Comparison of phosphodiesterase type V inhibitors use in eight European cities through analysis of urban wastewater

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Abstract

In this work a step forward in investigating the use of prescription drugs, namely erectile dysfunction products, at European level was taken by applying the wastewater-based epidemiology approach. 24-h composite samples of untreated wastewater were collected at the entrance of eight wastewater treatment plants serving the catchment within the cities of Bristol, Brussels, Castellón, Copenhagen, Milan, Oslo, Utrecht and Zurich. A validated analytical procedure with direct injection of filtered aliquots by liquid chromatography-tandem mass spectrometry was applied. The target list included the three active pharmaceutical ingredients (sildenafil, tadalafil and vardenafil) together with (bio)transformation products and other analogues. Only sildenafil and its two human urinary metabolites desmethyl- and desethylsildenafil were detected in the samples with concentrations reaching 60 ng L^{-1}. The concentrations were transformed into normalized measured loads and the estimated actual consumption of sildenafil was back-calculated from these loads. In addition, national prescription data from five countries was gathered in the form of the number of prescribed daily doses and transformed into predicted loads for comparison. This comparison resulted in the evidence of a different spatial trend across Europe. In Utrecht and Brussels, prescription data could only partly explain the total amount found in wastewater; whereas in Bristol, the comparison was in agreement; and in Milan and Oslo a lower amount was found in wastewater than expected from the prescription data. This study illustrates the potential of wastewater-

based epidemiology to investigate the use of counterfeit medication and rogue online pharmacy sales.

Keywords: erectile dysfunction; prescription drugs; LC-MS/MS; consumption; counterfeit; wastewater-based epidemiology

Highlights:

- Wastewater-based epidemiology approach expanded to investigate counterfeit medication
- Very sensitive analytical method allowed identification of target analytes at low ng L^{-1} level
- Different spatial trends in sildenafil use were found across Europe

1. *Introduction*

The chemical analysis of raw wastewater with advanced mass spectrometry techniques allows for the determination of human urinary biomarkers when these are excreted in sufficient concentrations and remain stable on their way along the sewer system (Castiglioni et al., 2013).The finding of specific biomarkers may reveal valuable near real-time information regarding a population's lifestyle, illness and exposure to external agents. Successful studies thus far have revealed the population's level of oxidative stress (Y. Ryu et al., 2016), its exposure to pesticides (Rousis et al., 2017), and to phthalate plasticizers (González-Mariño et al., 2017), its consumption of legal substances such as alcohol, nicotine or caffeine (Baz-Lomba et al., 2016; Gracia-Lor et al., 2017; Yeonsuk Ryu et al., 2016), its use of illicit drugs (Causanilles et al., 2017a, 2017c; Ort et al., 2014) and other psychoactive substances (Bade et al., 2017; Castrignanò et al., 2017; Causanilles et al., 2017b; González-Mariño et al., 2016), and its intake of certain pharmaceuticals (Causanilles et al., 2016).

The monitoring of active pharmaceutical ingredients (APIs) and their metabolites in wastewater offers an interesting value (van Nuijs et al., 2015) because these substances have gone through clinical trials before their final usage approval. Therefore, the information regarding the absorbed dose after drug intake, the biotransformation pathway and the excretion profile and rates in biological matrices is relatively well known (Abed, 2014). This information facilitates the selection of the appropriate target urinary biomarker in the application of wastewater-based epidemiology (WBE). Concentrations of the unchanged product and/or its metabolites in untreated wastewater, considered a collective, diluted pooled urine sample, can be converted into measured mass loads (ML) and then back-calculated into actual consumption estimates applying the appropriate correction factor. In addition, the number of dispensed pharmaceutical in the form of defined daily doses (DDD) or product quantities dispensed by pharmacies or doctors can also be obtained (in most cases, depending on the pharmaceutical and the country legislation). From these data, the average amount of the API that has been legally dispensed per day can be calculated and transformed into predicted loads (PL) (Carballa et al., 2008; Verlicchi et al., 2014).

The comparison between the actual consumption derived from ML and PL from prescription data can result in three different scenarios:

(i) Consumption estimated from measured wastewater loads is lower than the load expected from the dispensed data. This would represent the case of pharmaceuticals under consumption, with a lower usage that the quantity prescribed or defined by the DDD;

(ii) Consumption estimated from measured wastewater loads is similar to the expected from dispensed data, which represents the ideal situation, where there is no misuse;

(iii) Consumption estimated from measured wastewater loads is higher than the load expected from the dispensed data;

This third scenario represents the case of pharmaceuticals that are genuine but available from parallel import or in a counterfeit or falsified form and that can be acquired from other sources such as rogue online pharmacies or black market. This was the case observed for the phosphodiesterase type V (PDE5) inhibitor sildenafil, API in erectile dysfunction pharmaceuticals, in a study performed in the Netherlands in 2013 (Venhuis et al., 2014a). Results showed that only one third to one half of the consumption estimated from wastewater loads could be related to the acquisition of the drug from legal sources (Venhuis et al., 2014a).

However, the comparison needs to be handled with care, since other sources for discrepancy can be present. They might be related to the sewer system, with the incomplete release to the sewer system or elimination processes between the consumption point and the wastewater treatment plant (WWTP), namely (bio)transformation, sorption and sedimentation (McCall et al., 2016; Ramin et al., 2017, 2016; van Nuijs et al., 2015; Verlicchi et al., 2014). Alternatively, they could be related to other sources such as inaccurate or highly variable pharmacokinetic parameters between individuals, different applied dosages of the used API (which makes it difficult to compare it with a DDD), or no representative comparison (e.g. 1-week wastewater monitoring vs. monthly/yearly prescription data; national vs. local comparison).

Erectile dysfunction is estimated to affect 25 to 35 million men over the age of 18 in Europe, according to the European Federation of Pharmaceutical Industries and Associations (EFPIA, 2017). It is a disorder of increasing concern since an aging population will result in higher prevalence. Despite the high number of men affected, it is still highly stigmatized, and users usually tend to hide their related drug use. Illegal trading with products from the internet and with counterfeit medicines is increasing (Chiang et al., 2017). However, the individuals purchasing medicines via the internet are for the most part not sufficiently aware of the risks they run in doing so (Keizers et al., 2016). Concerns about the quality of these products may arise, specially towards the possible presence of impurities that may lead to poisoning if toxic, and an increased risk of side effects or overdosing.

