The Chlorination Behaviour and Environmental Fate of the Antiretroviral Drug Nevirapine in South African Surface Water

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Abstract

The wastewater treatment process, besides discharging pharmaceuticals into the environment, has been found to result in the formation of a variety of undescribed compounds. Here we investigate the laboratory scale chlorination of the commonly used anti-HIV drug Nevirapine, characterise its disinfection transformation products (DTPs), and using liquid chromatography with high resolution mass spectrometry, screen environmental surface water for these DTPs. Chlorination of Nevirapine was scaled up, fractioned by preparative chromatography and the

fractions were tested *in vitro* for toxicity and anti-HIV activity. Nevirapine was found to be resistant to degradation at relevant chlorination levels, which may partially explain its ubiquitous presence in South African surface water. During simulated chlorination, a variety of DTPs with varying properties were formed, some of which were detected in the environment, close to wastewater treatment plants. Interestingly, some of these compounds, although not as toxic as Nevirapine, retained antiviral activity. Further purification and synthesis is required to fully characterise these novel molecules.

Keywords

Nevirapine, chlorination, wastewater, LC-MS, surface water, LC-QTOF

Highlights

- Nevirapine is resistant to chlorination; may explain its environmental prevalence
- Nevirapine forms a variety of chlorination disinfection transformation products *in vitro*
- These compounds were detected in the environment by UHPLC-QTOF
- The disinfection transformation products were non-toxic *in vitro*, with slight antiviral activity
- Potentially biologically active modified pharmaceuticals are released into the environment

1. Introduction

Over the past two decades researchers have shown that pharmaceuticals and personal care products (PPCP) are released into water courses as a result of human use (Ternes et al., 2001; Peng et al., 2014).The effect these compounds, at low concentrations, have on human health or aquatic fauna and flora have yet to be determined fully (Petrie et al., 2014; Roden et al., 2015).

Furthermore, the development and promotion of drug resistance in bacterial populations has been postulated (Kümmerer, 2009).

Besides releasing pharmaceuticals into the environment, these compounds have also been found to be modified as a result of wastewater treatment. The resulting disinfection transformation products (DTPs), many of which are undescribed, are then released into the environment, which further complicates the impact of PPCPs on the environment. The mechanism and type of transformation product formation is dependent on the type of disinfection utilised. It has been found that pharmaceuticals may be modified by ozonation (Zimmermann et al., 2011), chloramination (Zhai et al., 2014) and chlorination (He et al., 2013; Bulloch et al., 2015). Chlorination is one of the more popular methods used to disinfect wastewater and has therefore received the most attention in the literature. In South Africa water disinfection is commonly achieved using chlorine gas. The Department of Water Affairs and Forestry (DWAF) requires that discharged wastewater should contain zero faecal coliforms per 100 mL with the caveat that residual chlorine may not be higher than 0.25 mg/mL (Leopold and Freese, 2009).

Chlorination, as a mechanism to treat wastewater and drinking water, has been the method of choice for a long time since it it is a cost effective and broad spectrum method of disinfection. In addition to this, chloramination of treated water ensures a longer duration of disinfection as chloramines have a longer half-life than free residual chlorine (Leopold and Freese, 2009).

Unfortunately chlorination has a number of drawbacks, such as the formation of disinfection by-products during the disinfection process. A large number of toxic compounds may be formed through the interaction between chlorine and dissolved organic matter. Compounds such as the trihalomethanes and the haloacetic acids have been identified in previous decades and are now strictly regulated (Richardson et al., 2007).

Very little is known about the chemical characteristics of pharmaceutical DTPs and their toxicity profiles cannot always be based on those of the parent compound. This was shown in the case of the chlorination of acetaminophen, which resulted in the production of the toxic compounds 1,4- benzoquinone and N-acetyl-p-benzoquinone imine (Bedner and MacCrehan, 2006).

Besides adding complexity to the potential toxicity profile, the biological activities of many of the degradation products are not known. The degradation products of antivirals or antibiotics may retain antimicrobial properties or even gain additional activities. Inroads into understanding the transformation of antibiotics are being made by various researchers and it has been found that while most antibiotics lose their activity during water disinfection, a few do form biologically active transformation products(Dodd et al., 2009; Escher and Fenner, 2011; Mestankova et al., 2012; Keen and Linden, 2013). In addition to understanding the chemistry behind their transformation, various technologies are in development to effectively remove pharmaceuticals and their disinfection transformation products from wastewater (Prasse et al., 2015). These technologies are however in their infancy and have yet to be adopted widely in “first world countries”, let alone in developing countries such as South Africa.

Recent research on the prevalence of HIV-1 antiretroviral compounds (ARVs) in South African surface water has shown that Nevirapine occurs ubiquitously in the environment (Wood et al., 2015). The drug is a non-nucleoside reverse transcriptase inhibitor (NNRTI) that is commonly used to prevent mother to child transmission of HIV and features as a first-line regimen for treatment of HIV-1 infection (Mofenson, 2010; Coovadia et al., 2012). Prasse and colleagues have shown that the compound also occurs in European surface water and its presence is attributable to inefficient removal during wastewater treatment (Prasse et al., 2010).

Vankova and co-workers showed that Nevirapine has low biodegradability in a closed bottle system (Vanková *et al.*, 2010). Although this theoretical finding addresses the compound’s

ubiquitous presence in South African surface water (Wood et al., 2015) , it does not describe how the compound reacts during wastewater treatment, if at all.

The antiretroviral class of compound has not been studied extensively in surface water across the world. This is most likely due to the regional prevalence of HIV. In addition to this, no research, to our knowledge, concerning the transformation behaviour of these drugs during the disinfection process has been described.

South Africa utilises more ARVs per capita than any other country in the world (WHO, 2013) which indicates that high amounts of these compounds would enter wastewater treatment works (WWTWs), that were not designed to remove pharmaceuticals. In addition to ineffective WWTWs, improper sanitation and illegal sewage release should also be considered. These factors, as well as the reduced expected dilution, in a water scarce region such as South Africa, lead one to expect that ARVs and their degradation products should be prevalent in the environment.

Here the reactivity of the antiretroviral drug Nevirapine to chlorine, in the form of sodium hypochlorite, is qualitatively studied. The degradation products that are formed as a result of chlorination are described and related to environmental water samples collected in South Africa. We proceed to show that although these disinfection transformation products of Nevirapine are not toxic, they may have the same or similar biological activity as the parent molecule. The environmental impact of releasing active, undescribed molecules from WWTWs has yet to be determined.

2. Materials and Methods

2.1 Chemical reagents

Nevirapine was purchased from the United States Pharmacopeia, through Industrial Analytical (Johannesburg, South Africa) and stock solutions (1 mg/mL) were made up in methanol and stored at -20°C until use. LC-MS grade water, methanol and dimethyl sulfoxide (DMSO) were purchased from Lab-Scan (Gliwice, Poland). Sodium hypochlorite from Merck (Johannesburg, South Africa), 10-14%, was diluted in water to 0.4 M and the concentration was found to be stable over time by iodometric titration. Monobasic and dibasic potassium phosphate (Merck) were used for buffering Nevirapine and NaOCl solutions to a final concentration of 10 mM. Ammonium Chloride, sodium thiosulphate and ascorbic acid were purchased from Radchem (Johannesburg, South Africa), formic acid from Sigma-Aldrich (Johannesburg, South Africa) and 20 mL borosilicate amber vials with PTFE caps from Macherey-Nagel (Düren, Germany). Pharmaceutical Nevirapine was obtained from Aspen (Johannesburg, South Africa) and utilized for large scale experimentation to reduce costs. Water and acetonitrile, each with 0.1% formic acid were obtained from Burdick & Jackson (Muskegon, USA). All buffers and reagents were formulated using LC-MS grade water (non-South African origin).

2.2 Chlorination reactions

Nevirapine (20 µg/mL) diluted in either LC-MS grade water, 10mM phosphate buffer pH 5.8 or 10 mM phosphate buffer pH 8 was combined in equal volumes with NaOCl diluted in either of the aforementioned solvents (to yield 50, 100, 200 or 500 µM NaOCl) and stirred at room temperature (20 °C +-1 °C). Aliquots were taken from the reaction at 1, 5, 10, 20, 30, 60 and 120 min and then again at 24 hours. Aliquots were analysed by HPLC-UV, UHPLC-QqQ and UHPLC-QTOF.

To identify an effective quenching agent, sample fractions (1 mL) for each time course were added to either sodium thiosulphate, ascorbic acid or ammonium chloride to yield a twofold molar excess (compared to NaOCl concentration), analysed by LC-MS plug injection and compared to unquenched data. In order to generate the most accurate data for a particular time point, unquenched reactions were incubated in the LC autosampler. Plug injections were performed using an Agilent 1290 series UHPLC coupled to an Agilent 6460 triple quadrupole (Agilent). Mobile phases consisted of water (A) and acetonitrile (B) both with 0.1% formic acid, held at 50% B at a flow rate of 0.4 mL/min. Sequential 15 µL plug injections (no column) of a sample incubated on the LC-MS autosampler, held at (20°C +-1°C), were analysed by mass spectrometry in MS2 scan mode by positive electrospray ionisation. Source conditions: gas temperature, 250^0C; gas flow, 8 L/min; nebulizer, 35 psi; sheath gas temperature, 300 ^{0}C; sheath gas flow, 10 L/min; capillary voltage, 3000 V and nozzle voltage, 0 V.

For kinetics studies Nevirapine (4 µM) diluted in either LC-MS grade water, 10mM phosphate buffer pH 5.8, 10 mM phosphate buffer pH 8 or WWTW effluent from the Zeekoegat plant was combined in equal volumes with NaOCl diluted in either of the aforementioned solvents (to yield 2 µM Nevirapine and 20 µM NaOCl) and stirred at room temperature (20 °C +-1 °C). Aliquots were taken from the reaction at 10 second intervals and combined with NH_4Cl to give a two-fold molar excess with respect to NaOCl. Samples were analysed by UHPLC-QTOF immediately after the last time course was sampled. An external 6 point calibration of Nevirapine in the matching reaction solvent was utilised for quantitative purposes and analysed in MassHunter Quant (Agilent).

2.3 LC-UV analysis of Chlorination Reactions

The LC-UV system consisted of an Agilent 1100 series binary pump LC coupled to an Agilent diode array detector (254 nm with 400 nm reference). A 1.8 µm Zorbax Eclipse Plus C18, 2.1x50mm, column was used to separate a 15 µL injection of the reaction products at a flow rate of 0.2 mL/min at room temperature. Mobile phase A consisted of water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid. The gradient was as follows: 0 min, 5% B; 20 min, 95%B; 22 min 95% B; 30 min, 5% B; 40 min, 5%B.

2.4 Large Scale Preparation of Nevirapine Chlorination Reaction Products

1.8 g of pharmaceutical Nevirapine (Aspen) tablets were crushed and resuspended in 25 % HCl. The solution was clarified by centrifugation (3000 rpm for 30 min) and the supernatant containing approximately 1 g of Nevirapine was collected. The yield of this acid extraction was determined by UHPLC-QTOF analysis as compared to an external calibration curve.

Acid extracted Nevirapine was diluted in either 100 mM phosphate buffer (pH 8) or 10 mM Phosphate buffer (pH 5.8) to 1.5 mg/mL, 0.4 M NaOCl was added daily for a period of four days and the reactions were monitored by UHPLC-QTOF analysis. Upon reaching the maximum diversity of reaction products, the mixtures were dried by rotary evaporation (Buchi, Switzerland) at 40^0C under vacuum. The total basic and acidic reactions were diluted in dimethyl sulphoxide to 1 mg/mL for toxicity studies.

