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Determination of Frequently Used Parabens in Shampoo and Conditioners using Validated HPLC Assay Method

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Parabens are esters of *p*-hydroxy benzoic acid and widely used as preservatives with a broad spectrum of antimicrobial activity in cosmetic, foods and pharmaceutical products. Methyl, ethyl, propyl and butyl paraben are commonly used in many cosmetic products. The concentration level of these parabens was restricted to maximum 1%. Recently, many cosmetic products such as shampoo and hair conditioners are available in the Indian market with label claims "no-parabens in the products". A HPLC method is developed for determining four frequently used parabens namely, methyl, ethyl, propyl and butyl paraben in shampoo and hair conditioners. The chromatographic separation was carried out on Phenomenex Kromasil C18 column (150 mm × 4.6mm i.d, 5 µm particle size) with water: methanol (60:40 v/v) as mobile phase and UV detection was performed at 254 nm. The method was validated with respect to specificity, linearity, accuracy, precision and robustness. The calibration curve was achieved to be linear and regression coefficient obtained was > 0.9999 for all the parabens. Accuracy of chromatographic method was evaluated by standard addition method; paraben content was estimated in eleven commercial shampoos and four hair conditioners available in Indian market which were claimed as no parabens were evaluated to assess the quality of claims of these samples. The results indicated that all the tested shampoos and hair conditioners met the requirements of the standards.

Keywords: Preservative, HPLC, Methyl paraben, Ethyl paraben, Propyl paraben, Butyl paraben.

INTRODUCTION

Cosmetic products and its stability are of particular relevance in our daily life, since the average adult is estimated to use at least seven different cosmetic products a day. An antimicrobial agent that plays a fundamental role in preventing the growth of microorganisms and also provides potency to cosmetics is chemical preservatives. In addition, external agents, such as air and sunlight also influence the stability and strangeness of cosmetics. Thus using compounds with antioxidant and light absorbent properties can help lengthen the life of cosmetics. However, according to the definition provided by the European Union (EU), cosmetic products have been regulated by European Union Council Directive 76/768/EEC, preservatives are only considered as substances, which may be added to cosmetic products for the primary purpose of inhibiting the development of microorganisms in such products and therefore, antioxidant and light absorbent are not considered as such as preservative. Hydroxybenzoates [methyl paraben (MP), ethyl paraben (EP), propyl paraben (PP) and butyl paraben (BP)] are alkyl esters of *p*-hydroxybenzoic acid with antimicrobial and antifungal properties. While the antimicrobial activity increases with expanding alkyl chain length of the ester group [1]. The antimicrobial activity may also be improved by combining two hydroxyl benzoates with short alkyl chains. Due to their broad antimicrobial spectra with relatively low toxicity, good stability and non-volatility, parabens are commonly used as preservatives to prevent alteration and degradation of cosmetics, pharmaceuticals and foods from microbial contamination and to protect the consumers [2,3]. Almost all types of cosmetic products containing parabens alone or combination, which may contact the skin, hair, scalp, lips, mucosae, axillae and nails, being used on a daily basis or occasionally in more than 13,200 formulations [4].

The European Union Regulation (EC) stipulate that parabens are regulated as preservatives in on cosmetic products under the denomination of 4-hydroxybenzoic acid and its salts and esters, with a maximum concentration of 0-4 % for single ester and 0-8 % for mixtures of esters. The use of butyl paraben and propyl paraben in finished cosmetic products does not exceed 0.19% as the sum of their individual concentrations [5]. Usage of parabens should be under great attention, because some studies mentioned that the use of preservatives can also produce other undesirable effects, which can appear either after first application or after years of cosmetic use. Parabens are reported as potent allergic contact dermatitis inducing agents. Several *in vitro* assays and *in vivo* animal model studies proves that parabens have estrogenic activity [6,7].

The European Cosmetic and Perfumary Association (Colipa) emphasizes that long term use of parabens are absorbed and retained in human body tissues without hydrolysis by tissue esterases to the common metabolite *p*-hydroxybenzoic acid. A wide range of applications of parabens has to be regulated. In consequence, it is important to develop analytical method to determine the concentrations of parabens in cosmetics that applied directly to the skin. Non-specific assay method (titration) with a long sample preparation time is reported in the United States Pharmacopeia (USP) [8].

