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DOI
10.1016/j.scitotenv.2016.04.158

Publication date
2016

Document Version
Final published version

Published in
Science of the Total Environment

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Citation for published version (APA):

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Download date: 28 Dec 2023
Determination of phosphodiesterase type V inhibitors in wastewater by direct injection followed by liquid chromatography coupled to tandem mass spectrometry

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HIGHLIGHTS

• A new analytical method based on LC-MS/MS was developed and validated.
• Drug usage patterns were discussed: “weekday” vs “weekend”, “normal” vs “atypical” week.
• Wastewater-based epidemiology approach was successfully applied.

ABSTRACT

A simple, fast and reliable analytical method for the determination of phosphodiesterase type V inhibitors in wastewater was developed and validated. The method was based on direct injection followed by liquid chromatography coupled to tandem mass spectrometry with triple quadrupole as mass analyzer. Transformation products and analogues were included in the target list besides the three active pharmaceutical ingredients (sildenafil, vardenafl and tadalafil). The method performance was thoroughly investigated, including the analyte stability in wastewater and matrix effect. All target compounds presented linear fits between their LOD and 500 ng/L. The quantification limits ranged from 1.6 to 30 ng/L for all compounds except for n-octylnortadalafil (LOQ: 100 ng/L); precision calculated as intraday repeatability was lower than 30%; accuracy calculated as procedural recovery ranged successfully between 85 and 105% in all cases. The method was applied to samples collected during three week-long monitoring campaigns performed in 2013, 2014 and 2015 in three Dutch cities. Only sildenafil and its two metabolites, desmethyl- and desethylsildenafil, were present with normalised loads ranging from LOQ to 8.3, 11.8 and 21.6 mg/day/1000 inh, respectively. Two additional week-long sets of samples were collected in Amsterdam at the time that a festival event took place, bringing around 350,000 visitors to the city. The difference in drug usage patterns was statistically studied: “weekday” versus “weekend”, “normal” versus “atypical” week; and results discussed. The metabolite to parent drug concentration ratio evolution during consecutive years was discussed, leading to several possible explanations that should be further investigated. Finally, wastewater-based epidemiology approach was applied to back-calculate sildenafil consumption.

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avoid a visit to the doctor and save money, as the official pharmaceuticals are more expensive (Schnetzler et al., 2010). But they are not aware of the involved health risk to which they expose themselves due to the unreliable origin and quality of these products (Venhuis et al., 2014c).

Analytical studies with PDE5 inhibitors as target compounds have been mainly addressed to the identification of new synthetic analogues since homosildenafil was first reported (Shin et al., 2003). The commonly applied techniques for structure elucidation and confirmation are liquid chromatography coupled to mass spectrometry (MS, HRMS, and MS/MS), UV detection, IR spectroscopy and NMR. Several recent examples can be found in the literature: structure elucidation of a thiono analogue of sildenafil in an alleged herbal aphrodisiac (Venhuis et al., 2008); isolation and structural elucidation of cyclopentanpyridine and n-octylnoctalafal in a dietary supplement (Hasegawa et al., 2009); identification of a nitrosated produg of aldendaf in a dietary supplement (Venhuis et al., 2011); identification of a new tadafal analogue, acetaminotadalaf, in a dietary supplement (Lee et al., 2013b). Besides, screening methods based on liquid chromatography coupled to tandem mass spectrometry have been validated in order to detect and quantify the presence of PDE5 inhibitors and a large list of analogues in potentially adulterated products. To date, >50 unapproved analogues have been reported as adulterants (Patel et al., 2014) and the number is still growing. Analysed samples are solid-based (pills, hard and soft capsules, bulk powders, herbal products), typically treated with a simple liquid–liquid extraction with an organic/aqueous mix solution and after centrifugation, the supernatant is diluted and/or filtered prior to injection to prevent contamination of the LC–MS system (Patel et al., 2014). When applied to potentially adulterated or counterfeit products, sildenafil and tadalafl as well as their analogues were the most frequently detected (Lebel et al., 2014; Lee et al., 2013a), although analogues were quantified at impurity levels which might indicate that their presence is due to by-product formation from the synthesis of the main pharmaceutical ingredient.

