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Short communication

LC-determination of five paraben preservatives in saliva and toothpaste samples using UV detection and a short monolithic column

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ABSTRACT

The present study reports the development and application of an HPLC–UV method for the simultaneous separation and determination of five paraben preservatives (methyl-, ethyl-, propyl-, *n*-butyl- and *iso*-butyl-paraben) in real samples. All analytes were separated efficiently in less than 20 min using a simple H_2O:ACN linear gradient and a short monolithic column (50 mm × 4.6 mm i.d.) at a flow rate of 3.0 mL min^{-1}. Phenoxyethanol was used as chromatographic internal standard. The method was validated for linearity, limits of detection and quantification, accuracy and precision. Human saliva and toothpaste samples were analyzed after SPE pretreatment on Licrolut RP-18 cartridges. The detection limits varied between 0.1 and 0.3 mg L^{-1} in all cases and the percent recoveries between 86 and 113%.

1. Introduction

Parabens are esters of p-hydroxybenzoic acid and according to the U.S. Food and Drug Administration (FDA) they are the most widely used preservatives in cosmetic products [1]. Industrial practice typically involves use of parabens' mixtures and in certain cases in combination with other types of preservatives to provide preservation against a broad range of microorganisms. The last few years there is an on-going debate on the safety and potential cancer risks from using paraben-containing products [2,3]. Additionally, parabens in personal care products, like mouth rinse solutions, react with free chlorine, usually contained in tap water to produce significant amounts of their chlorinated by-products in a few minutes. Further studies are necessary to evaluate the potential health risks and possible endocrine disrupter activity of halogenated parabens by-products [4]. On the basis of these facts, new analytical methodologies for the efficient quality control of parabens-containing cosmetics and formulations (the maximum allowed concentration for total parabens is 0.8% and for single parabens is 0.4%, w/w [5]) and analysis of biological material should be welcome.

The majority of previous reported LC methods for the determination of parabens employ conventional particulate-based columns. Such columns generally suffer from two main limitations. Slow mass transfer between the mobile and stationary phases and increased back-pressure at elevated flow rates. The recently commercialized monolithic materials can be an interesting and advantageous alternative [6,7] offering favorable properties for high-efficiency fast separations, such as low-pressure drop across the column, fast mass transfer kinetics and a high binding capacity [8]. It is characteristic that monolithic materials have been included in the L1 packing list of the U.S. Pharmacopoeia [9].

There are rather few reports on the use of monolithic materials for the analysis of parabens [10–19]. The main features of these methods are summarized in Table 1. The majority of the approaches are based on low-pressure separations by flow [10,11] or sequential injection chromatography [13,16,18,19]. Due to pressure limitations, the flow rates of such systems are restricted in most of the cases at values below 1.0 mL min^{-1} and thus fail to take into advantage the unique properties of monolithic materials [13,16,18,19]. Additionally, as can be seen in Table 1 a maximum of up to four parabens is separated. No method attempts to separate *n*-butylparaben from *iso*-butylparaben, despite the fact that this parabens-pair often co-exists in commercially available preservative mixtures (e.g. Phenonip® from Clariant [20]). Another interesting feature is that none of the methods reported in Table 1 was applied in biological material and were restricted to pharmaceutical and cosmetic products.

The purpose of the present study was to develop a simple and efficient HPLC method for the separation and determination of five parabens (methyl-, ethyl, propyl-, *n*-butyl- and *iso*-butyl-paraben) in real samples, including biological material. A short

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Table 1
Determination of parabens using monolithic columns.

