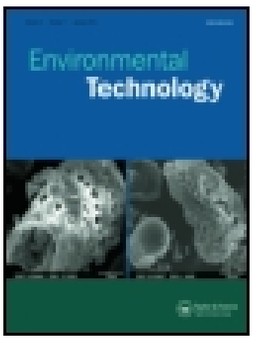

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The effect of urine storage on antiviral and antibiotic compounds in the liquid phase of source-separated urine

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ABSTRACT

The behaviour of pharmaceuticals related to the human immunodeficiency virus treatment was studied in the liquid phase of source-separated urine during six-month storage at 20°C. Six months is the recommended time for hygienization and use of urine as fertilizer. Compounds were spiked in urine as concentrations calculated to appear in urine. Assays were performed with separate compounds and as therapeutic groups of antivirals, antibiotics and anti-tuberculotics. In addition, urine was amended either with faeces or urease inhibitor. The pharmaceutical concentrations were monitored from filtered samples with solid phase extraction and liquid chromatography. The concentration reductions of the studied compounds as such or with amendments ranged from less than 1% to more than 99% after six-month storage. The reductions without amendments were 41.9–99% for anti-tuberculotics; <52% for antivirals (except with 3TC 75.6%) and <50% for antibiotics. In assays with amendments, the reductions were all <50%. Faeces amendment resulted in similar or lower reduction than without it even though bacterial activity should have increased. The urease inhibitor prevented ureolysis and pH rise but did not affect pharmaceutical removal. In conclusion, removal during storage might not be enough to reduce risks associated with the studied pharmaceuticals, in which case other feasible treatment practises or urine utilization means should be considered.

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HIV-treatment; pharmaceutical; removal; storage; urine

1. Introduction

The global target for sustainability and resource efficiency has raised the issue of developing the use of urine especially as a fertilizer for crop production,[1] as urine contains macronutrients (N, P, K) in relatively high quantities. Urine comprises 60–90% of N, P and K ingested by a person in liquid form,[2] in addition to many micronutrients. The present fate of urine ranges from discharges to the sewage, for example, in most cities with a modern wastewater treatment system, to conditions where urine is excreted into a hole in the ground where it infiltrates through the soil. However, in order to better manage the use of urine, the source separation of urine is increasingly studied and proposed as a method for promoting sustainable nutrient management. Source-separated urine can replace a large portion of nutrients applied in agricultural fields [3] and new opportunities are opening as a nutrient source, for example, in microbial electrochemical technologies [4] and microalgal cultivation.[5] Source-separated urine is typically stored before agricultural fertilizer use, and, for example, six months storage is recommended by World Health Organization [6] to remove pathogens from urine before use in fertilization purposes.

However, urine may contain pharmaceuticals as in humans they are to a great extent excreted in urine, which may be disadvantageous to its use, and, for example, in countries without proper sanitation, pharmaceuticals often end up straight in the environment. Various types of pharmaceuticals are used globally. Many studies have focused on compounds commonly associated with the high standards of living (painkillers, lipid modifiers, etc.) and the potential antibiotic resistance of bacteria at wastewater treatment plants (WWTPs). Their fate has been studied at WWTPs and receiving waters (for a review, e.g. [7]), while to our knowledge, pharmaceutical behaviour during long-term storage (e.g. six months) has not been sufficiently studied. Pharmaceuticals, which have been a little studied, are antivirals, which are commonly used as a combination in human immunodeficiency virus (HIV) treatment (lamivudine, zidovudine and nevirapine). [8–10] Antibiotics (trimethoprim and sulfamethoxazole) are used in combination with selected antivirals;[11] as

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well as antibiotics (ciprofloxacin and rifampicin) that are used to treat tuberculosis (referred to as anti-tuberculotics). Latent tuberculosis may be activated during HIV-infection which is why these pharmaceuticals are often used together, especially in developing countries. These pharmaceuticals have not been investigated as extensively although they are administered in large doses (hundreds of mg/d), but, for example, sulfamethoxazole, trimethoprim and ciprofloxacin have been detected in WWTP influent and effluents [12,13] (antivirals are practically not at all studied). It is not possible to obtain the real yearly consumption data globally, but the consumption goes hand in hand with the amount of confirmed HIV infections.

