Original Article

Evaluation of preservative effectiveness of *p*-coumaric acid derivatives in aluminium hydroxide gel-USP

Abstract

Background: Deterioration of pharmaceutical preparations due to growth of microorganisms is a great challenge and need of preservation becomes very important. Literature reports about various problems associated with the existing synthetic preservatives such as development of microbial resistance (in due course of time) and several serious side effects. **Aim:** The aim of the present study is to find out new preservatives synthesized from natural sources, which may have better efficiency than the existing synthetic preservatives. The derivatives of naturally occurring *p*-coumaric acid were subjected for their preservative efficacy study. Their preservative efficiency was evaluated and compared with the standard parabens. **Materials and Methods:** The selected amide, anilide and ester derivatives of *p*-coumaric acid were subjected to preservative efficacy testing in an official antacid preparation, (aluminium hydroxide gel-USP) against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Candida albicans* and *Aspergillus niger* as representative challenging microorganisms as per USP 2004 guidelines. **Results:** The selected derivatives were found to be effective against all selected strains and showed preservative efficacy comparable to that of standard and even better in case *E. coli, C. albicans* and *A. niger.* The 8-hydroxy quinoline ester derivative showed better preservative efficacy than standard as well as other derivatives. **Conclusion:** The newly synthesized *p*- coumaric acid preservatives were found to be effective in the proposed pharmaceutical preparation (Aluminium Hydroxide Gel – USP). Also, the synthesized preservatives have shown comparative and even better efficacy than the existing parabens and hence they have potential for use in pharmaceutical preparations.

Key words:
Amides, esters, log CFU/ml (Colony Forming Unit per ml), p-coumaric acid, preservative

Introduction

Non-sterile products such as pharmaceuticals, cosmetics, food items etc., with a high degree of water availability may be contaminated with microorganisms which may cause spoilage of the product with loss of therapeutic properties and if they are pathogenic, serious infections can arise.[1] To inhibit the growth of contaminating microorganism, antimicrobial preservative systems have been developed and introduced into the pharmaceutical, cosmetic or food products during manufacturing process and throughout its use by consumers.[2]

The commonly used chemical preservatives may cause very serious side effects such as the benzalkonium chloride may cause mucosal damage and was also reported as genotoxic and cytotoxic.[3,4] Thiomerosal used in ocular and nasal preparations was reported to be cytotoxic by Liao *et al.*, 2011.[5] The use of parabens may cause skin cancer, genotoxicity and breast cancer.[6]

In several cases, the microorganisms became resistant to antimicrobials and are able to degrade many commonly used preservatives especially *p*-hydroxybenzoates, e.g., parabens.[7] Microbial resistance to some of the existing commonly used chemical preservatives includes benzalkonium chloride, dibromodicyanobutane, chloramine, chlorhexidine, cholorophenol, benzoic acid, dimethyl oxazolidine, dimethyl dithiocarbamate, dimethoxy dimethyl hydantoin, formaldehyde, glutaraldehyde, hydrogen peroxide, iodine,

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methylene bischlorophenol, methylparaben, propylparaben, phenylmercuric acetate, mercuric salts, povidine-iodine, sorbic acid and quaternary ammonium compounds.[8]

The United States and British pharmacopoeias describe official methods for evaluation of preservative system.[9,10] Preservative efficacy test (challenge test) involves the artificial introduction of representative microorganisms including gram positive and gram negative bacteria, yeast and mold into the product under study, in sufficient amounts followed by the collection of kinetic information regarding the loss of their viability. The preservative potential of natural organic acids is well established in the literature *viz*. caprylic acid,[11] veratric acid,[12] 2,4 hexadienoic acid[13] and anacardic acid.[14] Also, the *p*-coumaric acid and its derivatives possess wide spectrum of biological activities like antimicrobial and antioxidant.[15,16]

