $^{1}DD_{298}$AT	0.9993	2.5–12.5	9.73×10^{-2}	-1.05×10^{-2}	0.04	0.14
AT as divisor $^{1}DD_{518}$CA	0.9986	5.0–25.0	15.07	−4.87	0.0007	0.002
ET as divisor $^{1}DD_{298}$AT	0.9993	2.5–12.5	9.73×10^{-2}	-1.05×10^{-2}	0.04	0.14
AT as divisor $^{1}DD_{534}$ET	0.9992	5.0–25.0	21.25	−4.56	0.0004	0.002
SSYas divisor $^{1}DD_{298}$AT	0.9993	2.5–12.5	9.73×10^{-2}	-1.05×10^{-2}	0.04	0.14
AT as divisor $^{1}DD_{520}$SSY	1.000	5.0–25.0	24.9	−0.33	0.0001	0.004
Differential derivative spectra						
$^{1\Delta}D_{264}$AT	0.9999	2.5–12.5	1.19×10^{1}	4.91×10^{-3}	0.102	0.34
$^{1\Delta}D_{571}$CA	0.9993	5.0–25.0	4.91×10^{-3}	2.32×10^{-3}	0.078	0.26
$^{1\Delta}D_{515}$ET	0.9996	5.0–25.0	3.32×10^{-2}	1.55×10^{-3}	0.013	0.04
$^{1\Delta}D_{522}$SSY	0.9996	5.0–25.0	1.37×10^{-2}	6.7×10^{-3}	0.02	0.07

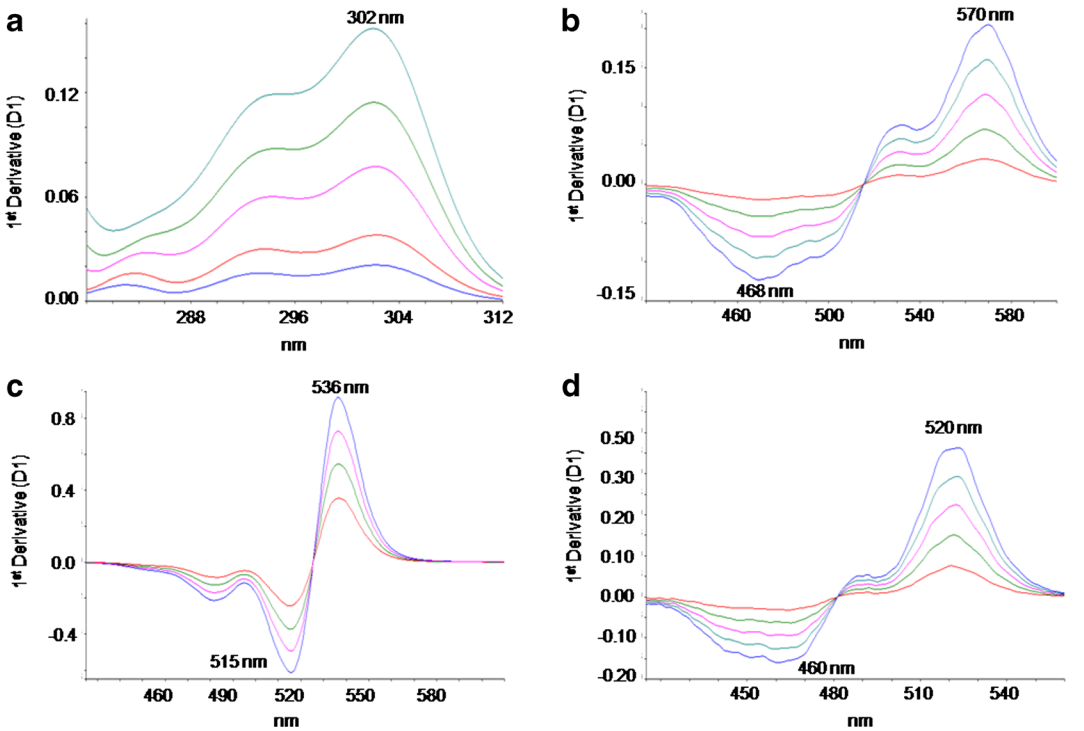

Fig. 1. 1st-order derivative spectra of standard **a** acetaminophen, **b** carmoisine, **c** erythrosine, and **d** sunset yellow

