

The Occurrence of Anti-Retroviral Compounds used for HIV Treatment in South African Surface Water

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Highlights

- A method for the simultaneous detection of 12 antiretroviral drugs using LC-MS/MS was developed.
- The presence of these compounds in South African surface water is described for the first time.
- Compounds occurred in the low to mid ng/L range, with compounds such as Nevirapine occurring ubiquitously across all the samples tested.
- Matrix effect played a notable role during the analysis of these compounds with a number undergoing signal enhancement; this was corrected for by using a modified standard addition method of quantification

Abstract

The study and quantification of personal care products, such as pharmaceuticals, in surface water has become popular in recent years; yet very little description of these compounds' presence in South African surface water exists in the literature. Antiretrovirals (ARVs), used to treat human immunodeficiency virus (HIV) are rarely considered within this field. A new method for the simultaneous quantification of 12 antiretroviral compounds in surface water using the standard addition method is described. Water samples were concentrated by a generic automated solid phase extraction method and analysed by ultra-high pressure liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). Substantial matrix effect was encountered in the samples and the average instrumental and method limits of detection were 1.2 ng/mL and 90.4 ng/L respectively. This is the first reported countrywide survey of South African surface water for the quantification of these compounds with average concentrations ranging between 5.1 and 431.4 ng/L.

Capsule:

This work represents the first quantitative description of anti-retrovirals, as a group, in surface water using a modified standard addition method and UHPLC-MS/MS.

1. Introduction

Concerns regarding the presence of personal care products (PCPs), such as pharmaceuticals, in water supplies have arisen recently with various researchers showing that a wide variety of pharmaceuticals are discharged into the environment as a result of inadequate wastewater

treatment (Ferrer and Thurman, 2012; Yu et al., 2012; Luo et al., 2014). This appears to be a global phenomenon (Kümmerer, 2009) and besides discharge from waste-water treatment plants (WWTPs) one should also consider alternative sources of contamination such as improper destruction of expired pharmaceutical stocks e.g. leachate from pharmaceutical landfilling (Peng, Ou, et al., 2014) or pit latrines (Graham and Polizzotto, 2013) in developing countries. There is a marked gap in the literature, regarding this global phenomenon, describing the situation in Africa.

Very little research has been carried out in South Africa to determine the presence of pharmaceuticals and their degradation products in surface water using mass spectrometry; as determined by searching the curated "US EPA Bibliographic Database of Publications Relevant to Pharmaceuticals and Personal Care Products" (Daughton and Scuderi, 2012). Also, South Africa utilises more anti-retroviral compounds per capita than any other nation in the fight against HIV/AIDS, with approximately 2 150 880 people receiving ARVs in 2012 (WHO, 2013). This presents a novel problem with regards to the presence and transformation of these compounds in the environment. Since South Africa uses more of these compounds than any other nation it has been theorized that these compounds should be present in the environment to a much greater extent. This phenomenon should also be exacerbated by the overall low rainfall and water scarcity in sub-Saharan Africa; which would lead to lower environmental dilution of the target compounds.

Anti-HIV compounds such as: nucleoside and non-nucleoside reverse transcriptase inhibitors, protease inhibitors, fusion inhibitors, entry inhibitors and integrase strand transfer inhibitors are used to treat HIV (“FDA Antiretroviral drugs used in the treatment of HIV infection,” 2014) and prevent mother-to child transmission (Mofenson, 2010). The breadth of the compound class therefore poses an interesting analytical challenge; and to our knowledge no other research addressing their simultaneous detection, in any environmental matrix, has been carried out.

Prasse and colleagues (2010) studied the presence of five anti-HIV compounds in addition to other anti-virals in the Hessian Reid river systems and found their presence as a result of WWTP discharge. Peng and co-workers (2014) utilised a similar methodology to detect antiviral drugs, including Stavudine and Zidovudine, in the Pearl River Delta in China; but could not detect these compounds in surface water. Given the global usage of these pharmaceuticals and since the compounds have been detected in European surface water and WWTP influent and effluent it is predicted that higher concentrations should be present in South African water supplies due to higher usage in the population. These compounds can be seen as additional candidates for consideration as emerging pollutants.

The main objective of this work was to develop a single LC-MS/MS method for the analysis of 12 commonly used anti-HIV compounds, concentrated by generic solid phase extraction (SPE), in order to for the first time quantitatively determine their prevalence in South African surface

water. This work also represents the first step in a nationwide survey for the detection of pharmaceuticals in surface water.

