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Stressed degradation studies of domiphen bromide by LC-ESI-MS/MS identify a novel promising antimicrobial agent

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ABSTRACT

Nowadays, parabens have been replaced by domiphen bromide, which is widely used in pharmaceutical and cosmetic products.

The main aim of this study was to investigate stressed degradation products of domiphen bromide by mean of a rapid, specific and reliable LC-ESI MS/MS since phenyl bromination may occur due to the oxidation of bromide counter ion under oxidative conditions. LC-ESI-MS/MS have characterized a new compound, *p*-bromodomiphen, as the only degradation product and structure elucidation was also confirmed by the synthesis of the standard. Notably, the resulting *p*-bromodomiphen bromide is more stable then domiphen bromide in oxidizing conditions since no di-bromoderivatives were detected by MS studies; both domiphen and its *p*-bromo derivative were tested for antibacterial activity and were more effective on Gram positive (*Staphylococcus aureus* ATCC25923 and *Bacillus cereus* DSM31) compared to Gram negative bacteria (*Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* DSM22644). In conclusion, stressed degradation studies by LC-ESI-MS/MS have characterized a new compound that comprises an alternative to domiphen bromide since its antimicrobial activity is comparable to, if not better than, the parental compound.

1. Introduction

Parabens –esters of parahydroxybenzoic acid, entered the market about a century ago. Since then, they have been employed as preservatives in a broad variety of products such as foods, injectables and topical formulations. Their proven antimicrobial properties, natural origin, non-mutagenic and non-toxic properties, odourless and colourless features, and their low cost, have made them the first choice as preservatives. However, in 1940 Poul Bonnevie published the description of allergenic contact dermatitis due to ethylparaben [1] and in the subsequent decades the sensitizing threat of parabens was confirmed [2,3,4]. Moreover, other publications asserted that parabens show estrogenic and antiandrogenic effects [5], and are potential endocrine disrupters that may contribute to the development of breast cancer [6,7] and male infertility [8,9]. Consequently, although there is no conclusive evidence regarding the carcinogenic role of parabens [10,11], both pharmaceutical and cosmetic companies are looking for substitutes to this benchmark preservative. In this context, alternative biocides such as isothiazolinones and phenol derivative, to name but a few, have been applied as paraben-replacers. The interest for new biocides is further fostered by the increasing trend of using natural substances that cannot realistically be sterilized and by the expansion of the global cosmetic preservatives market, which is expected to reach ~ 350 million USD by 2020 [12].

Domiphen bromide (i.e. (dodecyldimethyl-2-phenoxyethyl)ammonium bromide) is an aromatic quaternary ammonium salt used as antiseptic, antimicrobial and disinfectant.

Combined with a good workability, these properties make domiphen an attractive alternative for parabens such as that it is widely used in pharmaceutical and cosmetic preparations. However, we surmised that the presence of bromide as a counter ion, and of an aromatic ring with high reactivity towards electrophilic substitutions, may result in a side-reaction whereby bromide oxidation to bromine would further lead to phenyl bromination. As a matter of fact, bromination of aromatic compounds may occur under mild conditions, and in water, as reported by Podgorsek and Fine [13,14]. Therefore, assessing the chemical stability of

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domiphen bromide is critical in relation to an extended use of this compound, especially because its chemical profile has not been explored in depth. To the best of our knowledge, studies on domiphen bromide stability in general, and on bromination of its phenyl ring in particular, have not yet been reported.

In this study, we investigate the susceptibility of domiphen bromide to via an electrophilic addition attack to the activated phenyl ring, a modification that may occur in-situ via oxidation of its bromide counter-ion. Therefore domiphen bromide was exposed to stress oxidative conditions (ICH-recommended) and the resultant solutions were subjected to optimized LC-ESI-MS/MS to establish its degradation products. Furthermore, we unequivocally assigned the structure of the degradation product by comparison with synthetized standard. Finally, the antibacterial activity of *p*-bromodomiphen bromide was tested and compared to the antimicrobial profile of domiphen bromide.

2. Materials and methods

2.1. Instrumentations

A milli-Q water purification system (Millipore, Bedford, MA, USA) was used to further purify demineralised water.

HPLC analyses were performed on a Elite_La Chrom HPLC (VWR/HITACHI, Milan, Italy/Tokyo, Japan) apparatus equipped with a L-2130 high pressure quaternary gradient delivery system, a L-2455 diode array detector (DAD), a L-2300 column oven and a L-2200 autosampler. The separation was achieved on a XBridge™ column (4.6 mm X 150 mm, 5 μm) (Waters).

LC–MS experiments were performed on a Surveyor LC system, connected to a TSQ Quantum Ultra mass spectrometer through a Finnigan IonMax electrospray ionization (ESI) source

assembled with a stainless steel emitter (Thermo Fisher Scientific, Rodano, MI, Italy).

