

Detection of *Para*-Chloroaniline Resulting from the Interaction between Sodium Hypochlorite and Chlorhexidine Analyzed by Mass Spectrometry

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Abstract

5.1% of sodium hypochlorite (NaOCl) mixed with 2% of chlorhexidine (CHX) forms a brown precipitate that corresponds to *para*-chloroaniline (PCA), whether PCA is formed after the combination of NaOCl, CHX, and ethylenediaminetetraacetic acid (EDTA) by means of electron impact (high resolution and interlaced scanning) and ionization by fast atom bombardment (FAB), was analyzed. The brown precipitate, showed signals 127 and 153 Da, corresponding to *p*-chloroaniline and *p*-chlorophenyl isocyanate, respectively. These results were analyzed and compared with signals from the interlaced scanning program and confirmed with high resolution mass spectrometry analysis and compared with the NIST database. The mass spectra of this precipitated after different days confirmed the evolution of byproducts with the presence of a peak $m/z = 127$, due to the decrease of the fragment $m/z = 153$, which disappeared after 180 days. A blue and a white precipitate were observed by the addition of CHX (2%) with or without polyethylene glycol, respectively, EDTA (17%) and NaOCl (5.1%) precipitates contain chlorhexidine ($m/z = 505$), but no PCA was detected. We confirmed that PCA is not formed directly as a byproduct of CHX oxidation, but through the formation of the *para*-chlorophenyl isocyanate intermediate, which degrades slowly to PCA.

Keywords

Chlorhexidine, *Para*-Chloroaniline, *Para*-Chlorophenyl Isocyanate, EDTA, Sodium Hypochlorite

1. Introduction

Bacteria and their products are the major etiologic factors in the initiation, propagation, and persistence of periradicular periodontitis [1] [2]. The host's response to a microbial infection can consist of non-specific inflammation and/or specific immunological reactions. The infection is produced by the invasion of microbes causing tissue damage. Endodontic disease (pulpal and periradicular) is the result of both the pathogenic effects of the microbes and the response of the host. The goal of clinicians is to disrupt and destroy the microbial ecosystem associated with the disease process [3]. The aim of the root canal treatment is to eliminate bacteria from the infected root canal and to prevent reinfection. Biochemical cleaning and shaping of the root canal greatly reduce the number of bacteria [4]. The chemical debridement can take the form of intracanal medicaments, irrigants, or lubricants that facilitate the removal of the organic and inorganic components inside the root canal system. Antimicrobial rinses are used to decrease the microbial loads within the system before obturation and to reduce the potential for failure in the future [5]. Root canal cleaning and disinfection during chemomechanical preparation relies heavily on irrigants because of the anatomic complexities of the pulp canal system. Ideally, irrigants should have antimicrobial and tissue-dissolution actions, as well as other advantageous properties, such as lubrication, demineralization, and the ability to remove debris and the smear layer [6]. Sodium hypochlorite (NaOCl), at a concentration range from 0.5% to 6% [7] [8] [9], has been recommended as endodontic irrigant due to its ability to dissolve organic matter together with its broad antimicrobial action [10]. However, NaOCl has been shown also to have cytotoxic effects that can cause irritation and necrosis of periapical tissues [8] [11]. Furthermore, NaOCl and EDTA (ethylenediaminetetraacetic acid) have shown to be effective in the elimination of organic and inorganic debris [12] [13] [14].

On the other hand, chlorhexidine gluconate (CHX) is an antimicrobial agent that has been widely used as an effective antiseptic agent applied in endodontic treatment. CHX has also been shown to have antimicrobial substantivity in root dentin for up to 12 weeks [11] [15] [16]. The use of CHX in sequence with NaOCl can offer antimicrobial substantivity and tissue dissolution, respectively [8] [17]. Successive canal irrigation with CHX and NaOCl results in an orange-brown precipitate [18] [19] [20]. The presence of NaOCl in the canal when CHX is introduced produces a brown-colored precipitate that is very difficult to remove from the canal system [19]. Some of the previously published

studies claimed that the brown precipitate formed by the interaction between NaOCl and CHX contains *para*-chloroaniline (PCA) [18] [20] [21] [22]; however, other studies suggested that this precipitate did not contain PCA [5]. The generation of PCA is a potential consequence of the use of CHX [23]. The International Agency for Research in Cancer (IARC, 2006) classified PCA within the group 2B, which means that this agent is potentially carcinogenic for humans [24]. The literature reveals controversies regarding the formation of PCA, it is not clear whether PCA is formed after the interaction between NaOCl and CHX [25].