Regarding the analysis in environmental matrices: sildenafil has been included in multi-residue methods (Baker et al., 2014; Boleda et al., 2013), but very few studies have analysed the other 2 APIs tadalafl and vardenaf (Nieto et al., 2010), or have included metabolites and transformation products (Schröder et al., 2010). Recently, also photodegradation studies for the identification of new transformation products have been conducted (Eichhorn et al., 2012; Herbert et al., 2015) with special attention to those observed to be notably persistent, which might indicate a potential impact for the aquatic environment.

The aim of this work was to determine the actual use of PDE5 inhibitors in The Netherlands. To do so we used wastewater-based epidemiology (WBE) (Daughton, 2001; Ort et al., 2014), which is a powerful and objective approach for studying the health and lifestyle of the community living in the catchment area of the wastewater treatment plant where influent samples are collected. We present an analytical method based on liquid chromatography coupled to tandem mass spectrometry for the quantification of the three APIs present in ED pharmaceuticals, including transformation products and analogues (10 target compounds in total) in wastewater. Available information on pharmacokinetics plays an important role on the modelling, since the excretion ratio % with regard to the consumed/absorbed amount has to be applied. According to literature, after a single dose of Viagra® 92% of the drug is absorbed. From that absorbed dose, 27% is excreted as metabolites desmethyl- and desethylsildenafil with no detectable parent in either feces or urine (Muirhead et al., 2002a). However, the presence of sildenafil in municipal wastewater has been reported in several cities in the past (Baker et al., 2014; Boleda et al., 2013; Nieto et al., 2010; Schröder et al., 2010).

The method was applied to real influent samples collected during a series of week-long monitoring campaigns in the years 2013, 2014 and 2015 in the cities of Amsterdam, Eindhoven and Utrecht, and during two festival events in Amsterdam taking place in 2012 and 2014. Besides determining the environmental loads of the target compounds, an estimation of the sildenafil consumption was done as it has been previously done for environmental loads of other prescription pharmaceuticals (ter Laak et al., 2010) in The Netherlands (Venhuis et al., 2014b).

2. Material and methods

2.1. Reagents and standards

Sildenafil citrate, desmethylsildenafil, desethylsildenafil and noracetildenafil were obtained from LGC (Luckenwalde, Germany). Vardenaf dihydrochloride, n-desethyl vardenaf, tadafal, aminotadalaf, chloropretadalaf and n-octyl noctalafal were obtained from TRC Toronto Research Chemicals Inc. (Ontario, Canada). Two isotopically labeled internal standards (ILIS) were used as surrogates: sildenafil-d8 and desmethylsildenafil-d8, supplied by TLC Pharmachem (Ontario, Canada). All the above-mentioned standards were of high purity grade (>98%).

Individual stock solutions were prepared from powdered substance in methanol at a level ranging from 100 to 500 mg/L, and stored at −20 °C. A mix stock solution was prepared at a final level of 5 mg/L in methanol. Working solutions were prepared by dilution to the desired concentration with methanol, and stored at −20 °C. Calibration curve was prepared daily by diluting with ultrapure water the appropriate mix to a final composition water:methanol (90:10, v/v).

Methanol and acetone, both HPLC grade solvents, were supplied by Mallinkrodt Baker B.V. (Deventer, The Netherlands). Formic acid (50% in water) was obtained from Fluka Analytical (Sigma-Aldrich, Stenheim, Germany). The ultrapure water was obtained by purifying demineralized water in a Milli-Q system from Millipore (Bedford, MA, USA).

For the sample preparation, regenerated cellulose filters RC 0.2 µm were purchased from Phenomenex (Torrance, USA).

2.2. Sampling and sample preparation

Raw influent wastewater was collected after the sand trap from the WWTPs serving Amsterdam, Eindhoven and Utrecht, in The Netherlands. The sewer systems were characterized according to the questionnaire developed by Dr. Ort already used in previous WBE studies (Castiglioni et al., 2012).

For seven consecutive days 24-h volume-dependent influent composite samples (Castiglioni et al., 2012) were taken at each WWTP in 2013 (6th–12th March), 2014 (11th–18th March) and 2015 (4th–10th March). Each of these weeks was considered to reflect a “normal” week regarding the population’s drug use, as no big event or festivity took place during the weeklong monitoring. Two extra sets of samples collected at the WWTP in Amsterdam during festival events in 2012 and 2014 were also included in the study. These latter weeks were considered to reflect atypical behavior of the people involved regarding drug use, as a mass event took place during these two sampling weeks, lasting from Thursday to Sunday that attracted ~350,000 visitors to the city. The average sampling interval in all cases was lower than 15 min. WWTP characteristics were provided by the autosamplers located at the three WWTPs kept samples refrigerated at 4 °C during composite collection. Afterwards samples were stored in HDPE containers and frozen immediately after collection at −20 °C until analysis. The maximum time samples were stored prior to analysis was 2 years for the case of 2012 samples, 6 months for...
2.3. Liquid chromatography tandem mass spectrometry

A Thermo Scientific TSQ Vantage triple quadrupole mass spectrometer provided with a heated electrospray ionisation source (Thermo Electron, Bremen, Germany) was interfaced to a Surveyor HPLC system (Thermo Electron).

