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Research paper

Degradation of parabens by *Pseudomonas beteli* and *Burkholderia latens*

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ABSTRACT

p-Hydroxybenzoic acid esters (parabens) are commonly used antimicrobial preservatives in pharmaceutical formulations. Two microorganisms, isolated from non-sterile methyl paraben (MP) and propyl paraben (PP) solutions, were found to degrade the respective parabens. Identification by 16S rRNA partial gene sequencing revealed them to be *Pseudomonas beteli* and *Burkholderia latens*, respectively. The present work describes a previously unreported interaction of the parabens with *P. beteli* and *B. latens*. Degradation of MP at various concentrations by *P. beteli*, followed a logarithmic pattern, while that of PP by *B. latens* was found to be linear. It was subsequently observed that *P. beteli* could degrade only MP, while *B. latens* could degrade both the parabens. Absence of HPLC chromatogram peaks of expected degradation products indicated that the parabens were used up as a carbon source. The behaviour of pathogens (*Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans* and *Aspergillus niger*) of the pharmacopoeial preservative effectiveness test (PET), towards MP, showed that none had the ability to degrade the paraben. It was concluded that, for a paraben-preserved multi-dose ophthalmic formulation, the sole use of the four pathogens that are recommended by the pharmacopoeia for PET can falsely indicate the formulation to be effective against 'in-use' contamination.

1. Introduction

Non-sterile and multi-dose sterile pharmaceutical formulations contain antimicrobial agents to kill or limit the growth of any microorganism that are present initially or gain entry during use of the product. The presence of unwanted microorganisms in pharmaceutical products can lead to either spoilage of product or transmission of disease to the consumer. Spoilage can either be in terms of chemical degradation of drugs/excipients by the enzymes produced by the microorganisms or a physical breakdown of the product leading to visible, olfactory, taste or tactile changes, thus rendering the product unfit for its intended use [1]. It is also possible that the microorganisms, without causing spoilage, induce disease in the consumer [2]. This assumes further criticality in case of multi-dose sterile products like ophthalmic solutions where contaminated eye drops can pose a hazard to the patients' vision [3].

Ophthalmic solutions are formulated essentially as other pharmaceutical solutions [4] with the additional requirement of sterility [5–7]. Antimicrobial preservatives are a pharmacopoeial requirement for multi-dose ophthalmic solutions. Although the sterility is assured in the multi-dose ophthalmic product by using aseptic processes or terminal sterilization processes, additionally, antimicrobial preservatives need to be incorporated to prevent growth or destroy microbes introduced due to accidental 'in-use' contamination [1,8]. Incidentally, preservative-free multi-dose eye drops have been previously used and have shown contamination with potentially pathogenic microorganisms [9]. Some of the common antimicrobial preservatives for ophthalmic use include benzalkonium chloride, chlorhexidine digluconate, benzyl alcohol, chlorbutol and *p*-hydroxybenzoic acid esters (also known as parabens). The latter mainly include methyl paraben (MP) and propyl paraben (PP) and are essentially used in the concentrations of 0.015–0.2% w/v and 0.005–0.01% w/v, respectively [10,11].

A study in our lab was conducted to determine the effect of autoclaving on paraben content (0.015% w/v MP and PP) during in-house ophthalmic formulation development. The study revealed a substantial reduction in the concentration of parabens in non-sterile formulations after one month of accelerated stability studies (40 °C). Autoclaved formulations, however, did not show any loss of paraben content. Based on the published literature, it was postulated that presence of microbial contamination in the non-sterile formulations might have interacted with the parabens leading to their loss. Microbes from the above-mentioned MP and PP formulation were isolated. The present work was delineated with the aim to understand the effect of these two isolated organisms on parabens in solution and to comprehend the implications that resulted from it.

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2. Materials and methods

2.1. Materials

MP sodium salt (Alta Labs Ltd., India) and PP sodium salt (Gujarat Organics Ltd., India) were of analytical grade. Reagents and solvents used for chromatographic analysis were analytical grade or higher. Either water purified by reverse osmosis (Elga®) or double distilled water was used for all the experiments. Growth medium used for streak plating was antibiotic assay medium A (HiMedia Laboratories Pvt. Ltd., India).

2.2. Chromatographic determination of parabens

The concentrations of MP and PP in samples were determined by High Performance Liquid Chromatographic (HPLC) System (Shimadzu, Japan) using a modification of a previously reported method [12]. The sample was appropriately diluted and 10 μl was injected onto the chromatographic column (LiChroCART® 250-4, LiChroSpher 100 RP-18e, 5 μm, Merck). The compounds were eluted by pumping premixed acetonitrile – 50 mM phosphate buffer, pH 3.5 solution (50:50, v/v) at a flow rate of 1 ml/min and detected by UV photometer at the wavelength of 254 nm. The method was validated for precision, accuracy and linearity in the range employed (10–200 μg/ml). Area counts of the sample peak and standard peak were used to calculate the concentration of MP/PP in the sample. Suspected degradation products, namely, phenol and *p*-hydroxybenzoic acid, were also injected at a concentration of 100 μg/ml to verify for their presence in the inoculated paraben solutions.