The dried and crushed acidic reaction mixture was dissolved in water and fractioned by centrifugation into aqueous and non-aqueous fractions. These were dried, as before, and diluted in dimethyl sulphoxide to 1 mg/mL for toxicity studies.

Both the acidic and basic reactions were separated using preparative chromatography. 100 mg of each reaction in water (100 mg/mL) was loaded onto a Biotage SNAP Ultra C18 (12 g) column and separated on a gradient of acetonitrile and water (both with 0.1 % formic acid) over 30 min at a flow rate of 10 mL/min. Fractions were collected and dried by vacuum centrifugation (Martin Christ RVC 2-33IR) at 2 mBar, 40 ^{0}C, 900 rpm for 12 hours. Dried fractions were resuspended in DMSO (1 mg/mL) and subjected to toxicity and activity screening.

2.5 Environmental Sample Collection and Extraction

Grab samples were collected from all the major rivers and lakes (man-made) in South Africa as part of a multi-year water quality study. Samples were collected in “virgin” borosilicate Schott bottles and transported to the laboratory at room temperature. Sampling locations were chosen based on proximities to wastewater treatment works (WWTW) and human settlements. To provide a comprehensive picture of a specific body of water, multiple samples were taken from different locations in a sampling site. Samples were stored at -20°C until extraction after which extracts were stored at -20°C until analysis.

500 mL of each environmental sample was filtered using a 1 µm glass-fibre syringe driven filter (Pall) and extracted using the Smart Prep Extraction (Horizon, Salem, USA); an automated offline solid phase extraction instrument. Briefly 6 cc Oasis HLB, 500 mg (Waters) cartridges were conditioned with 4 mL methanol followed by 6 mL of HPLC Grade water. 500 mL of sample was then introduced at a flow rate of 10 mL/min after which cartridges were dried under nitrogen for three minutes. Cartridges were then eluted twice with 5 mL of methanol and dried under a gentle stream of nitrogen to 500 µL. All extractions were performed at 18 °C (+-2 °C).

2.6 UHPLC QTOF Analysis

Environmental concentrations of Nevirapine were reported previously and national samples were analysed by UHPLC-QTOF in full scan, “auto MS” and “all ions” mode. Similarly, chlorination reactions of Nevirapine at varying concentrations of NaOCl and pH (after 1 min) were analysed in order to characterise the resulting reaction products. In addition to these, the large scale chlorination reactions were monitored by UHPLC-QTOF.

1 µL of each sample was separated on an Agilent 1290 UHPLC using an HPH 2.1 x 100 mm Poroshell column (Agilent) with water and acetonitrile (both with 0.1% formic acid) as mobile phases at a flow rate of 0.5 mL/min. The gradient was as follows: 3 min, 2% B (organic); 22 min 100%; 25 min, 100%; 27 min, 2% and 30 min, 2%.

Eluting compounds were analysed by positive electrospray QTOF fitted with an iFunnel source. Source conditions: gas temperature, 200^0C; gas flow, 15 L/min; nebulizer, 40 psi; sheath gas temperature, 400 ^{0}C; sheath gas flow, 12 L/min; capillary voltage, 3500 V, nozzle voltage, 500 V and fragmentor, 365 eV.

Data obtained from full scan and auto-MS analysis of Nevirapine reactions were manually inspected using MassHunter Qual (Agilent) and exported to Mass Profiler (Agilent) for analysis. Features present in the control reactions (Nevirapine without NaOCl and NaOCl without Nevirapine) and blank injections were subtracted from the reaction data sets to determine unique reaction products. These features were inspected manually and exported to MassHunter PCDL Manager (Agilent) to create a database containing information on each reaction product’s accurate mass, retention time and MS/MS behaviour. This database was then applied to environmental samples to determine the presence of reaction products in the environment.

Molecular Structure Correlator (Agilent) was used to calculate precursor and fragment formulas and correlate actual MS/MS spectra with theoretical fragments of proposed structures. The structures were proposed (manually) for each of the major reaction products.

2.7 In Vitro Toxicity and Antiviral Activity

The CellTiter 96 AQueous One Solution Cell Proliferation Assay System (Promega, Madison, USA) was used to determine the toxicity (Barltrop et al., 1991) (Barltrop et al., 1991) of Nevirapine chlorination reaction product fractions in 293T cells (Graham et al., 1977). This was compared to the toxicity of pharmaceutical grade as well as analytical grade Nevirapine. Sample toxicity, as reflected by cell viability, was measured by the bioreduction of a MTS tetrazolium compound (MTS) to a coloured formazan product in the culture medium. The formazan product was spectrophotometrically quantified with a VesraMax microplate reader (Molecular Devices, Sunnyvale, USA). The degree of sample toxicity was related to the MTS-to-formazan conversion by the cells.

Sample titration into complete Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Johannesburg, South Africa) supplemented with fetal bovine serum (Hyclone, GE, Little Chalfont, UK), 1 M HEPES (Life Technologies, Johannesburg, South Africa) and 10 mg/mL Gentamicin (Sigma-Aldrich, Johannesburg, South Africa) provided a range of concentrations for toxicity testing. Unexposed cells were used as a negative control. Incubation commenced for 2 days at 37^{o}C under 5% CO_2 in a humidified atmosphere. After the addition of the MTS reagent, plates were incubated for 1.5 hours after which spectrophometric absorbances were read at 490 nm, with 690 nm as reference wavelength. A viability of 0% indicates total cells death while a viability of 100% indicates full cell viability. Cytotoxic concentration-50 (CC_{50}) values were calculated and indicate the sample concentration at which 50% of the cells are viable.

Anti-HIV activity was determined over non-toxic sample concentrations. Dilutions of each of the reaction product fractions were titrated in 96-well culture plates as described above. After the addition of cells and virus, the plates were incubated for 48 hours at 37^{o}C under 5% CO_2 in a humidified atmosphere. A standard HIV-1 subtype C isolate, MJ4, was used to screen for antiviral activity of the samples. A medium control (cells, virus and growth medium) was included. After incubation the Bright Glo™ Reagent (Promega) was used to assay for the expression of firefly luciferase in the plate wells (De Wet et al., 1987). Bioluminescence was quantified on a Victor-3 1420 Multi Label Counter (Perkin Elmer). The medium control was used as the uninhibited control. The percent viral activity was calculated as the factor of the bioluminescence of the test sample and that of the virus control.

A viral activity of 0% indicates complete viral inhibition while a viral activity of 100% indicates no inhibition (full viral activity). Inhibitory concentration-50 (IC_{50}) values were calculated and indicated the concentration of sample at which 50% of the viruses were inhibited. During the activity screening, a decrease in luciferase activity can be observed over toxic concentrations as a result of compromised cell viability, and not as a result of inhibition of the virus. An untreated cell control (media only) is used as a reference for 100% cell viability (no toxicity).

3. Results and Discussion

3.1 Chlorination Reactions

Various chromatographic systems were utilised throughout this research and although they differ by instrument type (UV detection, triple quadrupole and qTOF) a high level of correlation was found between the data. Three concentrations of Nevirapine were used in this research in order to represent environmental concentrations (low, 2 µM) as well as having concentrations

high enough to detect (medium, 37 µM) transformation products and purify them (high, 5620 µM).

The efficacy of ammonium chloride, sodium thiosulfate and citric acid as quenching agents was evaluated. Researchers (Bedner and MacCrehan, 2006; Soufan et al., 2012) have warned of the potential of sodium thiosulphate to reverse chlorination reactions, and this was found to be the case for Nevirapine. This was found by comparing quenched and unquenched LC-MS plug injection spectra in which the ion intensity of the Nevirapine pseudomolecular ion was compared. This highlights the fact that Nevirapine oxidation by NaOCl may be reversed (with the addition of a reducing agent such as sodium thiosulphate). Similarly, ascorbic acid was found to reverse Nevirapine chlorination reactions. Fortunately NH_4Cl was found to halt the reaction while not inducing additional reactions, as shown by the un-quenched control. Autosampler reactions (unquenched) and quenched stirred reactions were found to be comparable when analysed by plug injection, which indicates that the reaction is not adversely influenced by an increase in volume. Thus, large scale (10 mL) stirred reactions were utilised throughout in order to analyse identical samples multiple times across a number of analytical platforms.

UV analysis of chlorination reactions (37 µM Nevirapine to 50, 100, 200 and 500 µM NaOCl) showed that the compound reacts almost immediately (the earliest time course was one minute) and then remains relatively constant over the time course up to 24 hours. In Figure 1 the 1 minute reaction of Nevirapine with NaOCl is overlaid with the 24 hour reaction (at pH 8). The peak area of Nevirapine remains constant, whereas the reaction products show variability over time. The dynamic range of Nevirapine detection was verified by injection of a calibration curve and was found to be linear across the concentration range tested.

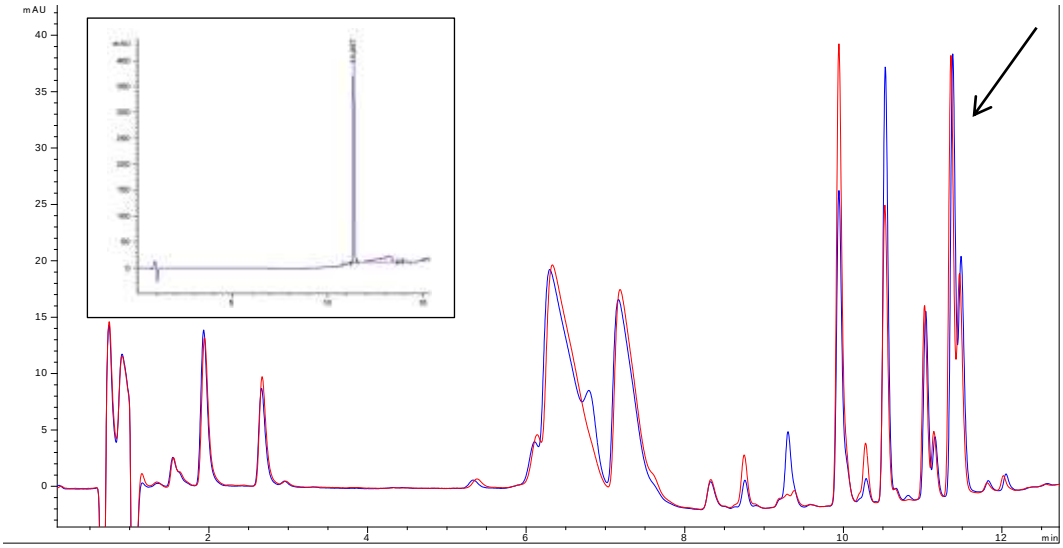


Figure 1: Overlaid LC-UV trace (254 nm) of 10 µg/mL Nevirapine (37 µm), indicated by an arrow, reacted with 100 µM NaOCl (pH 8) after one minute (blue trace) and 24 hours (red trace). The figure inset shows the LC-UV trace of unreacted Nevirapine (10 µg/mL in phosphate buffer).

