Chlorination of tramadol: Reaction kinetics, mechanism and genotoxicity evaluation

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HIGHLIGHTS

- The reactivity between TRA and chlorine was found to increase with increasing pH.
- The active species Cl_2 , Cl_2O and HOCl were considered during chlorine/TRA reaction.
- Eleven chlorination products were identified by UPLC–Q-TOF MS at various pH.
- Two possible reaction pathways were proposed under various pH conditions.
- The genotoxicity of TRA oxidation products increased with high dosage of chlorine.

GRAPHICAL ABSTRACT

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ABSTRACT

Tramadol (TRA) is one of the most detected analgesics in environmental matrices, and it is of high significance to study the reactivity of TRA during chlorination considering its potential toxicity to the environment. The chlorine/TRA reaction is first order with respect to the TRA concentration, and a combination of first-order and second-order with respect to chlorine concentration. The pH dependence of the observed rate constants (k_{obs}) showed that the TRA oxidation reactivity increased with increasing pH. k_{obs} can be quantitatively described by considering all active species including Cl_2 , Cl_2O and HOCl , and the individual rate constants of HOCl/TRA^0 , $\text{HOCl}/\text{TRAH}^+$, Cl_2/TRA and $\text{Cl}_2\text{O}/\text{TRA}$ reactions were calculated to be $(2.61 \pm 0.29) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, $14.73 \pm 4.17 \text{ M}^{-1} \text{ s}^{-1}$, $(3.93 \pm 0.34) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $(5.66 \pm 1.83) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, respectively. Eleven degradation products were detected with UPLC–Q-TOF-MS, and the corresponding structures of eight products found under various pH conditions were proposed. The amine group was proposed to be the initial attack site under alkaline pH conditions, where reaction of the deprotonated amine group with HOCl is favorable. Under acidic and neutral pH conditions, however, two possible reaction pathways were proposed. One is an electrophilic substitution on the aromatic ring, and another is an electrophilic substitution on the nitrogen, leading to an N-chlorinated intermediate, which can be further oxidized. Finally, the SOS/umu test showed that the genotoxicity of TRA chlorination products increased with increasing dosage of chlorine, which was mostly attributed to the formation of some chlorine substitution products.

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1. Introduction

The widespread occurrence of pharmaceuticals and personal care products (PPCPs) in water has gained much attention in recent years (Daughton and Ternes, 1999). Although PPCPs detected in the environment are at trace concentration levels (Daughton and Ternes, 1999), their potential risk to human healthy cannot be ignored considering their constant accumulation and high resistance to biodegradation (Joss et al., 2006). Tramadol (TRA, with chemical structure shown in Fig. 1) is a synthetic, centrally acting analgesic agent used for the relief of acute and chronic pain, which has been used in both human and veterinary medicine (Matthiesen et al., 1998; De Leo et al., 2009). In 2004, the total consumption of TRA was as much as 25.3 tons in Germany, and it is reported that 15–35% of TRA is excreted unchanged via urine (Kasprzyk-Hordern et al., 2007). The concentrations of TRA detected in secondary effluent and surface water were up to $97 \mu\text{g L}^{-1}$ and $6 \mu\text{g L}^{-1}$, respectively (Kasprzyk-Hordern et al., 2009). Additionally, according to subacute and chronic toxicity studies on TRA, exposure to TRA may lead to the clinical signs of intoxication, like behavioral disorders and convulsions (Matthiesen et al., 1998). Therefore, it is of high significance to understand the fate of TRA considering its potential toxicity to the environment.