As most of the commonly used preservatives belong to the class of antimicrobial(s) and antioxidant(s), hence the *p*-coumaric acid derivatives may be evaluated for their preservative potential. In view of the above mentioned facts, it became imperative to develop newer and stronger preservatives. Further, in view of the reported toxicity potential of common synthetic preservatives, it would be quite judicious to develop the preservatives based on the natural sources such as *p*-coumaric acid. In this context, amide, anilide and ester derivatives of *p*-coumaric acid were investigated for preservative efficacy in the present work. The preservative efficacy of most effective amide, anilide and ester derivatives of *p*-coumaric acid against gram positive *Staphylococcus aureus* MTCC 2901, *Bacillus subtilis* MTCC 2063, gram negative *Escherichia coli* MTCC 1652, fungal strains *Aspergillus niger* MTCC 8189 and *Candida albicans* MTCC 227 was investigated and compared them with the standard preservatives methyl and propyl paraben, in aluminium hydroxide gel-USP.[17]

Materials and Methods

Materials

Nutrient agar, nutrient broth, sabouraud dextrose agar and sabouraud dextrose broth were obtained from Himedia, Mumbai. Mannitol, methyl and propyl paraben were obtained from CDH, Mumbai.

Methods

Aluminum hydroxide gel-USP was used as the pharmaceutical product for evaluation of preservative efficacy testing.

Formula for preparation of aluminium hydroxide gel, gel USP 2004

Aluminium hydroxide gel, 36 g; Mannitol, 7 g; Methyl paraben, 0.2 g; Propyl paraben, 0.02 g; Saccharin, 0.05 g; Peppermint oil, 0.005 ml; Alcohol, 1 ml; Purified water q.s., 100 ml.

The weighed quantity of aluminum hydroxide gel and mannitol were triturated with 50 ml of water in a mortar. Methyl paraben, propyl paraben, saccharin and peppermint oil were dissolved in alcohol and added to above mixture and triturated well. The volume was made up to 100 ml with purified water followed by its sterilization by autoclaving.

For preservative efficacy testing, the aluminium hydroxide gel was prepared using the preservatives mentioned in Table 1 by replacing methyl paraben and propyl paraben from the above formula. The equimolar amount of selected preservatives [Figure 1] were calculated with reference to the amount of methyl paraben (0.0013 mol) and added into aluminum hydroxide gel.[18]

Strains

S. aureus MTCC 2901, *B. subtilis* MTCC 2063, *E. coli* MTCC 1652, *C. albicans* MTCC 227 and *A. niger* MTCC 8189 were used in this study were common contaminants and prescribed in USP for preservative efficacy testing in pharmaceutical preparations.

Preservative efficacy testing in aluminium hydroxide gel-USP 2004

The preservative efficacy test was performed essentially following the standard protocol described in USP-2004. In all cases, the preservative efficacy test was done in aluminum hydroxide gel-USP with and without the preservative system. The unpreserved product was used as a control to evaluate the viability of the inoculated cells and their ability to grow in the product.

Preparation of inoculum

The representative microorganisms were inoculated in

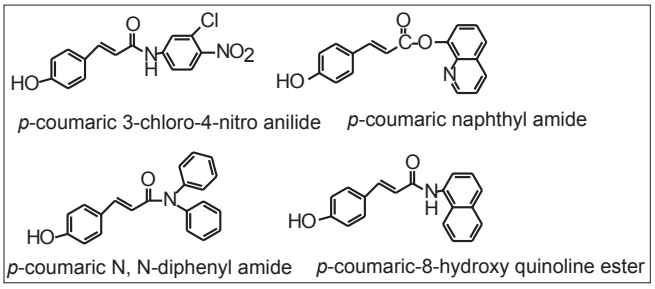

Figure 1: Structures of selected *p*-coumaric acid derivatives

Table 1: Amount of selected preservatives added in aluminum hydroxide gel-USP

Preservative	Amount (g)
p-coumaric-8-hydroxy quinoline ester	0.378
p-coumaric 3-chloro 4-nitro anilide	0.413
p-coumaric *N, N*-diphenyl amide	0.326
p-coumaric naphthyl amide	0.375