The chromatographic separation was achieved on an XBridge C18 column (150 mm × 2.1 mm I.D., particle size 3.5 μm) (Waters, Etten-Leur, the Netherlands) preceded by a KrudKatcher ULTRA HPLC in-line SS filter (0.5 μm × 0.1 mm I.D.) (Phenomenex, Torrance, USA) maintained at a temperature of 21 °C. From the sample aliquot 100 μL were injected into the system, and by using an optimized ternary gradient of ultrapure water (A), methanol (B) and acetonitrile (C), all three with 0.05% formic acid, the compounds were separated at a constant flow rate of 0.3 mL/min and introduced into the mass spectrometer. Because 6 out of the 10 compounds studied had a log Kow value within a narrow window (1.99–2.58, see Table 1) a ternary gradient was provided to sufficient chromatographic separation. The percentage of organic solvent B was changed as follows: 0 min, 0%; 4 min, 5%; 14 min, 20%; 15–17 min, 100% and the percentage of organic solvent C was changed as follows: 0–1 min, 0%; 4 min, 5%; 14 min, 30%; and 15–17 min, 0%. Between consecutive runs, the analytical column was re-equilibrated for 3 min.

The system was operated in positive mode. For the ionisation nitrogen gas was used. The source voltage was set to 2.5 kV, and the capillary gas was used. The source voltage was set to 2.5 kV, and the capillary temperature to 300 °C and 350 °C, respectively. The collision gas was used. The source voltage was set to 2.5 kV, and the capillary gas was used. The source voltage was set to 2.5 kV, and the capillary temperature to 300 °C and 350 °C, respectively. The system was operated in positive mode. The ionisation nitrogen gas was used. The source voltage was set to 2.5 kV, and the capillary gas was used. The source voltage was set to 2.5 kV, and the capillary temperature to 300 °C and 350 °C, respectively. The system was operated in positive mode. For the ionisation nitrogen gas was used. The source voltage was set to 2.5 kV, and the capillary gas was used. The source voltage was set to 2.5 kV, and the capillary temperature to 300 °C and 350 °C, respectively.

The method was validated in terms of linearity, limits of detection and quantification, precision intra-day (repeatability) and inter-day, procedural recovery and matrix effects by analyzing spiked wastewater samples with standard solutions at 6 different concentrations (0–500 ng/L) to investigate linearity. The concentration of analyte in a sample was obtained by comparing the peak area ratio of the analyte and internal standard to its corresponding ratio in the calibration curve.

Limits of detection and quantification (LOD and LOQ, respectively) were defined as the concentration that provides signal-to-noise (S/N) values of 3 and 10 for the quantifier ion of each analyte. The values were calculated at the lowest point of the calibration curve (in wastewater) correctly confirmed with qualifier ion ratio. Concentration values determined in the analysed real samples that were <LOQ were treated as follows: (1) if all values at a location for a certain compound were <LOQ, loads were set to zero; (2) if at least one value was >LOQ, values <LOQ were replaced with 0.5 × LOQ (Ort et al., 2014).

Intra- and inter-day precision were assessed at 4 levels: 20, 50, 100, 500 ng/L, with n = 7 replicates per level, and during 3 non-consecutive days. Procedural recovery (%) was calculated as the ratio of the signal of the analyte spiked to a sample after sample filtration (C) against the signal of the analyte spiked to the same sample before filtration (B): ([C]/[B] × 100). Matrix effect (%) was calculated as the ratio of the signal of solution B (where the signal of the native compound present in the used sample was subtracted) against the signal of the analyte spiked to ultrapure water (A): [B/A] × 100. The specificity was evaluated with two parameters, the retention time and the ratio...
between the quantifier (Q) and the qualifier (q): RT ± 2.5% and Q/q < 30%.