The reactivity of Nevirapine in the LC-UV data was validated by monitoring the intensity of the extracted *m/z* 267 ion (Nevirapine pseudomolecular ion) in LC-MS experiments and data were found to be comparable to LC-UV results. The extent of reaction was found to be dependent on NaOCl concentration as well as pH. The 37 µM Nevirapine was exhausted in as little as one minute when treated with 500 µM NaOCl in the buffered as well as unbuffered reactions. In the basic reaction (pH 8) with 200 µM NaOCl the Nevirapine is undetectable at 1 minute whereas it remains at a low level in the pH 5.8 and unbuffered reactions (Figure S1).

Although the reaction was found to be stable over time, it was found that pH greatly affected the reaction's speed well as the nature of the reaction products. LC-UV analysis (Figure S2) showed that a variety of distinct products were formed under basic and acidic conditions. In the mid-range concentration reactions (37 µM) Nevirapine was found to be resistant to degradation at the levels of chlorine typically used in WWTWs with up to 50% of the Nevirapine remaining intact after 24 hours when treated with a 5 fold molar excess of NaOCl. The concentration of Nevirapine in the samples were however much higher than what could be expected to be found

in the environment. The reactions were found to occur fastest at pH 8, although all reactions occurred rapidly and stabilised after one minute. It is also important to note that these experiments were carried out in a buffer with no other organic components.

As is the case with the chlorination of many pharmaceuticals, the kinetics of the Nevirapine reaction are first order with respect to each reactant, with second order overall (Deborde and von Gunten, 2008). Low Nevirapine (2 µM) concentrations treated with 10 fold free available chlorine (FAC) were utilised to simulate realistic environmental conditions and determine reaction kinetics. The reactions started within 10 seconds (the earliest measurement) and proceeded to completion with the assumption that free available chlorine (FAC) was in excess. The reaction of Nevirapine (NVP) with NaOCl as FAC can be described by the following equations,

$$\frac{\mathrm{d[NVP]}}{\mathrm{d}t} = -\kappa[\mathrm{FAC}][\mathrm{NVP}] \qquad (1)$$

$$\frac{\mathrm{d[NVP]}}{\mathrm{d}t} = -\kappa_{obs}.[\mathrm{NVP}] \qquad (2)$$

$$\ln\left(\frac{[\mathrm{NVP}]_t}{[\mathrm{NVP}]_0}\right) = -\kappa_{obs}.t \qquad (3)$$

where [NVP] is the total concentration of Nevirapine and [FAC] is the concentration of free available chlorine (in excess). κ is the second-order rate constant and the observed pseudo first-order rate constant is κ_{obs}, which was calculated based the slope of the linear component of the graph of equation (3); with $\kappa_{obs} = \kappa[\mathrm{FAC}]$ and $[\mathrm{FAC}] = [\mathrm{FAC}]_0$. The rates at various pH conditions at NaOCl concentrations similar to WWTW are shown in Table 1 as lower limit second order rate constants, as the reactions occurred too rapidly to measure in a batch format.

Table 1: Pseudo first order κ_{obs} and lower limit second-order Rate Constants, κ (M-1.s-1), for nevirapine (2 µM) reacted with 20 µM free available chlorine from NaOCl with phosphate buffer (pH 5.8 or 8) and without buffer.

Sample	Pseudo first order κ_{obs}	Second-order Rate Constants, κ ($M^{-1}.s^{-1}$), 20 µM FAC
pH 5.8*	0.0008	4.0×10^{1}
pH 8	0.0204	1.02×10^{3}
Unbuffered	0.1175	5.88×10^{3}

* The Nevirapine is not consumed entirely in this reaction.

The reaction occurred most rapidly in the unbuffered reactions, followed by reactions at basic pH, in acidic buffer and in WWTW effluent respectively. In the basic reaction the Nevirapine was completely consumed after 90 s, whereas in the acidic reaction the Nevirapine consumption ceases at 110 s, with up to 80 % Nevirapine remaining (Figure S3). The unbuffered reaction proceeds most rapidly, with total Nevirapine consumption. This is most likely due to the fact that the continued reactivity of the transformation products is unencumbered by the buffer and its resulting effect on compound speciation.

The wastewater matrix composition can greatly affect the efficacy of pharmaceutical transformation due to the chlorine demand exerted by dissolved organic molecules, nitrites and ammonia (Lee and von Gunten, 2010). During wastewater treatment, Nevirapine degradation would most likely be even less effective due to the increased chlorine demand caused by these molecules in the wastewater. Where, wastewater was used as a reaction matrix for kinetics studies, it was found that 95 % of the Nevirapine remained intact when treated with a 10 fold molar excess of chlorine. A second order rate constant could not be determined as the chlorine did not remain in excess due to the demand placed on it by dissolved organic matter,

pH was found to significantly affect the extent of Nevirapine degradation in all three reaction formats tested in this work, with greater reactivity seen at a high pH than in acidic reactions. The OCl^- species dominates at a basic pH and is a weaker oxidizer than HOCl. The increased reactivity of Nevirapine when OCl^- is predominant may therefore be as a result of the speciation (i.e. anionic, cationic or neutral) of Nevirapine.

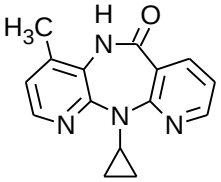
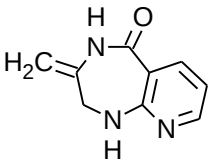
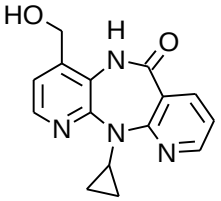
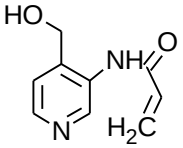
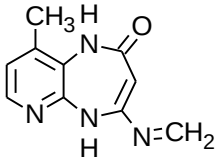
During wastewater treatment, chlorine is utilised as a disinfectant; and optimal disinfection is achieved when the HOCl species dominates (at low pH). As we have demonstrated these are the exact opposite conditions required for effective Nevirapine removal. The concept of reactivity related to pH is further confounded, as it was found that some compounds are effectively removed at low pH (e.g. sulfamethoxazole) while others (e.g. ciprofloxacin) are better removed at a high pH where the reaction is dependent on reagent speciation (Li and Zhang, 2012).These data provide credence to earlier research in that Nevirapine is one of the most ubiquitously occurring ARVs in the environment (Wood et al., 2015).

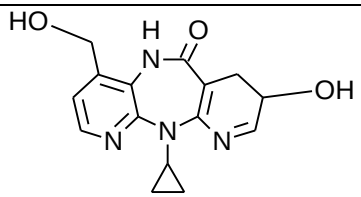
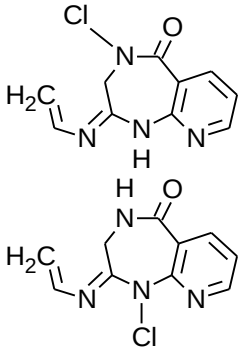
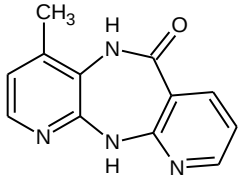
3.2 LC-qTOF Analysis

Accurate masses generated from analyses of various chlorination reactions were used to generate formulae for each mass. Formulae were then used to propose theoretical structures for the most prominent reaction products using the Nevirapine skeleton as a starting point (Table 2).

MS/MS spectra for each of these structures were then compared to theoretical mass spectra generated using Molecular Structure Correlator (MSC). When multiple proposed structures existed for a compound, MSC scores were utilised to discriminate between candidates. Only structures with scores greater than 98% (intact) and 90 % (for fragments) were accepted for further consideration.

Table 2: Accurate mass measurements and proposed structures for the most prominent chlorination reaction products of Nevirapine. Theoretical structures were compared to MS/MS data using Agilent Molecular structure Correlator.

Accurate Mass Measurement of the Pseudomolecular Ion and Proposed Formula	**Proposed Structures**
Nevirapine (267.1254) $C_{15}H_{14}N_4O$	
176.0808 $C_9H_{10}N_3O$	 
283.1185 $C_{15}H_{14}N_4O_2$	
179.0807 $C_9H_{10}N_2O_2$	
203.0921 $C_{10}H_{10}N_4O$	

301.1284 $C_{15}H_{16}N_4O_3$	
237.0538 $C_{10}H_9ClN_4O$	
226.0834 $C_{12}H_{10}N_4O$	

Data from the large scale as well as the 1 minute chlorination reactions were analysed to identify abundant and unique chemical entities (not present in the system or controls). The compounds' masses, retention times, MS/MS spectra and proposed formulae were collated into a searchable database containing 42 putative compounds (Table S1), using PCDL Manager (Agilent). It was found that several molecules yielding highly similar accurate mass measurements existed that were only distinguishable by their retention times. These are most likely stereoisomers as it was found that a variety of potential nevirapine reaction products share the same mass.

Although the chlorination of Nevirapine at chlorine levels similar to those used in WWTWs yielded a wide variety of major and minor reaction products, certain well described functional groups within the molecule may be used to predict transformation. Nevirapine contains a tertiary

amine, a moiety which reacts with chlorine as described in various studies (Prütz, 1998; Mitch and Schreiber, 2008; Shah et al., 2011; Selbes et al., 2012). Using these models the loss of cyclopropane as seen in a number of the DTPs may be explained (Figure 2). Unfortunately though, this may not be applied to all such compounds, as highlighted by Deborde and von Gunten (2008) in which Ciprofloxacin and Enrofloxacin (both contain a similar cyclopropane moeity) react preferentially with chlorine at another amine.

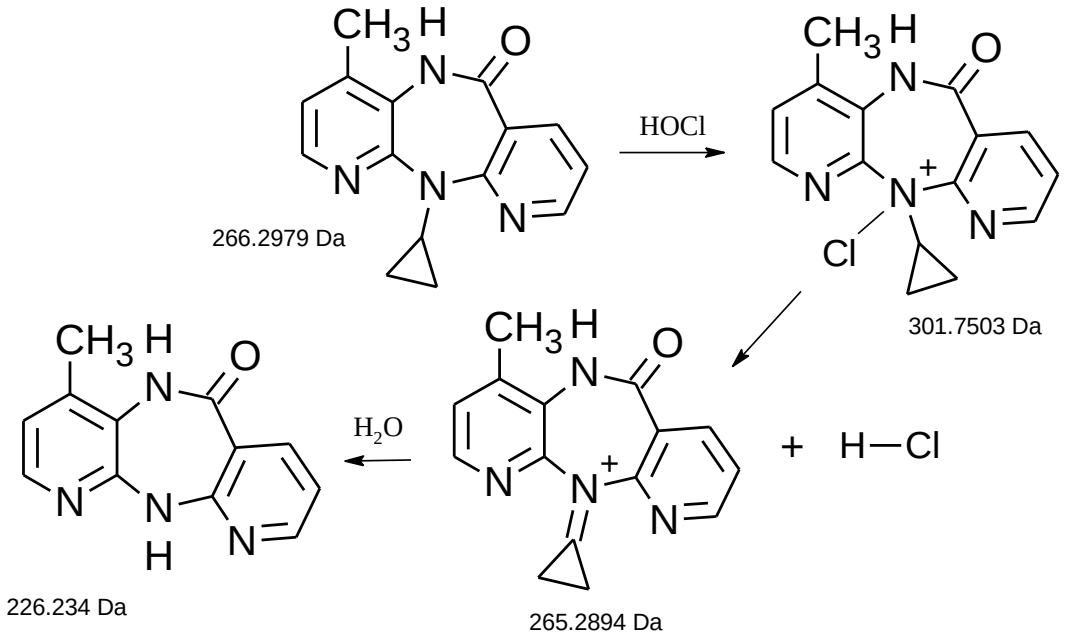


Figure 2: Proposed scheme for the reaction of the Nevirapine tertiary amine during chlorination.