A literature survey was undertaken to understand the state of the art of parabens quantification in various formulation matrixes. Separation and quantification of parabens in pharmaceutical and cosmetic products using capillary electrophoresis has been reported in the literature [9,10]. RP-HPLC methods have been reported for the assay of various parabens in cosmetic and pharmaceutical products [11-14]. Manojlovic *et al*. [15] reported RP-HPLC method for analysis of methyl paraben in multivitamin syrup. Method development and validation using HPLC with BDD (boron-doped diamond) electrode in an amperometric detector for the determination of commonly used parabens in shampoo matrix was reported [16]. HPLC-PDA-ESI/MS based method for the simultaneous quantification of recently banned preservatives was reported [17]. LC based method for the estimation of nine preservatives employing coagulants such as polyaluminium chloride and sodium hydroxide to minimize interferences in food matrices was reported [18]. Cosmetics containing four parabens were well separated using linear/gradient elution and FIC detection [19]. HPLC-PDA detection method reported for the effective screening of five parabens in ready-to-eat (RTE) food stuffs [20]. Literature survey indicates that presence and quantification of various parabens were attempted from various formulations. The reported methods involves cumbersome sample preparation steps, use of complexing agents, special electrodes/detector setup, *etc*. Moreover, there is no parabens estimation method following ICH guidelines [21]. Method validation based on ICH guidelines is paramount to ensure application across the laboratories.

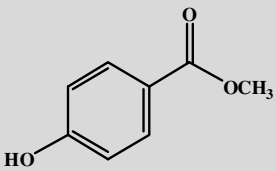
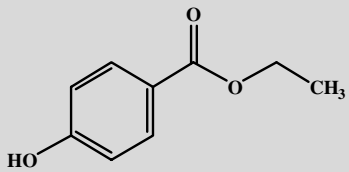
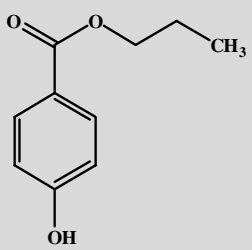
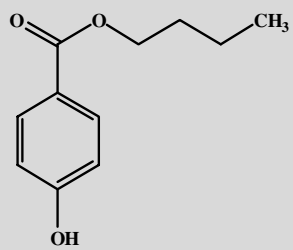
The main objective of this study was to develop a novel, selective and rapid analytical technique (HPLC) for the determination of most frequently used methyl, ethyl, propyl and butyl parabens in cosmetic products using the same stationary phase with suitable mobile phase based on ICH guidelines. The chemical structures of preservatives are presented in Table-1. In cosmetic and pharmaceutical industry, large quantities of reference standards are required to conduct all Current Good Manufacturing Practices (cGMP) work including instrument calibration, qualification and quantification. In this article, an attempt is made to develop a RP-HPLC method for the analysis of four parabens in eleven commercial shampoos and four hair conditioners available in Indian market. The proposed new analytical method (RP-HPLC) has been successfully validated as per ICH guidelines and has demonstrated to be accurate, linear, precise, reproducible, specific and robust. This novel method can be adopted and implemented in a quality control (QC) laboratory for the analysis of each of the four parabens.

EXPERIMENTAL

Methyl paraben, ethyl paraben, propyl paraben and butyl paraben were purchased from Sigma-Aldrich, USA. All the employed solvents were of HPLC analytical grade and obtained from Merck. All other chemicals were analytical reagent grade and deionised water used to prepare all solutions.

Samples: The eleven hair shampoos and four hair conditioners were used in this study and procured through online

TABLE-1
CHEMICAL STRUCTURE OF METHYL PARABEN, ETHYL PARABEN, PROPYL PARABEN AND BUTYL PARABEN

Preservatives	Chemical structure	Preservatives	Chemical structure
Methyl paraben		Ethyl paraben	
Propyl paraben		Butyl paraben	

portal from India. All the products contain label claim of no parabens.

Chromatogram conditions: Separation was performed using HPLC pump Shimadzu 10 Avp with autosampler and Shimadzu SPD-M10A DAD detector (Shimadzu Europe GmbH, Dulsburg, Germany) using column C18 (150 mm × 4.6 mm i.d, 5 µm particle size) with water:methanol (60:40 v/v) as mobile phase. Each solution (5 µL) was injected and separation was performed at 30 ºC with flow speed of 1 mL/min and with detection on wavelength maximal absorption at 254 nm. Total run time for each sample and standard was 20 min.

Stock solutions: The initial stock solutions of parabens were prepared by accurate weight of 8.32 mg of methyl paraben, 8.75 mg of ethyl paraben, 8.58 mg of propyl paraben and 8.96 mg of butyl paraben into 10 mL volumetric flasks. Methanol (5 mL) was added into volumetric flask and sonicate for 1 min and made up to the mark using methanol. Further diluted 1 mL into 100 mL of volumetric flask and made up to the mark with methanol.