In this work the WBE approach was applied to assess the use of PDE5 inhibitors in eight European cities accounting for almost 5 million inhabitant equivalents. 24-h composite influent wastewater samples were collected in each city for seven consecutive days and analysed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Measured concentrations in the samples were converted into mass loads and back calculated with known pharmacokinetic information to estimate consumption. In addition, available data at national level of the number of prescribed or dispensed erectile dysfunction pharmaceuticals were gathered to discuss their correlation.

2. Materials and methods

2.1. Chemicals and materials

The following analytes were selected in the study: sildenafil citrate, desmethylsildenafil, desethylsildenafil and noracetildenafil, purchased from LGC (Luckenwalde, Germany); vardenafil dihydrochloride, n-desethyl vardenafil, tadalafil, aminotadalafil, chloropretadalafil and n-octyl nortadalafil, purchased from TRC Toronto Research Chemicals Inc. (Ontario, Canada). Two isotopically labelled internal standards (ILIS) were used as surrogates: sildenafil-

d_8 and desmethylsildenafil-d_8, supplied by TLC Pharmachem (Ontario, Canada). All the above-mentioned standards were of high purity grade (>98%). Individual stock and working solutions were prepared in methanol and stored at -20 °C. Calibration curve was prepared daily by diluting with ultrapure water to a final composition water:methanol (90:10, v/v).

Methanol and acetonitrile HPLC grade solvents were supplied by Avantor Performance Materials 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 an Elga Purelab Chorus ultrapure water system (High Wycombe, United Kingdom). Regenerated cellulose filters RC 0.2 µm were purchased from Phenomenex (Torrance, USA).

2.2. Sample collection

A week-monitoring sampling campaign was performed in March 2015 in eight European cities. For seven consecutive days 24-h influent composite samples were collected at the entrance of the WWTPs serving the cities of Bristol, England; Brussels, Belgium; Castellón, Spain; Copenhagen, Denmark; Milan, Italy; Oslo, Norway; Utrecht, the Netherlands; and Zurich, Switzerland. The number of inhabitants included in the total catchment area under study represented almost 5 million people in Europe. **Table SI-1** compiles detailed information about the sample collection at the different locations: date of sample collection, influent flow (m^3 day^{-1}), sampling mode and frequency, average wastewater temperature (°C), pH, biological and chemical oxygen demand (BOD_5 and COD), total phosphate (P_{tot}), and nitrogen content as Kjeldahl (N_{tot}) and ammonia (NH_4-N).

2.3. Analytical methodology

The analytical methodology used to perform the wastewater chemical analysis was previously validated (Causanilles et al., 2016). All samples were collected in high-density polyethylene bottles, shipped frozen to KWR in Nieuwegein (NL) and stored in the dark at –20 °C until treatment. Samples were thawed and homogenized. Then a 10 mL aliquot was spiked with deuterated analogues to act as surrogate and filtered with regenerated cellulose syringe filters (0,2 µm). With no further pre-treatment, a 100 µL aliquot of each sample was injected into the liquid chromatography coupled to triple quadruple mass spectrometer (Thermo Scientific TSQ Vantage, Thermo Electron, Bremen, Germany). Chromatographic separation was achieved with a 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). The mobile phase consisted of an optimized water-methanol-acetonitrile gradient at 0.3 mL min^{-1} flow. The MS system operated in selected reaction monitoring (SRM) and positive ionisation mode during data acquisition. For each compound two transitions of the precursor ion $[M+H]^+$ were monitored, one for quantification and the second for confirmation purposes. Analyte concentrations were quantified using calibration with standards in solvent and the correspondent deuterated analogue. Additional details of the analytical method can be found in the Supplementary information: **Table SI-2** presents the specific LC-MS/MS parameters for compound identification, **Table SI-3** p shows the quality parameters of the method's performance, and **Figure SI-1** presents an illustrative chromatogram of a standard mixture of the selected PDE5.

2.4. Calculations

The quantitative chemical analysis of the wastewater samples included in the study resulted in the concentrations of each analyte expressed in ng L^{-1}. The daily mass loads were subsequently obtained by multiplying the measured concentration in each sample by the daily influent flow rate at the WWTP in m^3 day^{-1}. Loads, expressed as mg day^{-1}, were normalized dividing them by the population included in the catchment area.

Normalized loads were expressed as mg day^{-1} per 1000 inhabitants, allowing in this way the direct comparison of results among the different communities included in the study. In the case of concentration values in real sample below limits of quantification (LOQ), values were replaced by 0.5 × LOQ when at least one day in the week had a concentration value above the LOQ. Concentration values below limits of detection (LOD), as well as concentration values lower than LOQ when all values at that location were below LOQ, were set to 0.5 × LOD (Ort et al., 2014). Sildenafil actual consumption was estimated from measured ML as indicated elsewhere (Venhuis et al., 2014b) by summing the load of unchanged sildenafil and the absorbed dose back calculated from the metabolite load using the formula: [(Load desmethylsildenafil (moles) + desethylsildenafil (moles)) / 0,27]*474, and were expressed in mg $week^{-1}$ 1000 inh^{-1}. The calculation was based on the available pharmacokinetic data and the assumption that there were no elimination processes such as (bio)transformation or sorption between the consumption point to the WWTP or dumping of unused drugs. Further research of the biomarkers' behaviour in the sewer (see the introduction) would be required to verify this assumption. Earlier stability studies confirmed there was not a statistically significant decrease in concentration of the target compounds after 48 h storage at 4 °C (Causanilles et al., 2016).

PDE5 inhibitors are the API in pharmaceutical products used to treat erectile dysfunction (ED) and as pulmonary vasodilator antihypertensive (VA). Their classification within the ATC-system (Anatomic Therapeutic Chemical) corresponds to the group of genitourinary system and sex hormones (G), urological (04B), erectile dysfunction (E). The individual codes are necessary to find the national prescription and sales data of all formulations containing them as API despite the differences in brand name. The codes of the three approved substances included in the study and their established DDDs can be found in **Table 1**. DDD is defined as the assumed average maintenance dose per day for a drug used for its main indication in adults (WHO, 2017). Sildenafil does not only have a registration as erectile stimulant, but also for pulmonary arterial hypertension. For this treatment purpose, both the DDD and the number of prescriptions is lower. In the case of Belgium, only the prescription data for the application of sildenafil as VA was available. A similar trend in the prescription data was expected compared to the neighbouring country of the Netherlands and therefore the ratio ED/VA was extrapolated to estimate the number of prescriptions of sildenafil as erectile dysfunction drug in Belgium.