2.3. Effect of autoclaving

The effect of autoclaving on the paraben loss during stability studies was investigated. Batches of 0.015% w/v MP and PP were prepared using Elga® water. A pH of 5.0 ± 0.05 was obtained with the help of 0.1 N hydrochloric acid, and isotonicity was adjusted using sodium chloride. Each batch was filled in 5 ml washed and dried glass ampoules. After sealing, 10 ampoules of each batch were subjected to a sterilization cycle in an autoclave (15 psi, 121 °C for 20 min). The batches were labelled as follows:

(i) 0.015% w/v MP formulation without autoclaving – MPNS
(ii) 0.015% w/v MP formulation with autoclaving – MPAC
(iii) 0.015% w/v PP formulation without autoclaving – PPNS
(iv) 0.015% w/v PP formulation with autoclaving – PPAC

After the zero-day analysis, all the above batches were charged in a stability chamber (40 ± 2 °C) and analysed after every 30 days for 3 months.

2.4. Isolation and identification of microbes

MPNS and PPNS solutions were used to isolate the desired microorganisms that degraded MP and PP, respectively. In all the cases, unless otherwise mentioned, the agar medium used for plating the culture was antibiotic assay medium A fortified with 0.015% w/v MP or PP. The isolated paraben-resistant organisms were subsequently utilized to inoculate sterile, isotonic 0.015% w/v MP and PP solutions, to verify if they resulted in degradation. The microbes that degraded the parabens were taken forward in the study. The microorganism isolated from MP solution was labelled CMP and that from PP was labelled CPP. The organisms were outsourced to Microbial Type Culture Collection and Gene Bank (Institute of Microbial Technology, Chandigarh, India) for identification by 16S rRNA partial gene sequencing.

2.5. Preparation of inoculum

In all the cases, unless otherwise stated, the MP and PP solutions used were 0.015% w/v in strength, buffered (10 mM acetate, pH 5), isotonic (using sterile sodium chloride) and sterilized by autoclaving. The isolated organisms, CMP and CPP, were maintained as a suspension in MP and PP solutions at 4 °C. These suspensions were plated on the growth media and incubated for 24 h at a temperature of 40 °C. Fresh inocula of CMP and CPP colonies from the 24-h old culture were suspended in sterile Normal Saline (NS) in centrifuge tubes and centrifuged for 3 min at 3000 rpm. The supernatant was decanted and the pellet was resuspended in NS. The above process was repeated two more times and the final suspension in NS was diluted adequately to give an inoculum with a viable concentration of $2 \times 10^9 \pm 1 \times 10^9$ CFU/ml. An appropriate amount of this inoculum was added to different MP and PP formulations to give the desired concentration (CFU/ml).

2.6. Kinetics of paraben degradation

MP and PP solutions (buffered with 10 mM acetate buffer and made isotonic with sodium chloride) were prepared in four concentrations each, based on their recommended concentration range for ophthalmic solutions. The following concentrations were employed for MP (codes are mentioned in parenthesis): 0.58 mM (0.009% w/v) (MP-A), 1.09 mM (0.017% w/v) (MP-B), 6.86 mM (0.104% w/v) (MP-C) and 13.34 mM (0.203% w/v) (MP-D). The various concentrations for PP were 0.28 mM (0.005% w/v) (PP-A), 0.35 mM (0.006% w/v) (PP-B), 0.46 mM (0.008% w/v) (PP-C) and 0.58 mM (0.01% w/v) (PP-D). These formulations were filled and sterilized in glass vials. The MP and PP formulations were inoculated with CMP and CPP, respectively, to get a final viable count of $5 \times 10^7 \pm 1 \times 10^7$ CFU/ml. Vials of these formulations were stored at 40 °C in upright static condition for the duration of the study. A total of 0.5 ml of each formulation was withdrawn at 0, 24, 60, 96 and 132 h, appropriately diluted, and analysed by HPLC to determine the paraben content. Appropriate controls, without inoculation, were used to confirm the stability of the paraben formulations, for the duration of the study.