The molecule *m/z* 226 was detected a number of times in the analyses (of the chlorination reactions) but since it is one of the major MS/MS and CID fragmentation products of Nevirapine and its transformation products, its presence as a standalone molecule could not be confirmed with confidence in environmental samples, i.e. it may be a mass spectrometric fragmentation product and not an intact molecule.

3.3 Detection of Nevirapine and its chlorination products in the environment

The SPE technique utilised here was chosen for its universality. Unfortunately limits of detection and quantitation for the method could not be performed as reaction mixtures consisting of multiple components were used as standards. However, this work is largely qualitative with a lower limit of detection defined as an amount providing a signal to noise ratio of 3.

Samples from every major river and water body in South Africa (Table S2) were screened against a database of the most ubiquitous Nevirapine chlorination reaction products. From these data (Table S3), positive identification in the environment was only accepted if the mass of the pseudomolecular ion, the retention time and MS/MS spectra matched to the compound present in the *in vitro* chlorination reactions. As anticipated, the compounds were only detected in samples taken close to WWTWs in dense human settlements.

Full scan mass spectra provided a wealth of information regarding a particular sample and intense ions were automatically fragmented, in a separate injection, to provide MS/MS data. Fragmentation energy was set automatically based on the precursor's mass. Many of the

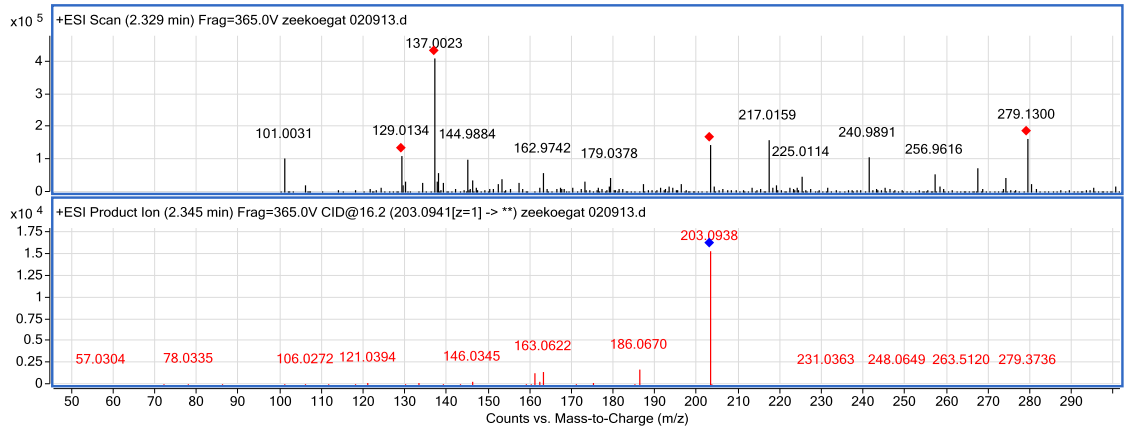


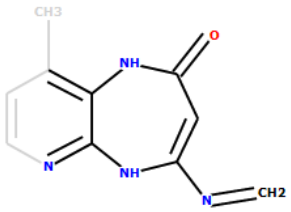
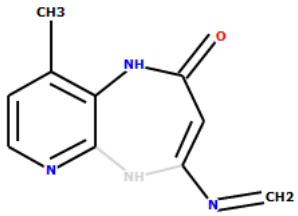
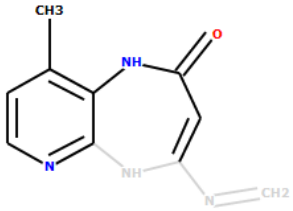
Figure 3: Full scan ESI spectrum (top) and ESI auto-MS/MS spectrum of *m/z* 203.0941+- 0.01 (bottom) of an SPE extract of water taken from the Zeekoegat WWTW discharge, analysed by UHPLC-QTOF (retention time: 2.239 minutes). Red squares (top) indicate ions automatically chosen for MS/MS and the blue square (bottom) indicates the precursor ion, fragmented at 16.2 eV.

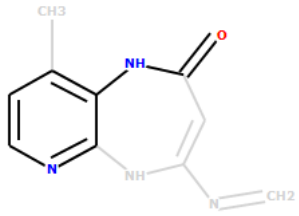
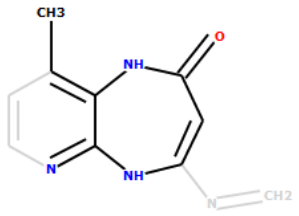
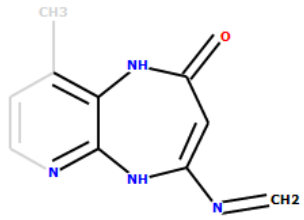
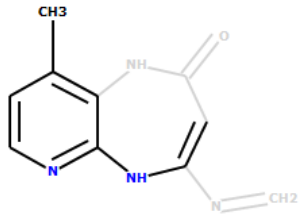
prominent chlorination products were detected in the environment. As an example, at the Zeekoegat sampling site, the compound with the *m/z* 203.0938 was detected (Figure 3).

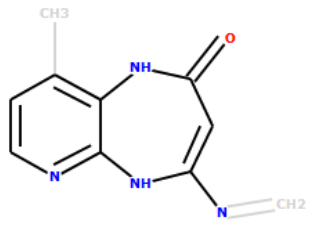
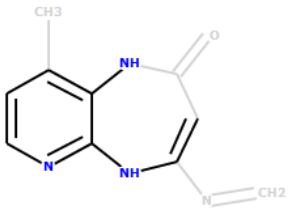
The QTOF operates in “auto MS/MS” mode in which intense ions are selected during full scan mode for MS/MS fragmentation. The instrument selects the collision energy to use on a case by case basis depending on the particular ion’s mass. Using this approach accurate mass measurements of the intact species as well as the resulting fragments (and their ratios) may be utilised to compare samples to analytical standards; or even generate structures by interpretation.

The MS/MS spectrum for this compound was predicted by the Molecular Structure Correlator (Agilent) software (Table 3). Although the software assumes that the pseudomolecular ion is fragmented fully, when proposing the ions’ nominal intensities, the ratio between the fragment ions agrees with the measured standard (Figure 4). Similarly, for this compound, and many like it, the ion ratios measured in the environment, match the “standards” generated in the laboratory.

Table 3: Measured mass spectrum compared to theoretical fragmentation generated for the MS/MS of *m/z* 203.0941 by Molecular Structure Correlator (Agilent) with the difference between measured and proposed masses and structures (grey text indicates fragmentation); for the 10 most intense ions.

Measured *m/z*	**Nominal Intensity**	**Proposed Formula**	**Mass difference (ppm)**	**Proposed Structure**
163.0615	100	$C_7H_7N_4O$	-0.4	
186.0664	83.11	$C_{10}H_8N_3O$	-1.1	
161.071	83.09	$C_9H_9N_2O$	-0.4	

121.0393	28.88	$C_6H_5N_2O$	2.8	
146.0353	22.62	$C_7H_4N_3O$	-2.8	
162.0536	16.33	$C_7H_6N_4O$	0.1	
133.0756	11.41	$C_8H_9N_2$	3.2	

175.0609	7.87	$C_8H_7N_4O$	3.1	
130.0397	7.53	$C_7H_4N_3$	2.1	
93.0441	5.26	$C_5H_5N_2$	6.7	

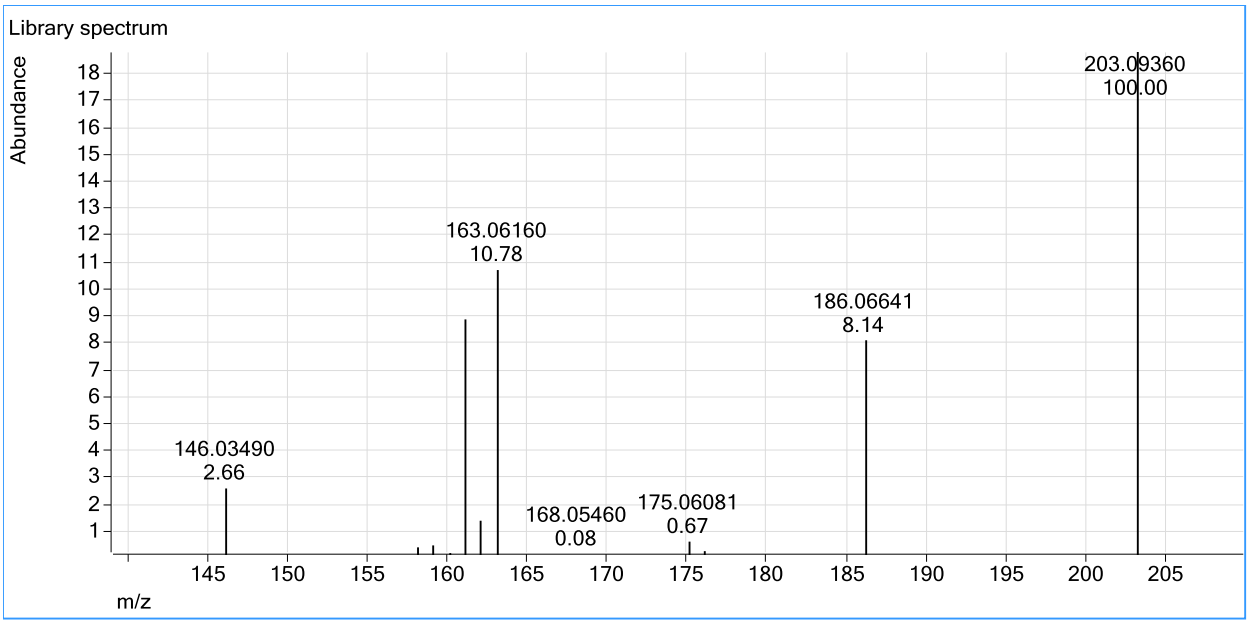


Figure 4: ESI auto-MS/MS spectrum of *m/z* 203.0941+-0.01, from a 1 minute chlorination reaction of Nevirapine, pH 8, 200 µM NaOCl; analysed by UHPLC-QTOF. The "standard" spectrum was exported from PCDL Manager (Agilent).

It was found, and it is self-evident, that the DTPs are more prevalent in highly populated areas that are near WWTWs. The prevalence of these compounds could also be related to Nevirapine concentration, in that samples that contained lower levels of Nevirapine were found to not contain detectable amounts of the DTPs. The majority of the DTPs were detected in the Roodeplaat system (Table 4), which is influenced by two of the three WWTWs that were sampled in this research. The environmental prevalence (Table S3), is therefore relatively low, compared to the ubiquitous distribution of the parent molecule (30 locations across South Africa were sampled and it was found that Nevirapine is detectable at the majority of the sites).

The concept of the minimal criteria required for identification of a compound by mass spectrometry is a widely discussed topic, as inter-laboratory LC-MS comparison is not always fully possible. This is because variability in instrumentation type and conditions would lead to

Table 4: An excerpt of Nevirapine chlorination products detected by positive ESI UHPLC-QTOF analysis of SPE extracts of surface water samples from the Roodeplaat Dam.