2. Materials and Methods

2.1 Chemicals and Reagents

Analytical reference standards obtained from the US, British and European Pharmacopoeia as well as Toronto Research Chemicals (Toronto, Canada) were purchased from Anatech (Johannesburg, South Africa). All compounds were of 97% purity or better. Zalcitabine, Tenofovir, Abacavir, Efavirenz, Lamivudine, Didanosine, Stavudine, Zidovudine, Nevirapine, Indinavir, Ritonavir, Lopinavir and caffeine stock solutions (1 mg/mL) were prepared in methanol and stored at -20 °C until use. ¹³C₃-trimethyl caffeine, 100 µg/mL in methanol, was obtained from Cambridge isotope Laboratories (Tewksbury, MA, USA) and diluted to 20 µg/mL in methanol before use. Standards were prepared and handled in a separate room from samples in order to prevent cross contamination. LC-MS grade acetonitrile, methanol and water were purchased from Lab-Scan (Gliwice, Poland) and formic acid from Merck (Johannesburg, South Africa). No South African-origin water was used as a reagent in the course of this research.

2.2 Environmental Sample Collection and Extraction

Grab samples were collected from various surface water sources in South Africa. Sampling locations were selected based on their proximity to wastewater treatment plants (WWTPs) and the distance from major bodies of water. Samples were collected in “virgin” borosilicate Schott

bottles while wearing nitrile gloves (to prevent the introduction of contaminants) and transported, protected from light, to the laboratory at room temperature. Samples were stored at -20°C until extraction after which extracts were stored at -20°C until analysis.

500 mL of each sample was filtered using a 1 µm glass-fibre syringe-driven filter (Pall, USA) and extracted using the Smart Prep Extraction System (Horizon, USA); an automated offline solid phase extraction instrument. The extraction procedure was modified from a method developed by Ferrer and Thurman in order to maintain a level of universality (Ferrer and Thurman, 2012). Briefly, 6 cc Oasis HLB, 500 mg SPE cartridges (Waters, Milford, MA, USA) were conditioned with 4 mL of methanol followed by 6 mL of HPLC-grade water. 500 mL of sample was introduced at a flow rate of 10 mL/min after which cartridges were dried under nitrogen for three minutes. Cartridges were eluted twice with 5 mL of methanol and the eluate dried under a gentle stream of nitrogen to 500 µL. All extractions were performed at 18 °C (+/- 0.5 °C) in a dedicated area.

The standard addition method was used to quantify all target analytes, with modification from the traditional approach (Conley et al., 2008). Each extracted sample (190 µL) was combined with 10 µL ¹³C₃-caffeine standard and divided into four aliquots of 45 µL each. To these, 5 µL of either 10 000 ng/mL, 1000 ng/mL, 100 ng/mL or 0 ng/mL standard mixture in methanol was added. The samples were analysed in triplicate in order of increasing concentration, with blank injections between each in order to prevent and evaluate carry-over. Standard addition data was analysed using Mass Hunter Quant (Agilent, Santa Clara, USA). The

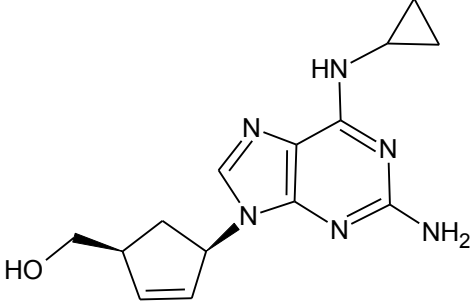
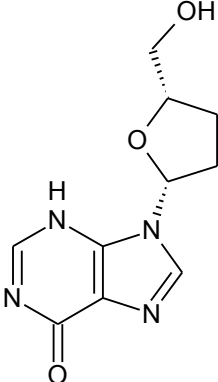
data obtained from these analyses were compared to an external calibration curve, generated by injecting a mixture of standards in methanol at 1, 10, 100 and 1000 ng/mL, in order to assess the effects of the matrix. Retention time reproducibility was checked periodically by injecting a standard mixture after every batch analysis.

2.3 LC-MS/MS Analysis

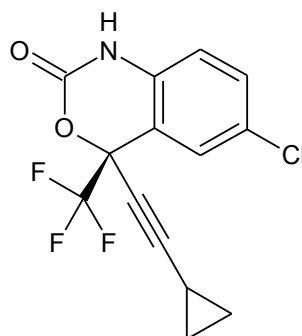
SPE extracts were analysed by LC-ESI-MS/MS. Target compounds were separated using an Agilent 1290 series UHPLC and mobile phases consisted of water (A) and acetonitrile (B) both with 0.1% formic acid. Following a 15 μ L injection onto a Zorbax Eclipse C8 XDB, 3.0x50mm, 1.8 μ m column the chromatographic gradient was executed at: 0% B, 3min; 100% B 20 min; 100% B 25 min; 0% B 30 min; 0% B 40 min. A flow rate of 0.4 mL/min was used and the column was maintained at 22 °C with no column effluent splitting. The UHPLC was coupled to an Agilent 6460 triple quadrupole, equipped with a Jet Stream electrospray ionization (ESI) source. All analyses were performed in positive ion mode.