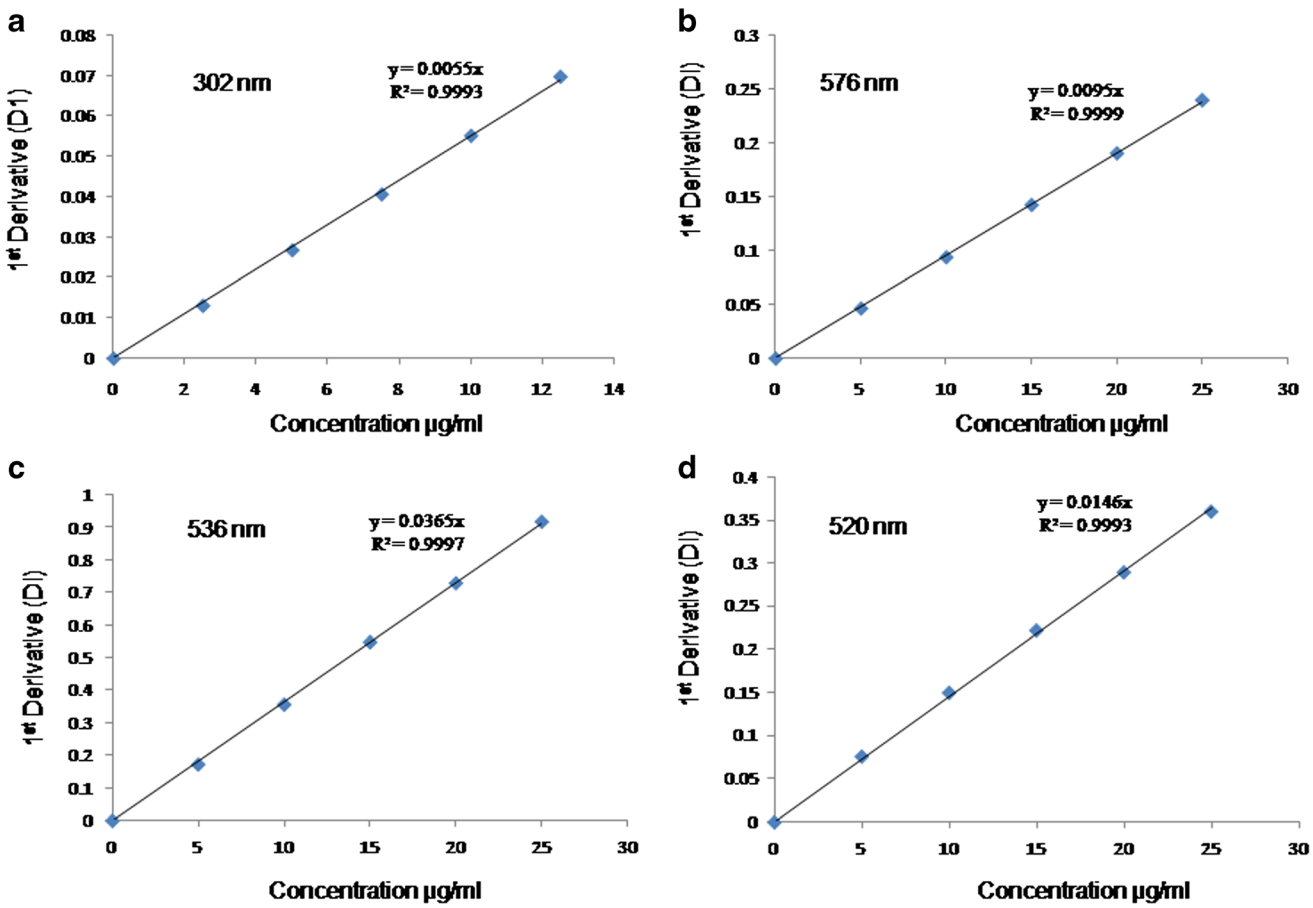

Fig. 2. Linear curve of 1st-order derivative spectra of standard **a** acetaminophen, **b** carmoisine, **c** erythrosine, and **d** sunset yellow

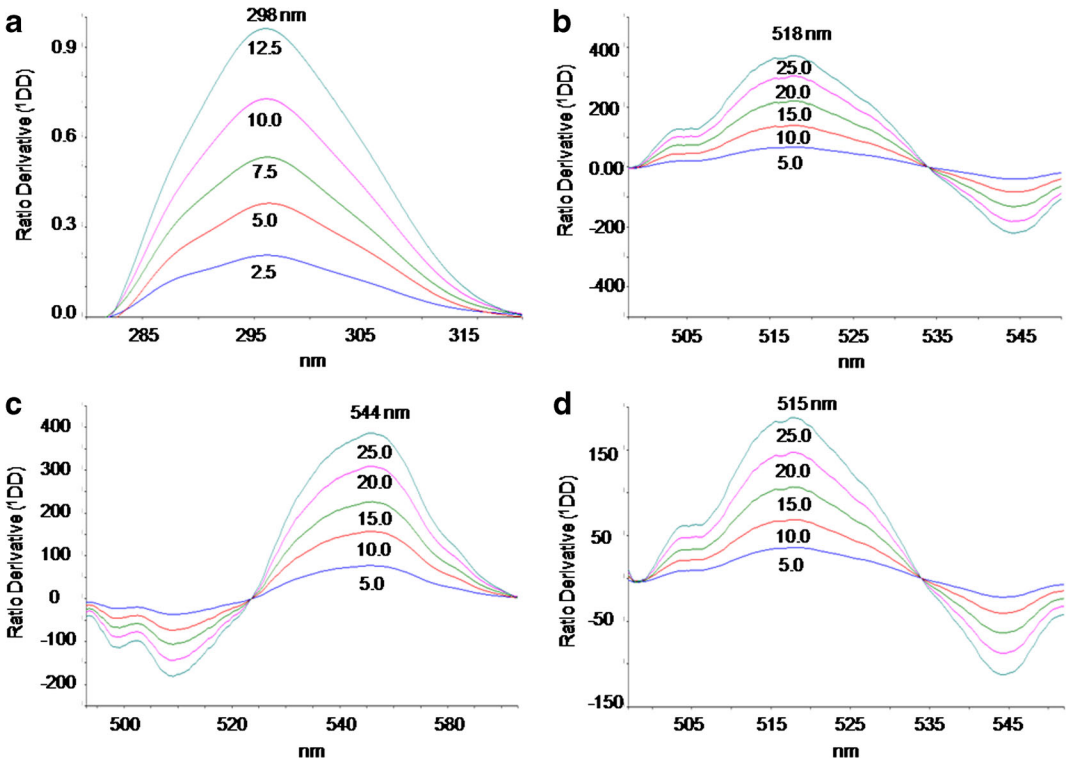

Fig. 3. Ratio spectra of standard **a** acetaminophen, **b** carmoisine, **c** erythrosine, and **d** sunset yellow

