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Research article

Quantification of selected pharmaceutical compounds in water using liquid chromatography-electrospray ionisation mass spectrometry (LC-ESI-MS)

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ABSTRACT

The detection and quantitation of pharmaceutical compounds (PCs) in different environmental matrices is still a challenge, due to their extremely low (ng-μg) concentrations and the lack of rapid and sensitive analytical techniques. A number of techniques, such as enzyme-linked immunosorbent assay (ELISA), chromatography, electrophoresis, and electrochemical methods have been explored. These methods are limited by their poor sensitivity. In this study, a hyphenated liquid chromatography-mass spectrometric (LC-MS) method was developed, validated, and tested for the detection and quantification of seven active pharmaceutical compounds, with solid-phase extraction for analytes recovery and separation of interference from the aqueous matrix. The sensitivity achieved for the method allowed for LODs (μg/L) of 0.0439, 0.0684, 0.1219, 0.0710, 0.1129, 0.0447, 0.0837 and LOQs (μg/L) of 0.1462, 0.2281, 0.4065, 0.2367, 0.3763, 0.1492, 0.2792, for lamivudine, acetaminophen, vancomycin, ciprofloxacin, sulfamethoxazole, diclofenac, and ivermectin, respectively, within a linear range of 0.01–0.1 μg/mL. Other ICH validation parameters are also discussed. The different PCs were positive in 61 % of the tested surface waters, with diclofenac present only in two of the sampling points. The concentrations at which they occurred were variable and ranged between ND and 398.98 μg/L.

1. Introduction

Among priority pollutants of environmental concern listed by the Environmental Protection Agency, EPA, are active pharmaceutical compounds (PCs) (McEniff et al., 2015). These compounds have been monitored and detected in several environmental compartments in many countries around the world (Dhar et al., 2019; Peng et al., 2019; Lee et al., 2019; Kleywegt et al., 2019; Fekadu et al., 2019; Rivera-Jaimes et al., 2018; Hossain et al., 2018; Fatoki et al., 2018). However, thorough and comprehensive studies are limited by the unavailability of rapid, sensitive, and specific analytical techniques that can detect at trace and ultra-trace environmental levels.

A significant consideration in the development of analytical methods for low concentrations of pharmaceuticals and many emerging contaminants is the chemistry of the analytes of interest, the efficiency of separation from interfering substances, and the method's sensitivity. These challenges could be due to the differences in the structures of pharmaceutical compounds (PCs), and those moieties of partially modified structure. These structures contain some chemical entities that affect their chemical properties and allow for behavior that makes it difficult to isolate or separate the PCs from potentiation or interaction with co-existing compounds. Potentiated and modified PCs such as common with β-lactams antibiotics could also facilitate a different environmental characteristic and even toxicity. This environmental speciation attributes necessitate additional sample preparation steps since most PCs hardly exist as stand-alone in the environment. Furthermore, certain pharmaceutical products referred to as combination drugs, formulated with more than one PCs exacerbates their environmental complexity, and thus, the need for separation of interferences.

Sample preparation processes for the recovery of PCs from different environmental matrices vary and subject to analyte type, perceived analytes' levels, and matrix type. The bioanalytical matrix, for example, is known to hold varying degrees of xenobiotic contaminants depending on the magnitude and extent of exposure. It is, therefore, essential to identify interfering substances and matrix effects to ensure high efficiency of extraction to improve the method's sensitivity. Sample preparation involving hydrolysis of some PCs (e.g. highly strained compounds) must be carried out in such a way that it does not result in the destruction of the structure of the analytes, as this and this can affect separation and detection (Sajonz et al., 2006).

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Early methods of extraction and recovery of organic analytes, including residues of pharmaceuticals, pesticides, phthalates, phenols, and many others from aqueous matrices, involve simple procedures such as liquid-liquid extraction (LLE), solid-liquid extraction (SLE), column chromatography and others. More advanced methods such as solid-phase extraction (SPE), solid-phase microextraction (SPME), stir bar sorptive extraction (SBSE), and supercritical fluid extraction (SFE), have also been reported (Kafeenah et al., 2018; Suazo et al., 2017; Silveira et al., 2013). Solid phase extraction has been reported to yield variable analyte recoveries, reaching 100% extraction recoveries in some instances, whilst sample matrix properties including pH value, salinity, and concentration of colloid and surfactant were carefully controlled (Lee et al., 2002). For example, hydrophilic-lipophilic balance (HLB) containing polystyrene-divinylbenzene-N-vinyl pyrrolidone terpolymer as solid phase column material provides optimal extraction of clofibric acid, ibuprofen, carbamazepine, naproxen, ketoprofen, diclofenac (DCF) and some other PCs from surface and wastewater, compared to other solid-phase extraction cartridges tested (Fatoki et al., 2018; Zhang and Zhou 2007; Kasprzyk-Hordern et al., 2007).

Separation and quantitation have primarily been based on liquid chromatography and molecular spectroscopy. These methods are employed because of the variability in the solubility and partitioning of PCs between different mobile phase solvents and stationary phase column materials, and the ease with which chromophores are developed with many groups of pharmaceuticals. The use of HPLC-UV-DAD (Fatoki et al., 2018; Olatunji et al., 2017), LC-TOF-MS (Al-Qaim et al., 2013), UPLC–MS/MS (Kafeenah et al., 2018; Kasprzyk-Hordern et al., 2007), LC-MS/MS (Fatta et al., 2007), GC–MS (Fatta et al., 2007; Lin et al., 2005), LC–ESI-MS–MS (Khan et al., 2015; Sekar et al., 2013), have been reported. It is important to note that the use of GC-MS is not very common since most pharmaceuticals are not volatile (Caban et al., 2015.

In this study, a new, sensitive, rapid, and accurate multi-residue analytical method was developed for the separation of the mixture of selected seven PCs comprising lamivudine, vancomycin, acetaminophen, ciprofloxacin, sulfamethoxazole, diclofenac and ivermectin in fresh surface waters and wastewater streams. The selection of the PCs which belong to different therapeutic pharmaceutical classes, including antiretroviral, antibacterial/antimicrobials, anthelmintic, and analgesics, was informed by drug volume usage speculations.

2. Materials and method

Acetaminophen (ACP), ciprofloxacin (CPX), sulfamethoxazole (SMZ), diclofenac (DCF), vancomycin (VMC), lamivudine (LMV), and ivermectin (IVT) standards were purchased from Sigma Aldrich, Germany. LCMS grade acetonitrile, LC grade methanol, and acetone were procured from Honeywell Company, Germany. All other chemicals and solvents used were of analytical grade. MilliQ water used in the LC analysis was prepared using RiOs™/Elix 5 Millipore Water Purifier (Model No PF05113).