MS/MS optimisation was performed automatically using the Agilent Optimizer software package (Table 1). These settings were then combined into a single dynamic MRM method with the following ESI Jet Stream source conditions: Delta EMV 400 V, gas temperature 250 °C, gas flow 8 L/min, nebuliser pressure 35 psi, sheath gas temperature 300 °C, sheath gas flow 10 L/min and capillary voltage 3000 V.

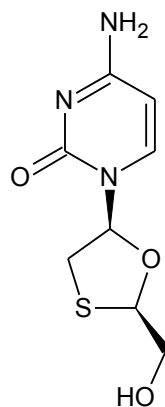
Table 1: Compound names, CAS numbers, precursor ions, product ions, collision energies, fragmentor voltages and structures of target pharmaceutical analytes.

Name (CAS No.)	m/z Precursor Ion	m/z Product Ion 1 (Collision Energy eV)	m/z Product Ion 2 (Collision Energy eV)	Fragmentor Voltage (V)	Structure
Nucleoside Reverse Transcriptase Inhibitors					
Abacavir (136470-78-5)	287.2	191 (17)	150 (29)	108	
Didanosine (69655-05-6)	237.1	137 (6)	76 (40)	80/76*	

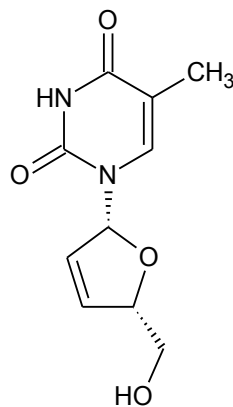
Efavirenz 316 299.1 (0) 237 (8) 80/84*
(154598-52-4)



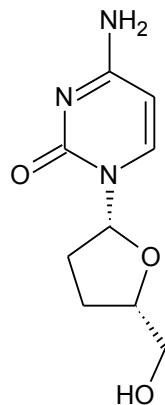
Lamivudine 230.1 112 (8) 95 (42) 144
(134678-17-4)



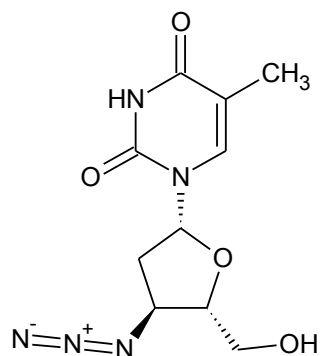
Stavudine 225.1 127 (10) 99 (0) 10
(3056-17-5)



Zalcitabine 212.1 112 (6) 95 (38) 40
(7481-89-2)

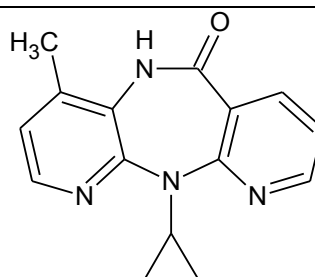


Zidovudine 268.1 127 (8) 110 (32) 76
(30516-87-1)



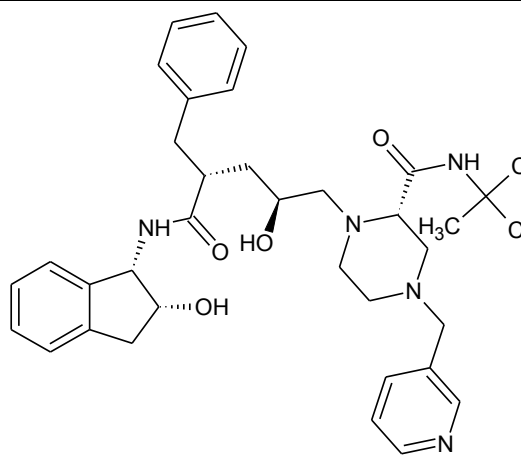
non-nucleoside reverse transcriptase inhibitor (nNRTI)

Nevirapine 267.1 226 (24) 80 (44) 120
(129618-40-2)



Protease inhibitors

Indinavir 614.4 421.3 (32) 97.1 (58) 164/160*
(150378-17-9)



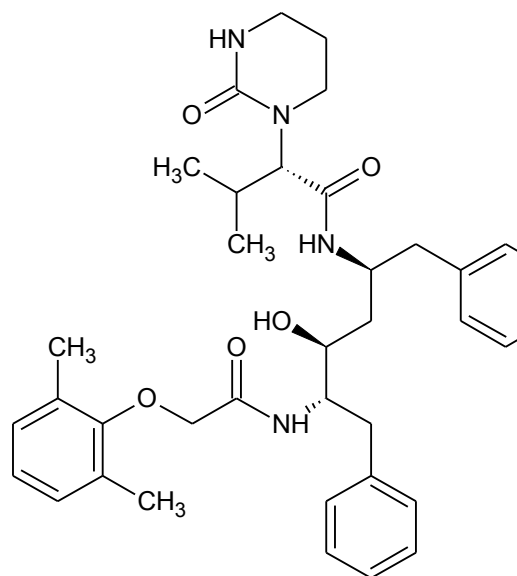
Lopinavir
(192725-17-0)

629

183 (20)

155 (40)