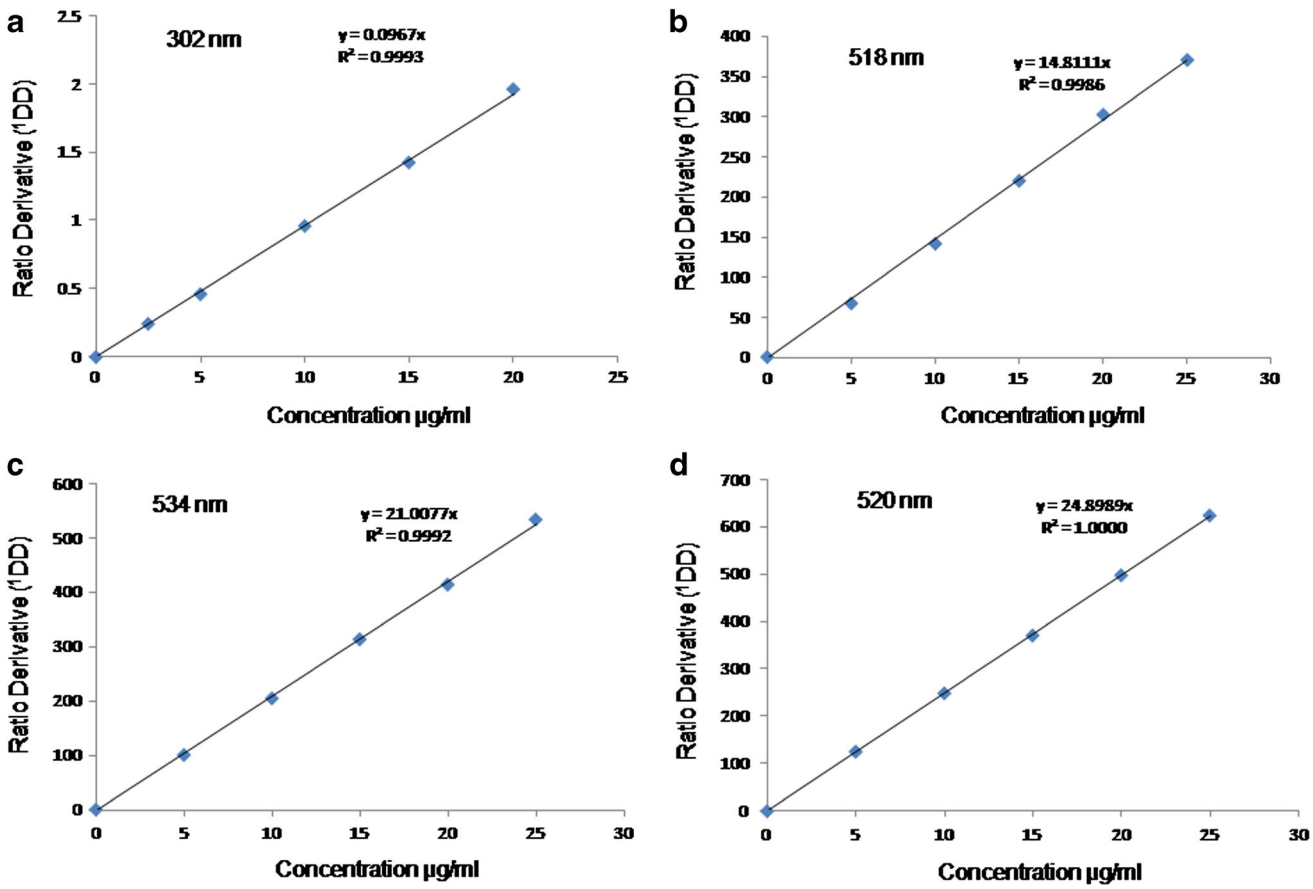

Fig. 4. Linear curve of ratio spectra of standard **a** acetaminophen, **b** carmoisine, **c** erythrosine, and **d** sunset yellow

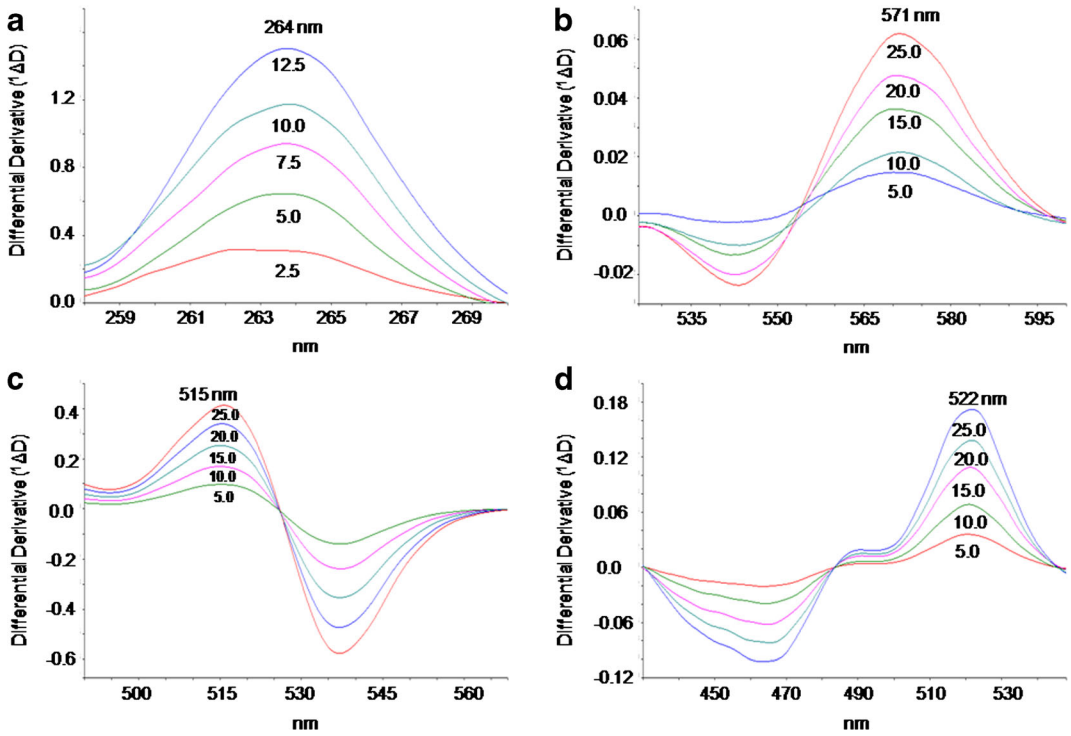

Fig. 5. Differential spectra of standard **a** acetaminophen, **b** carmoisine, **c** erythrosine, and **d** sunset yellow

water in double beam spectrophotometer. The signal of first derivative spectra of AT (obtained with a $\Delta\lambda$=8 nm and a smoothing over 17 experimental points) was measured at 302 nm (^{1}D 302, zero crossing point for first