Therefore, the aim of this study was to analyze the precipitate formed by the mixture of 5.1% NaOCl, 2% CHX, and 17% of EDTA by means of electron impact (high resolution and interlaced scanning), and ionization by fast atom bombardment (FAB).

2. Experimental

2.1. Reagents and Materials

Commercially available samples of 2% chlorhexidine gluconate in liquid presentation with polyethylene glycol (Consepsis Chlorhexidine Antibacterial Solution, Ultradent Products, Inc., South Jordan, UT, USA), imported by VAMASA, S.A. de C.V. Monterrey, NL, Mexico, 20% chlorhexidine digluconate solution in H₂O (Sigma Aldrich, St. Louis, MO, USA), and 9.8% PCA (4-Chloroaniline, Sigma_Aldrich, St. Louis, MO USA) were used.

2.2. Procedures

To prepare 2.0% CHX, with or without polyethylene glycol, was diluted with 90 mL distilled water. The chemicals analyzed in this study were: polyethylene glycol 400 (Fluka Chemika 81170), EDTA at 17%, (Aqueous Irrigant Roth International LTD, Chicago, IL, USA) and NaOCl (Cloralex, Alen del Norte S.A de C.V., NL, Mexico) at 5.1%, concentration, which was established by determining active chlorine and was obtained from commercial sources and used without further purification. Analyses were performed at the Department of Analytical Control of the School of Chemistry, UNAM. The experiments were performed at room temperature in the dark during the whole analyzed period.

CHX (2%) with (Consepsis) or without (Sigma) polyethylene glycol was added slowly to a solution of 3 mL of NaOCl (5.1%) in an Erlenmeyer flask under constant agitation during 1 min. The suspension obtained was introduced into a 15-mL polypropylene (PP) centrifuge tube. A brown precipitate was recovered from the suspension, which was filtered and washed with acetone to remove traces of polyethylene glycol. Then, the precipitate was collected with a spatula and placed into a flat-top 1.5 mL polypropylene microtube.

A combination of NaOCl (5.1%), EDTA (17%), and CHX gluconate (2%) with (Consepsis) or without polyethylene glycol (Sigma) was analyzed. This solution

was prepared by the addition of 3 mL of NaOCl and 3 mL of EDTA to an Erlenmeyer flask under constant agitation during 3 min, then 3 mL of CHX was added and stirred for 1 min. A blue-colored or a white precipitate was recovered from the above-described mixture, depending on the presence or absence of polyethylene glycol in the suspension. The suspension that contained NaOCl and CHX with polyethylene glycol was analyzed for 24 h, and for 10, 20, 60, 90, 150, and 180 days (**Table 1**). The analysis of the brown precipitate after 60 days was performed through the interlaced scanning experiment, which consists of choosing the signals of interest (127,153). The results showed signals that corresponded only to the fragments originated by the chosen signal, eliminating all other signals.

Table 1. Summary of the analyses of the different combinations.