2.4.1. Analyte stability

Few studies have addressed the stability in sewage of the target compounds, metabolites and transformation products (Baker and Kasprzyk-Hordern, 2011; van Nuijs et al., 2012). As these products are bioactive, further metabolization and transformation can occur after sample collection. Stability is therefore an important parameter to study for the correct quantification of the drugs, thus avoiding significant under estimations.

The stability experiments consisted of following the concentration change of analytes spiked in untreated raw (and unfiltered) wastewater over a definite period of storage at a temperature of 4 °C, as these are the conditions in which sample is kept in the autosampler during sampling. The experiment was carried out at the original pH (7.5–8). For each time interval (0, 3, 6, 9, 24, 30, 48 h), one blank and three independent sample aliquots spiked at 100 ng/L with all native compounds were prepared. Time 0 was considered as control. After the selected-time interval, ILIS were spiked to all samples, which were then homogenized and immediately filtered for the analysis as explained in Section 2.3.

3. Results and discussion

3.1. Method optimization: DI-HPLC-(QqQ)-MS/MS

Although influent wastewater is a very complex matrix that may require appropriate sample treatment for the removal of suspended matter and matrix components, direct injection is an interesting alternative because of a reduction of sample treatment steps that may lead to analyte losses and because of time saving. For direct injection obviously the sensitivity of the method should be considered beforehand. From pilot experiments and data published in literature (Nieto et al., 2010) we expected that concentrations in Dutch wastewater influents were such that direct injection could be applied. To make sure, we evaluated the sample procedure proposed by Nieto et al. (Nieto et al., 2010) with acetone ethylacetate for the elution of the Oasis HLB cartridge, as well as a direct injection procedure described for the determination of isothiazolinones (Speksnijder et al., 2010). Results showed similar performances (recoveries, satisfactory sensitivity, and matrix effects, results for SPE not shown here, see below for direct injection results) and therefore direct injection was selected in order to simplify the analytical methodology.

3.2. Method performance

Quality parameters are summarized in Table 2. All target compounds present a good linear fit within the studied range (LOD – 500 ng/L) with r-squared values higher than 0.999. Regarding the LOD and LOQ, compounds could be divided into three groups: sildenafil, desethylsildenafil, tadalafl and aminotadalafl with the lowest limits, LOQs ranging between 1.6 and 7.5 ng/L. The second group with LOQ ranging between 13.3 and 30.0 ng/L: desmethylsildenafil, noracetildenafl, chloropretadalafl, vardenafinafl and n-desethylvardenafinafl. Finally n-octylnortadalafl with highest LOQ at 100 ng/L. Intraday and inter-day repeatability is presented as RSD(%). For all compounds values were lower than 26% at the two higher levels. For the lower levels, sildenafil and its two metabolites, tadalafl, aminotadalafl, chloropretadalafl, n-octylnortadalafl and vardenafinafl presented in the same range whereas in the case of noracetildenafl and n-desethylvardenafinafl the RSD(%) for the lowest levels was 30%. Procedural recovery ranged successfully between 85 and 105 in all cases, with RSD(%) values lower than 25%. Regarding matrix effects appeared to be concentration dependent. At lower concentrations of the spike, invariably matrix enhancement was observed, up to 5-fold (desethylsildenafil), whereas at higher concentrations much lower enhancement and also sometimes
suppression of the signal (down to 46% for noracetildenafil) was observed (see Table 2).

3.2.1. Analyte stability

The results of the stability test are presented as percentage of change from the initial concentration, and plotted in Fig. 1. The error bars correspond to the standard deviation of triplicates. The statistical evaluation of the results was performed using ANOVA in order to assess a statistically significant change of the signal along the time. For two compounds a statistically significant (p-value < 0.05) decrease was observed: noracetildenafil (p = 0.001) and n-octynortadalafil (p = 0.048) over 48 h. For both compounds, p-value below 0.05 was observed from the 24-h point meaning that stability starts decreasing within the 9 to 24 h range. All other compounds tested did not experience any statistically significant decrease and can therefore be considered stable. Given that the composite sample collection lasts 24 h, noracetildenafil and n-octynortadalafil might experience some degradation. However this is minimized since the samples are frozen immediately after collection.