The number of DDDs prescribed in the year 2015 in each country (see **Table 1**) was multiplied by the DDD value, in mg, and divided by the country's population to normalize to 1000 inhabitants, and 52 weeks in a year (van Nuijs et al., 2015). In this way, PLs were estimated, expressed in mg week^{-1} 1000 inh^{-1}. Next, the ratio PL/ML was calculated to enable the comparison between prescription-derived data and actual consumption from wastewater loads (Verlicchi et al., 2014). Statistical analysis of the data, using ANOVA to compare differences between cities and between weekdays and weekends was performed using GraphPad Prism 5.

Table 1. Information on the investigated pharmaceuticals and national prescription data.

Pharmaceutical	ATC code	DDD[a] value (use)	Total number of DDDs prescribed in 2015				
			Belgium[1]	England[2]	Italy[3]	the Netherlands[4]	Norway[5]
Sildenafil	G04BE03	50 mg (ED) 20 mg (VA)	602,596[b] (ED) 106,648 (VA)	23,572,110 (ED) 198,800 (VA)	13,314,239 (ED+VA)	2,190,688 (ED) 387,710 (VA)	1,949,770 (ED+VA)
Tadalafil	G04BE08	10 mg (ED)	85,276	9,120,725	13,314,239	1,570,918	2,203,956
Vardenafil	G04BE09	10 mg (ED)	n.a.	1,262,350	n.a.	159,520	338,096

VA: Vasodilator Antihypertensive

ED: Erectile Dysfunction

n.a.: not available

[a] defined by the WHO Collaborating Centre for Drug Statistics Methodology, www.whocc.no

[b] Estimated from the ED/VA ratio observed in the Netherlands

Information source indicated with numbered superscript:

[1] National Institute for Health and Disability Insurance, www.riziv.be

[2] National Health Service, www.nhsbsa.nhs.uk

[3] Agenzia Italiana del Farmaco, www.agenziafarmaco.gov.it

[4] Dutch Foundation for Pharmaceutical Statistics, www.sfk.nl

[5] The Norwegian Institute of Public Health, www.norpd.no

3. *Results and discussion*

3.1. *Measured concentrations*

Results from the week-monitoring sampling campaign are reported in **Table 2**, together with the LODs and LOQs. Measured concentrations per city are presented as the 7-day mean with standard deviation, expressed in ng L^{-1}. Sildenafil and its two human metabolites were present at levels above the LOD in all cities and could be quantified in most of the samples. The parent compound was detected at a level between LOD and LOQ in the samples from Castellón and Milan, while in the city of Oslo it was at about the LOQ level only in the Sunday sample. When sildenafil was quantifiable, its concentrations were in the range of 4 to 19 ng L^{-1}. Desmethylsildenafil, the less abundant sildenafil metabolite, could not be quantified in the cities of Castellón, Milan, Oslo and Zurich. In Copenhagen and Utrecht on 2 and 4 days, respectively, levels were <LOQ, and these were therefore replaced by 0.5 × LOQ for the calculation of the city's average. Values were found in the range of 14 to 36 ng L^{-1}. Desethylsildenafil, the most abundant metabolite of sildenafil, was quantified in all samples, with concentrations between 5 and 51 ng L^{-1}. Neither the other two APIs included in the study, tadalafil and vardenafil, nor their metabolites nor analogues were found above their LOD.

The metabolite to parent concentration ratio was calculated when available. The ratio of desethylsildenafil to sildenafil ranged from 1.7 to 3.6 (6 cities, 2.8 ± 0.8). These results were in line with the range of ratios observed in the Dutch cities of Amsterdam, Eindhoven and Utrecht in the years 2013 to 2015 (Causanilles et al., 2016). The ratio of desmethylsildenafil to sildenafil ranged from 0.9 to 2.3 (4 cities, 1.6 ± 0.6). These results confirm literature findings: a lower ratio is expected for desmethylsildenafil, since it is the less abundant urinary metabolite (Muirhead et al., 2002).

Table 2. Measured concentrations (MCs) expressed in ng L^{-1} with standard deviation (± SD) for 7 sampling days, n=7.

Compounds	**LOD, ng L^{-1}**	**LOQ, ng L^{-1}**	**MC (mean ± SD), ng L^{-1}**							
			Bristol	**Brussels**	**Castellón**	**Copenhagen**	**Milan**	**Oslo**	**Utrecht**	**Zurich**
Sildenafil	2	6	12 ± 4	19 ± 3	(<LOQ)	14 ± 5	(<LOQ)	4 ± 2[a]	15 ± 4	9 ± 2
Desmethylsildenafil	5	18	26 ± 7	36 ± 2	(<LOQ)	19 ± 8[a]	(<LOQ)	(<LOQ)	14 ± 4[a]	(<LOQ)
Desethylsildenafil	1	2	28 ± 8	33 ± 5	13 ± 3	51 ± 7	5 ± 1	8 ± 4	51 ± 4	32 ± 5
Noracetildenafil	6	20	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)
Tadalafil	2	8	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)
Aminotadalafil	2	6	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)
Chloropretadalafil	4	13	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)
N-octylnortadalafil	30	100	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)
Vardenafil	7	24	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)
N-desethylvardenafil	9	30	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)	(<LOD)

[a] At least one value out of 7 is >LOQ; then the values <LOQ are replaced by 0.5 × LOQ

3.2. Daily loads and actual consumption

Measured concentrations were translated into normalized loads in mg day^{-1} per 1000 inhabitants to allow a better comparison between the cities included in the study. The 7-day average data for each city together with standard deviation is presented in **Table 3**. The highest normalized sildenafil load was found in the city of Brussels closely followed by Zurich and Copenhagen. Compared to these cities, a medium load was found in Bristol and Utrecht, and the lowest levels were observed in Milan and Castellón. For the metabolites a similar trend was found, in accordance with their excretion ratios. The daily variations are presented in **Fig. 1,** expressed as percentages of the total load. No statistically significant increase in loads was found in weekend samples compared to weekday samples, suggesting the use of sildenafil as needed and not with a clear recreational aim. The "weekend effect" is however very typical for some illicit drugs such as cocaine or ecstasy (MDMA) (Bijlsma et al., 2014; Causanilles et al., 2017c; Salvatore et al., 2015). Interestingly, in the case of sildenafil, the highest load is detected on Sunday whereas for the two metabolites the maximum is detected on Monday (**Fig. 1**). This could be explained by the metabolites being excreted later in time than the unchanged parent.