Analysis	Technique	Precipitate	<i>m/z</i>	Products
CHX 2% (Sigma) Without polyethylene glycol	Fast atom bombardment (FAB)	—————	505	Chlorhexidine (not shown)
CHX 2% (Consepsis) with polyethylene glycol	Electron impact	—————	89, 133, 177, 219, 459, 50, 547	CHX was not observed due to the high polyethylene glycol content (not shown)
CHX 2% (Consepsis) with polyethylene glycol	FAB	—————	505	CHX (Figure 1)
Polyethylene glycol (Fluka)	Electron impact	—————	89, 133, 177, 219, 459, 503, 547	Polyethylene glycol spectra (not shown)
NaOCl + CHX (Sigma) without polyethylene glycol Time "0"	FAB	Brown 	127 ↓ 153 ↑	<i>p</i> -chloroaniline <i>p</i> -chlorophenyl isocyanate (Figure 3(a))
NaOCl + CHX (Consepsis) with polyethylene glycol 24 h	Electron impact	Brown 	153	Complex spectrum due to the large amount of polyethylene glycol <i>p</i> -chlorophenyl isocyanate (not shown)
10 days	Electron impact	Brown	127 ↓ 153 ↑	<i>p</i> -chloroaniline <i>p</i> -chlorophenyl isocyanate (not shown)
20 days	Electron impact	Brown	127 ↑ 153 ↓	<i>p</i> -chloroaniline <i>p</i> -chlorophenyl isocyanate (not shown)
60 days	Linked scans	Brown	127 ↑ 153 ↑	<i>p</i> -chloroaniline <i>p</i> -chlorophenyl isocyanate (not shown)
90 days	Electron impact	Brown	127 ↑ 153 ↓	<i>p</i> -chloroaniline <i>p</i> -chlorophenyl isocyanate (not shown)
150 days	Electron impact	Brown	127 ↑ 153 ↓	<i>p</i> -chloroaniline <i>p</i> -chlorophenyl isocyanate (not shown)
180 days	Electron impact	Brown	127 ↑ 153 ↓ (almost disappeared)	<i>p</i> -chloroaniline (Figure 3(b))

Continued

180 days	Collision induced dissociation (CID) using linked scans(LS)	Brown	127 ↑	<i>p</i> -chloroaniline (Figure 3(c))
NaOCl + EDTA + CHX (Sigma) without polyethylene glycol Time "0" 10 days	FAB	White 	505 505	Chlorhexidine Chlorhexidine (not shown)
NaOCl + EDTA + CHX (Consepsis) with polyethylene glycol Time "0" 10 days	FAB FAB	Blue 	505	Chlorhexidine (not shown)

2.3. Instruments

Mass spectra were obtained using a JEOL JMS-SX102A instrument with *m*-nitrobenzyl alcohol as the matrix (FAB⁺ mode). EIMS and CID-LS measurements were made using a JEOL JMS-700 M Station (Tokyo, Japan) mass spectrometer.

3. Results

The structure of chlorhexidine (Consepsis) was confirmed using the positive FAB (fast atom bombardment) ionization mass spectrometry technique. Its molecular weight is $m/z = 504 + 1$ Da as shown in Figure 1. The mass spectra of PCA (Sigma-Aldrich) showed the mean peak at $m/z = 127$ (not shown).

The color change and formation of a precipitate were observed after adding 2.0% of CHX to NaOCl (Table 1). The composition of the brown precipitate was studied through FAB⁺ mode and additionally, high resolution mass spectrometry analyses were performed. The results obtained after the FAB⁺ analysis, agree with the reported database for the aforementioned structures (see Figures 2(a)-(d) in supporting information). Mass spectra of the brown precipitate initially showed the mean peak at $m/z = 153$ with 100% of relative abundance and a secondary peak $m/z = 127$ corresponding to *p*-chlorophenyl isocyanate and *p*-chloroaniline, respectively as shown in Figure 3(a), Table 1.

The mass spectra analysis of the brown solution performed at days 20, 60, 90, 150, and 180 showed the base peak $m/z = 127$ as a result of the consumption of fragment $m/z = 153$, which almost disappeared after 180 days Table 1. This brown precipitate was also analyzed by collision induced dissociation (CID) using linked-scans (LS) at constant B/E to detect daughter fragments of *p*-chloroaniline ($m/z = 127$) as shown in Figure 3(b) and Figure 3(c), Table 1.

Whereas the addition of 2.0% of CHX with or without polyethylene glycol plus 17% EDTA and 5.1% NaOCl induced a blue and a white precipitate, respectively, corresponding to CHX with a peak $m/z = 505$ (Table 1).