3.3. Analysis of wastewater influents

Sample concentrations in WWTP influents were transformed into daily loads of analytes entering the WWTP using flow rates provided by the WWTP operators (see Tables S1–S5). The results were expressed in normalized loads (mg/day/1000 inh) according to Eq. (1), where the concentration in the sample in ng/L is multiplied by the registered flow during the corresponding 24-h period and divided by the number of inhabitants within the catchment area. Census data were used for normalization to number of inhabitants connected to the sewer system.

\[
\text{load mg/day/1000 inh} = \frac{\text{Flow (10^3 L/day)} \cdot \text{Conc (10^{-6} mg/L)}}{n \cdot \text{inh} \cdot 10^3}
\]

3.3.1. Weekly variations

Sildenafil and its two transformation products desmethyl- and desethylsildenafil were the only active ingredients detected in the samples collected. This finding was in accordance with prescription data, as the three erectile dysfunction pharmaceuticals are similarly prescribed in The Netherlands but with lower daily doses: Viagra® at 50–100 mg compared to Levitra® + Cialis® at 10–20 mg. Therefore, vardenafila and tadalafil (and their corresponding analogues and transformation products) could be expected at a lower level below their respective LODs. Tables S6–S8 present the normalized daily loads determined in the samples collected during the three week-long monitoring campaigns performed in Amsterdam, Eindhoven and Utrecht in 2013, 2014 and 2015. Sildenafil was present in all samples analysed, with loads ranging from 1.6 to 8.3 mg/day/1000 inh. Temporal trends in the city of Amsterdam showed an increase from 2013 to 2014 and 2015, from a weekly average of 3.4 to 6.3 and 6.8 mg/day/1000 inh. In the case of Eindhoven, the weekly average remained constant in 2013 and 2014, followed by a decrease in 2015: 3.8, 3.8 and 2.6 mg/day/1000 inh, respectively. In the city of Utrecht an increase was observed from 2013 to 2014, and then a decrease in 2015 with weekly averages being 2.4, 3.1 and 2.4 mg/day/1000 inh, respectively. Desethylsildenafil is the most abundant metabolite of sildenafil in urine (Muirhead et al., 2002a). It was detected in all but two samples in 2013, which represents 97% of the total number of samples analysed. Temporal trends showed an increase in Amsterdam over the years, doubling from 2013 to 2014. Values corresponded to 6.3, 15.7 and 19.2 mg/day/1000 inh, respectively. In the cities of Eindhoven and Utrecht, a remarkable increase was observed from 2013 to 2014, but thereafter levels remained constant from 2014 to 2015. Values corresponded to 4.3, 11.1 and 11.1 mg/day/1000 inh. in Eindhoven and 2.1, 7.8 and 8.0 mg/day/1000 inh. in

Fig. 1. Stability plot for PDE5 inhibitors in wastewater at natural pH and 4 °C. Results have been normalized as percentage of initial concentration. Error bars correspond to the standard deviation of triplicates.

Fig. 2. (A: sildenafil; B: desmethylsildenafil; C: desethylsildenafil). Daily variations expressed as the percentage of the total load, combined results from 2013, 2014 and 2015. The box represents the median, 25% and 75% percentile values and the whiskers extend to the minimum and maximum values. The dotted line represents the average per city.
Utrecht. Desmethylsildenafil is the second metabolite detected, and known to be less abundant in urine than desethylsildenafil (Muirhead et al., 2002a). The time trend for this compound showed an increase over the years in Amsterdam, doubling from 2013 to 2014. Values corresponded to 3.9, 8.4 and 11.2 mg/day/1000 inh. respectively. In the cities of Eindhoven and Utrecht, it could not be detected in 2013, and a decrease from 2014 to 2015 was observed. Values corresponded to 5.5 and 3.4 mg/day/1000 inh. in Eindhoven and 3.6 and 2.2 mg/day/1000 inh. in Utrecht. Daily variations expressed as a percentage of the total weekly load of the individual compounds are presented in Fig. 2 (A: sildenafil; B: desmethylsildenafil; C: desethylsildenafil). The box represents the median, 25% and 75% percentile values and the whiskers extend to the minimum and maximum values. This way of presenting the results allows the rapid observation of a difference between weekdays (Mon–Fri) and weekends (Sa–Su), regarding the recreational use of the drug. Depending on the human excretion rates of individual drugs and the sewer residence time, the “weekend peak” may appear earlier or later. In the case of sildenafil and its two metabolites, an ANOVA single factor was performed to evaluate whether the difference among different days and the difference between weekdays and weekend were statistically significant. The result showed that there was no statistically significant difference, which can be interpreted as a non-recreational usage pattern.