2.1. Instruments and software

Shimadzu LCMS 2020 system consisting of a quaternary pump was operated on ASCII software application. The software on coded communication with the instrument was used for chromatogram conversion and integration, while Excel 2016 was used for regression analysis of the results obtained. A Mettler Toledo analytical balance was used for all weightings (massing) of pharmaceutical standards. pH measurements were taken using a pre-calibrated pH meter. An Ultra-sonicator (Scientech) was employed for the easy dissolution of analytes. The sampling map was created using all sampling point coordinates with the aid of the Google earth pro, 2020 application.

2.2. Preparation of stock solution of analytes

About 0.01g each of LMV, ACP, VMC, CPX, SMZ, DCF, and IVT were accurately weighed and dissolved in methanol in different 10 mL volumetric flasks and made up to mark to achieve the equivalent of 1000 mg/L stock solution of each of the PCs. The stock solutions were stored in a refrigerator at approximately 4 °C until use. Standard mixtures at different concentrations ranging between 0.01 to 0.10 μg/mL were prepared by serial dilution of an appropriate volume of the stock solutions in methanol to achieve the concentration range needed to develop the calibration curves for each PC.

2.3. Method development

In order to develop a single method that could detect, separate, and quantify seven PCs simultaneously, a single run for a defined concentration range of each analyte was first carried out by elution in Shimadzu C_{18} reversed-phase column on a Shimadzu 2019 Liquid Chromatograph coupled to a Mass Spectrometer (LC-MS). This procedure was carried out to determine the optimum instrument parameters (mobile phase composition and mobile phase pH, column oven temperature, injection volume, and flow rate) with which the selected analytes could be detected. Also, the procedure would help ascertain the retention time for each compound under the optimized instrument parameter set up. Thereafter, a combination of the seven PCs was injected online the LC-MS to confirm the resolution of the targeted compounds. The efficient separation of the PCs was achieved by gradient elution. After that, calibration curves for each analyte PCs were plotted to ascertain instrument response to changing concentration. The developed method was validated according to the International Conference on Harmonization, ICH (2005) standard validation procedures.

2.4. Method validation and suitability test

Data generated from instrument calibration and chemical analyte recovery were subjected to the prescribed International Conference on Harmonization (ICH, 2005)guideline procedure for validation and suitability evaluation to establish that the developed method meets the minimum requirement for its usefulness as well as system performance. This approach was to ensure that the method meets acceptable conformance specifications for its intended use.

The suitability assessment conducted include determination of tailing factor/symmetry factor, $S = \frac{W_{0.05h}}{2f}$; peak resolution, $R = 1.18 \times \frac{tR_b - tR_a}{W_{0.5ha} + W_{0.5hb}}$, Where tR_a and tR_b represent retention times of peaks a and b while $W_{0.5ha}$and $W_{0.5hb}$ represent peak width at half height of peaks a and b respectively); system precision (%RSD) and theoretical plates, $N = 5.54(\frac{t_R}{W_{0.5h}})^2$, all calculated for each PC peak on the chromatograms (Shimadzu, 2019). The validation parameters tested, include sensitivity, specificity, selectivity, linearity, linear range, detection limit (LoD), quantitation limit (LoQ), repeatability and reproducibility.

Stability evaluations were performed on stock solutions. The quality control standards containing 0.04 and 0.10 μg/mL of the PCs were subjected to a stability check of the analytes. The stock solutions were stable for more than one week at 4 °C. All stock dilutions in methanol and water mixture were stable at room temperature. All the samples were analyzed under the optimized chromatographic performance conditions to determine the peak intensities/areas of the analytes.

2.5. Sampling collection

Water samples were collected from in four sampling points at five sampling stations including Msunduzi River (30° 38.101′ E and 29° 39.672 S′) before articulation with Umgeni River, Umgeni River (30° 41.086′ E and 29° 39.423′ S), Inanda Dam Inlet on Umgeni River (30° 48.040′ E and 29° 39.005′ S), Inanda Dam Outlet on Umgeni River (30°

52.173′ E and 29° 42.934′ S) and at the Blue Lagoon (31° 02.157′ E, 29° 48.711′ S) estuary area. Umgeni River, of which Msunduzi River is a significant tributary adding a substantial quantity of water to it, is a significant source of drinking water supply, as well as water for agricultural and industrial use in KwaZulu-Natal. The Umgeni River discharges its content into the Indian Ocean via the Blue Lagoon estuary. These rivers were tested due to previous reports about increasing activities and sewage contamination of the rivers in the last few years (Matongo et al., 2015b; Still, 2006). The water samples were collected in 0.1 % HNO_3 pre-cleaned 2.5 L amber bottles. The samples were well labeled and held in ice-chest coolers, for onwards transfer to the laboratory. All samples were kept in the laboratory refrigerator at 4 °C and analyzed within one week of collection. Physicochemical characteristics of water were measured in-situ at the different sampling points.

2.6. Extraction/recovery efficiency of pharmaceutical compounds from aqueous system

The recovery of an analyte, usually expressed as a percentage, measures the ability with which a known amount of recoverable analyte carried through sample extraction and processing steps of a new or known analytical process is achieved.

A solid-phase extraction (SPE) procedure was also developed for the recovery of the 7-PCs from an aqueous matrix. Briefly, SPE columns consisting of polymeric weak cation cartridges (Strata) (200 mg/6 mL) were conditioned by eluting with 5 mL MeOH, followed by sequential elution with 50 % and 30 % MeOH/double distilled water through the column at a flow rate of 0.5 mL/min as described by Olatunji et al. (2017). After column conditioning, 300 mL of tap water spiked with the PCs analytes at low, medium, and high concentrations were loaded on the pre-conditioned column and eluted at a flow rate of 2 mL/min. The adsorbed analytes were thereafter recovered with 5 mL, 0.5 % formic acid/MeOH at 0.5 mL/min flow rate. Eluates were concentrated to near dryness and reconstituted to 1 mL in 0.5 % buffered MeOH. The recovered extracts were concentrated under compressed air, reconstituted in 1 mL acidified MeOH, and analyzed. The efficiency with which the PCs were recovered was validated by calculating their recoveries for the spiked concentrations.

3. Result and discussion

The effectiveness of chromatographic separation relies on the provision of adequate resolution. An excellent separation resolution minimizes, to a large extent, ambiguities associated with peak purity as a result of co-elution, peak pairing, as well as reduce qualitative retention time shifts and quantitative errors. An empirical procedure for achieving good separation and quantitation of complex mix involves adequacy of selected column, and a series of optimization including column conditioning, mobile phase composition, temperature control, mobile phase pH control, use of mobile phase modifiers (additives), and use of ion-pair reagents where necessary. These factors exert different influences depending on the complexity of the mixture/compounds to be separated, and the system used. The PCs investigated consist of different classes, including LMV, an active antiretroviral compound, ACP and DCF, analgesics, VCM, CPX, SMZ, all in the class of active antibiotics compounds, and IVT, an anthelmintic.