The behaviour of pharmaceuticals during storage is an important factor to take account in order to minimize risks when planning urine re-use options, such as fertilizer applications. During the storage of urine, pharmaceutical concentration reduction could occur either through precipitation and/or adsorption, but also via chemical and biological pathways, the latter indicated by increased pH and thus bacterial activity. During the storage, urea in urine, typically in a few months, undergoes ureolysis (mainly due to bacterial urease enzymes) where urea transforms into ammonia with a simultaneous increase in pH.[14] The increased pH may result in ammonia volatilization thus decreasing the fertilizing value of urine. Urine contains bacteria from the urinary tract, and typically those originating from faeces, which is commonly present as contamination;[15] thus, bacteria may be responsible for pharmaceutical degradation during storage. However, from a practical point of view, whether the compounds are biologically degraded or remain in the storage vessel is not very crucial.

To study the effect of ureolysis and thus storage pH on pharmaceutical removal, an urease-inhibiting compound can be added into the urine, and subsequently urea transformation into ammonia is avoided. The treatment prolongs the fertilization impact and decreases the loss as volatilized ammonia. The prevention of ureolysis also prevents the pH rise in the urine, which might prevent the decay of bacteria at high pH that might play a role in the behaviour of pharmaceuticals during urine storage. A urease-inhibiting agent nBPT [*N*-(n-butyl)-thiophosphoric triamide] was found to decrease ureolysis.[16] Thus, testing its effect on microorganism survival via lowering the urine pH is of interest, as well as its effect on pharmaceutical behaviour.

The aim of this study was to evaluate the effect of urine storage of six months on selected pharmaceuticals, which were studied as divided into three therapeutic groups referred to as antivirals, antibiotics and anti-tuberculotics, and as individual compounds with no amendments. The effects of faecal contamination and urease inhibitor to affect the compounds' behaviour in urine were also examined. The results obtained from this experiment can be used to evaluate the safety aspects of urine re-use options.

2. Materials and methods

2.1. Urine, chemicals and materials

Urine samples were collected from eight healthy volunteers, both male and female, receiving no medication, while the initial pharmaceutical concentrations in collected urine were not investigated. The samples were stored in 0.5–1.0 L, sterilized polypropylene jars for a maximum of one day (in 4°C) before they were all combined and carefully mixed in a volumetric flask, after which homogenized urine was divided into 150 mL portions in experimental jars. The adsorption onto polypropylene jars was assumed to be negligible, since polypropylene does not adsorb, for example, carbamazepine, trimethoprim or sulfamethoxazole.[17]

The eight studied pharmaceuticals (United Corporation Ltd., Kenya) were antivirals lamivudine (3TC), zidovudine (ZDV) and nevirapine (NVP); antibiotics trimethoprim (TRI) and sulfamethoxazole (SMX); and anti-tuberculotics ciprofloxacin (CIP) and rifampicin (RMP), which belong to three therapeutic groups, referred to as antivirals (3TC/ZDV/NVP), antibiotics (TRI/SMX) and anti-tuberculotics (CIP/RMP). Carbamazepine (CBZ) was included in this experiment as a well-studied recalcitrant pharmaceutical reference compound.

Urease inhibitor nBPT dissolved in water-soluble organic solvent was received as a ready-made StabilureN®-solution (Agra Group, Czech Republic). Faeces were mixed with deionized water (MilliQ-water, Millipore; 18.2 mΩ cm conductivity) to produce a stock concentration of 10 $g_{fresh\ weight}$/L. Autoclaving, which is often used to prevent biological activity, of the control was not performed, since when tested it changed the composition of urine by transforming urea into ammonium.