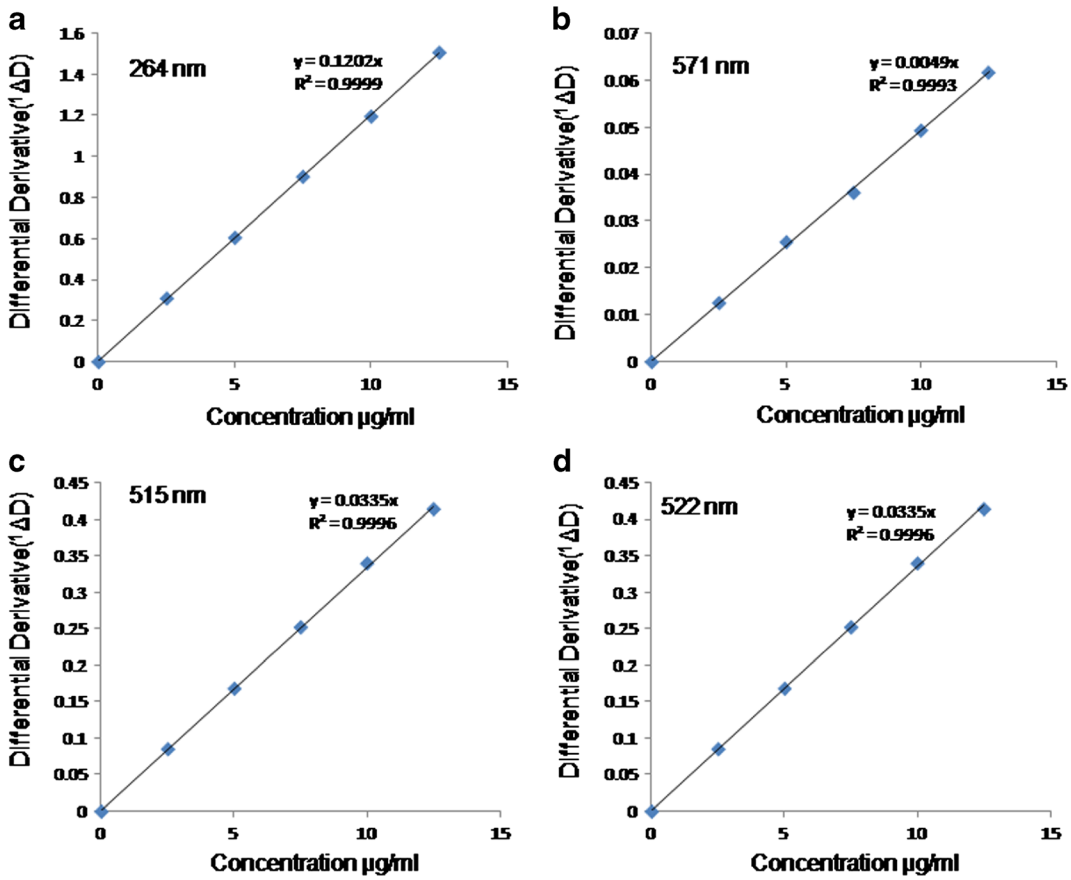

Fig. 6. Linear curve of differential spectra of standard **a** acetaminophen, **b** carmoisine, **c** erythrosine, and **d** sunset yellow

derivative spectra of ET, CA, and SSY), and by using an appropriate calibration curve, the concentration of AT was determined.

The CA content was determined by the first derivative signal measured at 570 nm (^{1}D570, zero crossing point for first derivative signal of AT), while ET and SSY content was determined by measuring at 536 and 520 nm, respectively (^{1}D 536 and 520, both zero crossing point for first derivative spectra of AT). These calibration graphs were prepared by varying the concentration of a colorant, without the presence of the other two colorants.

Ratio Spectrum-Zero Crossing Derivatives. The main advantage of ratio derivative method is the ability to measure the signals on both side of the zero value (a maximum or a minimum) with respect to wavelength. Moreover, the presence of more than one maxima and minima is another advantage of this method, which gives an opportunity for the determination of active compounds in the presence of other compounds and ingredients that may possibly interfere in the assay (49–51).

This method is applied on direct absorption spectra, to determine one component (either drug or color) while the other component is used as divisor. The ratio spectrum is obtained by dividing the absorption spectra of the mixture (containing drug and one of the three colors) by a standard spectrum of one of the component. The first derivative of the ratio spectrum is obtained to remove the spectral contribution of the component used as divisor; the component to be determined is measured at the zero crossing points of the other. The determinations carried out by this method are as follows:

i. When the divisor is a spectrum of one of the three dyes (CA, ET or SSY) whose concentration is 15 μg/ml, AT was determined at 234 nm for CA and 298 nm for both ET and SSY (^{1}DD234, ^{1}DD298, zero crossing point for ratio spectra derivative of either of the three dyes).
ii. When a standard spectrum of 7.5 μg/ml of AT is used as divisor, CA, ET, and SSY was determined at 518, 544, and 515 nm, respectively (^{1}DD518, 544 and 515, zero crossing point for ratio spectra derivative of AT).
iii. In order to obtain these first derivative spectra, a $\Delta\lambda = 4$ nm and a smoothing function of 23 experimental points were used.

Differential Derivative Spectra. Differential spectroscopy (ΔD_1) method is based on pH changes that have been reported to be useful in the determination of binary mixtures. The method depends on the utilization of difference absorption spectra corresponding to the same compound obtained at two different pH. The procedure comprises the measurement of ΔD_1 of a component in acidic solution against their respective alkaline solution as blank. The ΔD_1 has been successfully used