USP – United States Pharmacopoeia

nutrient agar I.P. (*S. aureus, B. subtilis, E. coli*) and sabouraud agar I.P. (*C. albicans, A. niger*). The seeded plates were incubated at 37°C for 24 h (*S. aureus, B. subtilis, E. coli*), 37°C for 48 h (*C. albicans*) and 25°C for 7 d (*A. niger*). After the incubation period, suspensions of microorganisms were prepared in sterile saline solution (0.9% w/v NaCl) to give a microbial count of 1×10^4 CFU/ml.[12]

Test procedure

Aluminum hydroxide gel-USP in their final container was used in the challenge test. The preparation was inoculated with the microbial cell suspension with a cell count of 1×10^4 CFU/ml. The inoculum never exceeded 1% of the volume of the product sample. Inoculated samples were mixed thoroughly to ensure homogeneous microorganism distribution and incubated. The CFU/ml of the product was determined at an interval of 0, 7, 14, 21 and 28 days on an agar plate. The log values of the number of CFU/ml [Tables 2-6] of aluminum hydroxide gel was calculated and compared as per the guidelines of USP 2004.

Criteria of acceptance for preservative system

As per USP 2004 requirement for antacid made with an aqueous base, preservative effectiveness is met if there is no increase from initial calculated count at 14 d and 28 d in case of bacteria, yeast and moulds and where, no increase is defined as not more than 0.5 $\log_{10}$ higher than the previous value measured.[10]

Results and Discussion

The results of preservative efficacy testing were performed in triplicate and were reported as mean values in Tables 2-6. According to Table 2 the ester, amide and anilide derivatives of *p*-coumaric acid as well as the standard showed the log CFU/ml values within limits on 14 d and 28 d and passed the selection criteria of the preservative effectiveness test and hence they were active and comparable to the standard against *B. subtilis*.

The *N, N*-diphenyl amide derivative of *p*-coumaric acid showed a change of more than 0.5 log value on 14 d and hence did not passed the criteria of preservative effectiveness test whereas the 8-hydroxyl quinoline ester, 3-chloro-4-nitro anilide and naphthyl amide derivatives of *p*-coumaric acid as well as the standard showed the results of log CFU/ml within the prescribed limits and hence were found to be effective preservative against *S. aureus* [Table 3].

There was an increase in log CFU/ml on 14 d for 3-chloro-4-nitro anilide derivative of *p*-coumaric acid and hence was not effective against the *E. coli*. All other ester and amide derivatives of *p*-coumaric acid showed the log CFU/ml values on 14 d and 28 d within the limits as per the criteria of preservative effectiveness testing against *E. coli* [Table 4]. 8-hydroxyl quinoline ester, 3-chloro-4-nitro anilide and *N, N*-diphenyl amide derivatives of *p*-coumaric acid were found as effective preservative agents against the *C. albicans* as per the criteria of preservative effectiveness testing while the naphthyl amide derivative of *p*-coumaric acid showed an increase of more than 0.5 log CFU/ml on 14 d and

Table 2: Bacterial count of *Bacillus subtilis* in aluminium hydroxide gel-USP supplemented with preservatives

Preservative added	Log CFU/ml (time in days)				
	0	7	14	21	28
p-coumaric-8-hydroxy quinoline ester	0.385	0.523	0.778	0.970	0.763
p-coumaric 3-chloro 4-nitro anilide	0.651	0.959	0.903	0.748	0.763
p-coumaric *N, N*-diphenyl amide	0.327	0.260	0.266	0.350	0.388
p-coumaric naphthyl amide	0.452	0.699	0.535	0.505	0.740
Standard	0.602	0.301	0.000	0.301	0.477
Control	0.903	0.477	0.602	0.778	0.845

USP – United States Pharmacopoeia; CFU – Colony Forming Units

Table 3: Bacterial count of *Staphylococcus aureus* in aluminium hydroxide gel-USP supplemented with preservatives

Preservative added	Log CFU/ml (time in days)				
	0	7	14	21	28
p-coumaric-8-hydroxy quinoline ester	0.824	1.038	0.748	0.623	0.852
p-coumaric 3-chloro 4-nitro anilide	0.149	0.165	0.271	0.243	0.390
p-coumaric *N, N*-diphenyl amide	0.495	0.903	0.341	0.544	0.586
p-coumaric naphthyl amide	0.523	0.477	0.377	0.544	0.732
Standard	0.602	0.301	0.000	0.301	0.477
Control	0.903	0.477	0.602	0.778	0.845