Owing to its low cost, chlorine is one of the most frequently used disinfectants for drinking water disinfection (Deborde and von Gunten, 2008). Prior studies reported that chlorine can easily react with some PPCPs, like sulfamethoxazole, ciprofloxacin, enrofloxacin, diclofenac, carbamazepine and tetracycline (Dodd and Huang, 2004; Wang et al., 2011; Soufan et al., 2012, 2013). Generally, the former studies considered hypochlorous acid (HOCl) and hypochlorite (ClO^-) as the main chlorine species, and the degradation reactions followed second-order kinetics, first order in the concentration of target compound, and first order in the chlorine concentration (Dodd and Huang, 2004; Soufan et al., 2012). Recently, some researchers pointed out that some other active species such as chlorine monoxide (Cl_2O) and molecular chlorine (Cl_2) take part in the chlorination disinfection process as well, in addition to HOCl/ ClO^- (Sivey et al., 2010; Sivey and Roberts, 2012). Despite their relatively lower concentrations, such species (Cl_2 , Cl_2O) possess much higher reactive activity with organic products compared with HOCl/ ClO^- (Sivey et al., 2010; Sivey and Roberts, 2012; Cai et al., 2013). Therefore, the contributions of HOCl/ ClO^- , Cl_2 and Cl_2O should all be considered during the PPCPs chlorination process. Additionally, chlorine attack generally leads to the generation of halogenated organic compounds, which typically possess relatively higher toxicity compared with the original organic products (Richardson and Ternes, 2011). Hence, evaluation of the toxicity of chlorination products should not be overlooked.

Recently, the kinetics and mechanism of the oxidation of TRA by ferrate and ozone have been investigated, where the apparent second-order rate constants were calculated to be $7.4 \pm 0.4 \text{ M}^{-1} \text{ s}^{-1}$ and $(4.2 \pm 0.3) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ at pH 8. The lone electron pair of the amine-N has been identified as the

predominant site of oxidant attack (Zimmermann et al., 2012). However, the fate of TRA during the chlorine disinfection process is unclear. Accordingly, detailed kinetics experiments were conducted from pH 4 to 9 during this work. Then the basic reactions considering all active species (including HOCl/ ClO^- , Cl_2 and Cl_2O) were employed to calculate the individual kinetic constants. Additionally, the degradation products under various pH conditions were detected and analyzed with Ultra Performance Liquid Chromatography–Quadrupole-Time of Flight–Mass Spectrometry (UPLC–Q–TOF–MS). Then, possible reaction pathways and mechanisms were proposed. Finally, the potential genotoxicity of the TRA chlorination products was evaluated.

2. Materials and methods

2.1. Chemicals

Tramadol was obtained from Sigma–Aldrich (Saint Louis, USA) with purity more than 99%. Sodium hypochlorite solution with 10% available chlorine concentration was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). The solvent acetonitrile was HPLC grade (Fisher Scientific, USA), and other reagents (CH_3COOH , CH_3COONa , $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, H_3BO_3 , $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, NaOH, H_2SO_4 , NH_4Cl), all obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China), were at least analytical grade. Reaction solutions were prepared using ultrapure water produced by a Millipore Water Purification System (Advantage A10, Millipore, USA). Free-chlorine stock solution was prepared by diluting initial sodium hypochlorite solution to yield about 100 mg/L free available chlorine and standardized by the DPD (N,N-diethyl-p-phenylenediamine, Sigma–Aldrich, Saint Louis, USA) colorimetric method (APHA, 1998).

2.2. Experimental procedures

To investigate the chlorination kinetics of TRA, batch experiments were conducted following pseudo-first-order conditions, with at least 10-fold excess of chlorine. Reactions were initiated by injecting an aliquot of chlorine stock solution into the prepared TRA solution (0.03 mM) containing 10 mM buffer solution. The previous studies reported that thiosulfate may convert the N-chlorinated intermediate back to the parent compound (Dodd et al., 2005; El Najjar et al., 2013). In order to avoid the potential interference of thiosulfate, NH_4Cl as a “soft” quenching reductant (1 mM), was used during this experiment (Dodd et al., 2005). At predetermined time intervals, samples (1 mL) were withdrawn and quenched. The resulting solutions were filtered ($0.22 \mu\text{m}$) and then analyzed by HPLC to measure the residual concentration of TRA as soon as possible. The reaction pH ranged from 4 to 9, which was maintained by 10 mM acetate buffer for pH 4–5, phosphate buffer for pH 6–8 and borate buffer for pH 9, respectively. A small quantity of H_2SO_4 or NaOH was used to control the pH if necessary. Unless otherwise specified, all experiments were conducted at $20 \pm 2^\circ\text{C}$. It should be noted that although the environmental concentration of TRA has been reported to be at the ng L^{-1} – $\mu\text{g L}^{-1}$ level, a relatively high concentration was chosen in this work to facilitate the detection of TRA loss and the identification of degradation products. Since rate constants are independent of the initial TRA concentration, this discrepancy would not affect the kinetic results.