2.7. Degradation pattern studies

This section deals with the assessment of degradation capability of CMP for PP and CPP for MP. This was carried out by inoculating MP and PP solutions with CMP and CPP individually, to give a final viable count of 2×10^8 CFU/ml. Degradation studies of MP and PP solutions were also carried out using repeatedly sub-cultured CMP and CPP inocula (four times at an interval of 7 days on antibiotic assay medium A) to study the effect of subculturing on the ability of microbes to degrade the respective paraben solutions. In all the cases, the content of paraben was determined using HPLC. The chromatogram of the degraded solution was subsequently checked for the presence of suspected hydrolysed products: phenol and *p*-hydroxy benzoic acid.

2.8. Growth pattern studies

Growth of both the isolates in presence of MP and PP was checked using (i) streaking experiment and (ii) pour plate experiment.

(i) *Streaking experiment:* CMP inoculum was used to streak on agar medium which was fortified with MP and PP, respectively. In a similar manner, the growth of CPP in presence of MP and PP was checked. All the four plates were incubated at 40 °C for 24 h.

(ii) *Pour plate experiment:* Molten agar medium was poured in a petridish containing 20 μl of the CPM inoculum and swirled to mix the contents. After the agar was set, 10 μl drops ($n = 5$) each of MP and PP solution were allowed to dry on it. Similarly, pour plate with CPP inoculum was prepared and drops of MP and PP solution were placed on the set agar. Both the plates were incubated at 40 °C for 24 h.

2.9. Degradation studies with PET microorganisms

MP, which was degraded by both the isolated organisms, was investigated for its susceptibility towards degradation by the microorganisms recommended for the preservative effectiveness test (PET) in the British pharmacopoeia [13]. The methodology was similar to that in Section 2.6. In this study, 0.015% w/v, buffered, isotonic and sterile (as in previous sections) MP solutions were inoculated individually with *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger*. The MP content was monitored at regular intervals and the study was extended to 720 h.

3. Results and discussion

3.1. Effect of autoclaving

During the development of ophthalmic formulations in our laboratory, as a part of routine exercise, the effect of a steam sterilization cycle (121 °C for 20 min) on the individual components of the formulation is checked. Hence, MP and PP at a pH of 5 were subjected to a steam sterilization cycle. The concentrations used were deliberately kept low to enhance the detection ability of HPLC towards any loss in MP.

Non-sterile MP gave 100% loss at the end of 3 months as against 0.5% loss in autoclaved MP solutions (Table 1). Non-sterile PP gave close to 81% loss at the end of 3 months as against 2% loss in autoclaved PP solutions. The results indicated a positive effect of autoclaving on the stability of parabens. This could be due to microbial degradation of parabens in non-sterile preparations. The literature reports a few incidences of contaminated products preserved by parabens. These contaminants were found to be resistant to parabens and also degraded them [2,14,15]. A similar case was assumed here, and microorganisms were isolated from the MP and the PP formulations.

3.2. Isolation and identification of microbes

Four visually different types of colonies were isolated from MP solution: (i) opaque pink, (ii) opaque yellow, (iii) transparent green which turned brown after a few days and (iv) opaque white, which turned transparent after a few days. Each type of colony was inoculated in fresh MP solution. After a period of 10 days, only the opaque white colony was able to degrade methyl paraben in solution (as determined by the HPLC method described in Section 2.2), and therefore, these microbes were taken up for further studies. This isolate was coded as CMP. From the PP solution, only one type of colony was isolated (opaque white, which turned transparent after a few days), which was also able to degrade fresh PP solution. It was coded CPP and taken ahead for further studies.

16S rRNA partial gene sequencing revealed isolate CMP to be *Pseudomonas* sp. with maximum pairwise similarity (99.015%) with *Pseudomonas beteli* which is an aerobic Gram-negative rod [16]. Isolate CPP was identified as *Burkholderia* sp. with maximum pairwise similarity (99.778%) with *Burkholderia latens* (aerobic Gram-negative rod). It is a recently identified novel species that forms a part of the *Burkholderia cepacia* complex (Bcc) [17]. In humans, Bcc is well known for causing infections in immunocompromised patients [18].

The sequence of the strains CMP and CPP is presented in Fig. 1.

For convenience, the use of the assigned codes for the isolates (i.e. CMP and CPP) will be continued in further sections.

Table 1
Effect of autoclaving on the degradation profile of MP and PP. % Loss in MPNS (MP, non-sterile), MPAC (MP, autoclaved), PPNS (PP, non-sterile) and PPAC (PP, autoclaved) is shown as average ±SD, $n = 3$.