The stock solutions of pharmaceuticals, 5.0 g/L, were prepared in methanol, except CIP in MilliQ-water due to low solubility in methanol. CBZ (antiepileptic) stock solution (in methanol) was 1 g/L. Stock solutions of pharmaceuticals were added in the sample jars to produce concentrations (10–80 mg/L) which could be present in source-separated urine originated from HIV and tuberculotic patients (see Table 1). These concentrations were calculated using typical pharmaceutical concentrations used per person per day as described in WHO database, [18] and as the literature indicates 2.7–70% of the administered dosages of different pharmaceuticals (Table 1)

Table 1. The administered dosage (per person per day) for the selected pharmaceuticals in this study, their approximate excretion percentages as a parent compound and the corresponding amount excreted into urine (per 1.5 L) per day.

Compound	Typical dosage (mg d^{-1})[a]	Excretion to urine % (unchanged)	Amount excreted in urine (mg L^{-1} d^{-1}) (1.5 L urine d^{-1})	Amount spiked in urine samples (mg L^{-1})
Lamivudine (3TC)[b,c]	300	70.0	140	50
Zidovudine (ZDV)[d]	600	16.0	64	80
Nevirapine (NVP)[e,f]	400	2.7	7	10
Ciprofloxacin (CIP)[e]	1000	45.0	300	50
Rifampicin (RMP)[e]	600	15.0	60	70
Sulfamethoxazole (SMX)[e]	320	20.0	43	50
Trimethoprim (TRI)[e]	1600	44.0	469	50
Carbamazepine (CBZ)[g]	1000	3.0	20	10

[a][18], [b][19], [c][20], [d][21], [e][22], [f][23], [g][24].

are excreted in urine. Thus, the used concentrations illustrate conditions when pharmaceutical-containing urine is not excessively diluted with urine containing no pharmaceuticals.

2.2. Experimental set-up

The experiment consisted of the following four different storage assays with pharmaceuticals, each performed in triplicate (Figure 1): (1) eight pharmaceuticals were stored separately and (2) in three therapeutic groups (antivirals, antibiotics and anti-tuberculotics), which were stored as such and with amendments of (3) faeces (final concentration of 9 mg/L, an average concentration shown to be present in source-separated urine [15]) and (4) urease inhibitor (final nBPT-concentration of 2 mM, [manuscript in preparation]). In addition, urine was stored as a control as such or with amendments, but no pharmaceuticals were added (Figure 1). All assays were performed as triplicates and altogether 60 jars were amended with 150 mL urine (jars closed tightly with a lid) and were stored for six months in the dark at room temperature.

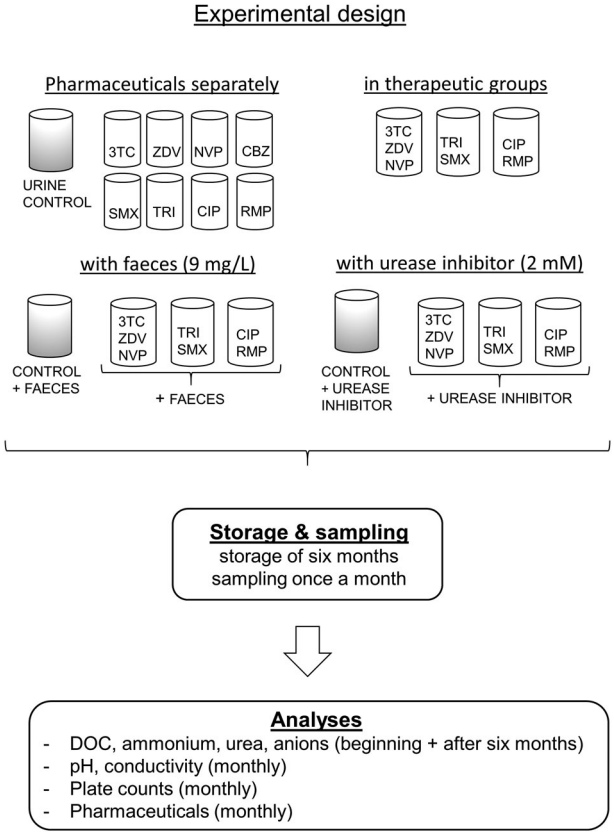

Figure 1. Schematic of the experimental design. Pharmaceuticals were added into urine as separate compounds and in therapeutic groups as such and with different amendments. All 20 experiments were performed as triplicate resulting in 60 experimental jars.