Sampling Location (GPS Coordinates)	*m/z*	RT	RT Diff (Tgt)*	Diff (DB, mDa)**	Mass	Proposed Formula***
Roodeplaat Dam Outflow (-25.608244, 28.367231)	160.0854	3.222	0.006	0.45	159.0783	$C_9 H_9 N_3$
Roodeplaat Dam (-25.623345, 28.349842)	160.087	3.18	-0.036	-0.84	159.0795	$C_9 H_9 N_3$
Roodeplaat Dam (-25.626404, 28.345692)	160.0855	3.238	0.022	0.9	159.0778	$C_9 H_9 N_3$
Roodeplaat Dam Outflow (-25.608244, 28.367231)	160.0858	3.225	0.009	1.4	159.0773	$C_9 H_9 N_3$
Pienaars River (-25.678677, 28.357116)	188.0825	4.662	-0.061	-0.49	187.0756	$C_{10} H_9 N_3 O$
Roodeplaat Dam (-25.618238, 28.358642)	188.0822	4.673	-0.05	-1.69	187.0768	$C_{10} H_9 N_3 O$
Roodeplaat Dam (-25.618238, 28.358642)	188.0823	4.656	-0.067	-1.65	187.0767	$C_{10} H_9 N_3 O$
Pienaars River (-25.678677, 28.357116)	317.0813	7.234	-0.017	-1.13	316.0742	$C_{15} H_{13} Cl N_4 O_2$
Roodeplaat Dam (-25.618238, 28.358642)	203.0922	2.288	-0.007	1.15	202.084	$C_{10} H_{10} N_4 O$
Roodeplaat Dam (-25.626404, 28.345692)	351.1577	5.312	-0.019	1.62	175.0737	$C_9 H_9 N_3 O$
Zeekoegat WWTW Outflow (-25.624620, 28.341890)	319.1144	9.616	-0.026	2.42	296.1254	$C_{16} H_{16} N_4 O_2$
Roodeplaat Dam (-25.626404, 28.345692)	319.1142	9.632	-0.009	2.63	296.1252	$C_{16} H_{16} N_4 O_2$

* RT Diff (Tgt) - The difference between the measured retention time and that of the standard (minutes)

** Diff (DB, mDa) The difference between the measured mass and that of the true mass of the "standard"

*** Proposed formula, generated by MassHunter Qual (Agilent)

variability of data generated for identical compounds (Rivier, 2003) . Therefore in this work, even though DTP "standards" were not isolated and characterised in pure form and only analysed as a mixture, a positive identification of a DTP in the environment was only accepted

if: retention time matched within 0.1 min, MS/MS spectra matched, the accurate mass difference was no greater than 1 ppm and that the overall MassHunter match factor was greater than 80 % (a value that encompasses all of the aforementioned factors).

It is exceptionally important to bear in mind that the lower molecular weight species described during the chlorination reactions and subsequently found in surface water, may not necessarily originate from the degradation of Nevirapine. This is because the probability of a shared feature between Nevirapine and another molecule increases as the fragment size decreases.

3.4 Large Scale Preparation and Separation of Chlorination Reaction Products

The small-scale chlorination studies could not be scaled-up to maintain the molar ratio between nevirapine and NaOCl, as it would have lead to very high volume reactions; and it was for this reason that the products of the reactions differed significantly. Compounds identified in the small scale reactions were not present in the scaled-up versions and visa-versa. Similarly, a plethora of novel compounds were found in the scaled-up reactions that were not present in the small scale reactions. This phenomenon could be attributed to not only the transience of some of the reaction products, but also the low concentrations of the small scale reactions. This is important since many researchers utilise this small scale *in vitro* approach to simulate an industrial process (wastewater treatment); and it may not provide a true reflection of the myriad of potential reactions that pharmaceuticals undergo in this situation.

A total of 45 and 29 fractions were prepared for the basic and acidic reactions, respectively. From UHPLC-QTOF analysis it was found that each fraction contained multiple compounds. This is due to the high levels of similarity between them, as they all arose from the same parent molecule. Chromatographic separation of these highly similar compounds would therefore prove

to be challenging. Nevirapine was also present in a number of fractions and its presence was borne in mind when attributing antiviral activity to a particular fraction.

Nevirapine, and subsequently the total chlorination reaction was found to be highly insoluble in water and the majority of commonly used laboratory solvents. The varying levels of solubility of the reaction components further confounds chromatographic purification.

3.5 In Vitro Toxicity and Activity

The total and preparative chromatography samples of both acidic and basic chlorination reactions of Nevirapine were subjected to toxicity and activity studies, *in vitro*. The concentration at which 50% of the cells' growth is inhibited (IC_{50}), is inversely proportional to the level of toxicity of a test compound. Thus, a low IC_{50} value is indicative of a high level of toxicity. This is determined by comparing the spectrophotometric absorbance, generated through the measurement of MTS, of the test sample to an untreated control. The ratio is then presented as a percentage to indicate cell survival. The total reactions were much less toxic than Nevirapine (Table 5) and none of the preparative fractions were found to be more toxic than the parent molecule in 293T cells.

Table 5: Inhibitory concentrations (µg/mL) at which 50% of 293T cells *in vitro* are killed, as determined by MTS assay. The total chlorination reactions of Nevirapine (in basic or acidic phosphate buffer; pH 8 or 5.8 respectively) as compared to a Nevirapine control. Standard deviation presented in brackets.

	Average IC_{50} (µg/mL)
Nevirapine basic reaction	73.7 (0.4)
Nevirapine acid reaction	34.1 (2.1)
Nevirapine control	0.03 (0.01)

That is not to say however that the compounds would not produce toxicity in another fashion (e.g. hepatotoxicity, carcinogenicity etc.) or exhibit novel aspects environmental toxicity. This should be determined by further *in vitro* studies. But, this provides a heartening indication that the chlorination products do not represent yet another anthropogenic source of toxicity that is being discharged into the environment.

The inhibition of viral replication was determined in a single cycle of infection. Virus-like particles were used that contain HIV-1 subtype C reverse transcriptase, integrase and protease, as well as the RNA transcript of the firefly luciferase protein. Once the virus infects the cell, the firefly RNA is reverse transcribed by the HIV-1 reverse transcriptase to a complementary DNA (cDNA) and integrated into the host cell's chromosomal DNA by HIV-1 integrase. Upon integration, the firefly luciferase gene is expressed to produce active firefly luciferase that can quantified by measuring its bioluminescence. In the absence of inhibitors, this signal is directly proportional to the number of infectious virus particles present in the initial inoculum. Since inhibitors (e.g. DTPs) decrease the number of firefly luciferase gene copies that are integrated into the host's genome, a decrease in the amount of bioluminescence will also be observed. The

bioluminescence ratio between an exposed sample and an unexposed control is presented as a percentage to indicate the effect of a test compound on viral activity.

During antiviral activity studies a number of the preparative fractions (Tables S4 and S5) showed antiviral activity. The majority of these however can be attributed to the presence of intact Nevirapine. This is because many of the novel compounds share structural similarity with Nevirapine and because of Nevirapine's insolubility, they could not be separated effectively by preparative chromatography. This could be circumvented by comparing the ratio of the UHPLC-QTOF extracted ion (*m/z* 267.1) peak area to the antiviral activity intensity (Figure S4) between the fractions. The fractions that had antiviral properties not attributed to Nevirapine could then be identified as outliers (i.e. an increase in antiviral activity not associated with an equivalent increase in Nevirapine concentration).

Since many of these compounds either share the Nevirapine "backbone" or are only slightly modified, it is reasonable to speculate that they would also share Nevirapine's structure activity relationship. Nevirapine displayed an IC_{50} value of 0.03 µg/mL, which is substantially lower than the levels at which the total chlorination reaction showed antiviral properties. The various purified fractions however had wide ranging IC_{50} values (0.02 to 20 µg/mL). Higher values may be due to reduced activity properties in the particular molecules or due to the fact that the compounds were not tested in pure form; thereby providing aberrant compound mass-to-activity results

Once these compounds are fully characterised by mass spectrometry (e.g. MS^n studies) and Nuclear Magnetic Resonance (NMR), they may be synthesised in their pure form. This will lead to a deeper understanding of their mechanism of action.

4. Conclusion

From kinetics studies, where chlorine was in excess, it was found that Nevirapine would not be degraded effectively during wastewater treatment because of: increased chlorine demand by dissolved organic matter and reduced degradation at the acidic pH used in wastewater treatment. The latter is most likely due to the speciation of the Nevirapine molecule. This serves to explain the ubiquitous environmental prevalence in South African surface water, as shown in earlier research (Wood et al., 2015). Although the molecule is relatively persistent, in this work it was shown that Nevirapine is still subject to modification by chlorination, producing a number of DTPs, and degrades entirely at a basic pH.

Through a UHPLC-QTOF analysis of South African surface water it was shown that the DTPs described from *in vitro* analysis are present in the environment. But, the national prevalence of these compounds is exceptionally low since the parent molecule is only found in trace amounts. This is because only a small proportion of an already low concentration of Nevirapine reacts to form these compounds and the distribution is further limited due to the dilution of WWTW discharge.

The chlorination reaction of Nevirapine was scaled up in order to isolate the DTPs identified in nature and in the small-scale stirred reactions; yet it was found that the scale of the reaction affects the nature of the reaction products. This serves to highlight that laboratory scale chlorination may not always be truly representative of industrial wastewater treatment, in that compounds identified in laboratory-scale reactions may not be present in wastewater purification scale reactions and vice versa. This is largely due to concentration differences between the two as well as the addition of a number of uncontrollable variables (e.g. reactive chemicals) in wastewater. With that said though, selected compounds that were identified in the

small scale reactions (lower total concentration of both Nevirapine and NaOCl) were incorporated into a database and subsequently detected in WWTW effluent.

Through *in vitro* toxicity and activity testing it was found that none of the nevirapine DTPs are more toxic than the parent molecule. It was however also found that antiviral activity is retained in some of the isolated fractions. Whether this can be attributed to a single molecule or through synergistic effects will only be clarified once individual DTPs are isolated or synthesized.

It is important to consider the fact that pharmaceuticals may be modified and still retain biological activity. It is therefore clear that we need to consider the total impact of not only discharging pharmaceuticals but also their reaction products into the environment. The wastewater treatment process should be scrutinized to not only remove pharmaceuticals through chemical degradation but also their resulting byproducts. Various authors have noted that pharmaceuticals may form biologically active transformation products during wastewater purification (Dodd et al., 2009; Escher and Fenner, 2011; Mestankova et al., 2012; Keen and Linden, 2013). Further investigation into the purification and characterization of the reaction products identified in this work is required in order to obtain a clear picture of how the disinfection of these types of compounds affect the environment.