Formulation code	% Loss of paraben content		
	30 days	60 days	90 days
MPNS	99.82 ± 0.26	95.66 ± 3.77	99.99 ± 0.00
MPAC	0.15 ± 0.47	0.81 ± 0.34	0.56 ± 0.34
PPNS	45.59 ± 3.15	73.72 ± 3.58	81.66 ± 5.90
PPAC	0.19 ± 0.11	0.17 ± 0.07	1.99 ± 0.24

3.3. Kinetics of paraben degradation

Various concentrations of MP and PP were employed to determine the effect of CMP and CPP on the paraben degradation. The concentrations of MP and PP used, approximately covered their recommended concentration range for ophthalmic solutions [10,11]. Fig. 2 shows the amount of MP lost in different solutions (MP-A through D). Curve fitting and regression analysis was carried out for profiles of Fig. 2A (between time points 24 h and 132 h) based on which the curve was designated as roughly exponential or logarithmic. At lower concentrations (MP-A, MP-B and MP-C), the plot of concentration lost vs. time (Fig. 2A) shows logarithmic profiles, while at higher concentration (MP-D), the curve fitting leads to an exponential fit. Table 2 shows that the values of MP concentrations, that have been lost in 132 h for different formulations, increase with the concentration of the formulation. However, it cannot be said that the loss is dependent on original concentration since the '% decrease' for all formulations is not constant but reduces with the increase in concentration of the formulation. Also, a plot of Log(concentration) vs. time (graph not shown) does not show linearity confirming the absence of first-order degradation kinetics.

Fig. 3 shows the amount of PP lost in different solutions. Here, at all the concentrations (PP-A, PP-B, PP-C, PP-D), the plot of concentration lost vs. time (Fig. 3A) shows almost linear profiles. The values of PP concentrations that have been lost in the four formulations (Table 2) remain almost same, indicating that the degradation of PP by CPP is not dependent on the concentration of the substrate and follows zero-order kinetics.

Since the rate of degradation in case of MP was not linear, the calculation of the degradation rate of MP by CMP isolate has not been attempted. The degradation rate for PP was calculated from the slopes of the best fit linear curves and was found to be 805 ± 190 nM/h at 40 °C.

At the end of 132 h, for the MP formulations, only MP-A and MP-B showed growth. The rest two formulations did not show any viable microorganisms. However, in case of PP, all the four formulations showed increase in the viable count. Table 2 shows the changes in pH and % increase in viable count of the different formulations. The formulations showing presence (and an increase) of microorganisms also showed an increase in pH from the initial value of five (before the study) to a value close to pH 7.8 at the end of the study.

MP at a concentration above 1.09 mM does not sustain the viability of isolate CMP. The reasons for this observation could be that MP above a concentration of 1.09 mM is bactericidal to CMP or that the absence of a nitrogen source and other micronutrients in the formulation eventually led the viable count to be zero. However, PP at all concentrations tested, not only keeps the CPP microorgan-

CMP: 814 bp of the aligned 16S rRNA gene

TGCAGTCGAACGGCAGCACAGGAGAGCTTGCTCTCTGGGTGGCGAGTGGCGGACG
GGTGAGGAATACATCGGAATCTACTCTGTCGTGGGGGATAACGTAGGGAAACTTACG
CTAATACCGCATACGACCTACGGGTGAAAGCAGGGGACCTTCGGGCCTTGCGCGATT
GAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACG
ATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAG
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCA
GCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAA
GAAATCCAGCCGGCTAATACCTGGTTGGGATGACGGTACCCAAAGAATAAGCACCGG
CTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTA
CTGGGCGTAAAGCGTGCGTAGGTGGTCGTTTAAGTCCGTTGTGAAAGCCCTGGGCT
CAACCTGGGAACTGCAGTGGATACTGGGCGACTAGAATGTGGTAGAGGGTAGCGGA
ATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAAGGC
AGCTACCTGGACCAACATTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTT
GGCACGCAGTATCGAAGCTAACGCG

CPP: 900 bp of the aligned 16S rRNA gene

TGCAGTCGAACGGCAGCACGGGTGCTTGCACCTGGTGGCGAGTGGCGAACGGGTG
AGTAATACATCGGAACATGTCCTGTAGTGGGGGATAGCCCGGCGAAAGCCGGATTAAT
ACCGCATACGATCCACGGATGAAAGCGGGGGACCTTCGGGCCTCGCGCTATAGGGT
TGGCCGATGGCTGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCA
GTAGCTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACT
CCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCA
ATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCCGGAAAGAAA
TCCTTGGCTCTAATACAGTCGGGGGATGACGGTACCGGAAGAATAAGCACCGGCTAA
CTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGG
GCGTAAAGCGTGCGCAGGCGGTTTGCTAAGACCGATGTGAAATCCCCGGGCTCAAC
CTGGGAACTGCATTGGTGACTGGCAGGCTAGAGTATGGCAGAGGGGGGTAGAATTC
CACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAATACCGATGGCGAAGGCAGC
CCCCTGGGCCAATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAG
ATACCCTGGTAGTCCACGCCCTAAACGATGTCAAGGTAGTTGTTGGGGATTCATTTCC
TTAGTAACGTAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTA
AAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAAT

Fig. 1. Sequence of strains CMP and CPP.

isms viable but also supports their growth. It is possible that propyl paraben acts as a carbon source for isolate CPP. This can be confirmed by following the growth kinetics of the isolate in minimal media having PP as the only source of carbon. However, ascertaining the ultimate fate of parabens in the presence of the above-mentioned isolates was not part of the objective of the present study.