50



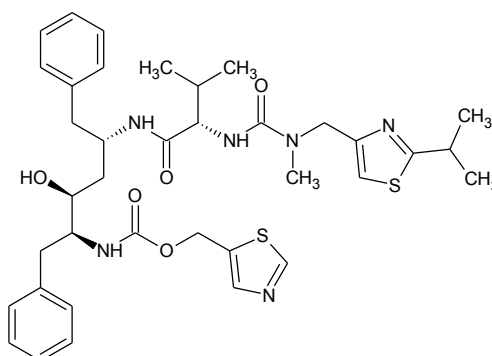
Ritonavir
(155213-67-5)

721.3

296 (14)

140 (58)

144



nucleotide analogue reverse transcriptase inhibitors

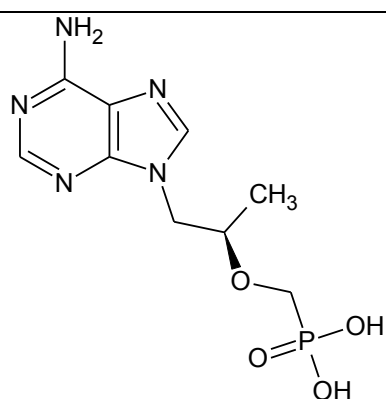
Tenofovir
(147127-20-6)

288.1

176.1 (24)

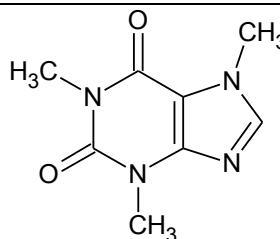
159 (32)

50

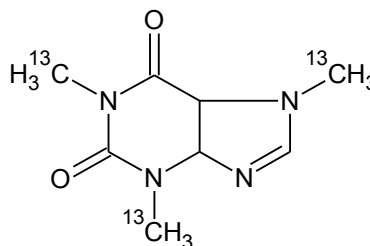


Xanthines

Caffeine (58-08-2)	195	138 (16)	110 (20)	100
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Caffeine (Trimethyl 13C3) (78072-66- 9)	198.2	140.2 (16)	112.1 (20)	100
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* Product ion 1 Fragmentor Voltage / Product Ion 2 Fragmentor Voltage

2.4 Method Validation

To estimate the rate of recovery, limit of detection and limit of quantification as well as the potential carry-over of the system, a mixture of the target compounds was serially diluted in 10 fold increments in Milli-Q water (Millipore, Billerica, MA, USA) in a range between 0.1 ng/L and 10 µg/L. 500 mL of each spike sample was extracted in triplicate with appropriate blank extractions between each. Residual target concentrations carried over into the blank samples were determined and extractions were performed from the highest to the lowest concentration in order to determine the maximal amount of expected carry-over.

Instrumental limits of detection and quantification (LOD and LOQ) were determined by repeat injection of a low concentration mixture of the targets diluted in methanol. The LOD and LOQ were defined as concentrations yielding a signal to noise ratio of 3 and 10 respectively

(ICH, 2008). Chromatographic variability was determined by calculating the relative standard deviation percentage (%RSD) for retention times of each compound.

Method detection limits (MDL) were determined by processing 24 environmental water samples from the Hennops River. Half of these were spiked at 100 ng/L and quantified by standard addition. The average concentration of each of the analytes from the unspiked samples were subtracted from these values and the MDL for each compound was calculated using the following equation (Conley et al., 2008):

$$\text{MDL} = T_{(n-1, \alpha=0.01)} \times S$$

where $T = 2.718$ for 11 degrees of freedom with $\alpha = 0.01$ and S is the standard deviation of the averaged triplicate concentrations of the blank-subtracted spiked samples. In addition to this, LOQ was calculated as described previously.

3. Results and Discussion

3.1 Chromatography and Mass Spectrometry

A variety of chromatographic programs, columns and solvents were tested; yet it was decided to sacrifice optimal conditions for the sake of universality. A number of the compounds are polar and were not effectively retained on C18 columns. A short C8 column (50 mm) was therefore chosen in order to retain more polar compounds and reduce elution times of non-polar compounds. A shorter column maximises the effect of column flushing at the end of each run since it was found that narrow bore columns rapidly became fouled, regardless of sample filtration steps. A chromatographic programme, with longer holds and flushing times, was

chosen in order to maximise column lifetime and ensure chromatographic reproducibility across analyses since the use of short gradients could promote the co-elution of interfering compounds that may enhance matrix effect; thereby eliminating the inherent purification properties of chromatography.

The retention time for each of the compounds on six different 50 mm columns from two major manufacturers; calculated from averaged triplicate injections of a 1 µg/mL mixture of the targets were utilised to compare column suitability (Figure S1, Supporting Information). The columns are highly comparable yet it was found that the Agilent XDB-C8 column yielded the best peak shape. When comparing compounds with the least retention time difference, the Agilent XDB-C8 column showed the highest resolution (0.19 min). Stavudine and Tenofovir display poor peak shapes on the majority of columns; with Stavudine eluting in the dead-volume on the Kinetix XB-C18 column (Phenomenex). The XDB-C8 column was also chosen because it is amenable to the addition of a greater variety of target compounds at a later stage.