In order to identify reaction intermediates and products, a series of higher concentration TRA solutions (0.05 mM) were treated with 10-fold dosages of chlorine from pH 4 to pH 9. These experiments were conducted for sufficient time (24 h) to reach

Fig. 1. Chemical structure of tramadol (TRA).

completion, and the resulting solutions were then filtered and analyzed by UPLC–Q-TOF-MS.

In order to examine the potential risk of TRA chlorination products, an ISO recommended SOS/umu bioassay was performed (ISO, 2000; Oda et al., 1985). The TRA solutions (0.05 mM, 200 mL) were treated with different amounts of chlorine (0-, 1-, 5-, 10-, 15- and 20-equiv to TRA concentration). After 24 h reaction, these solutions were first filtered and then extracted by solid phase extraction (Oasis HLB cartridges, Waters, Massachusetts, USA). The columns were pretreated with 6 mL dichloromethane, 6 mL methanol and 6 mL ultrapure water sequentially before use. Then the organics retained on the columns were washed with 6 mL dichloromethane, 6 mL dichloromethane/methanol (9:1) and 6 mL dichloromethane/methanol (5:5) sequentially, followed by a gentle nitrogen flow. The residues were dissolved in dimethyl sulfoxide solution (DMSO, Sigma–Aldrich, Saint Louis, USA) and stored below -20°C before the umu test. The SOS/umu test was performed according to the method of Oda et al. with some modifications (Oda et al., 1985; Elisabeth et al., 1998), and the specific procedures are provided in Supplemental Information (Text S1). Although the former studies demonstrated that the chlorine has inherent genotoxicity using a plant test (Feretti et al., 2003), controls considering the chlorine and buffer solutions were conducted, showing no obvious influence on the genotoxicity after the pretreatment processes (data not shown).

2.3. Analytical methods

The solution pH was measured by an Orion 720A digital pH meter equipped with a pH electrode acquired from Thermo Scientific Inc. (Massachusetts, USA). The concentration of TRA was measured by an Agilent 1260 HPLC/DAD system (High Performance Liquid Chromatography/Diode Array Detector, California, USA) coupled with a C18 column (250×4.6 mm, $5 \mu\text{m}$). The mobile phase was 0.1 M ammonium formate–acetonitrile (65/35, v/v) with a flow rate of 1 mL min^{-1} . The detection wavelength was 271 nm and the injected volume was $50 \mu\text{L}$ for each sample.

The degradation products of TRA chlorination were analyzed by a Waters UPLC/DAD system equipped with a Q-TOF MS (Massachusetts, USA). Separation was performed by an Agilent C18 column (2.1×50 mm, $1.7 \mu\text{m}$) at the flow rate of 0.2 mL min^{-1} . The mobile phase contained A (0.1 M ammonium formate) and B (acetonitrile). Gradient elution was as follows: 0–1 min, 90% A and 10% B; 1–10 min, linear gradient to 10% A and 90% B; 10–11 min, linear gradient to 90% A and 10% B; 11–12 min, 90% A and 10% B. The MS and MS/MS analysis was conducted with positive electro-spray ionization (ESI⁺) with a mass scan scope of m/z 50–1200.