3.4. Degradation pattern studies

Isolates CMP and CPP were introduced in sterile MP and PP formulations, respectively. They degraded these freshly prepared paraben solutions (Table 3). Degradation of parabens by isolates CMP and CPP can be a part of a resistance mechanism towards these preservatives. HPLC chromatograms of the above inoculation study did not reveal any extra peaks of phenol and *p*-hydroxybenzoic acid (Fig. 4) after degradation. Previously, degradation of parabens by a resistant strain of *Enterobacter cloacae* has been reported by Valkova, et al. [15], wherein they have studied the aerobic transformation of parabens into phenol. Close and Nielsen [14] have reported the degradation of MP and PP by a strain of *Pseudomonas cepacia*, where they have mentioned the appearance of the peak of *p*-hydroxybenzoic acid. In the present case, it is possible that after the hydrolysis of the paraben into its respective alcohol and *p*-hydroxybenzoic acid, the latter is used up by the microorganism via the β-ketoadipate pathway [19]. This pathway is one of the routes by which a microorganism can attack the six-carbon nucleus of an aromatic compound and convert it into an aliphatic acid. This is further broken down to acetyl-CoA and succinate, which then enter the TCA cycle. *Pseudomonas* sp. (isolate CMP) are one such kind of microorganisms which can dissimilate *p*-hydroxybenzoate through the β-ketoadipate (mainly by ortho-cleavage) pathway [16]. *Burkholderia* sp. (isolate CPP) are known for their versatility in using a variety of aromatic organic compounds as carbon sources [20–22]. *B. cepacia* (previously known as *P. cepacia*) has been previously reported to degrade parabens [2,14]. Utilization of *p*-hydroxybenzoic acid as a carbon source can explain the absence of its peak in the HPLC chromatograms of the microbiologically degraded solutions.

CMP is resistant to both 0.015% w/v MP and PP, but could degrade only MP (100%) significantly in 10 days (Table 3). It degraded PP to a very less extent (5.12%). CPP was resistant to both 0.015% w/v MP and PP, and could degrade both parabens. A lack of specificity of the enzyme responsible for paraben degradation, present in CPP, could account for such a behaviour.

Subculturing CMP and CPP on antibiotic assay medium A for four consecutive times led to a loss of degradation ability of the microorganisms as shown in Table 3. The reason for this could be the inducible nature of the enzymes that degrade the parabens [14]. These 10-days-old MP and PP solutions, which did not show degradation in the presence of sub-cultured organisms, were streaked on petri-dishes containing medium A. Ready growth of these organisms within 24 h at 40 °C established that subculturing did not result in the loss of resistance by these organisms towards the respective parabens solutions.

A detailed study involving isolating, characterizing and monitoring the degradation products formed at shorter time intervals is required to conclude whether these isolates simply transform the parabens or use them up as a carbon source for their growth. Such a detailed study to elucidate the degradation pathway is beyond the scope of this article.

3.5. Growth pattern studies

(i) *Streaking experiment*: During isolation, it was established that CMP could grow uninhibited on agar media fortified with 0.015% w/v MP. Further streaking experiments