Considering the MLs for sildenafil and its two metabolites, it was possible to back-calculate into actual sildenafil consumption by the population connected to the studied sewer system. This estimation was done as explained elsewhere (Venhuis et al., 2014b). The estimated consumption of sildenafil, in mg $week^{-1}$ 1000 inh^{-1}, back-calculated from wastewater loads (see **Table 3**) arranged the cities in the following order from a higher to a lower estimated use (including previously published results from other Dutch cities (Causanilles et al., 2016): 1st Amsterdam, with 872 mg $week^{-1}$ 1000 inh^{-1}; 2nd Copenhagen; 3rd Brussels; 4th Zurich; 5th Eindhoven, 432 mg $week^{-1}$ 1000 inh^{-1}; 6th Bristol; 7th Utrecht; 8th Oslo; 9th Castellón; and 10th Milan.

Table 3. Averaged normalized loads for sildenafil and its two metabolites with standard deviations (± SD) for 7 consecutive sampling days. Sildenafil actual consumption estimated from ML, and PL calculated from prescription data.

	Loads (mean ± SD), mg day^{-1} 1000 inh^{-1}							
	Bristol	Brussels	Castellón	Copenhagen	Milan	Oslo	Utrecht	Zurich
Sildenafil	2.8 ± 1.1	5.1 ± 1.0	0.2 ± 0.1 [b]	3.8 ± 1.2	0.4 ± 0.1 [b]	1.7 ± 0.7 [a]	2.4 ± 0.7	4.2 ± 1.5
Desmethylsildenafil	6.2 ± 1.7	9.4 ± 1.3	0.6 ± 0.1 [b]	5.3 ± 1.9 [a]	1.0 ± 0.2 [b]	1.2 ± 0.1 [b]	2.1 ± 0.9 [a]	1.1 ± 0.2 [b]
Desethylsildenafil	6.6 ± 2.1	8.5 ± 1.2	3.0 ± 0.6	13.7 ± 1.7	2.1 ± 0.5	3.7 ± 1.5	8.0 ± 0.5	13.9 ± 3.1
Sildenafil actual consumption, ML (mg week^{-1} 1000 inh^{-1})	365	517	100	542	87	145	292	439
Sildenafil predicted consumption, PL (mg week^{-1} 1000 inh^{-1})	415	55	n.a.	n.a.	211	361	133	n.a.

[a] At least one value out of 7 is >LOQ then when <LOQ replaced by 0.5 × LOQ

[b] All values <LOQ then replaced by 0.5 × LOD (SD was obtained from the different daily flow rate)

n.a. not available

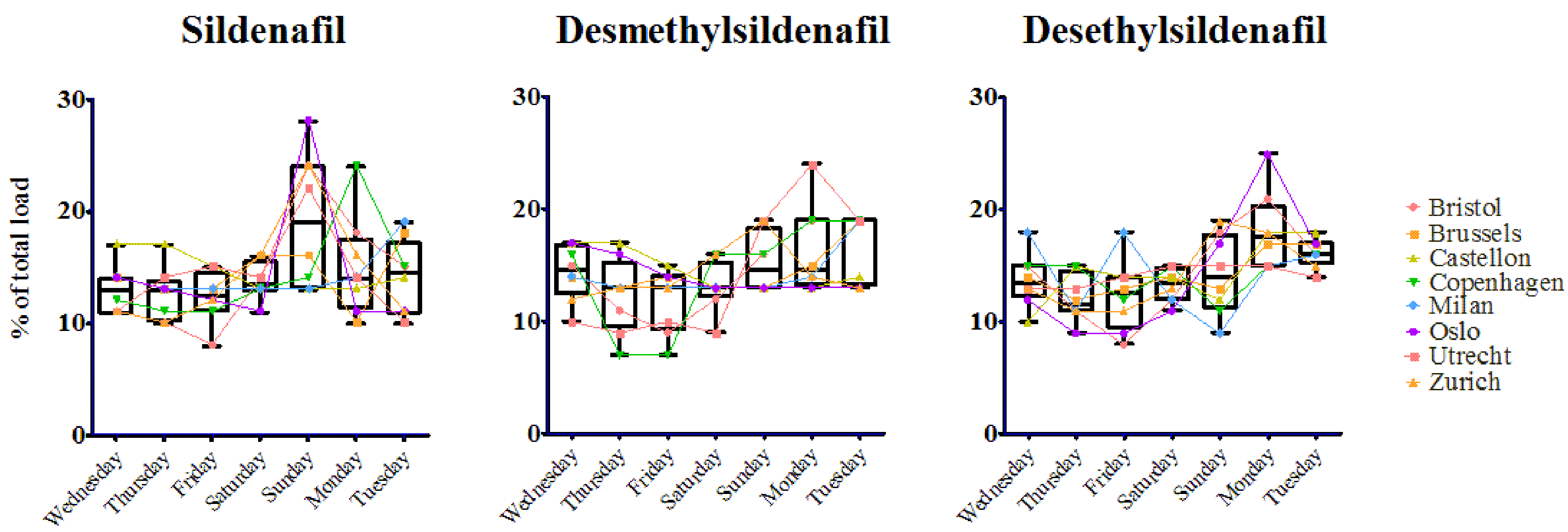

Fig. 1. Daily variations expressed as the percentage of the total load, combining results for the 8 cities. The box represents the median, 25% and 75% percentile values and the error bars extend to the minimum and maximum values. The coloured lines represent each of the cities.

3.3. Comparison between predicted and measured loads

The predicted loads (PLs) for the unchanged API sildenafil and its two urinary metabolites desmethyl- and desethylsildenafil are presented in **Table 3** (the yearly prescribed mg are shown in **Table SI-4**). The highest PL was found for Bristol, followed by Oslo, Milan and Utrecht with similar values, and the lowest was for Brussels. PL were not calculated for tadalafil and vardenafil, since the literature indicates that only a minor amount of the unchanged form was putatively identified in urine. This would result in an expected concentration close to zero, which would be below the LOD in wastewater for this compound.