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Supplementary Information

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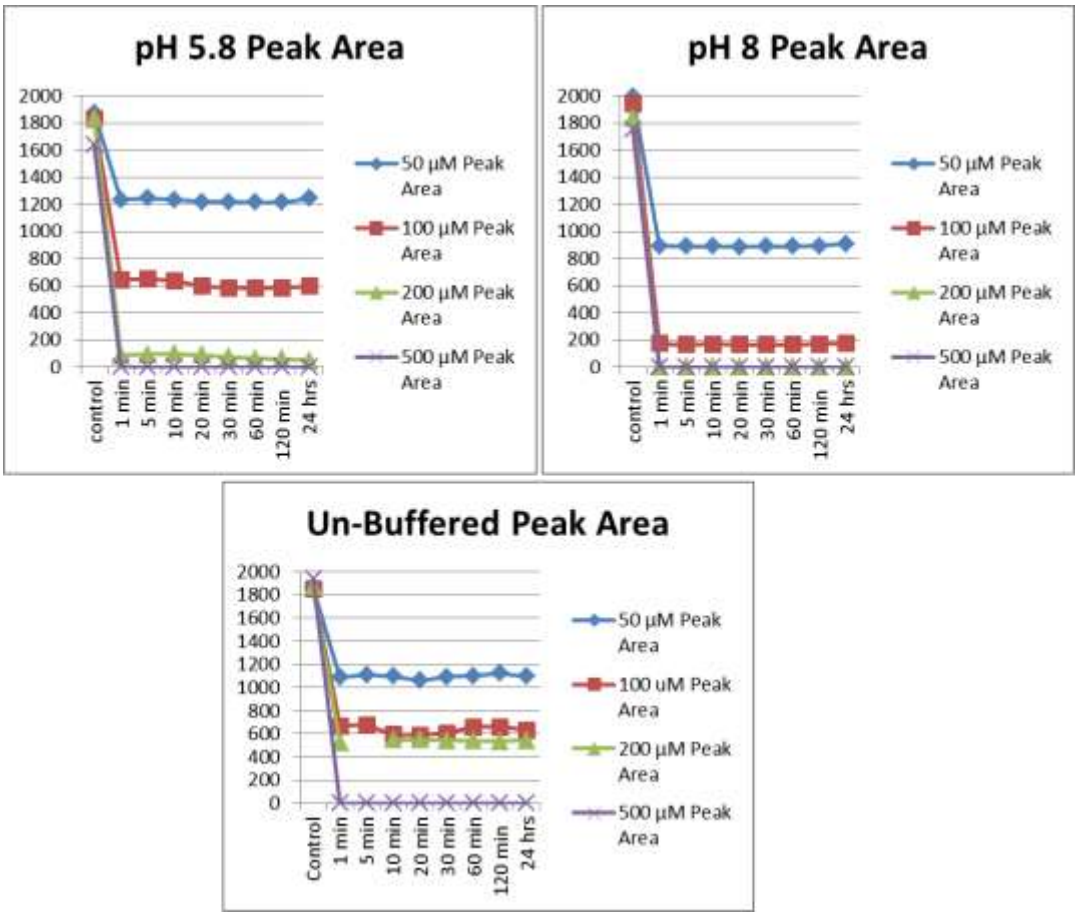

Figure S2: The pseudomolecular ion (m/z 267) peak area of Nevirapine (37 µM) reacted with varying NaOCl over time at pH 5.8 or pH 8 in 10 mM phosphate buffer or un-buffered, analysed by LC-QqQ.

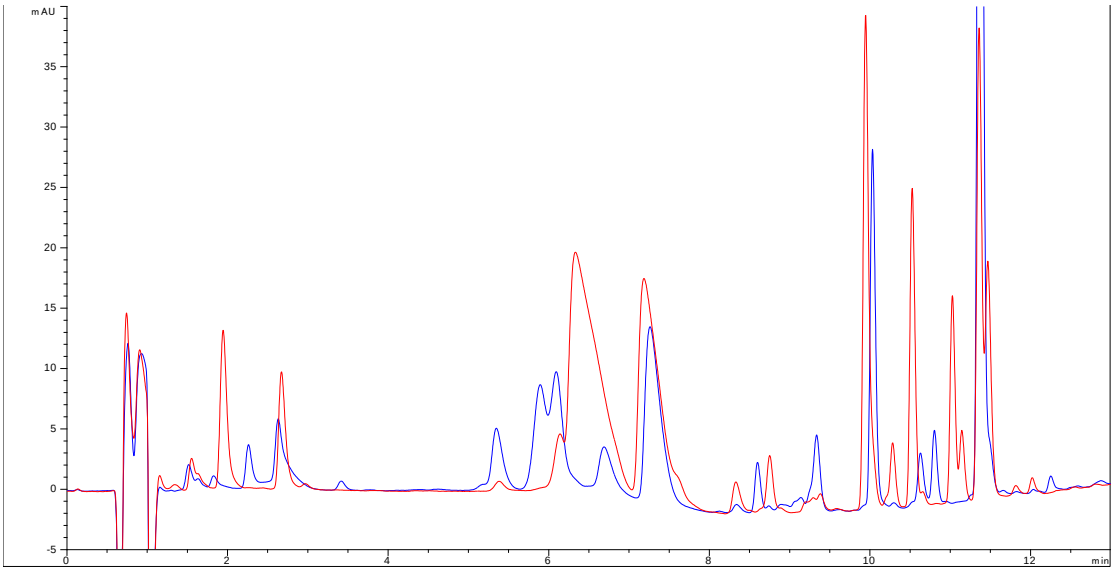

Figure S3: Overlaid LC-UV trace (254 nm) of Nevirapine reacted with 100 µM NaOCl at pH 8 (blue trace) and pH 5.8 (red trace) in 10 mM phosphate buffer after 24 hours.

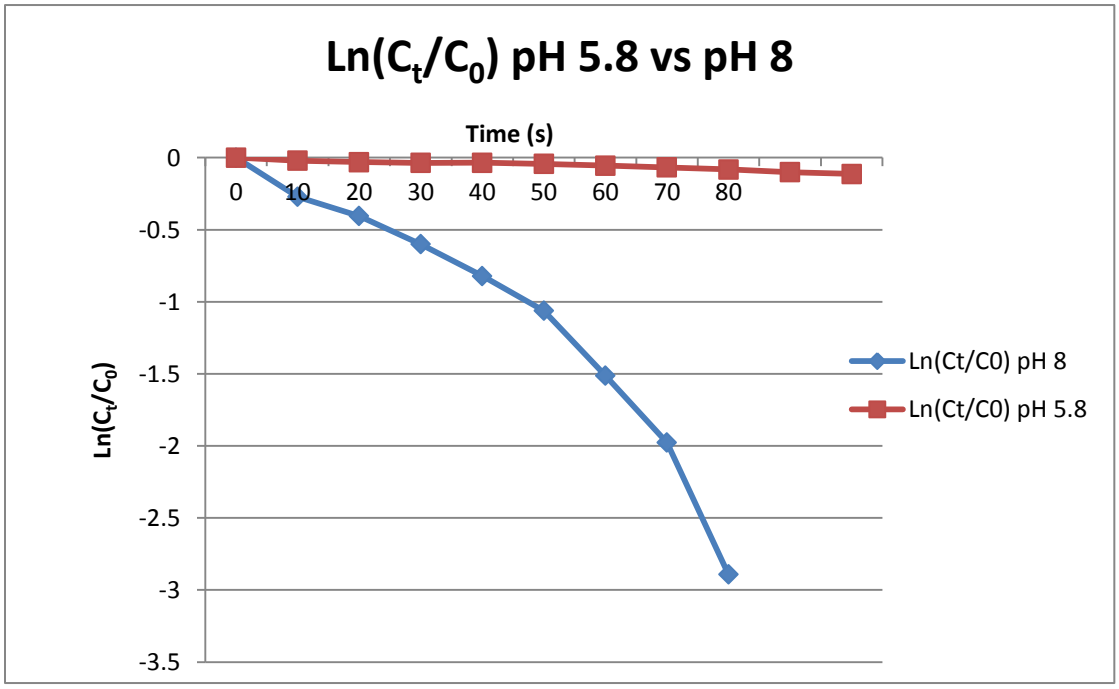

Figure S4: Ln(Ct/C0) over time for kinetic measurements of Nevirapine (2 µM) degradation at pH 5.8 or 8 when chlorine is at a 10 fold excess (20 µM).

Table S6: The most abundant unique entities identified from the Nevirapine chlorination reactions with proposed formulae, accurate mass, retention time (RT) and MS/MS fragment ions collated into a searcheable database with Agilent PCDL Manager.

Compound Name	Proposed Formula*	Measured Mass	RT (min)	MS/MS Fragment Ions
1	-	121.9176	0.442	
2	$C_9H_9N_3$	159.0787	3.216	120.0547, 97.9662, 56.9407
3	$C9H_8N_2O$	160.0665	0.663	133.0762, 78.0338, 118.0638, 121.0403
4	$C_9H_9N_3O$	175.0753	5.331	136.0503, 135.0425, 148.0509, 116.9766, 120.0565
5	$C_9H_{10}N_2O_2$	178.0742	1.211	136.9316, 118.9201, 56.9641
6	$C_{10}H_9N_3O$	187.0751	4.723	148.0505, 171.0556, 133.0751, 93.0435
7	$C_{11}H_8N_2O_2$	200.0593	5.588	80.0491, 122.023, 183.0555
8	$C_{11}H_8N_2O_2$	200.0596	6.304	122.0236, 183.0550, 186.0431, 173.0721
9	$C_{10}H_{10}N_4O$	202.0851	2.295	163.0616, 186.0664, 161.0711, 121.0391
10	$C_{10}H_9N_3O_2$	203.0704	5.698	161.0708, 204.0760, 133.0755, 78.0330, 118.06490
11	-	205.8791	0.442	-
12	-	207.8773	0.44	-
13	-	211.8456	0.468	-
14	$C_{10}H_{10}N_4O_2$	218.0828	0.631	161.0468, 219.0881, 201.0775
Nevirapine	$C_{15}H_{14}N_4O$	266.1168	7.178	226.0858, 107.0605, 161.0712, 198.0906
15	$C_{14}H_{13}N_3O_3$	271.0964	6.226	254.0923, 214.0608, 161.0707, 272.1019, 186.0657
16	$C_{14}H_{13}N_3O_3$	271.0965	6.351	254.0929, 214.0610, 161.0708, 186.0656, 272.1019, 133.0763, 118.0654
17	$C_{15}H_{14}N_4O_2$	282.1117	5.409	-
18	$C_{15}H_{14}N_4O_2$	282.1117	6.206	161.0710, 242.0793, 214.0845, 123.05520
19	$C_{15}H_{14}N_4O_2$	282.1118	6.853	255.1243, 243.0877, 242.0798, 213.0777, 215.0938, 161.0701
20	$C_{16}H_{16}N_4O_2$	296.1278	9.642	282.1106, 297.1339, 267.0869, 137.0705, 256.0960
21	$C^{15}H_{13}N_3O_4$	299.0911	6.498	122.0233, 96.0439, 217.0618, 187.0499, 189.0659
22	$C_{15}H_{16}N_4O_3$	300.1222	5.37	161.0712, 133.0764, 78.0338, 106.0303
23	$C_{15}H_{15}N_3O_4$	301.1058	5.588	122.0235, 94.0280, 98.0601, 96.0446
24	$C_15H_{15}N_3O_4$	301.1061	6.303	-
25	$C_{15}H_{15}N_3O_4$	301.1061	6.305	-
26	-	309.8221	0.523	212.8518, 174.8963, 94.9287
27	$C_{15}H_{14}N_4O_4$	314.1014	6.033	269.1039, 315.1080, 297.0994, 241.1089, 203.0943
28	$C_{15}H_{14}N_4O_4$	314.1018	5.099	161.0710, 133.0756, 118.0660, 78.0326, 105.0456
29	$C_{15}H_{14}N_4O_4$	314.102	6.43	269.1038, 315.1081, 229.0721, 297.0972, 241.1080, 242.0794
30	$C_{15}H_{13}ClN_4O_2$	316.0731	7.251	161.0708, 276.0408, 158.0240, 248.0462, 157.0166
31	$C_{15}H_{15}N_3O_5$	317.1013	6.498	-
32	$C_{19}H_{12}O_5$	320.0676	6.853	208.0979, 252.3384, 321.1343, 92.9652

33	$C_{16}H_{17}N_3O_5$	331.1167	7.565	-
34	$C_{16}H_{12}N_8O$	332.1136	1.501	227.0923, 333.1186, 287.1137, 245.1028, 251.0925, 186.0565
35	-	347.7782	0.49	-
36	$C_{21}H_{14}O_6$	362.0781	5.47	283.1199, 345.0749, 161.0715, 242.0800
37	$C_{21}H_{14}O_6$	362.0785	5.409	-
38	$C_{21}H_{14}O_7$	378.0731	5.134	243.0877, 188.0815, 161.0699, 379.0804, 253.1089
39	-	379.7677	0.452	-
40	-	379.7678	0.454	-
41	$C_{18}H_{12}N_2O_8$	384.0605	5.419	283.1188, 305.0983, 161.0695, 265.1084
42	-	483.7118	0.504	-

* - denotes an entity for which a formula could not be proposed with greater than 90 % certainty and/or cases for which automatic MS/MS did not record spectra for a particular compound.