3. Results and discussion

3.1. Reaction order of TRA chlorination

As shown in Fig. 2, chlorination experiments exhibited pseudo-first order dependence on the TRA concentration ($[\text{TRA}]$) in the presence of 10-fold excess of chlorine, demonstrated by the linear time-course plot of $\ln([\text{TRA}]_t/[\text{TRA}]_0)$. Accordingly, the following equation can be expressed for each experiment:

$$\ln \frac{[\text{TRA}]_t}{[\text{TRA}]_0} = -k_{\text{obs}} t \quad (1)$$

where $[\text{TRA}]_t$ and $[\text{TRA}]_0$ represent the concentration of TRA at time t and 0 respectively, and k_{obs} stands for the pseudo-first order rate constant.

Fig. 2. Pseudo-first-order kinetics plot of TRA degradation at pH 4–9 ($[\text{TRA}]_0 = 0.03 \text{ mM}$, $[\text{chlorine}] = 0.3 \text{ mM}$, $T = 20^{\circ}\text{C}$).

The reaction order corresponding to chlorine was then assessed by studying the variation of k_{obs} under varying free chlorine concentration ($[\text{chlorine}]$). Under several pH conditions, the variation of k_{obs} was not directly proportional to the varying free chlorine concentration (data not shown). Then in order to determine the reaction order with respect to chlorine, $\log k_{\text{obs}}$ versus $\log [\text{chlorine}]$ was examined. As shown in Fig. 3, $\log k_{\text{obs}}$ exhibits a good

Fig. 3. k_{obs} variation along with the change of $[\text{chlorine}]$ (as $\log k_{\text{obs}}$ vs $\log [\text{chlorine}]$) at pH 4–9.

linear relationship with $\log [\text{chlorine}]$ at different pH conditions ($R^2 > 0.97$). Accordingly, the pseudo-first order rate constants can be expressed as Eq. (2):

$$k_{\text{obs}} = k[\text{chlorine}]^n \quad (2)$$

A log transformation yields Eq. (3),

$$\log k_{\text{obs}} = n \log[\text{chlorine}] + \log k \quad (3)$$

where k represents the apparent rate constant and n is the reaction order with respect to chlorine.

The reaction order in $[\text{chlorine}]$ at various pH ranged from 1.01 to 1.67, indicating that reactions appearing first-order and second-order in $[\text{chlorine}]$ simultaneously existed during the TRA chlorination process. Recent works demonstrated that chlorine monoxide (Cl_2O) and molecular chlorine (Cl_2) could also be identified as active chlorination agents during the chlorination process (Sivey et al., 2010; Sivey and Roberts, 2012). Cl_2 can serve as a chlorination oxidant in the presence of chloride especially under low pH conditions (as Eq. (4)), and Cl_2O can be generated via dehydration of 2 equiv of HOCl as in Eq. (5) (Beach and Margerum, 1990; Sivey et al., 2010). Therefore, the concentration of Cl_2O ($[\text{Cl}_2\text{O}]$) is proportional to $[\text{chlorine}]^2$ and the reaction order was second-order in $[\text{chlorine}]$. While for HOCl/ ClO^- and Cl_2 , the reaction order was first-order in $[\text{chlorine}]$. This is in accordance with the present finding that reaction order corresponding to $[\text{chlorine}]$ is a combination of first-order and second-order, considering all these active species. Similarly, Sivey et al. studied the dimethenamid chlorination process, where the reaction orders in $[\text{chlorine}]$ determined ranged from 1.10 ± 0.13 to 1.78 ± 0.22 (Sivey et al., 2010). During the chlorination of PPCPs like antipyrine, reaction orders in $[\text{chlorine}]$ at various pH (6.53–7.62) ranged from 1.13 ± 0.15 to 1.59 ± 0.08 , which is consistent with our results as well (Cai et al., 2013).

3.2. Effect of pH

Fig. 4 shows the pH dependence on the observed rate constants (k_{obs}), where k_{obs} increases with increasing pH values. A similar tendency was observed during the O_3/TRA reaction, mostly

Fig. 4. Effect of pH on rate constants of TRA chlorination reaction ($[\text{TRA}]_0 = 0.03 \text{ mM}$, $[\text{chlorine}] = 0.3 \text{ mM}$, $T = 20^\circ\text{C}$). The solid line represents the model curve fitted with Eq. (14).

attributed to the fact that the deprotonated amine moiety possessed a much higher reactive tendency with O_3 than the protonated amine moiety. In contrast, the overall reactivity of TRA during ferrate oxidation decreased with increasing pH, which was ascribed to the decreasing reactivity of ferrate with increasing pH (Zimmermann et al., 2012).