3.1. Mobile phase optimization and instrument parameters for efficient separation

The mobile phase consisting of different ratios of mixtures methanol, acetonitrile, and Milli Q water were tested as eluent for the separation of LMV, ACP, VCM, CPX, SMZ, DCF, and IVT mix. The separation of the seven PCs was achieved by gradient elution by eluting the compounds mix through a C_{18} column (2.1 × 100 mm, 3 μm), vis-a-viz changing the composition of the mobile phase consisting of 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B), over the run time of 19 min. The suitable gradient program condition that resolved the mixture of 7-PCs started with 5% B to 80 % B in 12 min, then reducing to 50 % in 14 min, followed by an increase to 65 % in 17 min, and finally down to 5 % in 19 min.

The optimal instrument parameter settings for the effective separation of the PCs were flow rate, 0.4 mL/min; column temperature, 38 °C; and injection volume, 1 μL. The elution power and selectivity for each of the analytes were influenced by modification of mobile phase pH via a shift from an aprotic to a protic solvent. The viscosity of the mobile phase combination per time appeared to induce variations in analyte solubilities. This results in the differentials in the capacity of the column to retain the analytes, and thus the resolution of the separated compounds.

The MS chromatogram indicating the separation characteristics, retention time, and efficiency of separation of the seven compounds in the PCs mix: LMV, ACP, VCM, CPX, SMZ, DCF, and IVT using the optimized instrument parameters is as shown in Figure 1.

The retention times (min) for LMV, ACP, VMC, CPX, SMZ, DCF and IVT were 1.47, 3.22, 3.72, 4.71, 5.96, 9.85 and 14.29, respectively (Table 1).

The MS analyses were performed in the positive electrospray ionization mode ESI (+) for all pharmaceuticals. The SRM m/z characteristic fragmentation pattern of each of the individual PC analyte was determined through eight online injections at 230, 152, 725, 332, 254, 296, and 897 for LMV, ACP, VMC, CPX, SMZ, DCF, and IVT, respectively (Table 1).

3.2. System suitability

System suitability tests rely on the concept that equipment and analytical operations are an essential scheme in a systematic method validation process. The suitability of the chromatographic system is evaluated from the accuracy and precision of the characteristics of the analytes peak (Shabir, 2003). These tests mainly include evaluation of tailing factor (also known as symmetry factor), resolution, system precision, and theoretical plates. The tailing factor (S), resolution (R), system precision (% RSD of retention times of 7-PC analytes), and theoretical plates (N) were calculated for each PC chromatographic peak, from eight sample injections (n = 8). All these were assessed to confirm the system's suitability and performance.

3.2.1. Tailing factor

The tailing factor was calculated using the formula; $S = \frac{W_{0.05h}}{2f}$, where $W_{0.05h}$ is the peak width at 5 % the peak height above peak baseline while f value is obtained when a vertical line is drawn from the peak baseline at 5 % peak height above peak baseline ($W_{0.05h}$), the distance along that horizontal line from the leading edge of the peak to the vertical line is taken as the f value. Thus, the tailing factors obtained for the peaks of LMV, ACP, VMC, CPX, SMZ, DCF, and IVT were 1.03, 1.01, 1.01, 1.05, 1.04, 1.05, and 1.03, respectively (Table 1). According to the Center for Drug Evaluation and Research (CDER 1994), the acceptance criteria for tailing factors of a chromatographic peak should be ≤2 while system precision for APIs should be ≤5 %.

3.2.2. Peak resolution

Furthermore, the resolution between the peaks was calculated using the equation described in 2.4. The separation resolutions achieved within the chromatographic peaks of the 7-PC mix were 7.03, between LMV and ACP peaks; 2.34 between ACP and VMC peaks; 5.43, between VMC and CPX peaks; 7.32, between CPX and SMZ peaks; 22.71, between SMZ and DCF peaks, and 20.98, between DCF and IVT peaks (Table 2).

According to CDER (1994), the accepted value for the resolution between two closest eluting chromatographic peaks should be >2, although >1.5 is sufficient.

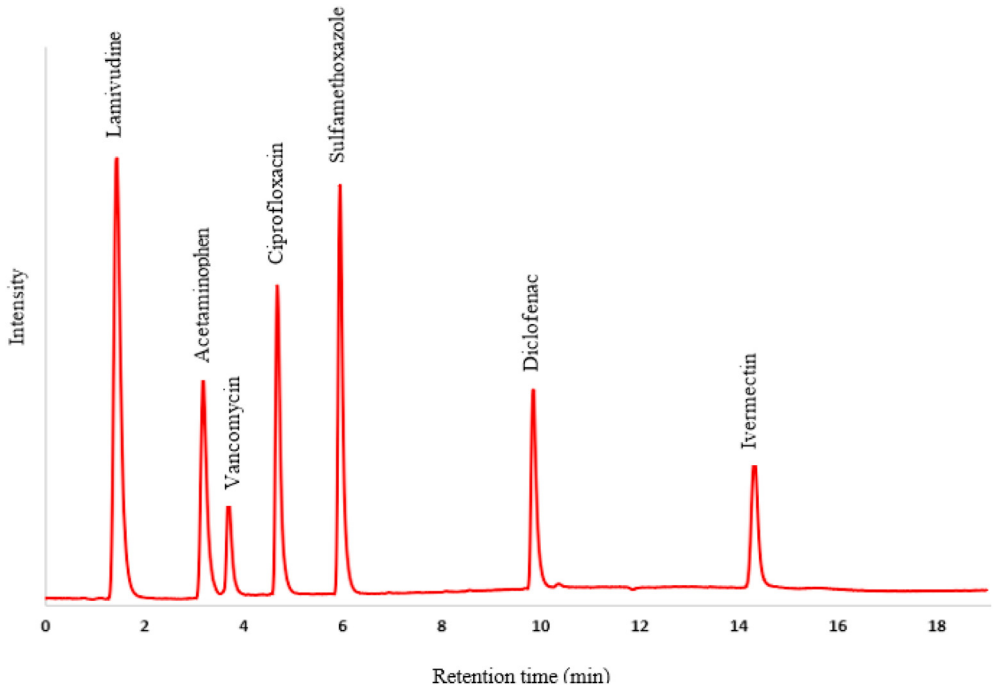

Figure 1. Chromatogram of the separation of the 7-PCs.

Table 1. Molecular formula, molecular weight, and tailing factors of the separated PCs by ESI-MS detection.

S/N	Analyte	Retention Time (min) Mean ± S.D (n = 8)	Retention Time (min) % RSD	Molecular Formula	Molecular weight ($gmol^{-1}$)	Positive ion	Molecular/ Fragment ion (m/z)	$W_{0.05h}$ Value (n = 8)	f Value (n = 8)
1	LMV	1.473 ± 0.033	2.25	$C_8H_{11}N_3O_3S$	229.26	230	$[M + H]^+$	0.373	0.18
2	ACP	3.221 ± 0.044	1.37	$C_8H_9NO_2$	151.16	152	$[M + H]^+$	0.363	0.18
3	VMC	3.723 ± 0.019	0.52	$C_{66}H_{75}Cl_2N_9O_{24}$	1449.30	725	$[M + 2H]^{2+}$	0.269	0.13
4	CPX	4.710 ± 0.007	0.43	$C_{17}H_{18}FN_3O_3$	331.35	332	$[M + H]^+$	0.291	0.14
5	SMZ	5.963 ± 0.023	0.38	$C_{10}H_{11}N_3O_3S$	253.28	254	$[M + H]^+$	0.264	0.13
6	DCF	9.851 ± 0.036	0.36	$C_{14}H_{11}Cl_2NO_2$	296.15	296	$[M + H]^+$	0.275	0.13
7	IVT	14.296 ± 0.073	0.51	$C_{48}H_{74}O_{14}$	875.10	897	$[M + Na]^+$	0.300	0.15

Table 2. Resolution between the chromatographic peaks of the PCs.