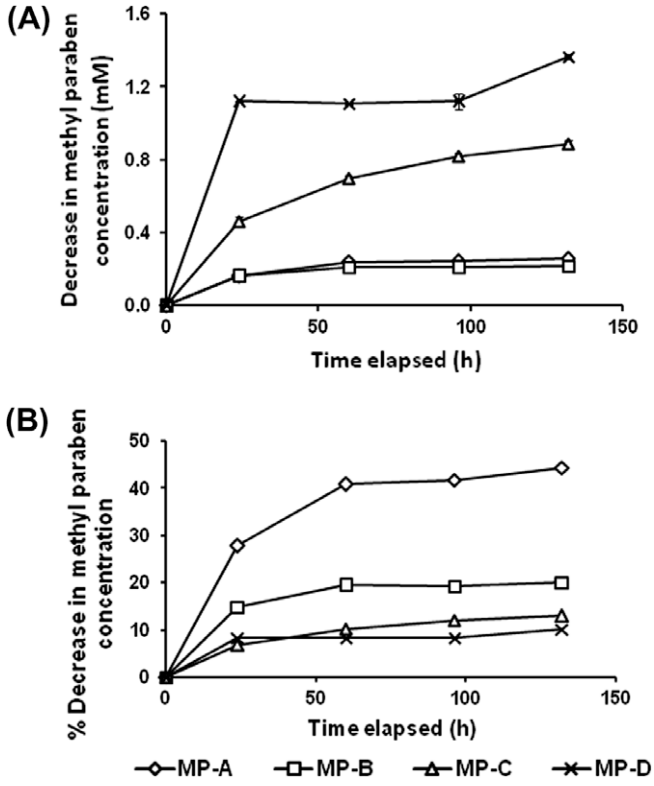

Fig. 2. Loss of MP, due to isolate CMP, in formulations with different concentrations: 0.009% w/v (MP-A), 0.017% w/v (MP-B), 0.104% w/v (MP-C) and 0.203% w/v (MP-D). These formulations were inoculated with CMP to give a final viable count of $5 \times 10^7 \pm 1 \times 10^7$ CFU/ml. Figure shows decrease in paraben concentration from zero-time in mM (A) and % decrease in paraben concentration from zero-time (B). Values represent average of ±SD, $n = 3$.

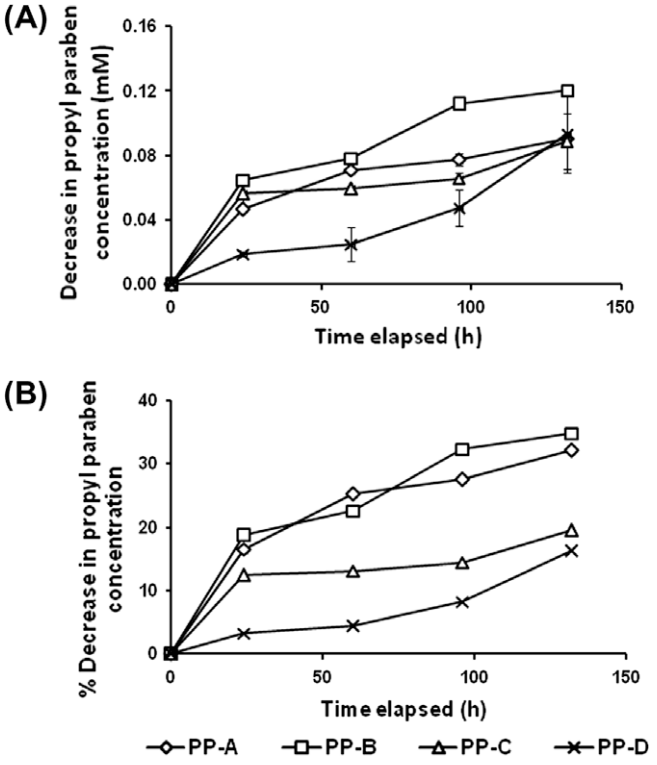

Fig. 3. Loss of PP, due to isolate CPP, in formulations with different concentrations: 0.005% w/v (PP-A), 0.006% w/v (PP-B), 0.008% w/v (PP-C) and 0.010% w/v (PP-D). These formulations were inoculated with CPP to give a final viable count of $5 \times 10^7 \pm 1 \times 10^7$ CFU/ml. Figure shows decrease in paraben concentration from zero-time in mM (A) and % decrease in paraben concentration from zero-time (B). Values represent average of ±SD, $n = 3$.

established that isolate CMP could also grow uninhibited on media which was fortified with 0.015% w/v PP. Similarly, CPP was found to grow on media fortified with 0.015% w/v MP as well as PP.

(ii) *Pour plate experiment:* The results of the above experiment were corroborated using pour plate experiments. The plate with CMP inoculum gave a uniform growth of colonies without any zone of inhibition at or around the drops of MP and PP, indicating its resistance to 0.015% w/v MP and PP. Similarly, a uniform growth of CPP without any zone of inhibition around the drops of MP and PP drops confirmed that CPP was resistant to both, 0.015% w/v MP and PP.

Table 3
Degradation of MP and PP by CMP and CPP after 10 days.

Formulation	Isolate used[b]	% Loss of paraben after 10 days at 40 °C
0.015% w/v MP	CMP	100
0.015% w/v PP	CMP	5.12
0.015% w/v MP	CPP	70.05
0.015% w/v PP	CPP	88.07
0.015% w/v MP	CMP[a]	0
0.015% w/v PP	CPP[a]	0

[a] After subculturing on antibiotic assay medium A, consecutively, for four times.
[b] Initial viable count in all cases was $2 \times 10^8 \pm 1 \times 10^8$ CFU/ml.