Table II. Intra- and Inter-Day Precision of the Drug and Three Colorants

Component	Amount (mg L^{-1})	Intra-day precision[a]			Inter-day precision[a]		
		Average signal	%RSDr	SE	Average signal	%RDS_R	SE
1st derivative							
Acetaminophen	2.5	0.013	0.95	0.0001	0.013	1.71	0.0001
	5.0	0.027	1.12	0.0002	0.026	1.92	0.0003
Carmoisine	5.0	0.047	1.33	0.0004	0.047	1.40	0.0004
	10.0	0.096	1.88	0.0010	0.094	1.67	0.0009
Erythrosine	5.0	0.171	1.12	0.0011	0.173	1.47	0.0015
	10.0	0.355	1.60	0.0075	0.355	0.59	0.0012
Sunset yellow	5.0	0.077	1.43	0.0006	0.077	1.80	0.0008
	10.0	0.149	1.21	0.0010	0.152	0.83	0.0007
Ratio derivative							
Acetaminophen	2.5	0.022	0.77	0.0001	0.022	1.78	0.0002
	5.0	0.054	1.93	0.0006	0.054	1.11	0.0010
Carmoisine	5.0	66.5	1.28	0.49	66.8	1.72	1.82
	10.0	143.5	1.48	1.22	141.7	1.38	1.95
Erythrosine	5.0	41.0	1.52	0.83	41.2	1.47	0.83
	10.0	85.6	1.92	2.43	86.2	1.83	2.41
Sunset yellow	5.0	127.1	1.37	1.00	125.2	1.00	2.17
	10.0	250.4	0.72	1.03	254.0	2.00	2.93
Differential derivative							
Acetaminophen	2.5	0.308	1.64	0.0029	0.314	1.53	0.0028
	5.0	0.597	1.26	0.0043	0.594	1.45	0.0050
Carmoisine	5.0	0.0128	1.46	0.0001	0.0117	0.98	0.0001
	10.0	0.0251	1.98	0.0003	0.0243	1.17	0.0002
Erythrosine	5.0	0.0834	1.96	0.0009	0.0794	0.83	0.0004
	10.0	0.0168	1.31	0.0013	0.1667	0.70	0.0007
Sunset yellow	5.0	0.0352	1.49	0.0003	0.0351	1.83	0.0004
	10.0	0.0664	1.69	0.0006	0.0675	1.90	0.0007

[a] Data is derived from triplicate values

to eliminate interferences in syrup formulations. There are reports on utilization of this technique for the estimation of individual drug and for combined preparations (48,52,53).

The difference spectra between the acidic (0.1 N HCl) solution and respective equimolar basic (0.1 N NaOH) solution of pure colorant(s) and AT were recorded from 200 to 700 nm by placing the acidic solution in the sample compartment and the basic solution in the reference compartment. A first derivative spectrum of each of the differential curves was subsequently recorded in Double Beam Spectrophotometer.

Validation of the Method

To support regulatory action, a method must be shown to be accurate, sensitive, and able to identify analyte with high selectivity. For this purpose, evaluation of the analytical method included determination of linearity, limit of detection (LOD), limit of quantification (LOQ), precision (reported as relative standard deviation (RSD)%), and recovery (reported as percentage recovered). The validation of the present three methods was performed as per ICH guidelines (54).

Linearity and Calibration Standard

The linearity of the assay was checked by analyzing different concentration of each component by 1st derivative, ratio derivative, and differential derivative method, and the calibration graph was obtained by plotting signal *versus* concentration.

Limit of Detection and Limit of Quantification

Limit of detection (LOD) and limit of quantification (LOQ) were based on the standard deviation of the response and the slope of the corresponding curve using the following equations;

$$\mathrm{LOD} = 3s/m;$$
$$\mathrm{LOQ} = 10\,s/m,$$

where "s" is the standard deviation of the derivative amplitude of the blank and "m" is the slope of the related calibration graphs (45).

Recovery, Repeatability, and Reproducibility

Different concentration of synthetic mixtures ranging between 2.5–12.5 μg/ml of AT along with either CA, ET, and SSY were prepared. The recovery and repeatability were determined by performing the experiment in triplicates and expressed as mean±SD.

Validation with HPLC

The syrup samples containing the drug and one of the three colors were measured by 1st derivative, ratio derivative, and differential derivative methods, and the results were

Table III. Percentage Recovery of the Acetaminophen and the Three Colorants

	Acetaminophen			Carmoisine			Erythrosine			Sunset yellow		
Amount added (ug)	Amount recovered (ug)	% recovery	%RSD	Amount recovered (ug)	% recovery	%RSD	Amount recovered (ug)	% recovery	%RSD	Amount recovered (ug)	% recovery	%RSD
1st derivative												
2.5	2.53	101.0	1.89	2.56	103.0	0.76	2.47	99.0	1.85	2.44	97.8	1.97
5.0	5.19	103.7	0.92	4.92	97.3	1.74	4.98	99.6	0.53	5.19	103.8	1.25
7.5	7.45	99.4	0.11	7.53	99.7	0.90	7.31	97.4	1.12	7.52	100.3	0.60
10.0	9.87	98.7	1.16	10.03	99.8	0.69	9.87	98.7	1.27	9.83	98.3	1.14
12.5	12.76	102.1	1.23	12.58	99.8	1.23	12.39	99.1	1.61	12.49	99.9	0.67
Ratio derivative												
2.5	2.58	103.1	1.59	2.56	102.3	0.68	2.54	101.5	1.19	2.47	98.8	1.42
5.0	5.06	101.3	1.18	4.84	96.8	1.93	5.17	103.5	1.03	5.16	103.2	1.12
7.5	7.44	99.2	1.07	7.42	99.0	1.50	7.46	99.4	0.84	7.55	100.6	0.84
10.0	10.22	102.2	0.31	9.95	99.6	1.42	10.14	101.4	1.05	9.89	98.9	1.41
12.5	12.72	101.7	1.64	12.35	98.8	1.21	12.68	101.4	3.05	12.67	101.3	0.97
Differential derivative												
2.5	2.38	95.2	1.19	2.56	102.5	1.85	2.51	100.2	1.11	2.54	101.8	0.81
5.0	4.87	97.3	1.51	4.89	97.8	1.82	4.97	99.4	1.69	5.15	103.1	1.56
7.5	7.44	99.2	1.37	7.62	101.5	1.14	7.30	97.3	0.58	7.45	99.4	0.88
10.0	9.75	97.5	1.00	9.70	97.0	0.59	9.88	98.8	0.73	10.1	100.8	1.99
12.5	12.34	98.7	1.60	12.1	97.0	1.99	12.6	100.8	1.49	12.7	101.4	1.21

Table IV. Result of the Amount of Acetaminophen in Commercial Syrup Formulation Purchased from the Market