Only in the case of Brussels (where the prescription data was estimated by extrapolating the Dutch trend) and Utrecht, the actual sildenafil consumption estimated from wastewater-based approach was higher than the expected by the national prescription data (see Table 3). Thus, in Brussels the PL of sildenafil was much lower than the actual ML in wastewater. This difference might be due to unregistered use of sildenafil (case (iii), see introduction), but one should bear in mind that, in this particular case, for the calculation of PL the estimation of prescribed DDDs was obtained by extrapolation from the Dutch ED/VA trend, because actual DDD data were lacking. The actual ED/VA ratio for Belgium may be different of course. Another possible reason for obtaining relatively low PLs, e.g. heavy rainfall during the sampling week, was discarded, as it did not occur. The second observation that can be made corresponds to the three cities, Bristol, Milan and Oslo, where PL/ML ratios for sildenafil were much higher than in Brussels and Utrecht. This translates into MLs lower than PL estimated from national prescription data. This could be explained by the non-consumption of the total prescribed amount, or by any of the other sources of discrepancy mentioned in the introduction such as a higher (bio)transformation or sorption of the compounds in the local sewer systems, or a less representative comparison between local and national prescription data. We currently do not have evidence to substantiate the likeliness of higher rates of in-sewer degradation in these countries. Overall, the comparison results must be handled with care since this study was performed only in one city per country in a limited time period (7 consecutive days), and therefore the extrapolation of results to the whole country's prescription data will be surely biased by the specific spatial and temporal profiles of that city (versus other areas within the countries).

In the cities of Amsterdam and Eindhoven, previously reported results (Causanilles et al., 2016) showed an even higher consumption, that could not be explained by national sales data (at least

60% of the wastewater loads of sildenafil were not explained by legitimately prescribed sildenafil (Venhuis et al., 2014a)). In Bristol, the predicted and measured values were in good agreement, while in Milan and Oslo the estimated consumption from wastewater was lower than the expected from prescription data. The final evaluation of the correlation between wastewater data and prescription data was found to be non-significant by Spearman's correlation coefficient ($\rho = -0.30$) with p-value above 0.05 ($p = 0.68$) (see **Fig. 2**).

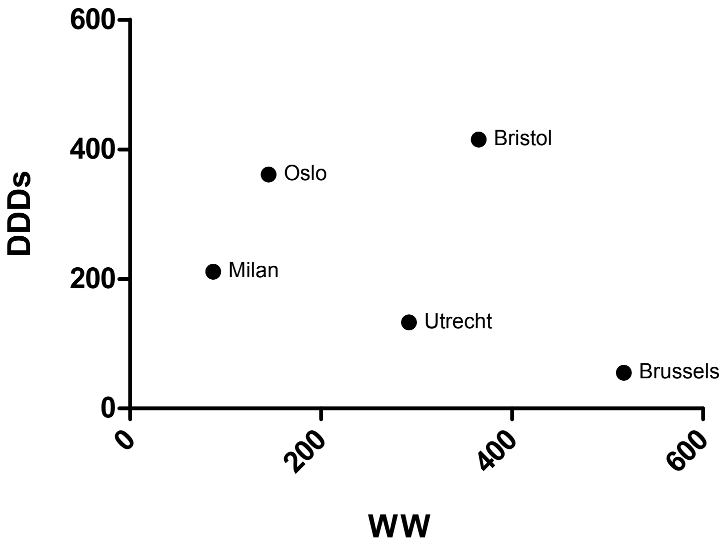

Fig. 2. Relationship between the predicted loads (PL) of sildenafil, calculated from the prescription data (DDDs), and actual sildenafil consumption estimated from the measured loads (ML) in wastewater (WW), both expressed in mg $week^{-1}$ 1000 inh^{-1}. For Castellón, Copenhagen and Zurich, no prescription data were available.

4. *Conclusions*

The present study is the first to compare the use of the erectile dysfunction products in different European cities through chemical analysis of wastewater. The analysis of influents revealed the presence of sildenafil and its two human metabolites in all cities sampled with average loads varying between 0.2 and 14 mg day^{-1} 1000 inh^{-1}. None of the other ED products analysed were observed in concentrations above the method detection limits. While it is known that sildenafil is available in products from illegal sources such as internet shops, the results of the present study show that consumption beyond prescribed doses is not common across Europe. Despite the limitations related to the assessment of both predicted and measured loads, it seems that the populations in Utrecht (and also in other cities in The Netherlands) and in Brussels might be more inclined towards the use of products from illegal sources or rogue online pharmacies than in the other three European cities included in the study for which prescription data were available (Bristol, Milan and Oslo). After this first study illustrating the potential of wastewater-based epidemiology in this field, further research will allow to improve the application of this approach for investigating the use of rogue pharmacies and counterfeit medication.

Author's contribution

AC and DRC performed wastewater analysis. AC drafted the manuscript with significant contributions from FH and PdV. AC, RB, JABL, SC, EC, EGL, FH, BKH, JK, AKM, AvN, BGP, PR, NIR, YR and KT organised the collection of the wastewater samplers and provided relevant data for WBE calculations and national prescription data. All authors read and approved the final manuscript.

Acknowledgements

This work is part of the EU Marie Curie ITN SEWPROF (Marie Curie-FP7-PEOPLE, grant number 317205) and the financial support is gratefully acknowledged. The authors thank the people and agencies that assisted in the collection of the wastewater samples and the national prescription data. Alexander van Nuijs acknowledges the Research Foundation – Flanders (FWO) for his scholarship. Authors also wish to acknowledge dr. Christoph Ort from Eawag for his advice and contribution to discussions.

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Supplementary information

Comparison of phosphodiesterase type V inhibitors use in eight European cities through analysis of urban wastewater

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4 Tables

1 Figure

Table SI-1. WWTPs characteristics.