3.6. Degradation studies with PET microorganisms

Several cases of contamination are reported for the multi-dose 'preservative-free' sterile products [9]. A multi-dose sterile product, therefore, needs to be protected from possible 'in-use' contamination. Antimicrobial preservatives are used for this purpose and their efficacy to prevent microbial contamination is ascertained by a challenge test – the preservative effectiveness test (PET) recommended in the pharmacopoeia [13]. However, a limitation of the test is that it uses only four pathogenic microorganisms as

Table 2
Decrease in concentration values, changes in pH values and increase in viable count observed at the end of the study.

Formulation	Initial concentration (mM)	Decrease in paraben concentration in 132 h (mM)	Decrease in paraben concentration from zero-time (%)	Final pH (ΔpH)[a]	% Increase in viable count
MP-A	0.58	0.26	44.35	7.75 (2.75)	26
MP-B	1.09	0.22	20.15	7.87 (2.87)	3.7
MP-C	6.86	0.89	12.92	4.89 (−0.11)	No growth
MP-D	13.34	1.36	10.23	4.90 (−0.10)	No growth
PP-A	0.28	0.09	32.11	7.86 (2.86)	293
PP-B	0.35	0.12	34.80	7.89 (2.89)	290
PP-C	0.46	0.09	19.45	7.85 (2.85)	371
PP-D	0.58	0.09	16.19	7.88 (2.88)	264

[a] Initial pH value for all formulations was 5.00 ± 0.05.