		Results calculated by the method (mg/ml)							
		1st derivative		Ratio spectra		Differential derivative		HPLC	
S.No.	Amount of AT present (mg/ml)	Amount recovered	% recovery mean±SD	Amount recovered	% recovery mean±SD	Amount recovered	% recovery mean±SD	Amount recovered	% recovery mean±SD
1	25	24.3	97.1±1.30	24.3	97.1±1.36	24.9	99.7±0.83	25.2	100.7±1.57
2	20	19.1	95.6±1.33	20.7	103.3±1.17	19.7	98.4±1.15	19.6	97.9±1.99
3	50	49.6	99.2±1.54	48.9	97.8±1.90	49.5	98.9±1.93	50.0	100.1±1.67
4	20	19.8	98.8±1.16	19.4	96.8±1.37	19.5	97.4±1.78	20.1	100.4±1.78
5	50	49.4	98.8±1.55	50.2	100.5±1.39	48.4	96.9±2.59	50.3	100.6±1.98
6	25	25.1	100.2±1.38	24.8	99.8±1.05	23.6	94.5±1.55	24.7	98.8±1.20
7	25	24.6	98.3±1.21	24.4	97.5±1.93	24.4	97.8±2.09	24.6	98.3±1.64
8	25	23.8	95.1±1.22	24.3	97.2±1.55	24.2	97.0±1.88	24.4	97.7±2.11
9	25	24.2	96.7±1.57	24.6	98.4±1.70	24.2	96.7±1.51	24.6	98.4±2.12

compared with HPLC technique. The HPLC method followed for AT and the three colors was that of Microsolv application notes (55) and Dixit *et al.* (56), respectively.

Intake of Colors

The intake of colors was assessed based on the doses recommended on the syrup bottles. Amount of color was calculated based on a single dose (10–15 mg/kg) of the syrup formulation taken orally which is prescribed for a minimum of three times a day or a maximum of four times a day. Based on above calculation and the weight of children of various age groups (57), the intake of minimum and maximum color on a single day was assessed.

Actual intake of color (mg kg^{-1} bwt)=(amount of colored syrup consumed (ml)×concentration of color present in syrup (mg kg^{-1})/body weight (kg).

The color intake data were then compared with the respective acceptable daily intake (ADI) values of each color to arrive at the extent of saturation of ADI limits in different age groups.

Statistical Analysis

Results were expressed as mean±SD (n=3). The SD, %RSD, and coefficient of determinations (r^2) were determined using Microsoft Excel statistical software (Microsoft Corporation, Microsoft Office Excel 2007).

RESULTS AND DISCUSSION

Quality Control Data of Colorants and Acetaminophen

The LOD of 1st derivative method of all the components including drug and color(s) ranged from 0.011–0.31 μg/ml, while LOQ ranged from 0.04–1.04 μg/ml (Table I). In ratio spectra method, LOD ranged from 0.0001–0.04 μg/ml and LOQ was in the range of 0.002–0.14 μg/ml of all the components. Regarding differential derivative method, LOD of all the components ranged between 0.013–0.10 μg/ml and LOQ was in the range of 0.04–0.34 μg/ml). Thus, LOD and LOQ values of acetaminophen in the present study are either similar or lower than the reported values by other methods (46,47).

The 1st derivative spectra of all the colorants and AT are illustrated in Fig. 1. The calibration curves were obtained by plotting the derivative values *versus* concentration for AT along with either CA or ET and or SSY in the concentration range mentioned in Table I. The calibration curve of 1st derivative exhibited good linear relationship with regression coefficient values of 0.9993–0.9999 and small intercepts

Table V. Result of the Amount of Color in Commercial Syrup Formulation Purchased from the Market

		Amount of color present (μg/ml), mean±SD			
S.No	Colors	1st derivative	Ratio spectra	Differential spectra	HPLC
1	Carmoisine	23.9±1.60	24.5±1.10	23.1±1.79	23.7±1.14
2	Carmoisine	31.4±1.53	32.2±1.44	32.3±2.19	35.9±2.01
3	Erythrosine	30.6±2.01	30.3±1.83	29.3±2.50	31.9±1.45
4	Carmoisine	20.8±1.50	19.2±1.60	21.3±1.39	21.5±2.01
5	Erythrosine	26.6±1.57	26.9±1.20	27.7±1.64	27.8±0.48
6	Erythrosine	42.8±1.20	43.6±1.03	43.0±1.58	42.2±1.61
7	SSY	291.4±2.79	290.9±1.48	290.8±1.03	291.4±2.80
8	SSY	253.1±2.05	251.8±1.55	249.8±1.16	254.7±2.07
9	SSY	236.2±2.66	237.4±1.81	239.2±1.45	235.9±2.08

ranging from -6.80×10^{-4} to 3.49×10^{-3} (Fig. 2). The ratio spectra of all the four components are given in Fig. 3. The regression coefficient values of ratio derivative method ranged between 0.9986–1.000 while that of intercept values from -1.05×10^{-2} to −4.87 (Fig. 4). The derivative spectra of AT, CA, ET, and SSY were measured at λ max of 264, 571, 515, and 522 nm, respectively (Fig. 5). A linear relationship of differential derivative method is illustrated by excellent regression coefficient (0.9993–0.9999) and small intercept values (Fig. 6). The regression coefficient reported by Hassan *et al.* (22), Joshi *et al.* (44), and Khanage *et al.* (47) for acetaminophen varied between 0.994–1.000 and showed close resemblance with our reported values of AT analyzed by three different proposed methods (0.9993–0.9999).

The efficiency of method was tested in terms of good RSD values for both intra-day and inter-day precision for the three derivative methods. In the 1st derivative, the intra-day precision (RSDr) varied from 0.95% for AT at 2.50 mg/L to 1.88% for CA at 10.0 mg/L, while the inter-day precision (RSD_R) ranged from 0.59% for ET at 10.0 mg/L to 1.92% for AT at 5.00 mg/L (Table II). The ratio derivative showed RSDr value from 0.72% (SSY) at 10.0 mg/L to 1.93% (AT) at 5.00 mg/L. The RSD_R ranged between 1.00% at 5.00 mg/L and 2.00% at 10.0 mg/L for SSY, while the concentrations of other colors and AT fall in between. The differential derivative RSDr ranged from 1.26% for AT at 5.00 mg/L and 1.98% for CA at 10.0 mg/L, while RSD_R precision varied from 0.70% for ET at 10.0 mg/L to 1.53% for AT at 2.5 mg/L. The good RSD values <2% for both intra-day and inter-day precision for all the three derivative methods showed accurate method efficiency. Khanage *et al.* (47) reported 0.37 and 1.24% RSDr and 0.68 and 1.10% RSD_R for AT following Q-absorbance and ratio spectra method. Similarly, Sawant *et al.* (46) reported 0.17% intra-day precision by simultaneous equation method and 0.58% intra-day precision by Q-absorption method, while inter-day precision was found to be 0.15 and 0.05% for the above two methods, respectively.