4. Discussion

The aim of this study was to analyze by mass spectrometry the products obtained by the degradation of CHX with NaOCl, as oxidant reagent, and the same reaction using polyethylene glycol and EDTA. Although, there are similar studies in the literature reporting the presence of PCA as the final product of the reaction in the precipitate formed with the mixture of CHX and NaOCl [19] [20] [26] [27] [28] [29] [30] in other studies, PCA formation is not detected in the reaction mixture even after long periods of time [5] [25]. Thomas and Sem

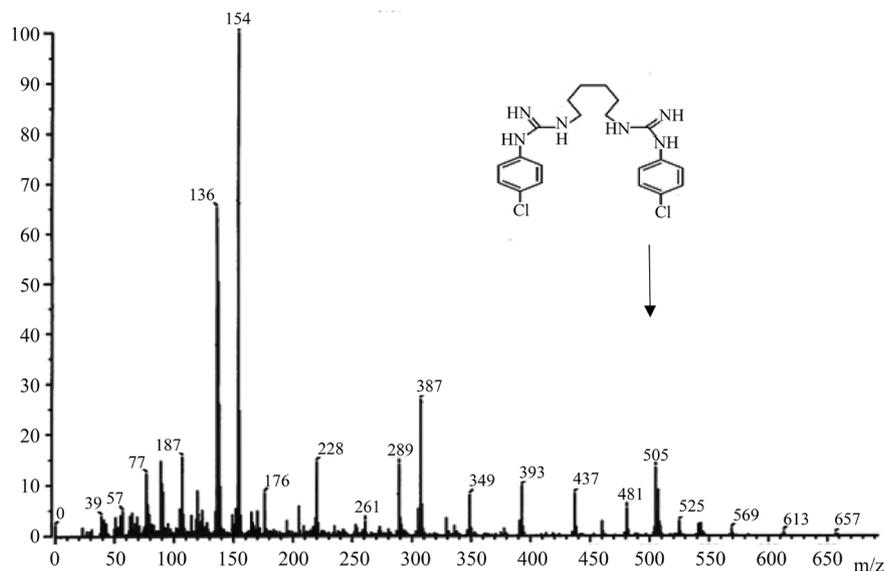


Figure 1. Mass spectra of chlorhexidine ($m/z = 505$) with polyethylene glycol (Consepsis) obtained by FAB^+ .

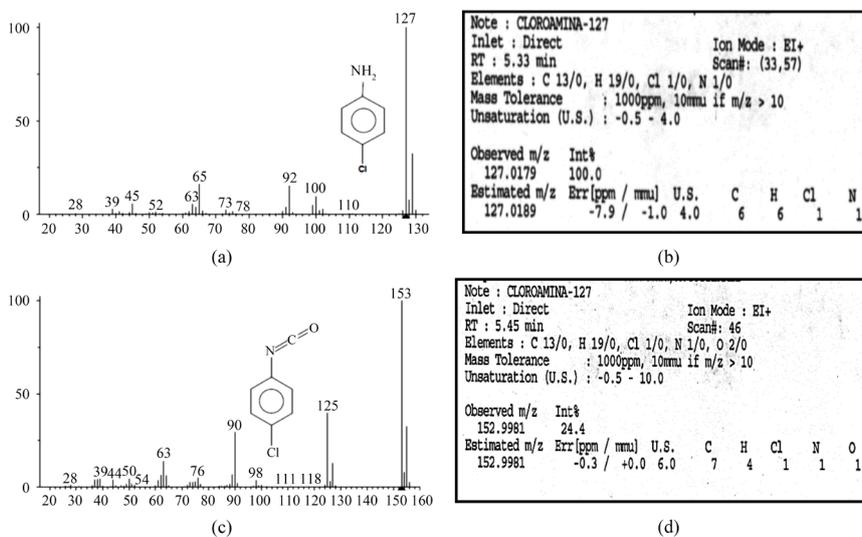


Figure 2. (a) Mass spectra of *p*-chloroaniline of NIST data base; (b) High resolution mass spectrometry analysis data of *p*-chloroaniline ($m/z = 127$); (c) Mass spectra of *p*-chlorophenyl isocyanate of NIST database; (d) High resolution mass spectrometry analysis data of *p*-chlorophenyl isocyanate ($m/z = 153$).