Analytes	Technique	Elution mode	Detection	Column	Flow rate (mL min^{-1})	Samples	Ref.
MP, EP, PP, *n*-BP	FIC	Stepwise gradient	CL	Chromolith RP-18 (5 mm × 4.6 mm i.d.)	2.6	Cleaning mousse and towels	[10]
MP, EP, PP, *n*-BP	FIC	Stepwise gradient	DAD	Chromolith RP-18 (5 mm × 4.6 mm i.d.)	3.5	Dehydrated soups, toothpaste, cola drinks, cleaning towels, hair mousse,	[11]
MP, PP	HPLC	Isocratic	UV at 254 nm	Chromolith RP-18 (100 mm × 4.6 mm i.d.)	4.0	Pharmaceutical gel	[12]
MP	SIC	Isocratic	DAD	Chromolith RP-18 (25 mm × 4.6 mm i.d.)	0.9	Nasal drops	[13]
MP, EP, PP, *n*-BP	CEC	–	UV at 200 nm	Home-made capillary column (100 μm i.d.)	–	Lotions, water-based ointments, oil-based ointments	[14]
MP, EP, PP, *n*-BP	HPLC	Isocratic and gradient	UV at 215 nm	Chromolith RP-18 (100 mm × 4.6 mm i.d.)	4.0	Tablets and lozenges	[15]
MP	SIC	Isocratic	UV at 245 nm	Chromolith RP-18 (50 mm × 4.6 mm i.d.)	0.48	Syrups and drops	[16]
MP, EP, PP	HPLC	Isocratic	UV at 225/233 nm	Chromolith RP-18 (25, 50, 100 mm × 4.6 mm i.d.)	2.0–5.0	Pharmaceutical gels	[17]
MP, PP	SIC	Isocratic	UV at 275 nm	Chromolith RP-18 (25 mm × 4.6 mm i.d.)	0.48–1.2	Pharmaceutical gel	[18]
MP, PP	SIC	Isocratic	UV at 243 nm	Chromolith RP-18 (25 mm × 4.6 mm i.d.)	0.6	Pharmaceutical cream	[19]
MP, EP, PP, *n*-BP, *iso*-BP	HPLC	Linear gradient	UV at 254 nm	Chromolith RP-18 (50 mm × 4.6 mm i.d.)	3.0	Saliva, toothpastes, synthetic saliva	This work

FIC/SIC: flow/sequential injection chromatography; CEC: capillary electrochromatography; CL: chemiluminescence.

monolithic column (50 mm × 4.6 mm i.d.) was selected as stationary phase. Applying a simple gradient elution program, all parabens – including the *n*- and *iso*-butylparaben isomers – were separated efficiently in less than 20 min, at a flow rate of 3.0 mL min^{-1}. The proposed method was validated for its application in human and synthetic saliva and toothpaste samples.

2. Experimental

2.1. Reagents and materials

HPLC grade solvents, methanol acetonitrile were used during all experiments and tests (Merck, Darmstadt, Germany). Ultra-pure water was produced by a Millipore system. Methylparaben (MP), ethylparaben (EP), propylparaben (PP), *n*-butylparaben (*n*-BP) and *iso*-butylparaben (*iso*-BP) working standards (purity >99%) were kindly supplied by Cosmopharm Ltd (Greece). Phenoxyethanol (chromatographic internal standard, I.S.) was also provided by Cosmopharm Ltd (purity >99%). Standard stock solutions of the above-mentioned analytes and the I.S. were prepared in MeOH at the 500 mg L^{-1} level. The stock solutions were found to be stable for at least two weeks if kept refrigerated. Working standards and mixtures were prepared freshly.

A SpeedRod® reversed-phase monolithic column (50 mm × 4.6 mm i.d., Chromolith, Merck) was employed throughout this study. Two types of RP-SPE cartridges were examined during method development; Abselut Nexus (30 mg/1 mL, Varian) and Lichrolut RP-18 (200 mg/3 mL, Merck).

2.2. Instrumentation

The HPLC setup comprised the following parts: a Spectra-Physics SP8800 ternary gradient pump; a Rheodyne 7725i manual injection valve, equipped with a 10-μL sample injection loop; a Schimadzu SPD-10A VP variable wavelength UV–Vis detector; a Spectra-Physics SP4290 integrator.

SPE was performed on a Supelco (St. Louis, MO, USA) Visiprep vacuum manifold system, having a 12-position capacity. Evaporation of solvents was carried out, at ambient temperatures, under a stream of nitrogen, by means of a model 18780 Reacti-Vap device (Pierce, Rackford, IL, USA).