Table S7: GPS co-ordinates of sampling areas.

Sample Name	**GPS Co-Ordinates**
Roodeplaat Dam System*	
Pienaars River Inflow	-25.678677, 28.357116
Zeekoegat WWTW Outflow	-25.624620, 28.341890
Angling Area	-25.626404, 28.345692
S.E Bank	-25.637763, 28.344150
Motorboat Launch	-25.618238, 28.358642
Rowing Club	-25.623345, 28.349842
Roodeplaat Outflow	-25.608244, 28.367231
Rietvlei Dam*	
Southern Bank	-25.881576, 28.268585
Northern Bank	-25.876767, 28.279846
Orange River System	
Orange River (Bethulie)	-30.534670, 26.022975
Gariep Dam Oviston	-30.692147, 25.761238
Gariep Dam (N.E)	-30.603858, 25.503609
Vaal confluence	-29.070882, 23.637209
Orange confluence	-29.072898, 23.638936
Confluence	-29.071810, 23.635868

Cape Region	
Eerste Rivier*	-33.941603, 18.857078
Theewaterskloof Dam	-34.027283, 19.208261
Vaal Dam	
Dam wall	-26.883278, 28.116047
Oranjeville	-26.999155, 28.214893
Vaal Dam Inflow	-27.020575, 28.608589
Vaal Dam Out Flow	-26.874950, 28.115583
Single system samples	
Hartebeesfontein WWTW Outflow*	-26.030715, 28.291084
Ditholo	-25.320242, 28.340728
Hartbeespoort Dam, Meerhof (2011)*	-25.760775, 27.891871
Hartbeespoort Dam, Meerhof (2014)*	-25.760775, 27.891871
Hartbeespoort Dam, Tap Water Sample*	-25.745594, 27.911238
Hartbeespoort Inflow, Crocodile River	-25.775818, 27.901601
Renosterkop	-25.108639, 28.887359
Inanda Dam	-29.673792, 30.854874
Inanda Dam offshore	-29.674016, 30.860239

* Indicates that the sample was taken in or near an urban environment.

Table S8: Nevirapine and its chlorination products detected by positive ESI UHPLC-QTOF analysis of SPE extracts of South African surface water samples.

GPS	Description	m/z	RT*	RT Diff (DB)**	Diff (DB, mDa)**	Mass	Peak Height	Proposed Formula
Roodeplaat Dam System								
-25.678677, 28.357116	Pienaars River before Roodeplaat Dam	170.0722	4.792	-0.069	-0.32	187.0754	51552	$C_{10} H_9 N_3 O$
-25.678677, 28.357116	Pienaars River before Roodeplaat Dam	188.0824	4.685	0.038	-0.16	187.0753	582284	$C_{10} H_9 N_3 O$
-25.678677, 28.357116	Pienaars River before Roodeplaat Dam	267.1248	7.142	0.036	-0.76	266.1175	3247866	$C_{15} H_{14} N_4 O$ (Nevirapine)
-25.678677, 28.357116	Pienaars River before Roodeplaat Dam	283.1192	6.159	0.047	-0.37	282.1121	1473596	$C_{15} H_{14} N_4 O_2$
-25.678677, 28.357116	Pienaars River before Roodeplaat Dam	317.0807	7.231	0.02	-0.67	316.0738	57884	$C_{15} H_{13} Cl N_4 O_2$
-25.626404, 28.345692	Roodeplaat Angling Area	267.1243	7.132	0.046	-0.22	266.117	1200003	$C_{15} H_{14} N_4 O$ (Nevirapine)
-25.626404, 28.345692	Roodeplaat Angling Area	188.0824	4.683	0.04	-0.23	187.0753	95604	$C_{10} H_9 N_3 O$
-25.626404, 28.345692	Roodeplaat Angling Area	375.1588	4.828	-0.105	-2.38	187.0775	81153	$C_{10} H_9 N_3 O$
-25.624620, 28.341890	Zeekoegat WWTW Outflow	188.0823	4.655	0.068	0.01	187.0751	321806	$C_{10} H_9 N_3 O$
-25.637763, 28.344150	Roodeplaat S.E. Bank	188.0829	4.626	0.097	-0.74	187.0758	99686	$C_{10} H_9 N_3 O$
-25.637763, 28.344150	Roodeplaat S.E. Bank	267.1252	7.153	0.025	-0.39	266.1172	1668882	$C_{15} H_{14} N_4 O$ (Nevirapine)
-25.618238, 28.358642	Roodeplaat Motor Boat Launch	188.0824	4.662	0.061	-0.22	187.0753	126361	$C_{10} H_9 N_3 O$
-25.618238, 28.358643	Roodeplaat Motor Boat Launch	267.1251	7.16	0.018	-1.16	266.1179	1742427	$C_{15} H_{14} N_4 O$ (Nevirapine)
-25.623345, 28.349842	Roodeplaat Rowing Club	188.0825	4.652	0.071	-0.33	187.0754	125822	$C_{10} H_9 N_3 O$
-25.608244, 28.367231	Roodeplaat Dam Outflow	267.125	7.15	0.028	-1.03	266.1178	1492262	$C_{15} H_{14} N_4 O$ (Nevirapine)
Orange-Vaal System								
-30.534670, 26.022975	Orange River (Bethuli)	267.1236	7.133	0.045	0.33	266.1164	21351	$C_{15} H_{14} N_4 O$ (Nevirapine)
-29.071810, 23.635868	Orange-Vaal River Confluence	267.124	7.133	0.045	-0.07	266.1168	46847	$C_{15} H_{14} N_4 O$ (Nevirapine)
-29.070882, 23.637209	Vaal River Confluence	267.1242	7.133	0.045	-0.24	266.117	44894	$C_{15} H_{14} N_4 O$ (Nevirapine)
-30.603858, 25.503609	Gariep Dam N.E Bank	267.1241	7.137	0.041	-0.06	266.1168	50227	$C_{15} H_{14} N_4 O$ (Nevirapine)
-26.999155, 28.214893	Oranjeville Bridge	283.1197	6.399	-0.193	-1.15	282.1128	47585	$C_{15} H_{14} N_4 O_2$
-26.874950, 28.115583	Vaal Dam Outflow	267.1253	7.14	0.038	-1.25	266.118	305071	$C_{15} H_{14} N_4 O$ (Nevirapine)
Hartbeespoort/Crocodile River System								
-25.760775, 27.891871	Hartbeespoort Dam, Meerhof	267.1251	7.134	0.044	-1.24	266.118	790461	$C_{15} H_{14} N_4 O$ (Nevirapine)
-25.775818, 27.901601	Hartbeespoort Inflow, Crocodile River	267.1251	7.087	0.091	-1.13	266.1179	429248	$C_{15} H_{14} N_4 O$ (Nevirapine)
-25.775818, 27.901601	Hartbeespoort Inflow, Crocodile River	283.1196	6.398	-0.192	-0.98	282.1127	560137	$C_{15} H_{14} N_4 O_2$

-25.775818, 27.901601	Hartbeespoort Inflow, Crocodile River	188.0822	4.613	0.11	-0.02	187.0751	81918	C_{10} H_9 N_3 O
Single Samples								
-26.030715, 28.291084	Hartbeesfontein WWTW	267.124	7.139	0.039	-0.08	266.1168	799749	C_{15} H_{14} N_4 O (Nevirapine)
-26.030715, 28.291084	Hartbeesfontein WWTW	283.1187	6.901	-0.048	-0.35	282.1122	36425	C_{15} H_{14} N_4 O_2
-26.030715, 28.291084	Hartbeesfontein WWTW	158.0723	5.175	0.156	0.03	175.0753	2041054	C_9 H_9 N_3 O
-25.881576, 28.268585	Rietvlei Dam	267.1248	7.128	0.05	-0.77	266.1175	744658	C_{15} H_{14} N_4 O (Nevirapine)
-25.881576, 28.268585	Rietvlei Dam	158.0715	5.166	0.165	0.75	175.0745	1145107	C_9 H_9 N_3 O
-29.673792, 30.854874	Inanda Dam Bank	267.1233	7.14	0.038	0.59	266.1162	247851	C_{15} H_{14} N_4 O
-29.674016, 30.860239	Inanda Dam Centre	267.1233	7.125	0.053	0.65	266.1161	319778	C_{15} H_{14} N_4 O

Table S9: The *in vitro* viral activity of the fractions of the basic Nevirapine chlorination reaction. A value of 100% or more indicates that a fraction has no antiviral properties. The concentration at which 50 % of the virus is inhibited (IC_{50}) was calculated from the average viral activity measured after exposure to each fraction at 10 µg/mL.