City		Bristol	Brussels	Castellon	Copenhagen	Milan	Oslo	Utrecht	Zurich
Residential Population		**886650**	**953987**	**180690**	**531000**	**1100000**	**580639**	**300000**	**410000**
Date of sample collection day 1	dd.mm.yyyy	16-3-2015	18-3-2015	25-3-2015	10-3-2015	10-3-2015	11-03-2015	4-3-2015	18-3-2015
Date of sample collection day 2	dd.mm.yyyy	10-3-2015	19-3-2015	26-3-2015	11-3-2015	11-3-2015	12-03-2015	5-3-2015	19-3-2015
Date of sample collection day 3	dd.mm.yyyy	11-3-2015	20-3-2015	27-3-2015	12-3-2015	12-3-2015	13-03-2015	6-3-2015	20-3-2015
Date of sample collection day 4	dd.mm.yyyy	12-3-2015	21-3-2015	28-3-2015	13-3-2015	13-3-2015	14-03-2015	7-3-2015	21-3-2015
Date of sample collection day 5	dd.mm.yyyy	13-3-2015	22-3-2015	29-3-2015	14-3-2015	14-3-2015	15-03-2015	8-3-2015	22-3-2015
Date of sample collection day 6	dd.mm.yyyy	14-3-2015	23-3-2015	30-3-2015	15-3-2015	15-3-2015	16-03-2015	9-3-2015	23-3-2015
Date of sample collection day 7	dd.mm.yyyy	15-3-2015	24-3-2015	31-3-2015	16-3-2015	16-3-2015	17-03-2015	10-3-2015	24-3-2015
Total influent day 1	m^3/24h	197493	234264	50228	148724	423110	333480	47740	157084
Total influent day 2	m^3/24h	204491	235442	49161	150936	403240	308279	45030	161005
Total influent day 3	m^3/24h	198950	234906	43728	147175	412310	277450	49530	161427
Total influent day 4	m^3/24h	197523	233096	38301	144840	402240	256766	46030	200010
Total influent day 5	m^3/24h	252682	230375	37243	145197	403020	250384	46900	243013
Total influent day 6	m^3/24h	220687	234774	37469	137793	422690	254570	45970	177167
Total influent day 7	m^3/24h	193194	359951	40476	137244	597470	252722	44580	160912
Sampling mode	-proportional	time	volume	time	volume	volume	volume	volume	volume
Sampling interval	m^3 or min	15 min	1300 m^3	15 min	2000 m^3	3800 m^3	1500 m^3	400 m^3	900 m^3
Sampling frequency day 1	min	15	8	15	19	13	6	12	8
Sampling frequency day 2	min	15	8	15	19	14	7	13	8
Sampling frequency day 3	min	15	8	15	20	13	8	12	8
Sampling frequency day 4	min	15	8	15	20	14	8	13	6
Sampling frequency day 5	min	15	8	15	20	14	9	12	5
Sampling frequency day 6	min	15	8	15	21	13	8	13	7
Sampling frequency day 7	min	15	5	15	21	9	9	13	8
Average wastewater temperature day 1	°C	n.a.	n.a.	13.1	n.a.	17.5	7.8	13.5	14.7
Average wastewater temperature day 2	°C	n.a.	n.a.	12.0	n.a.	17.6	8.0	n.a.	14.7

Average wastewater temperature day 3	°C	n.a.	n.a.	16.7	n.a.	17.6	8.2	n.a.	14.7
Average wastewater temperature day 4	°C	n.a.	n.a.	n.a.	n.a.	17.7	8.1	14.1	14.1
Average wastewater temperature day 5	°C	n.a.	n.a.	n.a.	n.a.	17.5	8.2	n.a.	12.6
Average wastewater temperature day 6	°C	n.a.	n.a.	17.5	n.a.	17.2	8.5	13.0	14.2
Average wastewater temperature day 7	°C	n.a.	n.a.	19.5	n.a.	16.0	8.7	n.a.	14.7
pH in sample day 1		n.a.	n.a.	7.4	8.0	8.0	7.5	8.6	7.8
pH in sample day 2		n.a.	n.a.	6.9	8.0	7.9	n.a.	8.3	8.1
pH in sample day 3		n.a.	n.a.	7.6	8.2	7.7	n.a.	8.3	8.3
pH in sample day 4		n.a.	n.a.	n.a.	8.1	7.8	n.a.	8.0	8.0
pH in sample day 5		n.a.	n.a.	n.a.	8.0	7.7	n.a.	8.1	8.0
pH in sample day 6		n.a.	n.a.	7.5	8.0	7.7	n.a.	8.3	8.1
pH in sample day 7		n.a.	n.a.	7.7	8.1	7.6	7.4	8.1	8.0
BOD_5 day 1	mg/L	n.a.	n.a.	245	411	183	103	n.a.	n.a.
BOD_5 day 2	mg/L	n.a.	n.a.	245	377	172	n.a.	n.a.	n.a.
BOD_5 day 3	mg/L	n.a.	n.a.	250	451	179	n.a.	n.a.	n.a.
BOD_5 day 4	mg/L	n.a.	n.a.	n.a.	423	n.a.	n.a.	n.a.	n.a.
BOD_5 day 5	mg/L	n.a.	n.a.	n.a.	456	n.a.	n.a.	n.a.	n.a.
BOD_5 day 6	mg/L	n.a.	n.a.	200	434	175	n.a.	n.a.	n.a.
BOD_5 day 7	mg/L	n.a.	n.a.	360	439	102	186	n.a.	n.a.
COD day 1	mg/L	n.a.	n.a.	516	909	372	273	530	n.a.
COD day 2	mg/L	n.a.	n.a.	516	585	344	n.a.	811	n.a.
COD day 3	mg/L	n.a.	n.a.	498	664	303	n.a.	530	n.a.
COD day 4	mg/L	n.a.	n.a.	n.a.	644	298	n.a.	568	n.a.
COD day 5	mg/L	n.a.	n.a.	n.a.	755	292	n.a.	598	n.a.
COD day 6	mg/L	n.a.	n.a.	677	693	385	n.a.	648	n.a.
COD day 7	mg/L	n.a.	n.a.	807	667	226	372	524	n.a.
Ntot day 1	mg/L	n.a.	n.a.	47.5	64.4	31.0	n.a.	n.a.	n.a.
Ntot day 2	mg/L	n.a.	n.a.	n.a.	61.7	29.4	n.a.	n.a.	n.a.
Ntot day 3	mg/L	n.a.	n.a.	n.a.	57.8	29.9	n.a.	n.a.	n.a.