2.3. HPLC procedure

The analytes were separated at a flow rate of 3.0 mL min^{-1} at ambient temperature and they were detected at 254 nm. The water/ACN gradient program included the following steps: 0–5 min: 3–10% ACN; 5–10 min: 10–20% ACN; 10–20 min: 20% ACN. Under the above-mentioned conditions the analysis cycle was completed in 20 min, followed by a 5-min post-gradient equilibration. Peak area ratio was used for signals evaluation, while each sample or standard was injected 3 times.

2.4. Sample preparation

Human saliva samples were collected from healthy volunteers who abstained for at least one week from using parabens-containing products. Prior to collection the donors had their mouths washed with a 5 g L^{-1} citric acid solution (saliva stimulator) and three times with doubly de-ionized water. The collected saliva was centrifuged at 2000 rpm for 5 min and was filtered through a 0.45 μm filter. A 2-mL volume of the sample was vortexed with 200 μL of either methanol (blank) or methanolic solutions of various concentrations of parabens (fortified samples for recovery experiments). The samples were further treated by a simple SPE protocol on Licrolut RP-18 cartridges (200 mg/3 mL, Merck) that

was consisted of the following steps: (1) activation of the cartridges with 1 mL methanol, (2) sample loading, (3) washing with 1 mL water and (4) sample elution with 1 mL methanol. The resulting solutions were evaporating to dryness, reconstituted in 200 μL of a methanolic solution of 100 mg L^{-1} of the I.S. and analyzed by the developed HPLC method.

The commercially available artificial saliva formulation was declared to contain MP and PP as preservatives (www.pharmagel.gr). The samples (0.5 mL) were diluted 20-fold in methanol, filtered through 0.2 μm syringe filters and analyzed without further pretreatment. Recovery experiments were carried out by spiking the samples with 100 μL of standard parabens' mixtures and 100 μL of a 10,000 mg L^{-1} methanolic solution of I.S. prior to dilution.

A 1-g quantity of a pooled sample of commercially available toothpastes was dispersed in 5 mL methanol and centrifuged for 15 min at 3000 rpm. 100 μL of the clear supernatant were diluted 20-fold in de-ionized water and treated with the SPE protocol described above. After elution, the resulting sample was evaporated to dryness and reconstituted in 2 mL methanol containing 100 mg L^{-1} of the I.S.

3. Results and discussion

3.1. Development of the HPLC protocol

Preliminary experiments were carried out in order to define the optimum wavelength for the UV detection of the analytes. Batch experiments using methanolic solutions (10 mg L^{-1} each analyte) showed that 254 nm was the λ_{max} for all parabens and was therefore selected for further studies.

The first attempts to separate the analytes were made under isocratic conditions using the SpeedRod (50 mm × 4.6 mm i.d.) monolithic column in all cases. Acetonitrile was used as organic modifier in all experiments. However, under isocratic conditions the BP isomers were not separated efficiently (R_s < 1.0) at any buffer, pH or aqueous/organic ratio tested, therefore a number of gradient elution protocols were tested. Using the program described in Section 2.3, *n*-BP and *iso*-BP were adequately separated (R_s = 1.5), while the analysis time was less than 20 min.

Phenoxyethanol was found to be a suitable chromatographic internal standard (I.S.), at a mass concentration of 100 mg L^{-1}. Under the selected gradient elution conditions, phenoxyethanol was eluted first and base-line separated from MP without causing interference with the analysis.

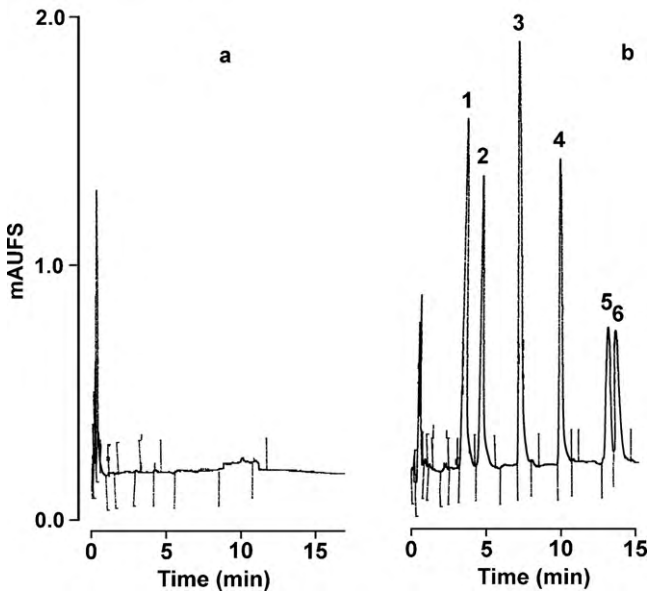

Fig. 1. Representative chromatograms of blank (a) and spiked (b) human saliva; 1: phenoxyethanol, 2: MP, 3: EP, 4: PP, 5: *n*-BP and 6: *iso*-BP.