Considering that the pK_a of TRA is 9.4, TRA presents two forms (the protonated form TRAH^+ and the neutral form TRA^0) in solution, whereas two major species of chlorine (HOCl/ClO^-) can be observed under experimental pH conditions as shown in Fig. S1 (Pospisilova et al., 1998; Chamberlain and Adams, 2006). Therefore, four parallel reactions co-existed during chlorine/TRA reaction.

In addition to the two major chlorine active species (HOCl/ClO^-), Cl_2 can also be formed mainly under acidic conditions as shown in Eq. (4) (Sivey et al., 2010). To confirm the existence of Cl^- in the initial solutions, ion chromatography (ICS-2000, Thermo, Massachusetts, USA) was applied to measure Cl^- in the stock solutions of chlorine and initial solutions without oxidants. The results indicated the initial concentration of Cl^- was nearly $0.1 \pm 0.02 \text{ mM}$. In addition, another minor species such as Cl_2O could act as an active oxidant as well during the chlorination process (Beach and Margerum, 1990). When pH is higher than 7, the formation of Cl_2 can be considered negligible due to the low concentration of H^+ (Deborde and von Gunten, 2008). Additionally, the contribution of Cl_2O also decreases at high pH values because of the low concentration of HOCl (Sivey et al., 2010).

It is well known that HOCl exhibits a higher reactivity with organic compounds compared with ClO^- (Dodd and Huang, 2004, 2007). Therefore, the contribution of ClO^- is negligible, and reactions (8) and (9) have been ignored during the chlorine/TRA reaction. Additionally, the nucleophile of protonated amine is unfavorable under chlorine attack (Ellis and Soper, 1954; Abia et al., 1998). However, TRA mostly exists as TRAH^+ and also possesses activity under low pH conditions. It could be concluded that TRA might possess other active sites besides the amine group, or that other active agents (Cl_2 and Cl_2O) could intervene in the reaction. Considering the overall chlorine reaction including reactions (6), (7), (10) and (11), the TRA chlorination kinetics was thus expressed as follows:

$$-\frac{d[\text{TRA}]_T}{dt} = (k_1[\text{HOCl}][\text{TRA}^0] + k_2[\text{HOCl}][\text{TRAH}^+] + k_{\text{Cl}_2}[\text{Cl}_2][\text{TRA}]_T + k_{\text{Cl}_2\text{O}}[\text{Cl}_2\text{O}][\text{TRA}]_T) \quad (12)$$

where $[\text{TRA}^0]$ and $[\text{TRAH}^+]$ represent the concentration of TRA^0 and TRAH^+ respectively, and $[\text{TRA}]_T = [\text{TRA}^0] + [\text{TRAH}^+]$.

Combining Eq. (12) and the distribution coefficients of each species,

$$-\frac{d[\text{TRA}]_T}{dt} = (k_1 a_2 b_1 [\text{HOCl}]_T + k_2 a_1 b_1 [\text{HOCl}]_T + k_{\text{Cl}_2} \frac{b_1}{k_{\text{Cl}_2}} [\text{Cl}^-][\text{H}^+][\text{HOCl}]_T + k_{\text{Cl}_2\text{O}} k_{\text{Cl}_2\text{O}} b_1^2 [\text{HOCl}]_T^2) [\text{TRA}]_T \quad (13)$$

where a_1 and a_2 are the distribution coefficients of TRAH^+ and TRA^0 respectively, and b_1 is the distribution coefficient of HOCl .