Ntot day 4	mg/L	n.a.	n.a.	n.a.	66.1	n.a.	n.a.	n.a.	n.a.
Ntot day 5	mg/L	n.a.	n.a.	n.a.	64.3	n.a.	n.a.	n.a.	n.a.
Ntot day 6	mg/L	n.a.	n.a.	76.0	63.2	31.6	n.a.	n.a.	n.a.
Ntot day 7	mg/L	n.a.	n.a.	n.a.	61.2	21.0	n.a.	n.a.	n.a.
Ptot day 1	mg/L	n.a.	n.a.	7.4	9.7	3.6	3.5	8.9	n.a.
Ptot day 2	mg/L	n.a.	n.a.	n.a.	8.9	3.5	3.5	9.9	n.a.
Ptot day 3	mg/L	n.a.	n.a.	n.a.	8.7	3.5	3.5	10.3	n.a.
Ptot day 4	mg/L	n.a.	n.a.	n.a.	9.0	n.a.	3.5	9.2	n.a.
Ptot day 5	mg/L	n.a.	n.a.	n.a.	10.1	n.a.	3.5	9.7	n.a.
Ptot day 6	mg/L	n.a.	n.a.	8.0	9.3	3.9	4.3	9.1	n.a.
Ptot day 7	mg/L	n.a.	n.a.	n.a.	9.5	2.4	4.3	9.7	n.a.
NH_4-N day 1	mg/L	n.a.	n.a.	n.a.	44.0	n.a.	15.9	40.7	20.9
NH_4-N day 2	mg/L	n.a.	n.a.	n.a.	41.0	n.a.	n.a.	55.8	26.6
NH_4-N day 3	mg/L	n.a.	n.a.	n.a.	41.0	n.a.	n.a.	41.9	23.0
NH_4-N day 4	mg/L	n.a.	n.a.	n.a.	45.0	n.a.	n.a.	38.7	21.1
NH_4-N day 5	mg/L	n.a.	n.a.	n.a.	44.0	n.a.	n.a.	43.3	17.8
NH_4-N day 6	mg/L	n.a.	n.a.	n.a.	42.0	n.a.	n.a.	41.1	20.8
NH_4-N day 7	mg/L	n.a.	n.a.	n.a.	41.0	n.a.	21.4	39.4	28.6

n.a. not available

Table SI-2. Selected PDE5 inhibitors and LC-MS/MS parameters used for compounds identification.

	CAS number	Molecular formula	Log Kow (*)	$[M+H]^+$	Product ions (m/z)	Collision energy (V)	S-Lens	RT (min)
Sildenafil (ILIS 1)	171599-83-0	$C_{22}H_{30}N_6O_4S$	2.30	475.2	58.2 (Q)	36	118	10.5
					100.2 (q1)	28		
					283.2 (q2)	36		
Desmethylsildenafil (ILIS 2)	139755-82-1	$C_{21}H_{28}N_6O_4S$	2.09	461.1	283.1 (Q)	35	130	9.6
					311.1 (q)	29		
Desethylsildenafil (ILIS 1)	139755-91-2	$C_{20}H_{28}O_4N_6S$	1.99	449.2	283.1 (Q)	36	138	9.4
					311.1 (q)	27		
Noracetildenafil (ILIS 1)	949091-38-7	$C_{24}H_{32}N_6O_3$	n.a.	453.2	97.1 (Q)	31	148	9.2
					113.1 (q)	31		
Tadalafil (ILIS 1)	171596-29-5	$C_{22}H_{19}N_3O_4$	0.04	390.0	204.1 (Q)	57	92	13.9
					268.1 (q)	14		
Aminotadalafil (ILIS 1)	385769-84-6	$C_{21}H_{18}N_4O_4$	-1.20	391.0	204.1 (Q)	56	87	11.9
					262.1 (q)	31		
Chloropretadalafil (ILIS 1)	171489-59-1	$C_{22}H_{19}ClN_2O_5$	2.58	427.1	274.1 (Q)	31	93	16.9
					135.0 (q)	19		
N-octyl nortadalafil (ILIS 1)	1173706-35-8	$C_{29}H_{33}N_3O_4$	5.22	488.2	366.2 (Q)	17	120	17.8
					169.1 (q)	39		
Vardenafil (ILIS 1)	224789-15-5	$C_{23}H_{33}N_6O_4S$	2.79	489.3	151.1 (Q)	41	159	9.6
					312.1 (q)	39		
N-desethylvardenafil (ILIS 1)	448184-46-1	$C_{21}H_{28}N_6O_4S$	2.09	461.2	151.1 (Q)	43	143	9.6
					312.2 (q)	33		
ILIS 1 Sildenafil-d_8	951385-68-5	$C_{22}H_{22}D_8N_6O_4S$	2.30	483.3	62.1 (Q)	37	126	10.5
					108.3 (q)	29		
ILIS 2: Desmethylsildenafil-d_8	1185168-06-2	$C_{21}H_{20}D_8N_6O_4S$	2.09	469.2	283.1 (Q)	37	160	10.7
					311.1 (q)	30		

n.a.: not available
(*) Log Kow (KOWWIN program estimates)

Table SI-3. Method performance: linearity, limits of detection and quantification, intraday and interday repeatability, procedural recovery and matrix effect.