3.2. Development of the SPE protocol

Preliminary experiments confirmed the necessity of applying an additional SPE pretreatment in order to improve the chromatograms of human saliva and toothpaste samples. For this reason, a simple SPE protocol was adopted as described in Section 2.4, using methanol as elution solvent. Reversed-phase cartridges from two different manufacturers were examined and compared in terms of analytes recoveries. The Abselut Nexus cartridges provided significantly lower recoveries for all analytes (23.9–53.0%) compared to the Lichrolut RP-18. The percent absolute recoveries [(peak area of extracted analyte versus peak area of un-extracted standard at the same concentration level) × 100] for all parabens using the latter SPE cartridges were satisfactory ranging between 78.9% (PP) and 85.4% (EP) and were selected for further experiments. No SPE-based treatment was necessary for the artificial saliva formu-

Table 2
Linearity and limits of detection and quantification of the developed method.

Analytes	Sample matrix	Regression equation [$R = (a \pm s_a) + (b \pm s_b) \times \gamma$(analyte)][a]	Linear range (mg L^{-1})[b]	Regression coefficient	LOD (mg L^{-1})[c]
MP	Aqueous	$R = (0.0140 \pm 0.0401) + (0.2528 \pm 0.0019) \times \gamma$(MP)	0.3–50	0.9998	0.1
	Human saliva	$R = (-0.0519 \pm 0.0479) + (0.1765 \pm 0.0033) \times \gamma$(MP)	0.5–50	0.9991	0.2
	Toothpaste	$R = (-0.0576 \pm 0.0512) + (0.2035 \pm 0.0035) \times \gamma$(MP)	0.5–30	0.9992	0.2
EP	Aqueous	$R = (0.0160 \pm 0.0377) + (0.2533 \pm 0.0018) \times \gamma$(EP)	0.3–50	0.9998	0.1
	Human saliva	$R = (-0.0421 \pm 0.0429) + (0.1867 \pm 0.0029) \times \gamma$(EP)	0.5–50	0.9993	0.2
	Toothpaste	$R = (0.0294 \pm 0.0491) + (0.1751 \pm 0.0034) \times \gamma$(EP)	0.5–30	0.9990	0.2
PP	Aqueous	$R = (0.0192 \pm 0.0396) + (0.2232 \pm 0.0019) \times \gamma$(PP)	0.3–50	0.9997	0.1
	Human saliva	$R = (-0.0539 \pm 0.0670) + (0.1857 \pm 0.0046) \times \gamma$(PP)	0.5–50	0.9984	0.2
	Toothpaste	$R = (0.0554 \pm 0.0329) + (0.1542 \pm 0.0023) \times \gamma$(PP)	0.5–30	0.9994	0.2
n-BP	Aqueous	$R = (0.0599 \pm 0.0609) + (0.1932 \pm 0.0027) \times \gamma$(*n*-BP)	0.6–50	0.9994	0.2
	Human saliva	$R = (-0.1821 \pm 0.0705) + (0.1911 \pm 0.0045) \times \gamma$(*n*-BP)	1.0–50	0.9988	0.3
	Toothpaste	$R = (0.0563 \pm 0.0282) + (0.1228 \pm 0.0018) \times \gamma$(*n*-BP)	1.0–30	0.9996	0.3
iso-BP	Aqueous	$R = (0.0496 \pm 0.0353) + (0.2127 \pm 0.0015) \times \gamma$(*iso*-BP)	0.6–50	0.9998	0.2
	Human saliva	$R = (-0.1026 \pm 0.0559) + (0.1919 \pm 0.0036) \times \gamma$(*iso*-BP)	1.0–50	0.9992	0.3
	Toothpaste	$R = (-0.0316 \pm 0.0606) + (0.1904 \pm 0.0039) \times \gamma$(*iso*-BP)	1.0–30	0.9991	0.3

[a] R: analyte/I.S. peak area ratio and γ(analyte): the mass concentration of the analytes in mg L^{-1}.
[b] The lower limit of the linear range corresponds to the LOQ of the method.
[c] The LOD and LOQ were estimated based on the S/N = 3 and 10 criteria, respectively.