The finding of the presence of sildenafil in wastewater, that has been reported before (Baker et al., 2014; Nieto et al., 2010; Schröder et al., 2010) is contradicting results from pharmacokinetic studies, which invariably conclude that no appreciable amounts of unchanged sildenafil are being excreted from the body after its consumption (Muirhead et al., 2002a; Walker et al., 1999). In another study however in the urine of two individuals dosed with sildenafil some unchanged compound was found (Strano-Rossi et al., 2010). In addition, phase-II conjugation possibly leads to excretion of a conjugated form of sildenafil that may be deconjugated in the sewer.

3.3.2. Festival event results

Besides the three week-long monitoring campaigns, two additional week-long sets of samples were collected in Amsterdam at the time that a festival event took place. This annually recurring event lasts from Thursday to Sunday bringing around ~350,000 visitors to the city. For these weeks the comparison among the loads within the city were expressed in g/day, since the normalization by 1000 inh. was difficult to apply. Tables S9–S10 present the daily loads determined in the samples collected during the two week-long monitoring campaigns performed in Amsterdam in 2012 and 2014.

At this point, and in order to better evaluate the observed trends along the years, it is worth mentioning that the patent that the pharmaceutical company Pfizer held for Viagra® expired in some European countries (including The Netherlands) in June 2013. For this reason, two periods can be differentiated. The first one including the results from the sampling campaigns performed in 2012 and 2013, when the patent was still valid; and the second including the results from the sampling campaigns performed in 2014 and 2015, after the expiration of the patent. Fig. 3 (A: sildenafil; B: desmethylsildenafil; C: desethylsildenafil) presents in box plots the daily loads during the five week-long monitoring campaigns performed in Amsterdam. To evaluate the difference between “event” weeks and “normal” weeks an ANOVA single factor was performed. Both the parent sildenafil and the transformation products showed similar temporal patterns: the daily loads in week 2013 were statistically significantly lower than those in other weeks (p = 0.05). Before the expiration of the event, it was more difficult to obtain the drug, and perhaps during the recreational event users took more trouble to obtain the drug (which would explain the statistical difference between the daily loads in the “normal” week in 2013 and in the event week in 2012). After the expiration it became easier to obtain the drug, and event weeks no longer showed statistical differences with “normal” weeks.

The “weekend peak” was also statistically investigated and resulted in a not significant difference of the loads between weekdays (Mon–Fri) and weekend (Sat–Sun).

3.3.3. Metabolite to parent concentration ratio

In order to evaluate the clearance of sildenafil, the daily ratios between the main transformation product and the parent drug obtained during one week are presented in Fig. 4. Only the ratio desethylsildenafil/sildenafil was evaluated, since the ratio desmethylsildenafil/sildenafil was not always available. The ratio desethylsildenafil/sildenafil showed a marked trend among cities and years. In the case of Amsterdam, the median experienced a slight increase of 1.5-fold from 1.7 in 2013, to 2.6 in 2014 and 2.7 in 2015. The increase was larger for the other two cities. In the case of Eindhoven the median of the ratio increased from 1.1 in 2013 to 3.2 in 2014 and 4.3 in 2015, which corresponded to a 4-fold increase. And in the case of Utrecht, the ratio increased from 1.2 in 2013 to 2.5 in 2014 and 3.5 in 2015, which corresponded to a 3-fold increase.

![Image](140)
The observed phenomenon is intriguing, since we expected the ratio to remain more or less constant along the years because of a stable transformation rate of the drug in the human body. To our knowledge, the change in the metabolite to parent drug concentration ratio has not been previously reported for PDE5 inhibitors. We propose two speculative explanations of this phenomenon.

3.3.3.1. Sewer characteristics. The first explanation is based on the effect of wastewater temperature. One of the parameters that could have an effect is the wastewater temperature. The average temperatures in the country during the weeks preceding the sampling campaigns averaged 2.5 °C in 2013, whereas in 2014 and 2015 they averaged 8 °C and 6.5 °C respectively (see daily temperatures in Tables S6–S8). We evaluated the stability of the analytes in wastewater at 4 °C in the laboratory and found no change in concentration over a 48 h period for the two compounds (see Fig. 1), but a difference of several degrees could already promote the further transformation of sildenafl into desethylsildenafl in the sewer. Besides, it is well-known in The Netherlands that often waste dumpings from illegal drug labs (Nu.nl “Vaker dumping van drugsresten in riool Nederlandse steden”) or under the pressure of a police raid (Emke et al., 2014) occur, and that the frequency of these dumpings has increased in the period 2013–2015 (Omroep Brabant). The disposal of this illegal waste can be either directly into the environment or into the sewer system, the latter affecting the wastewater conditions and possibly resulting in increased formation of sildenafl metabolite. More research on the degradation pathway of sildenafl in the sewer, and the effect of the different parameters involved in wastewater quality would be required to support the finding and this explanation.