USP – United States Pharmacopoeia; CFU – Colony Forming Units

Table 4: Bacterial count of *Escherichi coli* in aluminium hydroxide gel-USP supplemented with preservatives

Preservative added	Log CFU/ml (time in days)				
	0	7	14	21	28
p-coumaric-8-hydroxy quinoline ester	0.456	0.978	1.234	1.176	0.824
p-coumaric 3-chloro 4-nitro anilide	0.398	0.086	0.663	0.390	0.377
p-coumaric *N, N*-diphenyl amide	1.000	0.677	0.681	0.653	0.658
p-coumaric naphthyl amide	1.301	0.677	0.681	0.829	0.721
Standard	0.778	0.000	0.602	0.302	0.698
Control	0.845	0.602	0.778	0.954	1.041

USP – United States Pharmacopoeia; CFU – Colony Forming Units

Table 5: Fungal count of *Candida albicans* in aluminium hydroxide gel-USP supplemented with preservatives

Preservative added	Log CFU/ml (time in days)				
	0	7	14	21	28
p-coumaric-8-hydroxy quinoline ester	0.556	0.875	0.865	0.602	0.699
p-coumaric 3-chloro 4-nitro anilide	0.653	0.574	0.865	0.770	0.370
p-coumaric *N*, *N*-diphenyl amide	0.954	0.574	0.837	0.959	0.645
p-coumaric naphthyl amide	0.954	0.533	1.342	0.921	0.574
Standard	0.301	0.698	0.602	0.778	0.000
Control	0.477	0.778	0.845	0.845	0.903

USP – United States Pharmacopoeia; CFU – Colony Forming Units

Table 6: Fungal count of *Aspergillus niger* in aluminium hydroxide gel-USP supplemented with preservatives

Preservative added	Log CFU/ml (time in days)				
	0	7	14	21	28
p-coumaric-8-hydroxy quinoline ester	1.176	1.255	0.996	0.954	0.564
p-coumaric 3-chloro 4-nitro anilide	0.796	0.653	0.678	0.492	0.263
p-coumaric *N*, *N*-diphenyl amide	0.699	1.255	1.034	1.000	0.626
p-coumaric naphthyl amide	0.574	0.507	0.899	0.556	0.564
Standard	0.301	0.301	0.698	0.000	0.477
Control	0.698	1.079	0.954	1.000	1.079

USP – United States Pharmacopoeia; CFU – Colony Forming Units

hence was considered as less effective preservative against *C. albicans*. Also, the standard showed more than 0.5 log values on 28 d and was considered as less effective against *C. albicans* [Table 5].

There was a change of more than 0.5 log values on 28 d in case of *N, N*-diphenyl amide derivative of *p*-coumaric acid and hence was considered to be less effective preservative against *A. niger*. Also, the standard showed a change of about 0.477 log values on 28 d and thus can be considered as less effective against the *A. niger* while the 8-hydroxyl quinoline ester, 3-chloro-4-nitro anilide and naphthyl amide derivatives of *p*-coumaric acid were having the values of log CFU/ml within the prescribed limits and hence were considered as effective preservative agents against *A. niger* [Table 6].

Conclusion

The study has shown the preservative potential of 8-hydroxy quinoline ester, 3-chloro-4-nitro aniline, naphthyl amide and *N, N*-diphenyl amide derivatives of *p*-coumaric acid in pharmaceutical preparation. The selected amide, anilide and ester derivatives of *p*-coumaric acid were found effective against all selected strains and showed preservative efficacy comparable to that of standard and even better in case of *E. coli*, *C. albicans* and *A. niger*. The 8-hydroxy quinoline ester derivative showed better preservative efficacy than standard as well as other derivatives and it can be a better alternative to the existing preservatives for use in the pharmaceutical preparations.

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