These compounds are rarely analysed in a single run by mass spectrometry. Due to their divergent nature, average source settings were chosen to yield the most optimal conditions for the group, which in most cases is to the detriment of individual compounds' levels of sensitivity. The Agilent Mass Hunter Optimiser was used to automatically adjust fragmentator voltages and collision energies for each compound, which yielded a variety of product ions (Table S1, Supporting Information). Fragmentor and collision energy settings resulting in optimal

quantifier and qualifier ions (based on abundance and size) were chosen for each target compound.

3.2 Method Validation

The extraction and detection method was validated using a variety of techniques which would describe the effect of the matrix upon the analysis as well as the analytical limitations. Oasis HLB cartridges are described in the EPA method for the analysis of water for PCPs (Englert, 2007) and a number of researchers have adopted these to extract a variety of compounds with variations in cartridge sorbent mass, conditioning, load volumes and elution (Bijlsma et al., 2013; Cimetiere et al., 2013; He et al., 2013). A more universal approach developed by Ferrer and co-workers (2012) was automated and validated for ARV compounds (Table 2).

Instrumental limits of detection and quantification were based on the calculation of concentrations that would yield signal to noise ratios of 3 and 10 respectively. This was achieved through replicate (six) injections of calibration standards across the predicted detection range (0.1-1000 ng/L). Linearity for a five point curve proved to be acceptable with a lowest R^2 value of 0.994. The chromatographic retention time reproducibility was high for all compounds except Tenofovir (%RSD=2.07) which exhibited peak broadening during analysis.

Spiked MilliQ water was extracted in triplicate, and similarly LOD and LOQ was determined. These data showed that the “best case” extraction scenario without matrix proved to be

effective for all of the target compounds. The Horizon Smart Prep carryover was found to be lower than 0.5 % for the majority of compounds.

One of the major pitfalls of LC-ESI analyses is the effect of the matrix on analytical accuracy. Co-eluting compounds either suppress or enhance target signals leading to inaccurate quantitation. Analytical inaccuracy is a result of the synergistic effect of: sample components, compounds released during pre-treatment or extraction as well as mobile phase additives. Sample to matrix ratios, matrix type, extraction methodology, chromatography and mass spectrometer type all influence the extent of matrix effect (Gosetti et al., 2010). Samples may either be analysed by external calibration using matrix matched standards (Chen et al., 2010) or by standard addition (Ito and Tsukada, 2002; Conley et al., 2008; Cimetiere et al., 2013) . Matrix matched calibration has been found to be inaccurate in cases with high matrix load (Stüber and Reemtsma, 2004). Since matrix blanks are often unavailable and a single sample locality is chosen to provide matrix calibration for multiple locations; the incorrect assumption that all matrices are equivalent is made. Matrix effect can be accounted for by using isotopically labelled standards followed by correction. Unfortunately though, unless a standard for each target is used, one makes the incorrect assumption that all of the target compounds have identical chemical properties (Gosetti et al., 2010).

For these reasons a modified standard addition method (Conley et al., 2008) was applied to environmental analyses to account for matrix interference. Instead of diluting the sample with increasing volumes of standard, as is the accepted standard addition practice, the method used

here relies on adding equal volumes of standards with increasing concentrations. Isotopically labelled caffeine was introduced after extraction in order to correct for errors in pipetting small volumes, evaporation in the LC autosampler and variability in liquid chromatography.

Samples collected from the Hennopsriver were spiked with the target compounds at 100 ng/L, extracted 12 times and processed using the standard addition method. This river was chosen because it flows through an urban environment, and may present a “worst case” of matrix interference; and because of its proximity to the laboratory. The spike concentration was chosen as a value near the concentration of the compound with the highest instrumental LOQ (Stavudine). Linearity for the four point calibration was greater than $R^2=0.996$ and the method detection limit proved to be in the low ng/L range for the majority of compounds. The effect of the matrix on recovery was determined and these values were utilized to adjust the final environmental quantitation data (Table 2).

Table 2: Instrumental and method limitations with method efficacy parameters for the analysis of 12 ARVs and caffeine by solid phase extraction and LC-ESI-MS/MS.