Chromatographic peak	ΔRetention Time (min) (n = 8)	Resolution (N)
Peaks a-LMV and b-ACP	1.748	7.031
Peaks b-ACP and c-VMC	0.502	2.343
Peaks c-VMC and d-CPX	0.987	5.426
Peaks d-CPX and e-SMZ	1.253	7.322
Peaks e-SMZ and f-DCF	3.888	22.709
Peaks f-DCF and g-IVT	9.851	20.976

Table 3. Number of theoretical plates used for separation of PCs in the chromatographic column.

Chromatographic peaks	$W_{0.5h}$ Value (n = 8)	Theoretical plate (N)
LMV	0.156	513.89
ACP	0.141	3005.59
VMC	0.114	6000.21
CPX	0.101	12025.63
SMZ	0.101	19674.67
DCF	0.101	52588.12
IVT	0.151	53035.87

3.2.3. Theoretical plates

The efficiency of separation columns can be evaluated and ascertained by calculating the number of theoretical plates, using the formula $N = 5.54(\frac{t_R}{W_{0.5h}})^2$ (where, t_R = retention time). The more the number of theoretical plates, the better the efficiency of the column, and the more effective and reliable the separation method developed. The number of theoretical plates required for the separation of each of the PC in the 7-PC mix is presented in Table 3. The results revealed that the efficiency of the column used in this study is on the high reaching N = 53035.87 for the peak of IVT, which was the last to elute out of the column, with the least number of plates (513.89) required for the elution of first compound LMV. Overall, the separation of the 7-PCs was achieved on an average number of theoretical plates of 20977.71 ± 22637.99.

Although the theoretical plate number depends on elution time, it should generally be higher than 2000 (CDER 1994). The system precision represented by the %RSD of the retention times of the analytes for eight injections were in all cases ≥, 2.25. These values are within the acceptable range.

3.3. Method validation

Results from method validation are often used to judge the quality, reliability, and consistency of analytical results. The developed method

was subjected to the ICH validation procedure to establish whether the deployment of the analytical method/procedure for the intended PC analytes is suitable and sound for its intended application. Thus, the developed method was validated according to the ICH guidelines for specificity, linearity, limits of detection (LOD), and limits of quantitation (LOQ), range, accuracy, and precision.

3.3.1. Specificity

Specificity explains the ability of a method to accurately measure the analyte response in the presence of all potential sample components. The specificity of the method was determined by comparing the chromatograms obtained from the samples of mixed analytes with that obtained from blank, i.e., methanol, which was used for the preparation of all pharmaceutical standards. The solution mixture containing different concentrations of all analytes ranging from 0.01 – 0.06 g/L were injected into the column under the optimum chromatographic conditions to obtain the chromatographic peaks of all analytes. The t_R (Table 4) of the compounds remain the same over replicate injections (n = 18) of the PCs mix.

3.3.2. Linearity and linear range

The quality of the six-point calibration curve is a strong basis for acceptable quantitative analytical results. Six different levels of the mixture of the PCs of interest between a concentration range of 0.01 and 0.10 ng/μL were prepared, and each injected three times for each concentration (n = 18) on the LCMS. The calibration curves were prepared by plotting the peak areas of individual analyte versus their respective concentrations (S1 - Figures a–g). The coefficient of correlation (R^2) obtained for the incremental concentrations signals were: LMV, R^2 = 0.9999; ACP, R^2 = 0.9997; VMC, R^2 = 0.9998; CPX, R^2 = 0.9987; SMZ, R^2 = 0.9989; DCF, R^2 = 0.9997 and IVT, R^2 = 0.9998 (Table 4). These values are greater than the ICH (R^2 = 0.90) acceptable value. The regression equation for the best fit curve for each PC within the linear range of calibration concentrations 0.01–0.10 ng/μL (Table 4) indicate linear responses, although individual compound response varied with one and other. This, therefore, validates the proposed method for the intended use.

3.3.3. Limits of detection and quantification (LoD and LoQ)

The performance characteristics of the method developed for the separation, detection, and quantitation of the 7-PCs were validated by evaluating the limit of detection (LoD) and the limit of quantification (LoQ). LoD and LOQ describe the smallest concentration of an analyte that can be reliably measured by an analytical procedure (Shrivastava and Gupta 2011). The lowest level of an analyte that can be detected in a sample, but not necessarily quantified under a stated analytical test condition is often taken to be the LoD, while the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated conditions of the test is taken as the LoQ (Lu et al., 2016; Shrivastava and Gupta 2011).

The LoD and LoQ of the developed method were determined by calculating the signal to noise ratio obtained from a series of successive measurement of blank corresponding to 3 and 10 times the noise levels, respectively. The LoD and LoQ for each analyte were calculated using the formula below.

$$LOD = 3s/m \quad \text{Eqn. 1}$$

$$LOQ = 10s/m \quad \text{Eqn. 2}$$

Where s = Standard error of the mean; and m = slope.

The LoDs obtained for LMV, ACP, VMC, CPX, SMZ, DCF, and IVT were 0.044, 0.068, 0.122, 0.071, 0.113, 0.045 and 0.084 μg/L, respectively, while LoQs for the 7-PCs were 0.146, 0.228, 0.407, 0.237, 0.376, 0.149 and 0.279 μg/L, respectively (Table 4).

3.3.4. Precision and accuracy

Precision and accuracy are of utmost importance among the ICH validation parameters set for testing fits of a chromatographic test method (Betz et al., 2011). Accuracy measures the closeness of an experimental value to the true value (the actual amount of analyte in the matrix), while precision measures the closeness of individual measurements to one another. In this study, the precision of a set of results was assessed by repeatability and reproducibility analyses. Repeatability measured the variation in data generated in successive measurements under the same instrument condition parameter settings. In contrast, reproducibility measures the degree of variation in the entire study or experimental data over a measurement time sequence (usually for 5-days).

Three quality control samples were in this study. Five injections of each of the specified quality control samples at three different levels were injected for analysis, 5 consecutive times within 24 h for repeatability, and over a period of 5 days for reproducibility. The mean and relative standard deviations of the measurements were calculated and used to predict the accuracy and precision of the method/instrument. The results of repeatability (intra-day) and reproducibility (inter-day) analyses conducted to check the accuracy of the measurements using three quality control samples are presented in Table 5.