For a given pH and free chlorine concentration, k_{obs} can be expressed as a function of pH as follows:

$$k_{\text{obs}} = \left(k_1 a_2 b_1 [\text{HOCl}]_T + k_2 a_1 b_1 [\text{HOCl}]_T + k_{\text{Cl}_2} \frac{b_1}{k_{\text{Cl}_2}} [\text{Cl}^-][\text{H}^+][\text{HOCl}]_T + k_{\text{Cl}_2\text{O}} k_{\text{Cl}_2\text{O}} b_1^2 [\text{HOCl}]_T^2 \right) \quad (14)$$

Considering Eq. (14) and the observed experimental rate constants k_{obs} , the intrinsic constants k_1 , k_2 , k_{Cl_2} and $k_{\text{Cl}_2\text{O}}$ were calculated by multiple regression using origin 8.0 ($R^2 = 0.98$). The values of the rate constants k_1 , k_2 , k_{Cl_2} and $k_{\text{Cl}_2\text{O}}$ obtained from these calculations were $(2.61 \pm 0.29) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, $14.73 \pm 4.17 \text{ M}^{-1} \text{ s}^{-1}$, $(3.93 \pm 0.34) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $(5.66 \pm 1.83) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, respectively. The rate constant (k_2) between HOCl and TRAH^+ is several orders of magnitude lower than those for the other reactions, indicating that HOCl and TRAH^+ actually possess a relatively low reaction activity, in accordance with the former hypothesis. At pH 4, the TRA reactivity with chlorine showed a slight increase compared with pH 5, which could be attributed to the effect of Cl_2 . During the chlorination of trimethoprim, the increased reaction rate at pH < 5 also contributed to the generation of Cl_2 , which is in accordance with our result (Dodd and Huang, 2007). When pH > 5, the continuous increase of TRA reactivity can mostly be attributed to the increasing percentage of the deprotonated TRA (TRA^0). Considering the electrophilicity of the deprotonated TRA^0 , it should possess a much higher reaction rate with HOCl compared with the protonated form TRAH^+ (Zimmermann et al., 2012). On the other hand, although Cl_2 and Cl_2O possess much higher reactivity with TRA, the very low concentration of Cl_2 and Cl_2O may result in the lower k_{obs} under low pH conditions.

3.3. Degradation products

In order to analyze the degradation products of chlorine/TRA reaction, degradation experiments were conducted under different pH conditions (from 4 to 9). Several products were detected under diverse pH conditions, and eleven of them (named P1–P11) were identified. The possible structures of these products were deduced on the basis of the literature, the chlorination reaction mechanism and the analysis of the MS and MS/MS spectra (Lintz et al., 1981; Dodd and Huang, 2004, 2007; Deborde and von Gunten, 2008; Soufan et al., 2012; Zimmermann et al., 2012; Cai et al., 2013). A representative LC chromatogram of oxidized TRA at pH 7 is shown in Fig. S2. The detailed product names, MS/MS fragments and the proposed structures of these products are summarized in Table 1.

TRA: As shown in Fig. S3a, a strong liquid chromatogram peak appeared at 3.8 min, which was attributed to the TRA peak. The MS/MS spectrum (Fig. S3b) of TRA shows a strong molecular ion peak at m/z 264 (ESI⁺), and a weak fragment ion peak at m/z 246 resulting from the loss of H_2O (−18), which is in accordance with the previous studies (Radjenovic et al., 2012; Zimmermann et al., 2012).

P1, P2 and P3: P1, P2 and P3 were analyzed together, because of the obvious existence of one, two, and three chlorine atoms in the structure respectively according to the distribution of chlorine isotope peaks. The molecular ion peaks at m/z 298, 332, and 366 represent 34 Da, 68 Da and 102 Da addition compared with m/z 264, in line with the generation of mono-chlorinated, di-chlorinated and tri-chlorinated TRA products. Considering that the

Table 1

The detailed product names, MS/MS fragments and the proposed structures of products P1–P11.