3.3.3.2. Pharmacokinetics. The second explanation is based on the possibility that the expiration of the patent in 2013 has widened the number of currently available ED products, and that different formulations or doses experience different excretion ratios. It could be that sildenafl synthetic analogues not formally approved, but being the active ingredient in one of the counterfeit medicines or products such as dietary supplements, undergo a metabolic pathway leading towards the formation of desethylsildenafl. Further research in the counterfeit analogue area is required, not only regarding their identification but also their pharmacokinetics. Besides, the alteration in sildenafl pharmacokinetics has been studied as an effect of different parameters such as age, renal and hepatic functions (Muirhead et al., 2002b). The study reported a slower metabolism in elderly and patients with hepatic and renal failure, who showed a higher exposure to the drug because of a slower sildenafl clearance, compared to young men and healthy patients. Another possible reason to induce a change in the metabolism could be the prolonged use and the dose-dependence. It is well know that sildenafl users outside the official health system auto-medicate themselves (Rao et al., 2015) and prolong the (ab)use with high doses. Further research is required to study to which extent sildenafl pharmacokinetics might change for long-term users.

3.4. Back-calculation of sildenafl consumption

The obtained environmental loads can be used to back-calculate sildenafl consumption by the population connected to the sewer system (WBE). This was done based on pharmacokinetic data (Muirhead et al., 2002a) and the assumption that there were no losses in sewage due to degradation and no dumping of unused drugs (Venhuis et al., 2014a). Normalized results are shown in Fig. 5 for 2013, 2014 and 2015 and for the two festival events in 2012 and 2014. The data for 2013 have been used in a separate study in order to quantify illegal sales of sildenafl (Venhuis et al., 2014b). The data were expressed both as mg/week/1000 inhabitants and doses/week/1000 in. assuming that the recommended dose for an adults is 50 mg. The dose data for 2013, 2014 and 2015, where an increase is visible between 2013 and 2014 for all three cities, again suggest that the expiration of the Viagra® patent end of 2013 has led to increased sales and consumption of sildenafl.

4. Conclusions

An analytical method was developed and validated to determine the presence of ten PDE5 inhibitors in influent wastewater. Its application to wastewater influents collected from 2012 to 2015 in three Dutch cities resulted in the successful determination of sildenafl and its two main human metabolites. The presence of unchanged sildenafl in wastewater contradicted results from pharmacokinetic studies, whereas the other seven target analytes were not detected as expected from prescription data. Besides, sildenafl showed no recreational usage pattern, since the statistical study of the differences between weekdays (Mon-Fri) and weekend (Sat-Sun) loads was not significant. Two additional week-long sets of samples collected in Amsterdam at the time that a festival event...
took place were also included in the study. Only in the studied weeks before the patent expiration an increase in the load between “normal” and “event” was observed. The metabolite to parent drug concentration ratio was plotted, and showed a marked increase from 2013 to 2015, leading to several speculative explanations that open new research lines. Finally the environmental loads of sildenafil and its two metabolites were back-calculated into the estimated sildenafil consumption.

Acknowledgements

WWTPs’ staff is highly acknowledged: Mark Stevens and Jan Jonker from Hoogheemraadschap de Stichtse Rijnlanden (WWTP Utrecht), Peter van Dijk and Han van Happen from Waterschaps De Dommel (WWTP Eindhoven) and Alex Veltman and Peter Theijssen from Waternet (WWTP Amsterdam).

Dr. Cari Sängers’ (Kantisto BV) expert advice in pharmaceutical aspects is gratefully acknowledged.

Funding to support SEWPROF MC ITN entitled “A new paradigm in drug use and human health risk assessment: Sewage profiling at the community level” from the People Programme (Marie Curie Actions) of the European Union’s 7th Framework Programme FP7/2007-2013/ under REA grant agreement no. [317205] is acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.04.158.

References


Web references