Drug Name	Instrumental				Spiked MilliQ			Matrix Spike		
	R^2	LOD (ng/mL)	LOQ (ng/mL)	RT %RSD	LOD (ng/L)	LOQ (ng/L)	Carry-over (%)*	LOQ (ng/L)	MDL (ng/L)	% Recovery
Zalcitabine	0.998	0.01	0.04	0.00	7.0	23.3	0.1	0.1	21.9	16
Tenofovir	0.999	0.2	0.5	2.07	14.4	48.0	n.d	6.4	25.1	9
Lamivudine	0.996	3.1	10.4	0.99	0.5	1.7	0.03	1.3	13.4	20
Didanosine	0.999	0.1	0.3	0.07	0.06	0.2	0.2	0.04	40.4	66
Stavudine	0.998	8.1	26.9	0.19	5.4	18.1	n.d	18.2	36.6	49

Abacavir	0.994	0.02	0.06	0.03	0.002	0.01	0.1	0.04	43.1	89
Caffeine	0.995	0.1	0.4	0.06	0.3	1.07	n.d	0.07	35.3	62
Zidovudine	0.995	0.1	0.5	0.05	0.4	1.2	0.1	0.2	38.6	57
Nevirapine	0.995	0.03	0.1	0.04	0.01	0.02	0.1	10	92.7	74
Indinavir	0.991	0.2	0.6	0.34	1.4	4.5	4.0	0.04	35.3	44
Ritonavir	0.999	0.06	0.2	0.05	0.04	0.15	0.4	0.1	156.6	125
Lopinavir	0.999	0.09	0.3	0.03	0.15	0.5	0.3	0.05	117.2	88
Efavirenz	0.999	3.6	12.1	0.03	1.40	4.7	0.2	3	519.0	102

* The percentage carry-over is calculated as a function of the amount of analyte detected in the blank sample that follows directly after the highest concentration spiked sample. No detection is reported as (n.d).

Compound extraction efficiency and detection sensitivity varies within the group and may be seen as a function of the divergent nature of the compounds. Higher method limits of detection, when compared to instrumental limits, is most likely due to the variability introduced by variations in the SPE sorbent and the multiple pipetting steps of small volumes in the method. Also, a major source of variability is the final concentration step, in which volumetric estimation is performed by visual inspection. It is noteworthy that by using signal to noise to calculate the LOQ of the standard addition method, the sensitivity is much higher than when accounting for standard deviation between samples.

The spiked environmental samples were quantified using an external calibration curve (targets in HPLC-grade water) and these data were compared to concentrations derived from standard addition analysis (Figure S2, Supporting Information). Six of the 12 targets show marked signal enhancement (i.e. over estimation of concentration by external calibration) as a

result of the matrix. This over estimation is not due to the co-elution of a false positive compound as the unspiked samples showed no presence of signals similar to the target molecules. In addition to this, targets occurring in the un-spiked blanks were quantified and subtracted from the spiked samples. Tenofovir, Ritonavir and Efavirenz display substantial signal suppression with the chromatographic peak shape (spreading) of Tenofovir affected by the matrix. Nevirapine, the most frequently occurring target, appears to be unaffected by the matrix.

3.3 Environmental sample analysis

A variety of samples were collected during different weather conditions from dams and rivers across South Africa. Where logistically possible a body of water was sampled at different locations (Figure S3, Supporting Information). It must be borne in mind though that each sample only represents a “snapshot” at any given time. Inferences regarding the continual contamination of a particular water system can only be made if it is sampled continuously using passive samplers (Bartelt-Hunt et al., 2009) or over a long period of time (Kasprzyk-Hordern et al., 2008). The initial aim of this research however was to only to determine the presence of ARVs in South African surface water (Table 3).

Table 3: Pharmaceutical concentrations (ng/L) at various locations across South Africa as determined by automated solid phase extraction, standard addition and analysis by LC-ESI-MS/MS. Text printed in red represents quantitative data below the MDL but higher than instrumental LOQ.

		Drug Concentration (ng/L)												
	GPS Co-Ordinates	Zalcitabine	Tenofovir	Lamivudine	Didanosine	Stavudine	Abacavir	Caffeine	Zidovudine	Nevirapine	Indinavir	Ritonavir	Lopinavir	Efavirenz
Roodeplaat Dam System														
Pienaars River Inflow	-25.678677, 28.357116	n.d	243.2	242.3	n.d	778.9	4.2	149.8	339.2	1486.1	n.d	34.6	109.9	n.d
Zeekoegat WWTW Outflow	-25.624620, 28.341890	n.d	n.d	n.d	n.d	n.d	n.d	7.0	973.3	63.4	n.d	18.3	64.7	n.q
Angling Area	-25.626404, 28.345692	n.d	n.d	n.d	n.d	102.1	3.1	243.2	626.5	236.0	1.2	12.7	12.1	n.d
S.E Bank	-25.637763, 28.344150	n.d	n.d	184.2	8.4	n.d	4.6	396.8	298.3	337.1	n.d	0.7	2.7	n.q
Motorboat Launch	-25.618238, 28.358642	n.d	n.d	n.d	n.q	n.q	2.6	366.3	293.2	316.4	n.d	8.6	15.3	61.4
Rowing Club	-25.623345, 28.349842	n.d	n.d	150.7	3.8	n.d	4.0	294.8	305.7	294.2	n.d	5.4	9.3	n.d
Roodeplaat Outflow	-25.608244, 28.367231	n.d	n.d	35.4	0.8	413.2	2.8	303.5	223.9	347.1	6.7	5.1	15.5	n.q
Rietvlei Dam														
Southern Bank	-25.881576, 28.268585	n.d	n.d	131.7	n.d	n.d	n.d	181.2	155.5	87.6	n.d	n.d	2.3	n.d
Northern Bank	-25.876767, 28.279846	n.d	n.d	94.5	n.d	n.d	n.d	248.9	188.0	176.9	n.d	n.d	22.4	n.d
Orange River System														