Compound name	MS	MS/MS fragments	Proposed structure
P1	298.3	280.2, 235.2, 155.1, 58.1	
P2	332.2	314.2, 269.1, 189.1, 58.1	
P3	366.2		
P4	250.3	232.3	
P5	284.3	266.2, 235.2, 200.2, 193.2, 155.1	
P6	318.2	300.2, 269.2, 234.2, 189.1	
P7	278.3	260.3, 201.2	
P8	312.2	294.2, 235.2, 72.1	
P9	217.1	189.1, 135.2	Unknown
P10	249.1	231.1	Unknown
P11	223.1	155.1, 121.1	Unknown

electrophilic substitution reaction has been identified as one of most ubiquitous mechanisms during chlorination (Deborde and von Gunten, 2008), the most probable chlorine substitution site was on the aromatic ring which possesses relatively higher electron density (Soufan et al., 2012). The MS/MS spectra and the most possible structures of these products are shown in Figs. S4, S5 and S6, respectively.

P4, P5 and P6: The products P5 and P6 possess one and two chlorine atoms in the structure respectively, attributed to the existence of chlorine isotope peaks. The molecular ion peaks at m/z 250, 284

Fig. 5. The possible degradation pathway of TRA during chlorination process.

and 318 represent 14 Da loss compared with m/z 264 (TRA), 298 (P1) and 332 (P2), which is most likely corresponded to the loss of a methyl group. Accordingly, we deduced P4, P5 and P6 were demethylation products generated from TRA, P1 and P2 respectively, and the corresponding MS/MS spectra are shown in Figs. S7–S9.

P7 and P8: The molecular ion peak of product P7 at m/z 278 represents 14 Da addition compared with m/z 264, which is consistent with the addition of an O atom and loss of two H atoms. Therefore,

we proposed the existence of an aldehyde group in the product P7, which was generated from the oxidation of a methyl group. The product P8 (m/z 312) represents 34 Da addition compared with P7 (m/z 278), which was mostly attributable to the chlorine substitution. In addition, the MS/MS spectrum of P8 shows an important fragment m/z 72 mostly attributable to the $-N(CH_3)_2CHO$ group, which is an indirect evidence for the existence of an aldehyde group. Hence, the MS/MS spectra and the proposed structures of P7 and P8 are shown in Figs. S10 and S11, respectively.

Fig. 6. The variation of genotoxicity during TRA chlorination process under different chlorine dosage (chlorine concentration is 1-, 5-, 10-, 15- and 20-equiv to TRA concentration, pH = 7).

P9, P10 and P11: The products P9, P10 and P11 showed molecular ion peak at m/z 217, m/z 249 and m/z 223, respectively. According to the nitrogen rule, where an odd number of nitrogen atoms corresponds to an odd molecular weight, these products possess even molecular weight, indicating there is no nitrogen atom in these products. Although the exact structure of those products was not confirmed, we proposed that these products were obtained from oxidation of the amide group.

3.4. Reaction pathway and mechanism

During the oxidation of TRA by ferrate and ozone, the lone electron pair of amine-N has been considered as the predominant site of oxidation. An oxygen transfer mechanism can lead to the formation of N-oxide-TRA, and a one-electron transfer mechanism may result in the formation of N-centered radical cation intermediates, which can be further oxidized (Zimmermann et al., 2012). However, during UV and UV/H₂O₂ oxidation of TRA in the presence of chloramine, oxidation cleavage of methoxy and methoxybenzene moieties, O-demethylation, hydroxylation, and cyclohexane ring-opening were identified as major reaction mechanisms (Radjenovic et al., 2012). The former study concluded that there were three kinds of reaction mechanisms during chlorination of organic compounds, including oxidation reactions, addition reactions to unsaturated bonds and electrophilic substitution reactions at nucleophilic sites (Deborde and von Gunten, 2008). On the basis of the former TRA oxidation process and chlorination mechanism, a possible chlorine/TRA reaction pathway was proposed as shown in Fig. 5. The TRA chlorination process mostly takes place by two kinds of reaction pathways, including chlorine substitution and oxidation of the amine group.