	linearity	LOD	LOQ	Intraday repeatability (RSD (%) , n=7)				Interday repeatability (RSD (%), n=7, d=3)				Procedural Recovery ± RSD (%)				Matrix Effect ± RSD (%)			
	(r^2)	(ng/L)	(ng/L)	20 ng/L	50 ng/L	100 ng/L	500 ng/L	20 ng/L	50 ng/L	100 ng/L	500 ng/L	20 ng/L	50 ng/L	100 ng/L	500 ng/L	20 ng/L	50 ng/L	100 ng/L	500 ng/L
sildenafil	0.9997	1.8	6	16	10	5	5	24	9	10	9	93.1 ± 19.7	102.7 ± 10.4	100.1 ± 11.7	97.5 ± 14.7	241.9 ± 22.6	247.8 ± 15.9	82.3 ± 10.1	73.6 ± 12.2
desmethylsildenafil	0.9999	5.4	18	27	15	7	12	25	24	8	9	99.9 ± 20.2	100.4 ± 16.9	99.9 ± 12.5	90.8 ± 21.1	406.6 ± 35.0	437.1 ± 34.7	116.8 ± 12.6	82.0 ± 21.3
desethylsildenafil	0.9997	0.5	1.6	18	11	10	4	33	18	9	8	97.2 ± 22.1	100.7 ± 10.4	102.3 ± 11.4	93.2 ± 19.0	393.4 ± 30.9	549.0 ± 17.1	156.8 ± 14.7	99.8 ± 13.3
noracetil	0.9990	6	20	31	13	5	6	36	23	9	6	94.8 ± 57.7	102.7 ± 17.1	104.4 ± 13.9	99.0 ± 15.7	298.6 ± 85.3	216.1 ± 33.6	70.5 ± 51.0	46.7 ± 34.0
tadalafil	0.9998	2.3	7.5	10	11	11	7	13	13	13	11	89.3 ± 21.5	96.5 ± 7.8	96.0 ± 8.6	97.7 ± 12.7	246.6 ± 23.6	270.1 ± 14.2	84.0 ± 10.3	72.2 ± 12.5
aminotadalafil	0.9995	1.8	6	8	11	11	8	14	16	11	11	91.3 ± 16.5	100.9 ± 8.6	97.5 ± 8.8	98.2 ± 13.9	217.5 ± 15.7	251.0 ± 15.4	77.8 ± 10.8	69.1 ± 13.6
chloropretadalafil	0.9993	4	13.3	6	8	9	8	12	15	8	10	93.4 ± 15.4	87.2 ± 8.4	91.7 ± 10.2	92.4 ± 11.5	195.0 ± 20.6	243.8 ± 14.2	73.1 ± 10.2	64.9 ± 13.6
n-octylnortadalafil	0.9999	30	100	11	15	10	10	20	27	26	16	-	-	16.4 ± 20.5	27.4 ± 36.8	163.1 ± 19.3	234.0 ± 24.1	77.4 ± 18.8	75.3 ± 10.9
vardenafil	0.9998	7.2	24	17	18	9	5	22	20	14	7	92.2 ± 23.6	101.3 ± 12.2	102.1 ± 12.5	96.6 ± 12.1	320.5 ± 32.2	322.7 ± 24.9	96.5 ± 17.2	83.4 ± 12.3
n-desethylvardenafil	0.9998	9	30	26	16	9	8	37	30	15	13	95.4 ± 25.0	96.5 ± 14.4	98.9 ± 13.0	97.0 ± 16.7	607.0 ± 26.9	616.0 ± 26.0	152.1 ± 14.7	125.8 ± 13.0

Table SI-4. Amount of API prescribed in 2015, expressed in mg $year^{-1}$.

Country	Prescribed mg $year^{-1}$		
	Sildenafil [a]	Tadalafil	Vardenafil
Belgium	$3,23 \cdot 10^{7}$ [b]	$8,53 \cdot 10^{5}$	n.a.
England	$1,18 \cdot 10^{9}$	$9,12 \cdot 10^{7}$	$1,26 \cdot 10^{7}$
Italy	$6,66 \cdot 10^{8}$	$1,33 \cdot 10^{8}$	n.a.
the Netherlands	$1,17 \cdot 10^{8}$	$1,57 \cdot 10^{7}$	$1,60 \cdot 10^{6}$
Norway	$9,75 \cdot 10^{7}$	$2,20 \cdot 10^{7}$	$3,38 \cdot 10^{6}$

[a] total sildenafil

[b] Estimated from the ED/VA ratio observed in the Netherlands

n.a.: not available

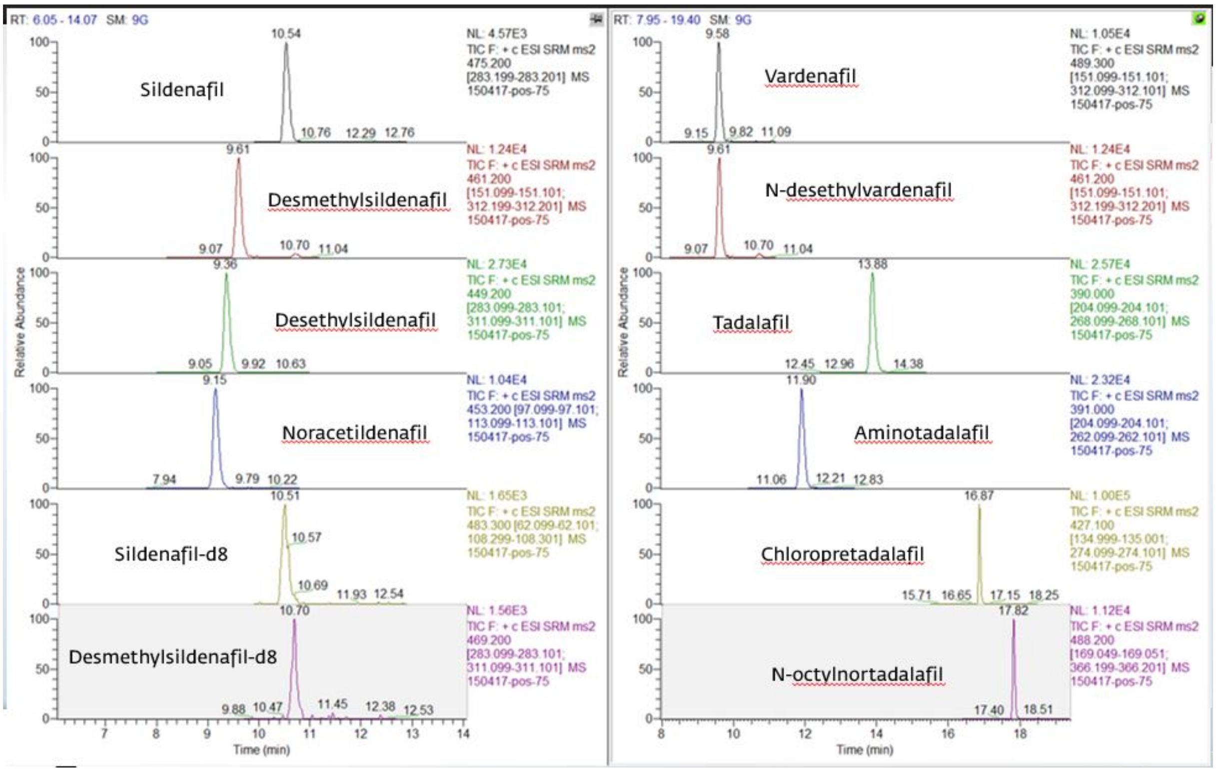

Figure SI-1. Chromatogram from a standard mixture of the selected PDE5 at 50 ng L^{-1} concentration level.