The precision and accuracy data obtained indicated that there was no interference from sample/instrument components. Inter-day, as well as intra-day replicates of the selected analytes, resulted in a % RSD value of ≤8.74 %. Vikram Singh et al. (2011) reported that precision values should be less than 15 %, as stated by CDER guidance for bioanalytical method validation. Hence the precision and accuracy of the proposed method are very high.

3.3.5. Stability

Stability was determined using the prescribed stability-indicating method (SIM) guideline for industry, analytical procedures, and methods validation of the FDA (2000). The SIM is a validated accurate, and precise analytical procedure used in measuring active drug ingredients, substance, or drug product ingredients that are free of interferences from the process, impurities, excipients, and degradation products. This SIM test was carried out to monitor the results obtained to guarantee methods efficiency and data quality.

Table 4. Retention time (t_R), linearity characteristics of calibration curve, LODs, and LOQs for selected pharmaceutical compounds.

Pharmaceutical Compounds	Retention time (min)	Standard Error of the mean (S.E) (n = 18)	Equation of the best fit	Slope	Coefficient of regression (R^2)	LOD (μg/L)	LOQ (μg/L)
Lamivudine	1.47	1052.65	y = 239937x + 50.075	239937	0.9999	0.0439	0.1462
Acetaminophen	3.22	841.76	y = 123013x – 150.11	123013	0.9997	0.0684	0.2281
Vancomycin	3.72	269.29	y = 22085x - 1.5689	22085	0.9998	0.1219	0.4065
Ciprofloxacin	4.71	1597.41	y = 224980x – 89.77	224980	0.9987	0.0710	0.2367
Sulfamethoxazole	5.96	4775.82	y = 423021x + 453.17	423021	0.9989	0.1129	0.3763
Diclofenac	9.85	710.11	y = 158700x – 120.79	158700	0.9997	0.0447	0.1492
Ivermectin	14.29	529.35	y = 63207x - 112.16	63207	0.9998	0.0837	0.2792

The stability of the analytes was established since the final assay values of PC analytes were found similar after five injections for five days. Table 6 shows the % RSD for the data obtained for the injection of each of the PCs, which was, in all cases ≥, 4.02 %.

This result showed that the data obtained were not out of trend over the intra-day replicates, as well as the 5-inter-day replicates measurement period, and are sensitive at least within the LoD and LoQ ICH threshold. According to Swartz and Krull (2004) and CDER (2006), a stability indicator is a powerful quality control tool that determines whether analytical results are within or out-of-specification (OOS).

Furthermore, the pharmaceuticals were tested for their ability to withstand analytical assay procedures stress (Snyder et al., 1997), such as the effect of temperature, stationary phase, and buffers/modifiers. While acid (0.1 % formic acid) and temperature (<30 °C) do not have a significant effect on the PCs, it does have an impact on the PCs' retention in the C_{18} column and the resolution of separation. Also, elevated temperatures above 30 °C, higher concentrations of mobile phase modifiers (acids buffers) may result in the degradation of PCs. Although we did not investigate the photo-stability of the 7-PCs, the structural characteristics of most of these compounds suggest that they may be photosensitive.

3.4. Recoveries of PCs from aqueous solution

3.4.1. Extraction efficiency (% recovery)

The recovery of an analyte, usually expressed as a percentage, measures the extraction efficiency achieved in the recovery of a known amount of an analyte carried through the newly developed or known analyte sample extraction analytical processing steps or method. The recovery was based on loading 5 mL methanol pre-conditioned (at a flow rate of 0.5 mL/min) weak cation polymeric reverse phase (Strata PRP, 200 mg/6 mL bed), and hydrophilic-lipophilic balance (Supelco HLB, 200 mg/6 mL bed) adsorbent column in solid-phase extraction (SPE). The adsorbed residues of the pharmaceutical were recovered from the column bed using an appropriate solvent. Of all the solvent (dichloromethane, pure methanol, buffered methanol, acetone and acetonitrile alongside buffered water) tested for the recovery of the column trapped PC residues in SPE cartridge column, 0.5 % formic acid in methanol proved to be the most efficient in extracting LMV, ACP, VCM, CPX, SMZ, DCF, and IVT.

The mean extraction efficiencies (Table 6) for three different concentrations ranged between an acceptable value of 84.70 %–116.31 %, while % RSD was in all cases ≤, 8.50. The selectivity of the SPE method for water samples showed no interfering compounds that reduce the ability to quantify the analytes.

3.5. Analysis of real samples

For each of the collected sample, physicochemical parameters such as pH, conductivity, total dissolved solids, and weather factors such as temperature (Tpt), dew point (DP), heat index (HI), humidity (Hm), and atmospheric pressure (AtP) were measured in-situ. The pH of the water ranged 6.47 and 7.51 (S2) across all sampling stations; while the conductivity and total dissolved solids (TDS) of the water ranged 285 and 378 μS except for Blue Lagoon which was >4000 μS; and 141–189 ppm with Blue lagoon having >2000 ppm. The high conductivity and TDS values observed in the Blue Lagoon water are characteristics of estuarine and marine waters. The Tpt, DP, HI, Hu and AtP across all sampling stations were ranged 21.78–25.17 °C, 10.7–15.8 °C, 22.2–27.6 °C, 34.6–74.3 %, and 736.3–769.4 mmHg, respectively. Statistical analysis (ANOVA and a linearity test) was conducted for the measured concentration of the PCs and these parameters. However, no significant differences ($p < 0.05$) were found for conductivity, TDS, and pH.

The developed method was used in the analysis of surface water collected from five major sites within the KwaZulu-Natal Province of Durban, South Africa. These sites include Msunduzi River, Inanda Dam (Inlet and Outlet), Umgeni River, and Blue Lagoon. Samples were collected from four different points within each sampling site into 0.1 N nitric acid pretreated 2.5 L amber bottles. The water samples were filtered and processed for the recovery of LMV, ACP, VMC, CPX, SMZ, DCF, and IVT, using the developed SPE method, and thereafter separated and quantified using the instrument optimized parameter setting on LC-ESI-MS. The extractable residue concentrations of the investigated PCs are presented in Table 7.