The jar lids were opened once a month when samples were taken thus resulting in the O_2-depletion in the jar head space and generating anoxic conditions before next sampling. Sample volume was 3 mL and the total volume of samples taken out during the experiment was 21 mL per jar. The effect of volume reduction due to volatilization in the jars was presumed to be small due to tight lids.

2.3. Analyses

Before measurements and sampling (3 mL) the jars were shaken vigorously a few times. The shaking of the sample did not have an effect on the precipitates (remained in the jars) and all samples taken for further studies were filtrated (0.2 µm Nylon filter, VWR) before analysis. pH and conductivity were measured from the sample jars; pH with WTW 3210 pH-meter with a SenTix 41 pH electrode and conductivity with WTW 3210 conductivity meter with a Tetraflon 325 electrode.

The temperature was monitored with Data Loggers (MSR Electronics GmbH, Switzerland) and data were analysed with MSR software (V5.12.04). The temperature was 19.5 ± 0.5 °C, (except peaks for 2–3 hours at 25°C during five days) during six months.

Dissolved organic carbon (DOC) was determined according to an SFS (Finnish Standard Association)

standard.[25] Urine samples, diluted with MilliQ-water and filtered through 0.45 µm prewashed syringe filter (Nylon, VWR), were analysed with TOC-5000 analyser (Shimadzu).

Anions (PO_4^{3-}, SO_4^{2-}, NO_3^-, NO_2^-) were determined with ion chromatography (Dionex ICS-1600) from filtered (0.45 µm nylon syringe filters, VWR) samples according to standard SFS-EN ISO 10304–1:en.[26] The separating column used was IonPac AS4A-SC anion exchange column with ASRS-300 suppressor (2 mm). The eluent was a buffer containing 1.9 mM Na_2CO_3 and 1.7 mM $NaHCO_3$.

The sum of ammonium (NH_4^+) and ammonia (NH_3) nitrogen concentrations was determined with an ammonia-selective electrode (Thermo-Orion). The electrode was calibrated with the standards of 100 and 1000 ppm solutions of (NH_4^+) − *N* from ammonium chloride. The ionic strength-adjusting solution (Thermo-Orion) was added to the samples and standards to convert ammonium to ammonia. The gas-sensitive membrane electrode measured the NH_3 concentration of the sample. Urea concentration was determined by measuring ammonia from the sample, then adding the urease enzyme (jack bean urease, *EC* 3.5.1.5, Sigma-Aldrich), and afterwards letting the sample stand for ca. 16 hours in room temperature, while the urease catalysed urea decomposition to ammonia. Subsequently, ammonia was measured and the difference between the two ammonia concentrations equals the amount of urea in the sample.