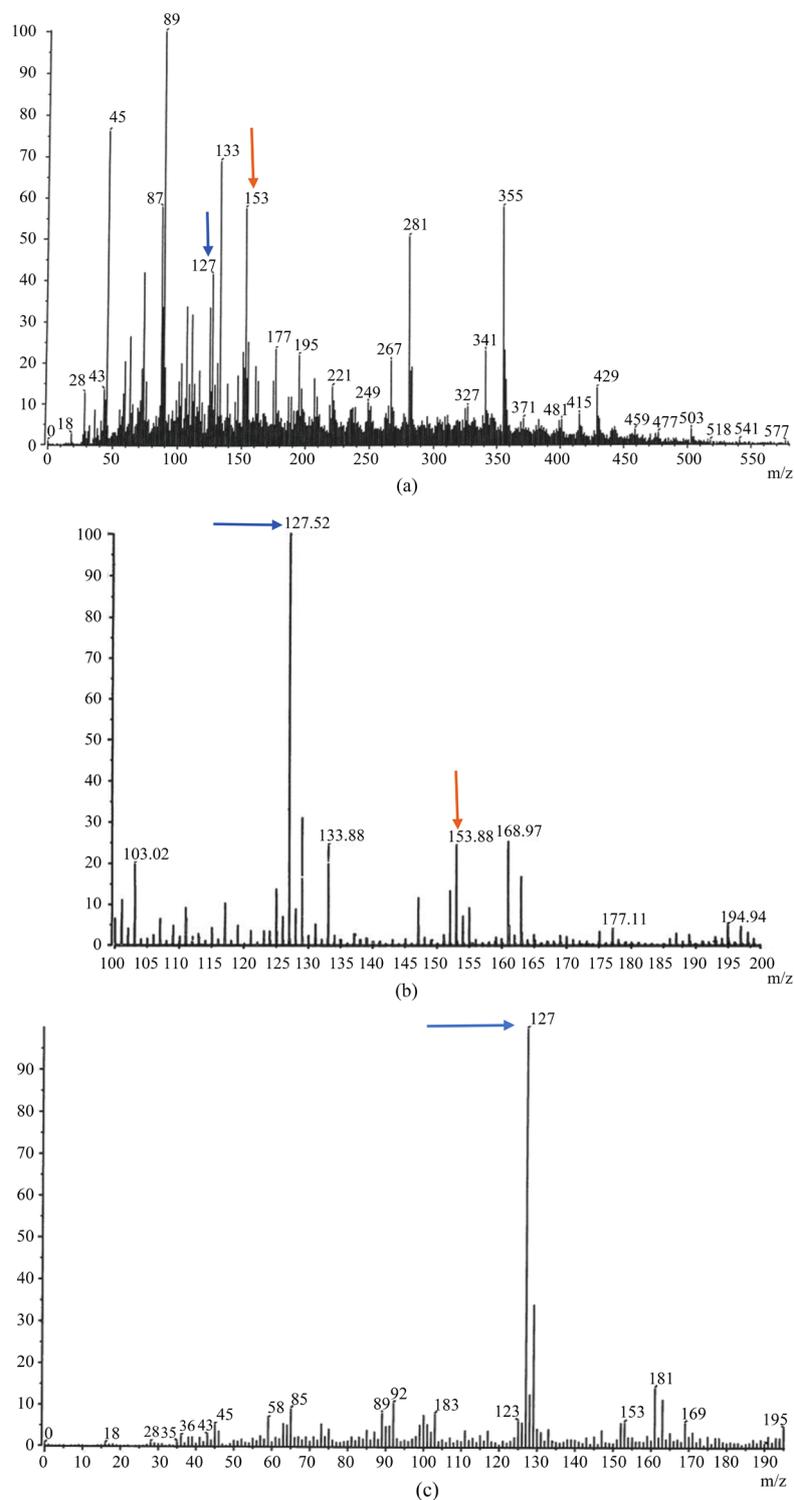


Figure 3. (a) Mass spectra of brown solution corresponding to the oxidation of the chlorhexidine (CHX) solution with sodium hypochlorite solution. $m/z = 127$ and 153 correspond to *p*-chloroaniline \longrightarrow and *p*-chlorophenyl isocyanate. \longrightarrow ; (b) Electron impact spectrum (EIMS) of the brown precipitate obtained from the combination of NaOCl with CHX (Consepsis) for 180 days; (c) Mass spectra of the brown solution after (180 days) analyzed by collision induced dissociation (CID) using linked-scans (LS) at constant B/E of $m/z = 127$.