Orange River (Bethulie)	-30.534670, 26.022975	71.3	189.0	n.d	n.d	n.d	10.3	53.9	2.8	10.2	21.8	5.7	n.d	n.d
Gariep Dam Oviston	-30.692147, 25.761238	n.d	n.d	n.d	54.0	n.d	13.6	5.8	3.0	14.8	17.0	4.8	3.3	n.d
Gariep Dam (N.E)	-30.603858, 25.503609	n.d	144.7	n.d	n.d	n.d	2.1	2.0	16.3	2.1	20.6	4.1	9.0	n.d
Vaal confluence	-29.070882, 23.637209	n.d	n.d	n.d	n.d	n.d	n.d	12.0	n.d	14.1	n.d	n.d	n.d	n.d
Orange confluence	-29.072898, 23.638936	n.d	n.d	n.d	n.d	n.d	n.d	4.0	n.d	10.6	n.d	n.d	n.d	n.d
Confluence	-29.071810, 23.635868	n.d	n.d	n.d	n.d	n.d	n.d	0.02	n.d	7.5	n.d	n.d	n.d	n.d
Cape Region														
Eerste Rivier	-33.941603, 18.857078	n.d	n.d	n.d	n.d	n.d	n.d	30.8	n.d	13.2	n.d	n.d	n.d	n.d
Theewaterskloof Dam	-34.027283, 19.208261	n.d	n.d	n.d	n.d	n.d	n.d	7.3	n.d	8.1	n.d	n.d	n.d	n.d
Vaal Dam														
Dam wall	-26.883278, 28.116047	n.d	n.d	n.d	n.d	n.d	n.d	78.1	0.5	8.7	n.d	6.0	8.1	n.d
Oranjeville	-26.999155, 28.214893	n.d	n.d	n.d	n.d	n.d	n.d	15.5	n.q	17.5	n.d	n.d	7.3	n.d
Vaal Dam Inflow	-27.020575, 28.608589	n.d	n.d	n.d	n.d	n.d	n.d	80.7	51.7	38.2	n.d	n.q	23.3	n.q
Vaal Dam Out Flow	-26.874950, 28.115583	n.d	n.d	n.d	n.d	n.d	n.d	158.0	22.6	43.00	n.d	n.d	21.7	n.q
Single system samples														
Hartebeesfontein WWTW Outflow	-26.030715, 28.291084	n.d	n.d	n.d	n.d	n.d	n.d	333.2	451.8	88.7	26.2	6.6	129.6	74.1
Ditholo	-25.320242, 28.340728	n.d	n.d	9.5	n.d	n.q	n.d	n.q	n.d	142.8	n.d	n.d	n.q	n.d

Hartbeespoort Dam, Meerhof (2011)	-25.760775, 27.891871	n.d	n.d	n.d	n.d	n.d	n.d	926.9	349.5	129.6	5.0	10.5	283.2	n.d
Hartbeespoort Dam, Meerhof (2014)	-25.760775, 27.891871	28.2	n.d	6.6	54.1	n.d	3.2	402.0	138.5	137.2	14.5	7.2	304.8	88.1
Hartbeespoort Dam, Tap Water Sample	-25.745594, 27.911238	8.4	n.d	n.d	n.d	n.d	n.d	263.3	72.7	23.2	n.d	21.6	39.7	28.2
Renosterkop	-25.108639, 28.887359	n.d	n.d	n.d	n.q	n.d	n.d	441.3	n.q	13.3	n.d	n.d	n.d	n.d
Inanda Dam	-29.673792, 30.854874	n.d	n.d	n.d	32.0	n.d	n.d	231.7	21.1	4.5	n.d	n.d	19.6	n.d
Inanda Dam offshore	-29.674016, 30.860239	n.d	n.d	n.d	32.3	n.d	n.d	40.7	22.7	52.2	n.d	n.d	3.7	n.d
Total no. of occurrences across all locations		2	3	8	7	3	10	28	21	29	8	15	21	4
Average concentrations detected (ng/L)*		36.0	192.3	106.9	26.5	431.4	5.1	188.5	217.0	144.3	14.1	10.1	50.4	63.0

* Average values determined from only samples in which targets compounds were detected.

n.d – Not detected; values below instrumental LOD.

n.q – Not quantified; values above instrumental LOD but below instrumental LOQ.