For the analysis, 3 mL of filtrated (0.2 µm Nylon filter, VWR) sample was taken, of which 1 mL was pre-treated for analysis and the rest was stored in the freezer (−18° C) for LC-ESI-MS/MS. Pharmaceuticals were determined from filtrated samples, which were pre-treated with solid phase extraction (SPE) and analysed with Hewlett-Packard Agilent 1100 HPLC with UV detection using a method optimized for the simultaneous detection of all eight compounds, described by Pynnönen and Tuhkanen.[27] They also presented the method suitability for the urine matrix and the recoveries for the present pharmaceuticals as well as limit of detection (LOD) and quantification (LOQ) values (Table 4). The effect of sample pH change on retention to SPE sorbent/HPLC column was taken into account by testing pharmaceutical retention to the SPE sorbent in different pHs (6–9) and constructing correction factors for different pHs based in compound peak areas (data not shown here). This was done as pH of the urine samples for HPLC-analysis was not adjusted as the addition of pH-adjusting agent into small sample volumes would have caused sample dilution that should have been accounted for. Monthly, the data from chromatographic separation regarding each urine treatment were studied and concentrations for pharmaceuticals were calculated according to previously prepared external standards. The HPLC-UV chromatograms were investigated to evaluate the behaviour of the compounds during storage by studying the parent compound peaks and screening for peaks with similar spectra as parent pharmaceuticals. Qualitative LC-ESI-MS/MS analysis (manuscript in preparation) with the same pre-treatment as described for HPLC-UV were used to evaluate possible transformation products from assays as well as to verify the concentration of the parent compound.

3. Results

Behaviour of eight pharmaceutical compounds during the six-month storage of urine spiked with individually, in therapeutic groups (antivirals, antibiotics and anti-tuberculotics and with different amendments (faeces and urease inhibitor) was studied using CBZ as a reference pharmaceutical. In addition, urine was stored as such (Table 2) and with amendments. In all assays, precipitates, either solid or both solid and floating, were visible after six months at the bottom of the jars, and in some cases already earlier.

pH and conductivity were followed as indicators of biological and ureolytic activity. During the six-month storage, the pH rose up to 8.7–9.6 in urine as such and with individual pharmaceuticals and in the groups of antivirals and antibiotics while with group of anti-tuberculotics pH remained lower (7.9) (see Table 3). In the presence of faeces, pH rise was similar or lower than without, while the presence of the urease inhibitor mitigated pH rise in all three groups as well as in urine control resulting in final pH 7.4–8.2. Changes in conductivity followed in general, changes in pH: when pH rose (up to 9) conductivity rose from initial ca. 9 up to 20–25 mS/cm, while with assays where pH was lower (6.5–8.2) conductivity remained lower as well (<15 mS/cm) (data not shown).

The pharmaceutical concentrations in the liquid phase were analysed once a month in all assays during

Table 2. Characteristics of fresh and six months stored urine.

Parameter (unit)	Fresh urine[a]	Stored urine[a]
pH	6.3 (0.0)	9.6 (0.0)
DOC (g/L)[b]	3.9 (0.0)	1.5 (0.0)
$NH_4^+ + NH_3$ -N (g/L)	0.26 (0.02)	1.04 (0.03)
Urea (g/L)	0.89 (0.05)	0.05 (0.03)
Conductivity (mS/cm)	9.1 (0.0)	25.9 (0.3)
NO_3^- (mg/L)[b]	32.4 (5.6)	n.d.
NO_2^- (g/L)[b]	0.04 (0.02)	0.77 (0.07)
PO_4^{3-} (g/L)[b]	0.93 (0.26)	0.35 (0.02)
SO_4^{2-} (g/L)[b]	1.61 (0.0)	0.91 (0.02)

Note: n.d. = not detected.
[a]mean (± stdev), $n = 3$.
[b]filtrated samples.

Table 3. pH after six months of storage. Data are mean (±standard error), $n = 3$.