Sample preparation: The tested cosmetic products, including shampoos and hair conditioners were prepared by weighing accurately 1 g into 50 mL volumetric flask. In each flask, added 40 mL methanol to dissolve the content by using sonicator for 10 min and made up to the mark with methanol. About 10 mL of this sample solution was filtered through 0.45 µm millipore membrane filters.

General approach: The alkyl portion of the ester group in parabens plays the significant role in the separation of homologus parabens on reverse phase columns. The retention times of parabens increase with increased hydrophobicity of the parabens. Due to the difference in hydrophobicity, C18 HPLC columns are ideal for the chromatographic separation of parabens. Based on this rationale, only C18 HPLC columns were selected for evaluation. A wavelength of 254 nm was selected for the detection of parabens.

Separating conditions and optimization: The objectives of this study were to determine optimal separating conditions for methyl, ethyl, propyl and butyl parabens in shampoos and hair conditioners. Selection for analytical determination and quantification of parabens depends on surfactant presented in tested samples. Quantitative analysis of parabens in shampoos and hair conditioners were performed using stationary phase C18 (150 mm × 4.6 mm i.d.) and mobile phase methanol: water (60:40 v/v), separation has been performed on wavelength with maximum absorption 254 nm.

RESULTS AND DISCUSSION

Validation of analytical method: Analytical method validation was performed according ICH Q2 (R1) guideline over linearity, accuracy, precision, robustness, LOD and LOQ. System suitability requirements were met prior to perform the method validation experiments. The assay values of samples were calculated by comparing their responses. To ensure system suitability during the sample run, the % difference between the bracketing standard injections was calculated and evaluated against the acceptance criterion. Chromatograms of individual parabens standard concentrations were recorded at 254 nm (Fig. 1).

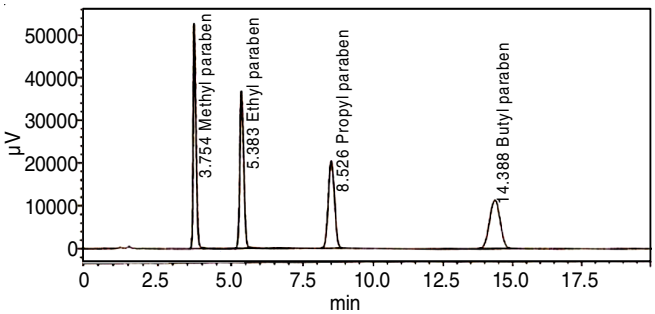


Fig. 1. Representative chromatogram of methyl paraben (MP), ethyl paraben (EP), propyl paraben (PP) and butyl paraben (BP) in a standard solution of 12.5 µg mL^{-1}

Specificity: The specificity and peak purity were carried out to determine whether there is any interference due to the presence of impurities and including degradation products and in order to prove the method is specific and selective, the standard peak of the drug and sample peak were compared to the RT against the blank and placebo chromatogram.

Sensitivity: The developed method was carried out for sensitivity studies based upon limit of detection (LOD) and limit of quantification (LOQ).The LOD and LOQ were calculated based on the standard deviation of *y*-intercept of the regression line (σ) and slope of the line (S), using the following equations:

$$LOD = 3.3 \times \sigma/S \text{ and}$$

$$LOQ = 10 \times \sigma/S$$

Precision: The precision of the method was carried out by repeatability (intra-day) and intermediate precision (inter-day) for methyl, ethyl, propyl and butyl parabens. The standard solutions were prepared and determined by measuring six replicates of three consecutive days. The retention time and peak area expressed and % RSD were calculated. The % RSD values are tabulated in Table-2.

Accuracy: Accuracy of the developed method was determined based on the recovery studies. Recovery studies were carried out by adding known concentration of standard solution at three different levels (50, 100 and 150%) of parabens in shampoo before starting the extraction procedures. The results of accuracy study are shown in Table-3.