2.2. Chemicals

HPLC, LC/MS grade acetonitrile, LC/MS grade ammonium formate and formic acid were purchased from Sigma-Aldrich (Milano, Italy).

2.3. Mobile phase preparations and experimental conditions

Mobile phase A and mobile phase B for the HPLC and LC/MS analyses were acetonitrile/buffer solution (3.0 mM ammonium formate (pH = 3.75)) 9/1 and buffer solution (3.0 mM ammonium formate (pH = 3.75))/acetonitrile 9/1 respectively. The buffer solution was prepared by dissolving ammonium formate (3.0 mM) in HPLC-grade water. The resulting solution was buffered to pH = 3.75 by formic acid. All the analyses were performed in isocratic conditions 65% of eluent A and 35% of eluent B, flow 1 mL/min.

Column temperature was fixed at 40 °C and chromatograms were extracted at 220 nm.

Mass spectrometric detection was performed by spraying the flow coming from the column directly into the detector. Nebulization was achieved by applying 3.5 kV spray voltage, 300 °C capillary temperature, sheath gas 45% and auxiliary gas 10%. Mass spectra were acquired in a 200–1200 m/z range in positive ion mode.

2.4. Antibacterial activity

Aqueous stock solutions of the two compounds were tested for antibacterial activity by the disc diffusion method [15] using the following bacteria: *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC25923, *Pseudomonas aeruginosa* DSM22644 (PAO1) and *Bacillus cereus* DSM31. All the strains were cultured on Tryptic Soy Agar (TSA, Scharlab, Barcelona, Spain) for 16–18 h at 30 °C (*P. aeruginosa*) or 37 °C. Following colony growth, several morphologically similar colonies were selected and suspended in sterile saline solution (0.85% NaCl w/v in water) to a turbidity of 0.5 McFarland standard, approximately corresponding to $1–2 \times 10^8$ CFU/mL. The bacterial suspension was then spread over Mueller–Hinton Agar (MHA, Merck KGaA, Darmstadt, Germany) plates by swabbing in three directions. The compounds stock solutions were both prepared by dissolving domiphen bromide and *p*-bromodomiphen bromide in sterile water to final concentration of 1000 mg/L, and then serially diluted two-fold for three times. Ten microliters of each dilution, equivalent to 10, 5, 2.5 and 1.25 mg/L respectively, were individually loaded onto sterile discs (6 mm diameter) and then placed on agar plates. After incubation of plates at 30° or 37 °C for 18–24 h, the diameters of the inhibition zones were measured. All the tests were performed in duplicates. The Minimum Inhibitory Concentration (MIC) was determined using a broth-micro dilution method in sterile flat-bottomed 96 well polystyrene microtiter plates according to guidelines [16]. The highest dilutions of the compounds that showed no turbidity in the assay were considered as MIC.

3. Results and discussion

3.1. Unequivocal structure elucidation of the found degradation product

Various methods have been described for the determination of domiphen bromide [17]. However, to the best of our knowledge the application of mass spectrometry (MS) has not been reported. Indeed, MS would be essential for the presumed degradation products of electrophilic addition, namely whereby bromine is covalently linked. We have thus applied an LC–MS analysis. The mobile phase composition was acetonitrile/buffer solution (3.0 mM ammonium formate (pH = 3.75)) 9/1 (eluent A) and buffer solution (3.0 mM ammonium formate (pH = 3.75))/acetonitrile 9/1 (eluent B) respectively and the isocratic condition was set at 65% of eluent A and 35% of eluent B to reach acceptable time of analysis and reliable separation.

To investigate the possible oxidation of bromide, and the following electrophilic addition, samples of domiphen bromide were exposed to stress oxidative conditions with 15% H_2O_2, 0.2 M (ICH) at different pH values since the disproportionation reaction of bromide is pH dependent. Along with the stability in oxidative conditions degradation products of domiphen bromide in hydrolytic, and thermal conditions were also investigated as reported in [18].

All HPLC analyses were performed under the same experimental conditions (see section 2.3). The chromatogram obtained from the HPLC analysis of domiphen bromide shows that the retention time of domiphen bromide is 4.44 min (see Supplementary Materials). However, the HPLC chromatogram of the sample of domiphen bromide treated at room temperature with hydroperoxide at pH = 1 showed two peaks. The first peak, at retention time of 4.32 min, is domiphen bromide, while a second peak, at retention time of 5.71 min, appeared immediately after the addition of the oxidizing agent and relates to an unknown stress degradation product (Fig.1a)). Notably, a complete conversion of domiphen was observed after 30 min as shown by the chromatogram in Fig. 1b.

HPLC chromatograms of the samples of domiphen bromide stressed with hydroperoxide at pH = 3 and pH = 6 showed a slower conversion of domiphen, as the side-product was clearly detectable only after one hour incubation at 40 °C (Fig.1c) and d) respectively). Overall, a complete conversion of domiphen bromine into this single side-product was observed at pH = 1, and room temperature,