Residues of the ACP (n = 60) and SMZ (n = 60) were the most frequently detected pharmaceuticals (n = 60) and found in all the tested water samples (100%) during the monitoring cycles. Lamivudine (LVM), VMC, CPX, and IVT occurred in 80%, 50%, 50%, and 40%, respectively, of the tested water samples collected from the sampling stations. Diclofenac (DCF) was not detected in the water samples, except in those collected at upstream points 1 and 2 on Umgeni River (5.29 and 51.94 μg/L, respectively. The concentration of SMZ was the highest and ranged between 2.65 ± 0.25 μg/L in P_3-Blue Lagoon water (n = 12) and 398.39 ± 0.90 μg/L in P_1-Inanda Dam outlet water (n = 12). ACP, with detected concentrations ranged; 56.70 ± 0.74 μg/L at P2-Inanda Dam Outlet and 177.55 ± 5.35 μg/L at P1-Inanda Dam Inlet followed this. The concentration of LMV, CPX, VCM and IVT in the tested surface waters collected ranged; not detected, ND, – 33.99 ± 1.89 μg/L at P3-Umgeni River; ND – 38.83 ± 2.09 μg/L at P4-Umgeni River; ND – 22.36 ± 3.97 μg/L at P3-Msunduzi River, and ND – 6.57 ± 0.91 μg/L at P3-Umgeni River, respectively. The observed concentration levels of the pharmaceuticals vary with the water stream types. However, the occurrence levels observed in the surface waters may not be unconnected with the pharmaceutical's class, their different physical and chemical properties, and those attributed to the water matrices in which they are resident.

Overall, the percentage of positive detection observed for each pharmaceutical at each of the water columns during the sampling period varied, except for DCF. The mean positive occurrence of the PCs in waters sampled across all sampling points in all sampling stations was found to be 62.85% (n = 60). There was no significant difference ($p > 0.05$) between positive detection of ACP, SMZ, and LMV, while there was a significant difference ($p < 0.05$) in positive detection of ACP, SMZ, LMV, and CPX, VCM, IVT. Although there was no particular location trend

Table 5. Repeatability (intra-day) and reproducibility (inter-day) precision at low, medium, and high PCs concentrations.

Pharmaceutical Compounds	Therapeutic Class	Retention Time t_R(min) Mean ± S.D	Precision (%RSD, n = 5) 0.04 ng/μL		Precision (%RSD, n = 5) 0.08 ng/μL		Precision (%RSD, n = 5) 0.1 ng/μL	
			Intra-day precision	Inter-day precision	Intra-day precision	Inter-day precision	Intra-day precision	Inter-day Precision
LMV	Antiretroviral	1.473 ± 0.033	1.28	0.93	1.58	0.77	1.04	1.34
ACP	Analgesic	3.221 ± 0.044	0.66	2.14	0.83	1.12	1.75	0.87
VMC	Antibiotic	3.723 ± 0.019	5.99	4.02	4.90	2.80	1.83	2.74
CPX	Antibiotic	4.710 ± 0.007	8.74	1.17	5.35	2.21	3.80	1.22
SMZ	Antibiotic	5.963 ± 0.023	2.34	2.20	1.20	0.36	1.41	1.69
DCF	Analgesic	9.851 ± 0.036	4.32	2.64	2.61	3.75	2.44	2.72
IVT	Anthelmintic	14.296 ± 0.073	5.29	3.43	2.66	2.74	6.12	1.31

Table 6. Percent relative standard deviation for the stability of the 7-PCs and percentage recoveries of analytes (%R) from spiked water.

Pharmaceutical Compounds	Stability of PCs				Percent recoveries of analytes (%R)					
	Average Peak Area and % RSD, n = 5 (0.04 ng/μL)		Average Peak Area and %RSD, n = 5 (0.10 ng/μL)		0.04 ng/μL (n = 3)		0.08 ng/μL (n = 3)		0.10 ng/μL (n = 3)	
					Mean ± S.D	%RSD	Mean ± S.D	%RSD	Mean ± S.D	%RSD
LMV	26642	0.93	66197	1.34	93.74 ± 6.55	6.99	98.46 ± 3.11	3.16	98.73 ± 1.30	1.32
ACP	40134	2.14	100668	0.87	92.99 ± 2.75	2.95	95.03 ± 4.11	4.33	94.10 ± 2.11	2.24
VMC	2634	4.02	6336	2.74	100.51 ± 4.86	4.83	102.09 ± 8.68	8.50	102.13 ± 6.41	6.28
CPX	16499	1.17	41166	1.22	84.70 ± 7.22	8.52	94.51 ± 2.03	2.15	116.31 ± 1.62	1.39
SMZ	41336	2.20	101608	1.69	98.48 ± 1.72	1.74	99.63 ± 1.30	1.30	99.59 ± 2.20	2.21
DCF	26140	2.64	62793	2.72	93.22 ± 4.79	5.13	99.26 ± 3.61	3.64	97.22 ± 5.33	5.48
IVT	1705	3.43	4020	1.31	98.19 ± 3.26	3.32	99.93 ± 3.07	3.08	103.20 ± 0.49	0.47

Table 7. Concentration levels (μg/L) of the 7-PCs detected in water collected from the different sampling point (n = 60).

	LMV		ACP		VMC		CPX		SMZ		DCF		IVT		Σ_iPCs	Mean	Std Dev
	Mean	stdev	Mean	stdev	Mean	stdev	Mean	stdev	Mean	stdev	Mean	stdev	Mean	stdev			
Msunduzi River																	
point 1	16.32	2.40	168.38	3.07	11.06	4.56	8.62	0.43	181.26	2.94	<0.04	-	5.43	1.65			
point 2	26.50	2.68	132.04	0.80	10.05	4.62	<0.07	-	161.20	0.44	<0.04	-	6.30	0.57			
point 3	29.98	0.88	138.83	4.91	22.36	3.97	<0.07	-	127.87	2.89	<0.04	-	<0.08	-			
point 4	21.39	2.09	151.35	4.50	14.68	7.88	<0.07	-	163.99	1.11	<0.04	-	1.16	2.01			
Σ_iPCs	94.19		590.6		58.15		8.62		634.32		-		12.89		1398.77	199.82	284.06
Inanda Dam Inlet																	
point 1	13.09	0.97	170.86	5.15	2.11	3.65	23.29	1.40	132.69	3.09	<0.04	-	<0.08	-			
point 2	13.65	0.43	144.05	4.61	<0.12	-	6.20	0.36	186.61	0.67	<0.04	-	5.76	0.67			
point 3	13.29	0.51	133.99	3.79	13.22	2.97	<0.07	-	184.40	1.49	<0.04	-	<0.08	-			
point 4	10.66	0.72	166.02	7.00	18.30	1.71	14.03	0.82	177.87	1.56	<0.04	-	2.22	3.85			
Σ_iPCs	50.69		614.92		33.63		45.52		681.57		-		7.98		1434.31	204.90	304.03
Inanda Dam Outlet																	
point 1	3.47	0.33	55.44	3.63	2.98	5.16	19.83	0.86	398.39	0.90	<0.04	-	<0.08	-			
point 2	2.47	0.45	54.56	0.71	<0.12	-	7.18	0.44	56.27	1.03	<0.04	-	<0.08	-			
point 3	3.11	0.69	71.73	3.29	<0.12	-	<0.07	-	98.20	1.10	<0.04	-	<0.08	-			
point 4	2.98	0.88	81.65	2.81	<0.12	-	<0.07	-	53.76	1.30	<0.04	-	<0.08	-			
Σ_iPCs	15.5		263.38		2.98		27.01		606.62		-		-		915.49	130.78	230.44
Umgeni River																	
point 1	26.60	1.53	96.70	6.84	<0.12	<0.12	5.80	0.65	128.08	1.37	5.29	0.27	<0.08	-			
point 2	33.75	2.14	139.74	4.17	<0.12	-	12.21	0.90	138.27	2.13	51.94	3.17	<0.08	-			
point 3	33.99	1.89	112.30	7.05	1.67	2.89	7.63	0.60	192.68	0.96	<0.04	-	6.57	0.91			
point 4	32.73	1.87	132.13	13.88	20.63	5.87	38.83	2.09	148.52	1.67	<0.04	-	0.00	0.00			
Σ_iPCs	127.07		480.87		22.3		64.47		607.55		57.23		6.57		1376.06	195.15	244.23
Blue Lagoon																	
point 1	<0.04	-	101.01	8.39	<0.12	-	<0.07	-	2.86	0.12	<0.04	-	<0.08	-			
point 2	<0.04	-	96.07	3.19	<0.12	-	<0.07	-	2.67	0.23	<0.04	-	<0.08	-			
point 3	<0.04	-	116.72	2.19	<0.12	-	<0.07	-	2.65	0.25	<0.04	-	<0.08	-			
point 4	<0.04	-	81.98	6.21	<0.12	-	<0.07	-	11.29	0.56	<0.04	-	<0.08	-			
Σ_iPCs	-		395.78		-		-		19.47		-		-		415.25	59.32	148.54
$\Sigma_i\Sigma_i$PCs	287.45		2345.55		117.06		145.62		2549.53		57.23		27.44				
Mean	57.49		469.11		23.41		29.12		509.91		11.45		5.49				
Std dev	53.16		144.79		23.86		26.42		275.85		25.59		5.53				