TABLE-2
PRECISION STUDIES OF METHYL PARABEN, ETHYL PARABEN, PROPYL PARABEN AND BUTYL PARABEN

Validation steps	Parameters (n = 6)	Methyl paraben		Ethyl paraben		Propyl paraben		Butyl paraben		Acceptance criteria
		RT	Area	RT	Area	RT	Area	RT	Area	
Intra-day precision	RSD (%)	0.13	0.26	0.15	0.17	0.15	0.1	0.13	0.25	< 2
Inter-day precision	RSD (%)	0.12	0.40	0.09	0.57	0.08	1.28	0.51	0.51	< 2

TABLE-3
ACCURACY STUDIES OF METHYL PARABEN, ETHYL PARABEN, PROPYL PARABEN AND BUTYL PARABEN

Preservatives	Level of concentration applied (% of target) (n = 3)		
	50	100	150
Methyl paraben	99.25 ± 0.09	99.83 ± 0.13	99.38 ± 0.14
Ethyl paraben	102.38 ± 0.21	100.79 ± 0.19	99.36 ± 0.12
Propyl paraben	98.49 ± 2.31	96.76 ± 0.38	97.2 ± 0.84
Butyl paraben	98.49 ± 2.31	96.76 ± 0.38	97.20 ± 0.84

Linearity: From the stock solution, suitable dilutions were prepared using methanol as solvent at six different concentrations in the range of 25, 50, 75, 100, 125 and 150% level of methyl paraben, ethyl praben, propyl paraben and butyl paraben by measuring against the blank solution. The corresponding concentrations levels of the standard solutions were injected in duplicate. The linear regression analysis of standard curve plotted against between the concentration *versus* peak area (Fig. 2) and the intercept, slope values are shown in Table-4.

Robustness: Robustness of the method was studied by injecting the standard solution into the chromatograph at varied conditions of flow rate ± 0.2 mL/min, mobile organic phase composition ± 5, wavelength ± 5 nm and column temperature by ±5 ºC and the results are shown in Table-5.

LOD and LOQ: Limit of detection (LOD) is the lowest concentration of analyte that can be detected whereas limit of quantification (LOQ) is the lowest concentration of analyte that can be quantified with suitable linearity and precision. The LOD and LOQ of parabens were calculated and its values are summarized in Table-6.

Applications: The analytical application of the proposed method was utilized to determine parabens including methyl, ethyl, propyl and butyl parabens in shampoos and conditioners. A total 14 market samples of shampoo and conditioner were tested. All the samples claim an absence of parabens in the label. Out of the 14 samples tested, two samples were found to contain methyl paraben at 0.15% and 0.03% level. Peaks were identified by comparison of retention times with analytical standard and ultraviolet absorption spectra of parabens. It is worth to mention that using the present method developed, the lower concentrations of parabens can be detected using HPLC with DAD detection at 254 nm. This method provides simple sample preparation and can be used to monitor the trace level of parabens in samples found in shampoo and conditioner matrix.