Nevirapine, Lopinavir and Zidovudine were found most frequently throughout the survey. The drug concentrations are in the low ng/L range with Stavudine, Nevirapine and Zidovudine showing the highest averages.

Greater attention was given to the Roodeplaat Dam system due to its proximity to the laboratory as well as the fact that two WWTW fed into the dam (Zeekogat and Bavianspoort). The concentrations of the compounds were found to vary between the two WWTW but a sample could not be taken upstream of the Bavianspoort plant therefore the background contribution made by the Pienaar's River (which flows through an urbanized area) could not be made. The majority of the target compounds were found in the Roodeplaat system at varying concentrations. This highlights the importance of collecting multiple samples from different points in a system before significant inferences can be made. This is further substantiated by the differences in concentration detected at the Orange-Vaal River confluence. Samples from the Vaal River, Orange River and from the confluence (all taken within 100 meters of each other) were found to differ significantly.

In the Roodeplaat system, in most cases, the drug concentration is lower at the outflow than any other point sampled within the dam. There are points of higher concentration within the dam e.g. at the recreational angling area. This may be due to the depth and mixing of the water in the area. The angling area is approximately 100 m from the Zeekoegat WWTW outflow, which did not contribute all of the compounds detected in the angling area. The data generated

form this body of water bears special consideration since the dam is used for recreation (fishing and water sport) as well as a source for potable water.

Nevirapine was detected in all of the surface water samples albeit only reliably quantified in nine out of the 24 sampling locations. The compound is a non-nucleoside reverse transcriptase inhibitor that is widely used for the treatment of HIV as well as the prevention of mother to child transmission (Coovadia et al., 2012). The prevalence of this compound can most likely be attributed not only to its frequent therapeutic use, but also the compound's persistence in the environment. The compound has been found to be non-biodegradable in a "closed bottle" *in vitro* system (Vanková and others, 2010). This type of "*in vitro*" research shows that it is most likely persistent in the environment.

Caffeine is commonly used an anthropogenic marker for surface water contamination by wastewater (Buerge et al., 2003) and it appears ubiquitously throughout the sampled areas. One does not however see a relationship between caffeine levels and target compound levels as determined by the variability in the ratio between target and caffeine concentration. Targets were detected in samples where caffeine could not be quantified. Caffeine may have a shorter environmental half-life than the targets or the compounds are not removed with equal efficiency by WWTW. Potentially, the WWTW in a specific area is effective or that the population is not consuming as many ARVs as other more urbanised areas. This is evident in the samples taken from rural areas where population density is lower. A large, impounded body of water may provide a cumulative picture of human activity. Even if target compound influx is not

detectable, due to the persistent nature of these compounds, concentrations may increase over time.

Additional considerations must be made in sub-Saharan Africa that may not be relevant to similar European studies. Inadequate sanitation in certain parts of the country, the use of pit latrines and malfunctioning WWTWs means that untreated human waste is quite often discharged into water systems. The presence of these compounds in the environment cannot be solely attributed to functional WWTW discharge.

The standard addition method was utilized since a specific quantitative answer was required of this research. The method is highly labor and cost intensive as a single sample analysis consists of a minimum of 17 injections. For screening purposes it is suggested that an external calibration curve consisting of spiked matrix in conjunction with isotopically labelled standards should be used. The standard addition method does not lend itself to routine screening but has proven to be vital when quantifying novel contaminants for the first time in a particular water system. It is proposed that once the linearity of a SAM calibration is established in a particular matrix, the number of calibrators used for routine work may be reduced, thereby reducing costs and analytical time.

The environmental relevance of the presence of antiretrovirals in water supplies is not immediately clear, as is the case with antibiotics, in that they may promote drug resistance. This is because HIV does not have a non-human host and does not occur in the environment, as

is the case with other viruses or bacteria. Also, the model of transfer of resistance genes between species cannot be applied when considering the virus. The influence of these compounds on environmental retroviruses has not been established in the literature and it should be borne in mind that although the presence of these compounds in the environment may not affect the target virus, they may still promote the development of drug resistance in other pathogens.

4. Conclusion

The LC-MS method presented efficiently separates and analyses the major members of the ARV drug class. The standard addition method accurately quantifies these compounds in complex matrices and is a more cost effective alternative to the use of isotopically labelled standards; many of which are not commercially available for this group of compounds. The extraction and analytical method was developed to remain generic and amenable to the addition of more compounds to the analytical paradigm. This work represents a nationwide survey of surface water and presents, for the first time, qualitative data for many of these pharmaceuticals in the environment. Compounds such as Nevirapine have been detected in European surface waters, yet the majority of the compounds targeted in this work have not been previously described in the literature. The ecotoxicity of these emerging pollutants and their degradation products has not been established and further research may aid in determining the consequences of discharging these compounds into the environment.

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Supporting Information:

The supporting information describes chromatographic optimisation, a map of sampling locations and a list of product ions for each of the compounds obtained by mass spectrometry.

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