Table 3
Precision and accuracy of the assay for human saliva and toothpaste analysis.

Analyte	Added ($mg\,L^{-1}$)	Human saliva			Toothpaste		
		Found (±SD) ($mg\,L^{-1}$)	RSD (%)	Recovery (%)	Found (±SD) ($mg\,L^{-1}$)	RSD (%)	Recovery (%)
MP	0.5	0.48 (±0.02)	4.2	96.0	0.56 (±0.02)	3.6	112.0
	1.0	1.03 (±0.03)	2.9	103.0	1.10 (±0.03)	2.7	110.0
	3.0	3.18 (±0.05)	1.6	106.0	3.10 (±0.06)	1.9	103.3
	5.0	5.51 (±0.09)	1.6	110.2	4.60 (±0.12)	2.6	92.0
	10.0	9.52 (±0.12)	1.3	95.2	10.3 (±0.18)	1.7	103.0
	20.0	19.9 (±0.35)	1.8	99.5	20.6 (±0.28)	1.4	103.0
	30.0	30.7 (±0.85)	2.8	102.3	29.5 (±0.63)	2.1	98.3
EP	0.5	0.53 (±0.02)	3.8	106.0	0.44 (±0.03)	6.8	88.0
	1.0	1.13 (±0.02)	1.8	113.0	0.94 (±0.03)	3.2	94.0
	3.0	2.73 (±0.06)	2.2	91.0	3.18 (±0.07)	2.2	106.0
	5.0	5.37 (± 0.19)	3.5	107.4	4.71 (±0.14)	3.0	94.2
	10.0	9.83 (±0.16)	1.6	98.3	11.0 (±0.23)	2.1	110.0
	20.0	19.8 (±0.41)	2.1	99.0	20.1 (±0.34)	1.7	100.5
	30.0	30.9 (±0.49)	1.6	103.0	29.6 (±0.32)	1.1	98.7
PP	0.5	0.56 (±0.03)	5.4	112.0	0.43 (±0.02)	4.7	86.0
	1.0	1.10 (±0.03)	2.7	110.0	0.95 (±0.04)	4.2	95.0
	3.0	2.65 (±0.07)	2.6	88.3	2.98 (±0.06)	2.0	99.3
	5.0	5.26 (±0.14)	2.7	105.2	4.65 (±0.16)	3.4	93.0
	10.0	9.26 (±0.34)	3.7	92.6	10.5 (±0.22)	2.1	105.0
	20.0	19.0 (±0.38)	2.0	95.0	20.5 (±0.28)	1.4	102.5
	30.0	29.9 (±0.54)	1.8	99.7	29.5 (±0.68)	2.3	98.3
n-BP	1.0	1.12 (±0.02)	1.8	112.0	0.89 (±0.05)	5.6	89.0
	3.0	2.68 (±0.07)	2.6	89.3	2.87 (±0.06)	2.1	95.7
	5.0	4.67 (±0.06)	1.3	93.4	4.89 (±0.09)	1.8	97.8
	10.0	11.1 (±0.46)	4.1	111.0	10.3 (±0.30)	2.9	103.0
	20.0	17.6 (±0.51)	2.9	88.0	20.5 (±0.44)	2.1	102.5
	30.0	31.7 (±0.32)	1.0	105.7	29.5 (±0.58)	2.0	98.3
iso-BP	1.0	1.12 (±0.03)	2.7	112.0	1.00 (±0.04)	4.0	100.0
	3.0	3.06 (±0.06)	2.0	102.0	2.73 (±0.08)	2.9	91.0
	5.0	5.18 (±0.12)	2.3	103.6	4.63 (±0.11)	2.4	92.6
	10.0	9.93 (±0.38)	3.8	99.3	10.5 (±0.26)	2.5	105.0
	20.0	19.8 (±0.29)	1.5	99.0	20.6 (±0.42)	2.0	103.0
	30.0	30.5 (±0.82)	2.7	101.7	29.5 (±0.95)	3.2	98.3

lation. The efficiency of the applied SPE clean-up procedure can be clearly seen from a representative chromatogram of blank and spiked human saliva (Fig. 1). Similar results were obtained for the other type of samples as well.