(2010) did not detect PCA in the reaction mixture analyzed by nuclear magnetic resonance (NMR), although they proposed the partial formation of PCA [5]. Nowicki and Sem (2011) analyzed the same mixture [31] and reported the presence of derivatives of benzene (*p*-chlorophenyl urea and chlorophenyl guanidine), which can be transformed into PCA [32] [33] [34]. On the other hand, the analysis by nuclear magnetic resonance spectroscopy and infrared spectroscopy of the precipitate formed after mixing NaOCl and QMix 2 did not reveal free PCA [35]. Although, Basrani reported, in 2007, the presence of characteristic peaks obtained from the fragmentation of CHX through mass spectrometry methods ($m/z = 127, 153, 170, \text{ and } 195$), identification of the fragments and the proposal of a mechanism were not performed [20]. The non-identification of PCA in the reaction mixture by certain techniques evidences that it is necessary to search for appropriate techniques for the analysis of this reaction. Herein, we showed that a useful method for the study of CHX degradation to PCA is mass spectrometry. The fragmentation pattern of the reaction mixture was subjected to FAB ionization mode. All fragments were confirmed by high resolution analysis, using electron impact mass spectrometry (EIMS). Finally, daughter ions of the main peak ($m/z = 127$) corresponded unequivocally to PCA that were detected by collision induced dissociation (CID) using linked-scans (LS) at constant B/E.

The mass spectrometry data (FAB⁺ mode) of the monitored reaction mixture showed characteristic peaks at $m/z = 127, 153, 170, \text{ and } 195$. We found that the $m/z = 153$, which corresponds to *p*-chlorophenyl isocyanate, is an initial byproduct of the oxidation reaction. However, the fact that its abundance is inversely proportional to the 127 m/z peak (PCA) suggests that *p*-chlorophenyl isocyanate ($m/z = 153$) is an intermediate than can react to finally produce PCA ($m/z = 127$).

Barbin *et al.* reported that many factors are involved in the degradation time of CHX to PCA [31]. Our study confirmed that the presence of polyethylene glycol in the reaction mixture decreases the reaction rate for the formation of PCA, as shown in **Table 1**.

On the other hand, our results show that the introduction of EDTA (17%) in the reaction mixture of CHX with NaOCl, in the presence or absence polyethylene glycol, inhibits immediately the formation of PCA even after 10 days. The ability of EDTA to inhibit the oxidation reaction can be explained by the presence of active chlorine in NaOCl that interacts with EDTA forming a stable compound, which does not react with CHX, as reported by Clarkson [36] [37]. Therefore, the use of EDTA as an intermediate irrigating agent applied between NaOCl and CHX is an efficient method to inhibit the reaction. We agree with Kolosowski [21] and Rasimick [38], who demonstrated that EDTA does not degrade CHX into PCA, hence, fostering the antimicrobial effect of both solutions.

5. Conclusion

Although further studies are necessary to determine the presence of minimal

concentrations of CHX in the mixture of the monitored oxidation reaction, we conclude, under the limitations of this study, that *p*-chlorophenyl isocyanate is an intermediate in the oxidation reaction of CHX into PCA. Furthermore, we demonstrated that polyethylene glycol decreases efficiently the reaction rate for the formation of PCA. Oppositely, the presence of EDTA inhibits completely the oxidation reaction due to its interaction with the active chlorine present in NaOCl inducing a compound without oxidation properties.

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Conflicts of Interest

The authors deny any conflicts of interest related to this study.

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