		pH							
		Separately		Therapeutic groups		Faeces		Urease inhibitor	
		Start	6 months	Start	6 months	Start	6 months	Start	6 months
Antivirals	3TC	6.3 (0.0)	9.3 (0.2)	6.3 (0.0)	9.6 (0.0)	6.1 (0.0)	9.5 (0.0)	6.5 (0.0)	8.2 (0.1)
	ZDV	6.3 (0.0)	9.6 (0.0)						
	NVP	6.3 (0.0)	9.5 (0.0)						
Antibiotics	TRI	6.4 (0.0)	8.7 (0.4)	6.4 (0.0)	9.2 (0.2)	6.2 (0.0)	7.8 (0.8)	6.5 (0.0)	7.4 (0.0)
	SMX	6.3 (0.0)	9.4 (0.2)						
Anti-tuberculotics	CIP	6.3 (0.0)	8.9 (0.1)	6.4 (0.0)	7.9 (0.2)	6.2 (0.0)	7.4 (0.2)	6.5 (0.0)	7.7 (0.5)
	RMP	6.3 (0.0)	9.4 (0.1)						
Reference	CBZ	6.4 (0.0)	9.6 (0.0)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Control	6.3 (0.0)	9.6 (0.0)	6.3 (0.0)	9.6 (0.0)	6.1 (0.0)	9.5 (0.0)	6.5 (0.0)	7.7 (0.0)

Note: n.a. = not available.

the six-month storage (Table 4). Sampling was performed monthly as it was speculated that not much difference can be observed in a shorter time span. In all assays and with all amendments RMP concentration reduced gradually during six months (Figure 2) below the LOD while the other pharmaceuticals including CBZ (Figure 2, shown as an example) exhibited 23–52% removals and 3TC even up to 76% when incubated alone or as therapeutic groups (Table 4). Faeces amendment resulted in a similar or lower (even less than <1%) reduction of the pharmaceutical concentrations than without faeces. Urease inhibitor reduced the removal of all pharmaceuticals except with CIP (Table 4).

The HPLC-UV chromatograms of monthly samples of all five assays were screened for peaks with similar spectra as parent pharmaceuticals to assess the presence of the initial pharmaceuticals and/or transformation compounds. For RMP, whose concentration reduced to below LOD, no parent compound peak was detected in the chromatograms (with or without different amendments) after six months (small parent compound peak was still present after five-month storage), which indicated its complete removal from the liquid phase as seen in Figure 2. As for 3TC, NVP, ZDV and TRI, no potential transformation product peaks were detected in chromatograms. On the contrary, anti-tuberculotic CIP (Figure 3(a)) and antibiotic SMX (Figure 3(b)) both produced a peak with almost similar a spectrum to the parent compound, after three to five months depending on amendment (additional peaks indicated with an arrow) suggesting degradation.

In addition to HPLC-UV, a qualitative LC-ESI-MS/MS technique was used to screen pharmaceuticals from the assays of individual compounds after six-month storage. The removal of RMP was confirmed while also four unidentified transformation products were observed in assays with RMP. In addition, various transformation products for CIP, SMX and 3TC were identified, while not any transformation products for NVP, ZDV and TRI (available in the literature) were detected (Table 4).

The qualitative analysis showed no marked differences between different amendments. For 3TC, one degradation product was found in three amendments while CIP had seven identified transformation products, of which one was observed in every amendment. In the case of SMX, one degradation product was seen in every amendment.

4. Discussion

The present results show that storage of urine-containing pharmaceuticals for six months varies depending on the compound and concentration reductions in the liquid fraction of urine range from small to marked

Table 4. Reduction of pharmaceuticals in the liquid phase after six months of storage. Data are mean (±standard error), $n = 3$. 'Individually' refers to just one pharmaceutical amended in urine.

		Reduction (%) in the liquid phase after six months				Transformation products detected	
	Pharmaceutical	Individually	Therapeutic groups	Faeces	Urease inhibitor	HPLC-UV	LC-ESI-MS/MS
Antivirals	Lamivudine (3TC)	75.6 (7.8)	51.4 (8.3)	28.9 (22.3)	<1	n.d.	+
	Zidovudine (ZDV)	51.5 (3.7)	45.6 (0.5)	<1	<1	n.d.	n.d.
	Nevirapine (NVP)	25.6 (6.2)	28.8 (3.1)	24.5 (2.9)	16.9 (5.0)	n.d.	n.d.
Antibiotics	Trimethoprim (TRI)	23.7 (1.7)	40.3 (4.8)	42.0 (3.6)	18.9 (1.6)	n.d.	n.d.
	Sulfamethoxazole (SMX)	24.0 (4.7)	32.2 (3.0)	<1	<1	+	+
Anti-tuberculotics	Ciprofloxacin (CIP)	51.1 (10.6)	41.9 (27.4)	38.5 (8.5)	44.2 (19.5)	+	+
	Rifampicin (RMP)	>99	>99	>99	>99	n.d.	+
Reference	Carbamazepine (CBZ)	26.8 (3.5)	n.a.	n.a.	n.a.	n.d.	+