The distribution of degradation products at various pH conditions was summarized as shown in Table S1. Although the exact structures of products P9–P11 were not proposed, they were all obtained from the oxidation of the amino group according to the above analysis of degradation products. Accordingly, the degradation products detected under alkaline pH conditions (as shown in Fig. S12) were all obtained from the oxidation of the amide group, not from chlorine substitution. Considering that the HOCl is the most active chlorine species under high pH conditions, we proposed that the amine group should be the initial attack site under alkaline conditions, where the deprotonated amine group is favorable for reaction with HOCl (Abia et al., 1998). The above kinetics

results also indicated that the HOCl/TRA⁰ reaction possessed a relatively high rate constant.

Under acidic and neutral pH conditions, however, both amine group oxidation products and chlorine substitution products were detected. Therefore, two possible reaction pathways have been proposed. One is an electrophilic substitution on the aromatic ring, leading to the formation of mono-chloro TRA derivative compounds. The mono-chloro TRA can be further transformed to generate other chloro-substitution products. Considering the aromatic ring possesses a relative high reactivity, we propose that the substitution mainly occurs on the aromatic ring. Chlorine substitution is ubiquitous during the chlorination process, and a similar oxidation mechanism has been widely observed during chlorination of other PPCPs, such as antipyrine, carbamazepine and trimethoprim (Dodd and Huang, 2007; Cai et al., 2013; Soufan et al., 2013). Another pathway is an electrophilic substitution on the nitrogen. This attack generally leads to N-chlorinated intermediate formation, which can generate a nitrenium cation followed by further oxidation, leading to the formation of demethylation products and products containing alcohol or aldehyde groups. The amino group has been reported as the primary attack site during the oxidation of TRA by ferrate and ozone (Zimmermann et al., 2012). During the diclofenac chlorination process, degradation products detected were all obtained from the electrophilic attack on the aromatic ring or amine function of diclofenac, which was in line with our results as well (Soufan et al., 2012).

3.5. Genotoxicity

The potential risk of chlorination products is a hot topic in water disinfection treatment (Richardson and Ternes, 2011). The genotoxicity of TRA chlorination products under various dosages of chlorine at neutral pH (pH 7) is shown in Fig. 6. The value of IR is greater than 2.0, indicating the existence of genotoxicity in the sample (Oda et al., 1985). The results showed that the initial TRA solution and that with low dosage of chlorine showed no obvious genotoxicity, while the genotoxicity increased with respect to the increasing dosage of chlorine. It has been reported that chlorinated organic compounds formed during chlorination process generally show higher toxicity than their parent compounds (Hu et al., 2002, 2003). Therefore, the increased genotoxicity of the TRA oxidation products was mostly attributed to the formation of several chlorine substitution products with higher genotoxicity than TRA.

4. Conclusions

In this work, detailed kinetics, mechanism and toxicity test were conducted to elucidate the fate of TRA during the chlorination disinfection process. From a kinetics point of view, the chlorine/TRA reaction is first order with respect to TRA concentration, and a combination of first-order and second-order with respect to chlorine concentration, which is attributed to the contribution of Cl₂O. The observed rate constants (k_{obs}) increased with increasing pH, which can be quantitatively described by considering all active species, including Cl₂, Cl₂O and HOCl. The Cl₂ and Cl₂O show a much higher reaction rate with TRA compared with HOCl, and the rate constant of the HOCl/TRA⁰ reaction is several orders of magnitude higher than the HOCl/TRAH⁺ reaction. From a mechanism standpoint, eleven degradation products were detected, and the corresponding structures of eight products found under various pH conditions were proposed. Under alkaline pH conditions, the amine group was proposed to be the primary attack site, where the deprotonated amine group is favorable for reaction with HOCl; while under acidic and neutral pH conditions, two possible reaction pathways have been proposed. One is electrophilic

substitution on the aromatic ring leading to the formation of chlorine substitution products, and another is an electrophilic substitution on the nitrogen, leading to formation of N-chlorinated intermediates, which can be further oxidized. Finally, the genotoxicity of TRA chlorination products increased corresponding with the increase in dosage of chlorine, mostly attributable to the generation of some chlorine substitution products.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.chemosphere.2015.06.034>.

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