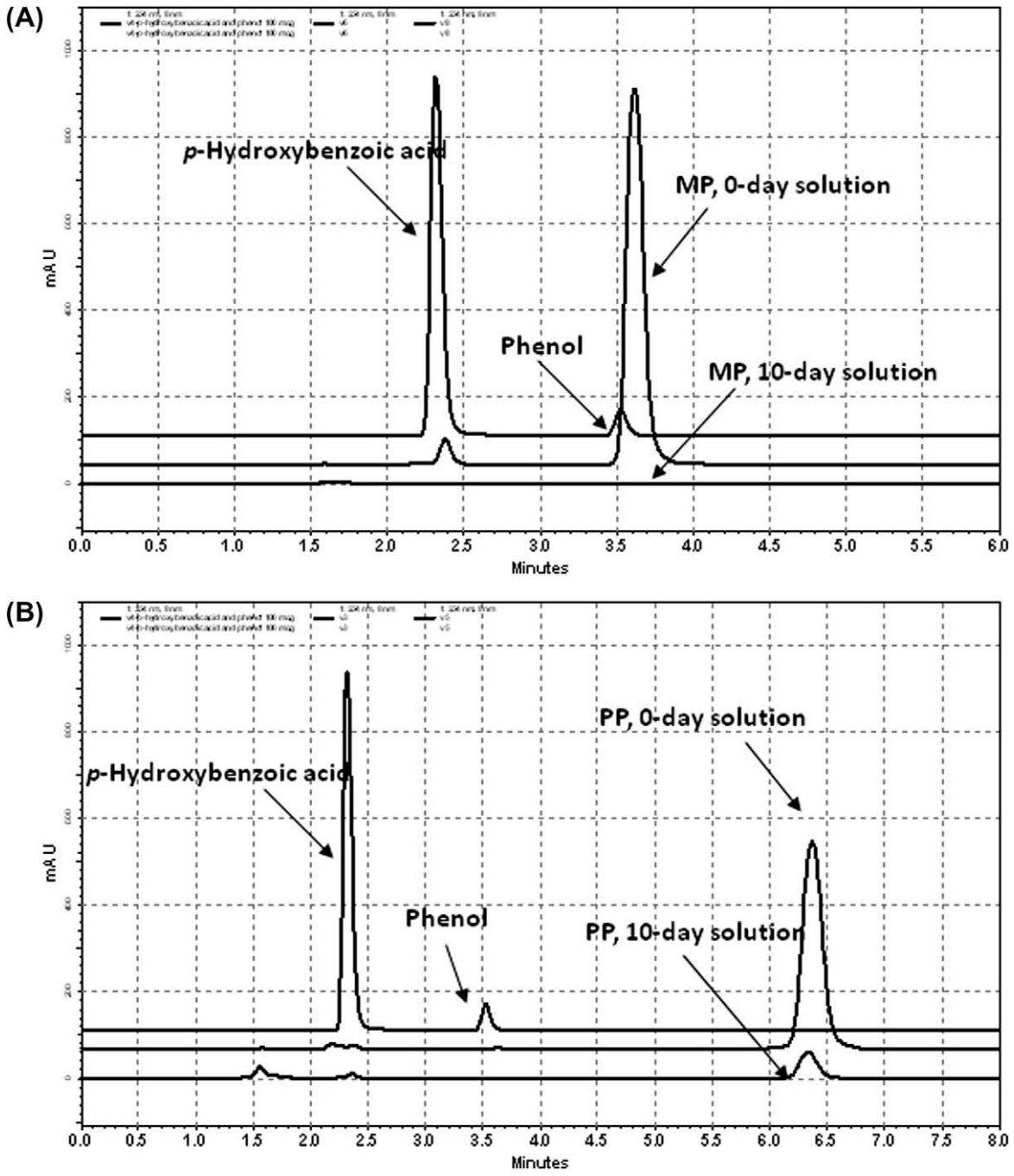

Fig. 4. HPLC chromatograms of MP solution degraded by CMP (A) and PP solution degraded by CPP (B). Chromatograms of *p*-hydroxybenzoic acid and phenol (100 μg/ml) are overlaid in both cases.

representatives of Gram-negative bacteria, Gram-positive bacteria and yeast (*P. aeruginosa*, *S. aureus*, *C. albicans*, and *A. niger*) to assess the microbicidal efficacy of the product. After a specific time period (maximum 28 days) of inoculation with the above-mentioned microbes, if the preserved product does not test positive for the presence of these microbes, it is concluded that the preserved formulation is safe for patient use. In real time, there is a possibility of the formulation to be contaminated by microorganisms other than those mentioned in the pharmacopoeia.

A study was conducted to understand the behaviour of pharmacopoeially recommended pathogens towards the parabens. MP solution (which was degraded by isolate CMP as well as CPP) was used as a prototype substrate. It was inoculated with *P. aeruginosa*, *S. aureus*, *C. albicans* and *A. niger*. It was found that none of the four organisms degraded the paraben, giving a 0% loss at the end of 30 days (720 h). It should be noted that the actual PET was not conducted here (the effect of the MP solution on the viability of the four organisms was not checked). It was expected to fail the test since the MP concentration used in this study is considered sub-optimal.

This study demonstrated that the organisms recommended by the pharmacopoeia will not always reflect the behaviour of real-time microbial contaminants. In real time, if a paraben-preserved product gets contaminated with CMP/CPP during patient use, it could degrade the antimicrobial preservative, making the formulation predisposed to subsequent contamination.

4. Relevance to pharmaceutical formulations

Two types of pharmaceutical formulations exist from a microbiological point of view – sterile and non-sterile. The sterile formulations are further categorized into single-dose and multi-dose formulations, out of which the latter has a propensity to get contaminated during patient use. Microbial growth is especially favourable in formulations having high water activity like aqueous solutions, aqueous suspensions, aqueous emulsions and gels. Therefore, non-sterile pharmaceuticals and multi-dose sterile pharmaceuticals are protected from microbial growth/contamination by the addition of antimicrobial preservatives. The parabens meet several of the criteria of an ideal preservative, including a broad spectrum of antimicrobial activity, safety (i.e. relatively non-irritating, non-sensitizing and of low toxicity) and stability over a wide pH range. However, there is increasing evidence of microbial resistance towards parabens and in a few cases even microbial degradation of the parabens. A previously unreported interaction of *P. beteli* and *B. latens* with parabens is presented in this article. The results are significant as the parabens are commonly used preservatives in multi-dose ophthalmics and other pharmaceutical formulations. 'In-use' contamination of paraben-preserved ophthalmic products by *P. beteli* and *B. latens* can thus prove detrimental to the antimicrobial preservative effectiveness of the formulation which will lead to an inability of the product to counter subsequent contamination by other microbes. Also, the above-mentioned organisms are known pathogens and cause serious infections, especially in immunocompromised patients. Process validation can take care of the initial sterility/bioburden of the product. However, 'in-use' contamination still remains an issue. Newer synergistic combinations of antimicrobial agents need to be explored in order to tackle this issue. Also, development of novel preservative systems which are effective against the resistant microbes can help in developing safe and robust non-sterile and multi-dose sterile pharmaceuticals.

5. Conclusion

The two isolated microorganisms, *P. beteli* and *B. latens*, were resistant to methyl paraben and propyl paraben and degraded them, respectively. Degradation of parabens by these resistant isolates was as high as 30–40% of the initial paraben content. However, the four microorganisms (*P. aeruginosa*, *S. aureus*, *C. albicans*, and *A. niger*) prescribed for the pharmacopoeial preservative effectiveness test could not degrade methyl paraben. These studies indicate that the microorganisms of the pharmacopoeial preservative effectiveness test may not always reflect the behaviour of real-time microbial contaminants in a paraben-preserved product. A large loss of parabens by microbial degradation can compromise the preservation effectiveness of the multi-dose ophthalmic formulation against successive microbial contamination and can prove hazardous to the consumer's health.

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