In order to evaluate the genuineness of the method, recovery experiments were performed. Synthetic mixtures were prepared of all the components in the range of 2.5–12.5 μg/ml, and spectra were recorded against respective blank. The 1st derivative and ratio spectra were calculated from the normal spectra, and differential derivative was calculated from differential spectra obtained by placing the acidic solution in the sample compartment and the basic solution in the reference compartment of all the components. The percentage recovery in 1st derivative method ranged from 97.3 to 103.8%, while in case of ratio derivative method, recovery varied from 96.8 to 103.5% (Table III). The differential derivative method showed recovery of all the components in the range of 95.2–103.1%. The values indicate an adequate recovery rate, and the recovery percentages found in the present study are in close resemblance to other methods (22,44,46,47).

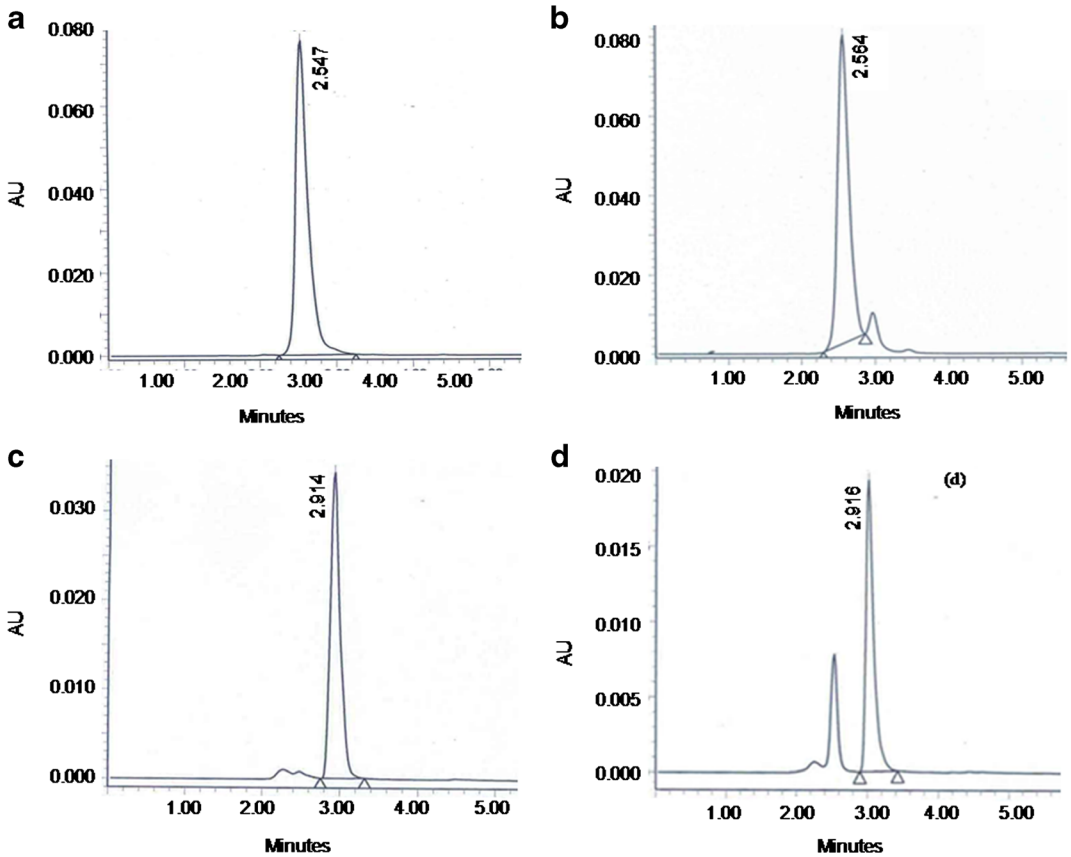

Fig. 7. HPLC chromatogram of standard AT (**a**), standard CA (**c**) along with the samples showing AT (**b**) and CA (**d**), respectively

Application to Real Samples and Their Validation with HPLC

The developed derivative spectroscopic methods were successfully applied to the commercial syrup formulation containing AT along with either of the colors: CA, ET, and SSY. The three derivative spectral methods were also validated by HPLC for the analysis of the same syrup samples (Tables IV and V). The HPLC chromatogram of standard AT and three colors along with samples are given in Figs. 7 and 8. The results of the three derivative methods showed ≥95% recovery, and the values were similar to those analyzed by HPLC method. Thus, the proposed derivative spectroscopic method is applicable for the routine analysis of color and drug, AT simultaneously in the syrup formulations.

Intake Assessment of Colors

The exposure assessment of three colors in different age group is shown in Table VI. Syrup medicines are generally prescribed to children up to age of 6 years due to inability or uneasiness of swallowing the tablets or capsules (2). Thus, only four age groups were taken into account for this study based on ICMR guidelines (57). Based on the dosage amount mentioned in the bottles, a minimum of three times a day and a maximum of four times a day were taken into account to calculate minimum and maximum intake. The intake of carmoisine saturated the ADI limits to an extent of 1.22% at the minimum consumption of drug and up to 1.63% at the maximum consumption of the drug (Table VI). However, Erythrosine saturated the ADI levels to an extent of 61.2 and 81.6% at the minimum and maximum intake levels, respectively. Since EFSA has reduced the ADI of SSY from 2.5 to 1 $mgKg^{-1}$ bwt (59), the intake of SSY was calculated according to new ADI value. It was found that SSY saturates the ADI from a minimum of 47% to a maximum of 63% on a single day when the drug is taken in the form of syrup.