observed for the detected concentrations, results from the Inanda Dam Inlet contained higher Σ_iPCs measured, followed by Msunduzi River water and then Umgeni River. Inanda Dam Outlet water generally contains lesser concentrations of all the measured pharmaceutical residues, than the Dam Inlet water except for the elevated levels of SMZ (398.39 ± 0.90 μg/L) at P1. Blue Lagoon contained the least Σ_iPCs, with residues of ACP (85.19 ± 6.45–121.29 ± 2.27 μg/L) and SMZ (2.65 ± 0.25–11.29 ± 0.56 μg/L) being the main PC mostly detected. Of all the water samples tested, those collected around multiple discharge points gave higher positives with a range of detection between 10% and 62%, with a percentage detection reaching 100% for ACP and SMZ, compared with those collected away from discharge points. The highest concentration of Σ_iPCs measured during this study was SMZ, while the least Σ_iPCs was IVT. The levels of pharmaceutical residues (Σ_iPCs) detected in the samples were in the order: sulfamethoxazole > acetaminophen > lamivudine > ciprofloxacin > vancomycin > diclofenac > ivermectin (SMZ > ACP > LMV > CPX > VCM > DCF > IVT).

The frequency of detection of ACP (n = 20) and SMZ (n = 20) suggests their extensive usage. For instance, ACP is one of the typical easy to come by over the counter (OTC) analgesic, as well as the most prescribed

pain-relieving drug, hence their wide occurrence (Figure 2). Also, SMZ is a cheap broad-spectrum active antibiotic that is largely used for the therapy of bacterial infections, and this probably accounts for its prevalence in all the sampling points. Huang et al. (2011) reported that SMZ is one of the common water-polluting sulfonamide antibiotics detected in surface waters worldwide.

A comparison between the observed ACP and SMZ concentration in water sampled at the different sampling stations revealed no statistically significant differences ($p > 0.05$). However, anomalously high SMZ concentration (398.39 ± 0.90 μg/L) was detected in P1 Inanda dam inlet water. The detection of LMV (an antiretroviral (ARV) PC) was also conventional. Diclofenac, an analgesic, was not detected in 80% of the water samples tested ($n = 20$). It was only discovered in sampling points P1 and P2 on the Umgeni River. However, there was a significant difference ($p < 0.05$) between the concentration observed in the two sampling points. The non-detectable levels found for DCF may be due to its relatively poor thermal and solar radiation stability. Bartels and Tumpling (2007) showed that diclofenac is sensitive to solar radiation in their study and that its concentration decreased with solar radiation. Therefore, diclofenac levels in almost all the surface waters tested may have decimated due to exposure to visible radiation (light), and thus decreasing its concentration to undetectable/no detection levels. This phenomenon, in turn, may be responsible for their near-zero positive occurrence frequency. It is therefore suggested that metabolites or fingerprints of DCF degradation product tracking may be an alternative means of DCF monitoring where the need to test DCF is contingent.

There were locational variations in the occurrence levels of the tested PCs within the sampling stations, especially in locations classified as low human activity areas, which indicate low PCs concentrations. In contrast, sampling sites traversing through communities of high social activities areas showed elevated levels. Thus, aside from Inanda Dam inlet and the Dam Outlet, humans, and urban influence on the water quality of all the rivers, especially in terms of the levels of pharmaceuticals detected, cannot be ruled out. It is important to note that, although Blue Lagoon sampling site has very high human and recreational activities about the sampling points, most of the Blue Lagoon water samples generally contained a non-detectable level of tested PCs during the study period, except ACP and SMZ. The deficient detection levels of the PCs in the Blue Lagoon estuarine waters may be attributed to the volume dilution effect, occasioned by the marine water intrusion. This attribute also had a significant impact on the salinity of the water. Statistical analysis conducted along with weather conditions showed that there were significant differences ($p < 0.05$) between Tpt (°C) and levels of observed DCF concentrations. Samples that contained a measurable concentration of DCF were collected on days when the temperature was less than 20 °C and detected only in samples collected in high activity areas along the Umgeni River. Significant differences ($p < 0.05$) were also observed for IVT concentration and TDS.

The measured concentration levels of PCs observed in all the fresh surface waters is consistent with findings from other studies. Reported concentrations of residues of PCs in many water bodies around the world are in agreement with this study's results. For example, study results is consistent with the occurrence levels of PCs reported in some surface waters in Spain, United Kingdom, South Korea, and Serbia (Grujic et al., 2009; Kim et al., 2007; Gros et al., 2006; Ashton et al., 2004). Higher concentration was reported in some other studies (Lacey et al., 2012; Martín et al., 2011). In studies reported by Jiang et al. (2011) and Dinh et al. (2011), sulfamethoxazole and diclofenac were listed among the commonly detected PCs in surface China and in downstream of WWTPs receiving waters respectively. Kasprzyk-Hordern et al. (2007) reported a similar frequency of PCs detection in rivers of the UK (mean percentage frequency of 74%). As with SMZ antibiotics, which was the PC with the highest Σ_{SMZ}PCs values, Iglesias et al. (2014) also reported residues of antimicrobial marbofloxacin as PCs with Fthe highest concentration values at a rural site located in the Miño River flow. Data on IVT, LMV and VMC are generally scarce within and outside Africa. The levels of the tested PCs were comparable with those reported in some earlier studies in South Africa, with ACP and SMZ recording 10-folds and 80-fold higher levels respectively. A report presented by Matongo et al. (2015) indicated an occurrence level of 5.32 μg/L for SMZ in some surface waters, which falls within this study 2.65 ± 0.25–11.29 ± 0.56 μg/L SMZ concentration range **(S3),** with SMZ detection in some discrete columns recording