3.3. Method validation and application

The proposed HPLC method was validated in terms of linearity, limits of detection (LOD) and quantification (LOQ), repeatability (within-day precision), intermediate precision (day-to-day precision) and accuracy.

Linearity was evaluated in standard solutions, human saliva and toothpaste matrices. The LODs and LOQs in each case were estimated based on the signal-to-noise ratio criterion (S/N = 3 and 10, respectively). The results including the regression equations, the linear ranges and regression coefficients are summarized in Table 2.

The precision and accuracy of the proposed HPLC method in aqueous solutions was validated both within the same day and during a period of seven days. All experiments were carried out at three concentration levels of the analytes (low-medium-high), namely 1.0, 5.0 and 30.0 $mg\,L^{-1}$. The within-day precision varied in the range of 0.5–2.2% and the day-to-day precision in the range of 0.5–5.8%. The relative errors were acceptable varying within the range of −13.0 and +10.0% and −7.0 and +6.3% for within-day and day-to-day experiments, respectively.

The precision and accuracy data from the application of the proposed HPLC method to the analysis of human saliva and toothpaste samples are summarized in Table 3. The accuracy was evaluated for a wide range of concentrations including values between the limits of quantification and the upper limits of the method for

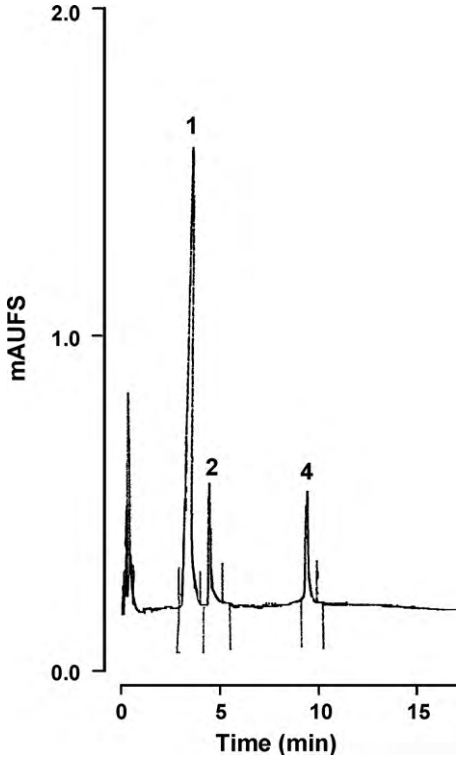

Fig. 2. Representative chromatogram of human saliva collected immediately after treatment with Xerotin®; 1: phenoxyethanol, 2: MP and 4: PP.

all parabens. The percent recoveries were acceptable in all cases ranging between 88.0 and 113.0% for human saliva and between 86.0 and 112.0% for toothpaste samples. As stated above, Xerotin® artificial saliva was analyzed without SPE pretreatment. The percent recoveries for MP and PP ranged between 95.1 and 108.0% with the RSD being <6.6% in all cases. In order to further demonstrate the bioanalytical character of this study, we analyzed human saliva collected directly after treatment of a human volunteer with Xerotin® according to the product instructions. A representative chromatogram is shown in Fig. 2.

4. Conclusions

The proposed HPLC method offers a simple and viable tool for the determination of paraben preservatives in real samples. The combination of a short monolithic column and a simple linear gradient elution protocol enabled the separation of all analytes including the *n*- and *iso*-BP isomers that are not typically included in previous publications. A simple SPE protocol enabled the successful application of the developed method to more complex matrices such as human saliva, compared to the majority of previous methods that are restricted to pharmaceuticals.

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