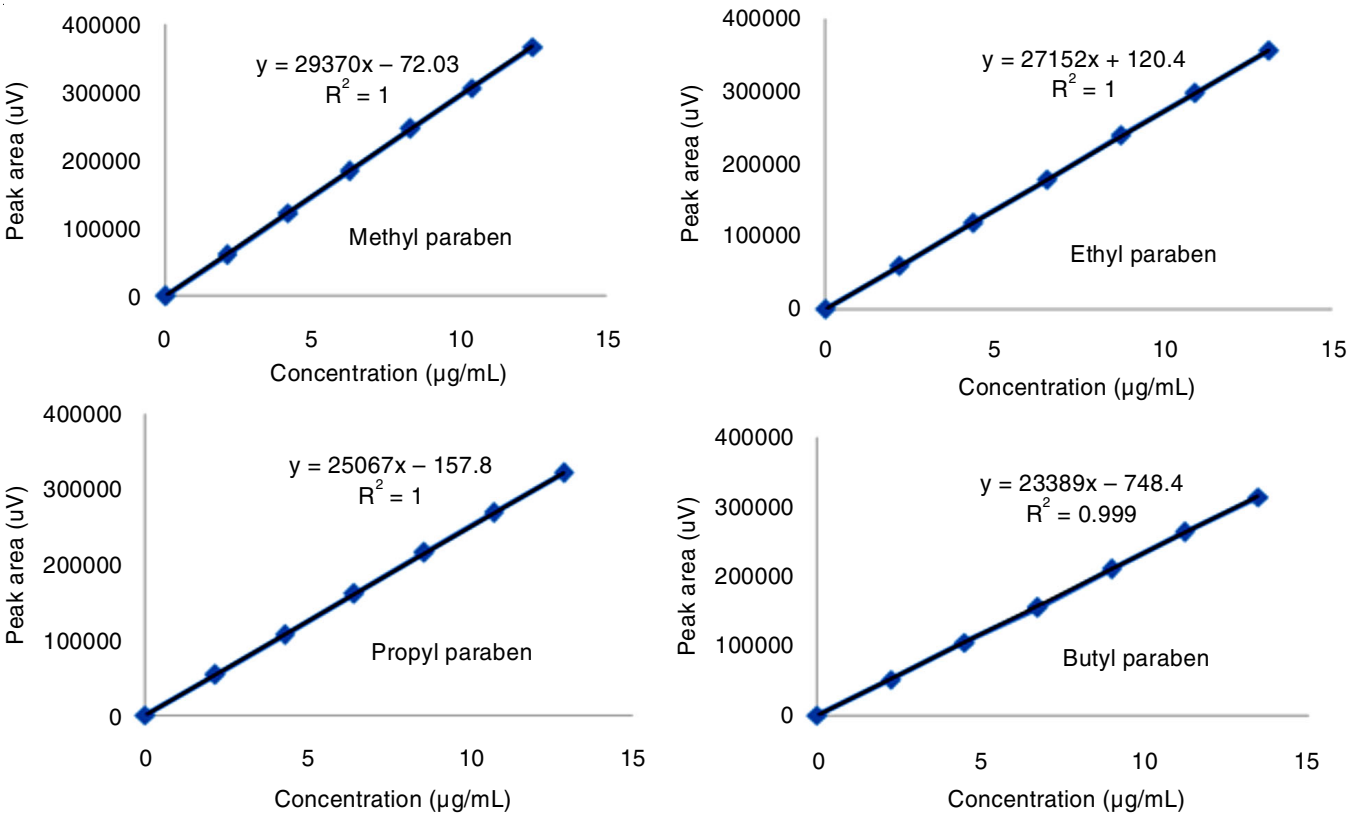


Fig. 2. Calibration curve for methyl paraben, ethyl paraben, propyl paraben and butyl paraben

TABLE-4
LINEAR REGRESSION CHARACTERISTICS OF METHYL PARABEN, ETHYL PARABEN, PROPYL PARABEN AND BUTYL PARABEN

Parameter	Methyl paraben	Ethyl paraben	Propyl paraben	Butyl paraben
Linearity range (µg/mL)	2.08-12.48	2.19-13.13	2.14-12.86	2.24-13.44
Regression equation (y = mx + b)	y = 29370x – 72	y = 27152x + 120	y = 25066x – 157	y = 23388x – 748
Correlation coefficient (R^2)	0.9999	0.9999	0.9999	0.999

TABLE-5
ROBUSTNESS CHARACTERISTICS OF METHYL PARABEN, ETHYL PARABEN, PROPYL PARABEN AND BUTYL PARABEN

Parameter	Change level	Methyl paraben			Ethyl paraben			Propyl paraben			Butyl paraben		
		RT	USP tailing	USP plate count	RT	USP tailing	USP plate count	RT	USP tailing	USP plate count	RT	USP tailing	USP plate count
Flow rate (± 0.2 mL/min)	0.80	4.688	1.240	7771	6.719	1.152	9857	10.637	1.068	11274	17.929	1.029	11579
	1.20	3.167	1.267	8091	4.547	1.147	8091	7.214	1.068	9867	12.202	1.036	10596
M.P organic composition	65:35	4.714	1.176	6288	7.310	1.105	7230	12.68	1.080	7441	23.394	1.076	7388
	55:45	3.308	1.261	5349	4.406	1.182	6536	6.382	1.106	7562	9.826	1.073	7945
Wavelength (± 5 nm)	249	3.787	1.232	7008	5.434	1.127	9085	8.614	1.003	10721	14.554	1.031	11150
	259	3.788	1.232	7007	5.434	1.124	9094	8.614	0.993	10667	14.554	1.029	11251
Temperature (± 5 °C)	35°	3.968	1.213	6044	5.712	1.135	7192	9.093	1.091	7902	15.399	1.069	8055
	45°	3.914	1.205	5774	5.625	1.122	6951	8.893	1.082	7645	14.915	1.063	7752

TABLE-6
LOD AND LOQ STUDIES OF METHYL PARABEN, ETHYL PARABEN, PROPYL PARABEN AND BUTYL PARABEN

Parameters	Methyl paraben	Ethyl paraben	Propyl paraben	Butyl paraben
LOD (µg/mL)	0.071	0.090	0.088	0.185
LOQ (µg/mL)	0.216	0.273	0.267	0.560

Conclusion

A quality control friendly, efficient, reproducible and robust HPLC method has been successfully developed and validated as per ICH guidelines for the determination of methyl paraben, ethyl paraben, propyl paraben and butyl paraben in various comm-ercial shampoos and hair conditioners. The stability of the method indicate as it can adequately separated all four parabens by method development validated analysis which enables the accuracy and the reliable quantification of parabens in cosmetic products. As the new method is a simple, rugged and reproduc-ible method, it can be easily implemented in a quality control laboratory.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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