Colors especially SSY have shown to cause irritability, restlessness, sleep disturbance, and hyperactivity in children (8–14). Our recent study has shown that non-cytotoxic dose of SSY causes immunomodulatory effects in splenocytes (60). Earlier studies showed that the intake of ET and SSY exceeded the ADI limits to an extent of 1.3 to 6 times in children at the average consumption of food commodities and average levels of detected colors (61). So additional ADI saturation from a minimum of 47% to a maximum of 82% with SSY and ET through medicinal syrups is quite alarming and need to be considered for modifications by the regulatory authorities. Colors are not a pre-requisite for syrup formulations and are added to match the flavor of the formulation. Hence, the colors in syrup formulations can be reduced to a minimal extent to safeguard the health of children as they are already vulnerable to ill effects of these chemicals.

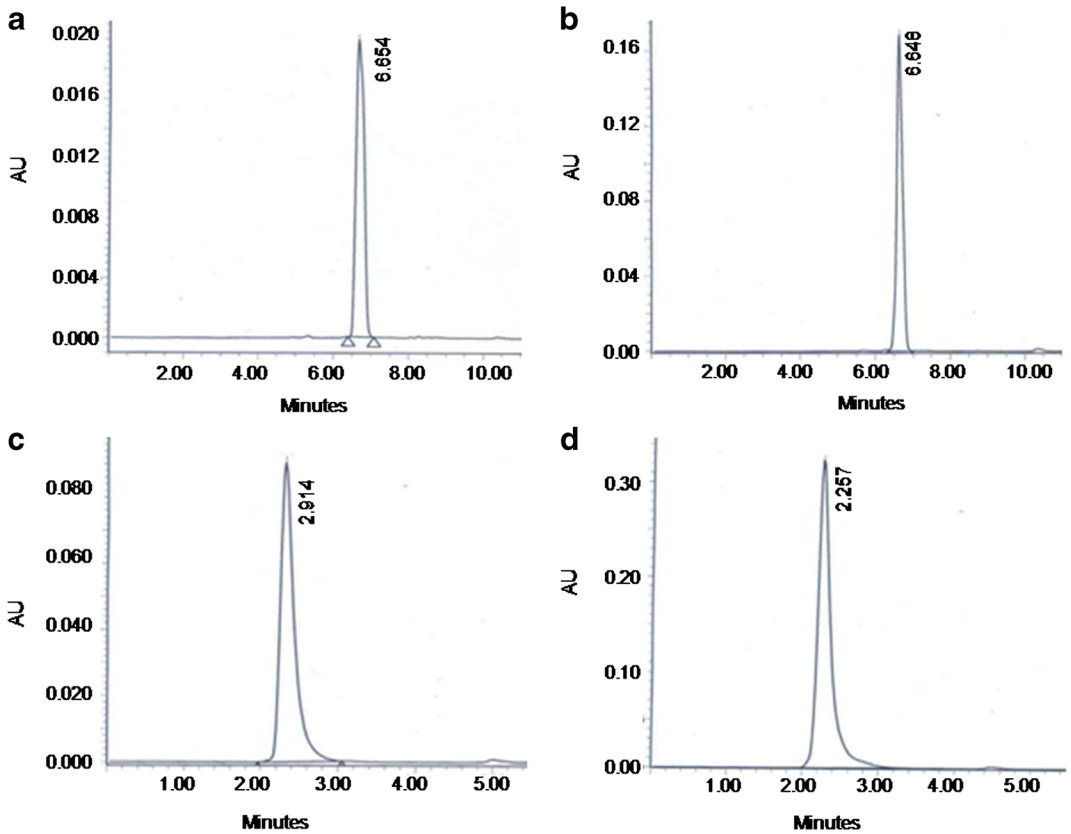

Fig. 8. HPLC chromatogram of standard ET (**a**) standard SSY (**c**) along with the samples showing ET (**b**) and SSY (**d**), respectively

Table VI. Exposure Assessment of Food Colors in Different Age Group of Children

Age[a] (yrs)	Intake of colors/day (μg)											
	Carmoisine[b]				Erythrosine[b]				SSYFCF[c]			
	Minimum		Maximum		Minimum		Maximum		Minimum		Maximum	
	Intake (μg)	% ADI saturation	Intake (μg)	% ADI saturation	Intake (μg)	% ADI saturation	Intake (μg)	% ADI saturation	Intake (μg)	% ADI saturation	Intake (μg)	% ADI saturation
0–0.5	263.4	1.22	351	1.63	331	61.2	441	81.6	2537	47.0	3383	62.6
0.5–1.0	409.8	1.22	546	1.63	514	61.2	685	81.6	3946	47.0	5262	62.6
1.0–3.0	629.3	1.22	839	1.63	790	61.2	1053	81.6	6060	47.0	8081	62.6
4.0–6.0	878.0	1.22	1171	1.63	1102	61.2	1469	81.6	8456	47.0	11275	62.6

[a] Age groups as per ICMR guidelines (57)
[b] ADI of carmoisine and erythrosine are 0–4 and 0–0.1 mg kg^{-1} bwt, respectively (58)
[c] ADI of SSYFCF was taken as 0–1 mg kg^{-1} bwt (59)

CONCLUSION

At present, no method is available to estimate simultaneously the drug and the color added as an excipient. The three derivative spectroscopic methods described in the present investigation provide simple, accurate, and reproducible quantitative determination of the drug, AT along with the color either CA, ET, and SSY in synthetic mixtures and commercial drug formulations without any interference from other ingredients. The present methods also offer a cost-effective and time-saving alternative that can be used for determination of the drug and the color simultaneously in pharmaceutical formulations. The present study also showed that colors like SSY and ET are saturating more than 50% of ADI value which is alarming and needs to be considered for modification by regulatory authorities to safeguard the health of children.

ACKNOWLEDGMENTS

We are grateful to the Director of CSIR-IITR, Lucknow, India for his keen interest in this study. SDR is thankful to Delhi Institute of Pharmaceutical Sciences & Research (DIPSAR), Delhi, India for forwarding her candidature as Research Trainee to IITR, Lucknow, India. The manuscript is IITR communication # 3229.

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