	Percent virus activity				
NVP Basic Fractions:	**1**	**2**	**Average**	**Standev**	**Calculated IC_{50} (µg/mL)***
Nev. Rxn. FC C1	103.6%	102.4%	**103.0%**	**0.8%**	-
Nev. Rxn. FC C2	97.7%	98.4%	**98.1%**	**0.5%**	19.6
Nev. Rxn. FC C3	60.8%	61.4%	**61.1%**	**0.5%**	12.2
Nev. Rxn. FC C4	4.7%	3.9%	**4.3%**	**0.6%**	0.9
Nev. Rxn. FC C5	4.9%	4.3%	**4.6%**	**0.4%**	0.9
Nev. Rxn. FC C6	8.7%	7.7%	**8.2%**	**0.7%**	1.6
Nev. Rxn. FC C7	14.0%	12.4%	**13.2%**	**1.1%**	2.6
Nev. Rxn. FC C8	18.4%	16.6%	**17.5%**	**1.2%**	3.5
Nev. Rxn. FC C9	8.5%	8.1%	**8.3%**	**0.3%**	1.7
Nev. Rxn. FC C10	26.2%	26.0%	**26.1%**	**0.2%**	5.2
Nev. Rxn. FC C11	29.4%	29.8%	**29.6%**	**0.2%**	5.9
Nev. Rxn. FC C12	36.6%	37.7%	**37.1%**	**0.8%**	7.4
Nev. Rxn. FC C13	12.4%	11.6%	**12.0%**	**0.6%**	2.4
Nev. Rxn. FC C14	2.5%	1.7%	**2.1%**	**0.6%**	0.4
Nev. Rxn. FC C15	4.5%	4.4%	**4.4%**	**0.0%**	0.9
Nev. Rxn. FC C16	21.1%	17.9%	**19.5%**	**2.2%**	3.9
Nev. Rxn. FC C17	38.5%	36.0%	**37.2%**	**1.7%**	7.4
Nev. Rxn. FC C18	45.6%	42.7%	**44.2%**	**2.1%**	8.8
Nev. Rxn. FC C19	42.7%	39.0%	**40.9%**	**2.6%**	8.2
Nev. Rxn. FC C20	29.2%	26.1%	**27.7%**	**2.2%**	5.5
Nev. Rxn. FC C21	29.8%	29.9%	**29.9%**	**0.1%**	6.0
Nev. Rxn. FC C22	50.8%	52.0%	**51.4%**	**0.9%**	10.3
Nev. Rxn. FC C23	59.1%	58.7%	**58.9%**	**0.3%**	11.8
Nev. Rxn. FC C24	50.7%	53.1%	**51.9%**	**1.7%**	10.4
Nev. Rxn. FC C25	71.2%	73.9%	**72.5%**	**1.9%**	14.5
Nev. Rxn. FC C26	89.6%	88.9%	**89.3%**	**0.5%**	17.9

Nev. Rxn. FC C27	89.1%	94.7%	**91.9%**	**4.0%**	18.4
Nev. Rxn. FC C30	83.4%	92.9%	**88.1%**	**6.7%**	17.6
Nev. Rxn. FC C31	89.6%	92.2%	**90.9%**	**1.9%**	18.2
Nev. Rxn. FC C32	91.4%	94.2%	**92.8%**	**2.0%**	18.6
Nev. Rxn. FC C33	100.6%	101.1%	**100.9%**	**0.4%**	-
Nev. Rxn. FC C34	92.2%	93.4%	**92.8%**	**0.9%**	18.6
Nev. Rxn. FC C35	94.9%	99.5%	**97.2%**	**3.2%**	19.4
Nev. Rxn. FC C36	91.3%	98.2%	**94.7%**	**4.9%**	18.9
Nev. Rxn. FC C37	94.3%	94.1%	**94.2%**	**0.1%**	18.8
Nev. Rxn. FC C38	95.5%	95.1%	**95.3%**	**0.2%**	19.1
Nev. Rxn. FC C39	101.4%	95.6%	**98.5%**	**4.1%**	19.7
Nev. Rxn. FC C40	90.2%	91.6%	**90.9%**	**1.0%**	18.2
Nev. Rxn. FC C41	94.4%	96.6%	**95.5%**	**1.6%**	19.1
Nev. Rxn. FC C42	95.8%	100.1%	**97.9%**	**3.0%**	19.6
Nev. Rxn. FC C50	95.2%	88.6%	**91.9%**	**4.7%**	18.4
Nev. Rxn. FC C51	99.1%	89.7%	**94.4%**	**6.7%**	18.9
Nev. Rxn. FC C52	99.5%	88.1%	**93.8%**	**8.0%**	18.8
Nev. Rxn. FC C53	92.4%	88.6%	**90.5%**	**2.7%**	18.1
Nev. Rxn. FC C54	114.8%	113.4%	**114.1%**	**1.0%**	-

* The calculation is based on the assumption that the relationship between antiviral activity and test compound concentration is linear; - where viral activity is ≥ 100 % at 10 µg/mL, the IC_{50} could not be calculated.

Table S10: The in vitro viral activity of the fractions of the acidic Nevirapine chlorination reaction. A value of 100% or more indicates that a fraction has no antiviral properties. The concentration at which 50 % of the virus is inhibited (IC_{50}) was calculated from the average viral activity measured after exposure to each fraction at 10 µg/mL.

	Percent virus activity				
NVP Acidic Fractions:	**1**	**2**	**Average**	**Standev**	**Calculated IC_{50} (µg/mL)***
Nev. Acid Rxn. FC D1	87.5%	84.7%	**86.1%**	**1.9%**	17.2
Nev. Acid Rxn. FC D2	101.1%	98.3%	**99.7%**	**2.0%**	19.9
Nev. Acid Rxn. FC D3	102.9%	105.6%	**104.2%**	**1.9%**	-
Nev. Acid Rxn. FC D4	101.8%	107.1%	**104.5%**	**3.8%**	-
Nev. Acid Rxn. FC D5	100.8%	97.5%	**99.1%**	**2.4%**	19.8
Nev. Acid Rxn. FC D6	97.5%	97.6%	**97.5%**	**0.1%**	19.5
Nev. Acid Rxn. FC D7	96.3%	96.8%	**96.5%**	**0.4%**	19.3
Nev. Acid Rxn. FC D8	100.5%	99.7%	**100.1%**	**0.5%**	-
Nev. Acid Rxn. FC D9	101.8%	98.4%	**100.1%**	**2.4%**	-
Nev. Acid Rxn. FC D11	105.4%	103.1%	**104.2%**	**1.6%**	-
Nev. Acid Rxn. FC D12	96.1%	94.5%	**95.3%**	**1.1%**	19.1
Nev. Acid Rxn. FC D14	102.1%	97.8%	**99.9%**	**3.0%**	20.0
Nev. Acid Rxn. FC D15	103.4%	95.2%	**99.3%**	**5.8%**	19.9
Nev. Acid Rxn. FC D16	100.9%	98.6%	**99.7%**	**1.6%**	19.9
Nev. Acid Rxn. FC D17	104.2%	100.6%	**102.4%**	**2.5%**	-
Nev. Acid Rxn. FC D18	110.7%	104.4%	**107.5%**	**4.4%**	-
Nev. Acid Rxn. FC D19	95.8%	92.9%	**94.4%**	**2.1%**	18.9
Nev. Acid Rxn. FC D20	50.8%	44.9%	**47.8%**	**4.2%**	9.6
Nev. Acid Rxn. FC D21	57.6%	54.3%	**56.0%**	**2.4%**	11.2
Nev. Acid Rxn. FC D22	26.2%	21.6%	**23.9%**	**3.3%**	4.8
Nev. Acid Rxn. FC D23	1.2%	0.9%	**1.1%**	**0.2%**	0.2
Nev. Acid Rxn. FC D24	0.0%	0.1%	**0.1%**	**0.0%**	0.02
Nev. Acid Rxn. FC D25	0.1%	0.1%	**0.1%**	**0.0%**	0.02
Nev. Acid Rxn. FC D26	16.6%	14.8%	**15.7%**	**1.3%**	3.1

Nev. Acid Rxn. FC D27	72.2%	65.1%	**68.7%**	**5.0%**	13.7
Nev. Acid Rxn. FC D28	99.9%	93.0%	**96.5%**	**4.9%**	19.3
Nev. Acid Rxn. FC D29	101.7%	89.6%	**95.7%**	**8.6%**	19.1
Nev. Acid Rxn. FC D30	107.4%	85.6%	**96.5%**	**15.4%**	19.3
Nev. Acid Rxn. FC D34	104.1%	91.1%	**97.6%**	**9.2%**	19.5

* The calculation is based on the assumption that the relationship between antiviral activity and test compound concentration is linear; - where viral activity is ≥ 100 % at 10 µg/mL, the IC_{50} could not be calculated.

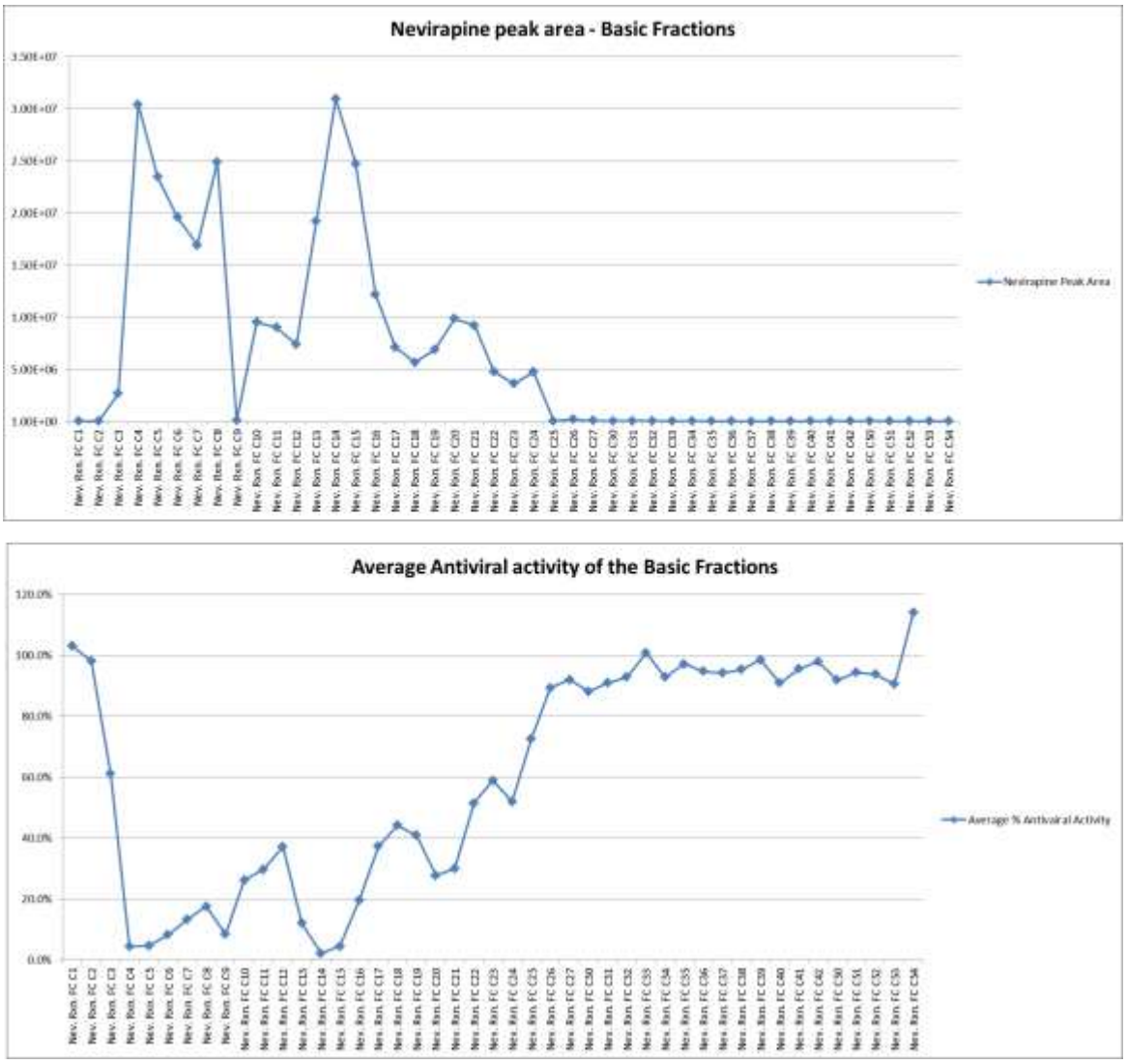

Figure S5: The peak area of the extracted Nevirapine ion analysed by UHPLC-QTOF (top) and the average antiviral activity (bottom) of each of the chlorination reaction preparative chromatography fractions.