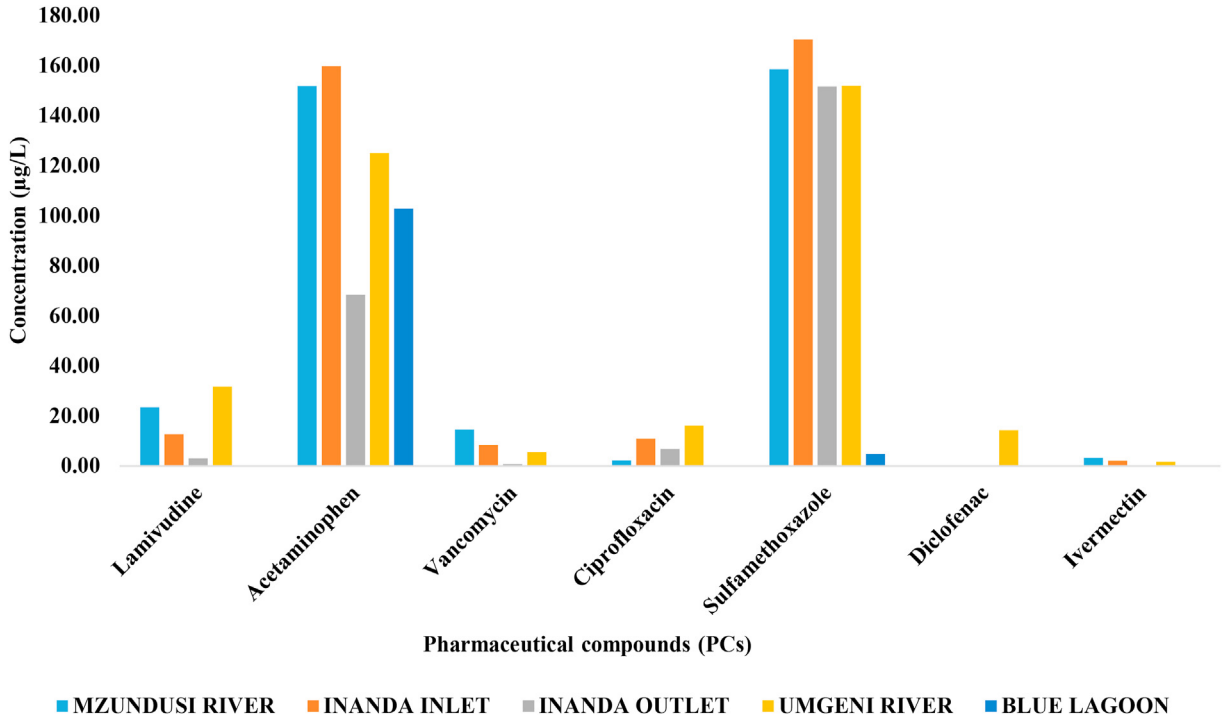

Figure 2. Concentrations (μg/L) of the PC analytes detected in selected surface water around KwaZulu-Natal Province.

80-folds levels reported by Matango et al. ACP concentration observed in this study were nearly 10-folds higher those reported (17.30 μg/L) by Gumbi et al. (2017). DCF occurred in a few ssamples, and the measure concenteartion were consistent with Madikizela and Chimuka (2017) who reoprted DCF range, ND to 51 94 μg/L in treated wastewater effluent in South Africa. While LMV was not detected in all samples, the measured values ($LMV_{max.}$, 33.99 μg/L) were far below levels reported by Abafe et al. (2018), Swanepoel et al. (2015), and Wood et al. (2015). The occurrence concentration of IVT (1.97 μg/L) and those of CPX in some farm wastewater receiving surface water in the Western Cape South Africa, and in surface water around KwaZulu-Natal, respectively (Fatoki et al., 2018; Agunbiade and Moodley 2016), is within the range ND to 6.57 μg/L observed in this study.

Unfortunately, the study is unable to ascertain the health and quality status of the river waters for residues of pharmaceutical compounds since there is no clear set of regulatory instructions, guideline values, or standards. Although the Σ_iPCs were ranged between 430.75 and 1456.39 μg/L across the sampling stations, the induction of toxic effects on native and migratory aquatic lives cannot be ruled out. This is because the low concentration of pharmaceutical compounds has been reported to elicit negative responses in exposed non-target organisms. The potential toxicity effect of SMZ on plants and soil organisms was reported by Klosterhaus et al. (2013), Jung et al. (2008) and Isidori et al. (2005), while the toxic impact of DCF, 17α–ethinylestradiol (EE2), and many others in exposed freshwater crustaceans, lower vertebrates and fish were variously reported by Kim et al. (2009) and Schwaiger et al. (2004). Therefore, the levels observed in the tested waters are of significant concern. Consequently, there is a need for constant monitoring as well as a necessity to develop methods for resolving residues of pharmaceutical compounds, especially in drinking water treatment plants to protect humans, animals, biotic and aquatic resources.

4. Conclusion

The present paper describes the development of a rapid LCMS method for the detection and separation of the mixture of 7 pharmaceutical drugs, i.e., lamivudine, acetaminophen, vancomycin, ciprofloxacin, sulfamethoxazole, diclofenac and ivermectin under an optimum analytical condition. The total run time was 19 min. The method was validated according to the ICH validation procedure and was found to be selective, sensitive, precise, and accurate for the LCMS assay of the 7 APIs. Following the validation acceptability criteria used in inferring the validity of the newly developed analytical procedure, the method is suitable for the intended use, since it meets the appropriate stipulated acceptable compound separation and detection criteria and satisfactory conformance to specifications for its intended purpose. This method was used for the environmental monitoring of the 7-PCs in selected surface waters and other aqueous media.

The occurrence of residues of pharmaceutical compounds in the aquatic environment is of human and environmental health concern. All the analytes except DCF were positively detected in the water of all the sampling points in all the sampling stations, at variable concentrations falling within the range ND and 398.98 μg/L. However, there was a DCF spike at two of the sampling points on the Umgeni River. Water samples collected from sampling sites located in low human activity areas had lower PCs positive concentrations compared with those along higher human activity areas with higher positive concentrations, except the Blue Lagoon, which had more negatives (80%) measurements. There were significant differences only between the occurrence levels DCF/IVT and other PCs tested. However, there was no significant difference between the concentrations of ACP, SMZ, and LMV. The statistical relationship between concentrations, sampling points, temperature conditions, pH, conductivity, and TDS indicated no significant differences.

Declarations

Author contribution statement

Olatunde Stephen Olatunji: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Elizabeth Oyinkansola Omotola: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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