Notes: For pharmaceuticals in urine [27]; LOD: 3TC 1.6 mg/L, ZDV 189 µg/L, NVP 71 µg/L, TRI 39 µg/L, SMX 115 µg/L, CIP 189 µg/L, RMP 503 µg/L, CBZ 41 µg/L. LOQ: 3TC 5.4 mg/L, ZDV 630 µg/L, NVP 237 µg/L, TRI 129 µg/L, SMX 383 µg/L, CIP 631 µg/L, RMP 1.7 mg/L, CBZ 136 µg/L. + = transformation product detected. n.d. = not detected. n.a. = not available.

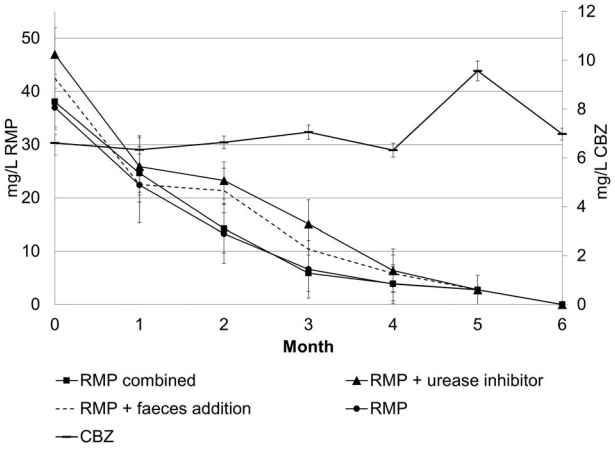

Figure 2. The concentration of rifampicin (RMP, left y-axis) and carbamazepine (CBZ, right y-axis) in monthly samples during the six-month storage of urine with different amendments. Combined refers to the results of urine amended with therapeutic groups. The error bars represent the standard error between three replicates.

(from 23% to 75%) while RMP was removed >99% in all assays. Furthermore, the potential for the formation of transformation products, which can be also harmful, is evident during storage. The concentration reductions were lower with faeces amendment and the lowest with urease inhibitor amendment. The results showed marked reductions for some compounds but under the studied conditions neither biological nor chemical mechanisms occurred to enable the complete removal of the studied pharmaceuticals from the liquid phase (except RMP). Thus, it appears that conditions in source separation and storage of urine do not favour the concentration reduction and six-month storage as recommended by WHO does not mean a complete removal of the studied pharmaceutical risks. Therefore, methods to actively decrease pharmaceutical concentrations in the source-separated urine should be studied to enable its use.

Although source separation of urine is not a new concept, there are currently only a few studies available on pharmaceutical behaviour during urine storage,[28–30] none of which has used the same pharmaceuticals as the current study, except for CBZ. The concentration reduction during 3–4-month storage of urine for CBZ was reported to be 20–80% [28–30] and the highest reductions for several pharmaceuticals (up to 80–90%) were observed during pH-controlled storage (3–4 months [28]). It was discovered indicating that a low pH facilitates concentration reduction during urine storage. However, it was acknowledged that the length of the storage period (3–4 months) was not enough to completely remove the pharmaceuticals,[28] a finding which concurred with the results presented in the current study. In the current study, room temperature and six-month storage were used, but parent compounds were still present in urine afterwards. Varying storage periods (from three months to a year), different pHs (from pH 2 to 11) and temperatures (from 4°C to 38°C) have been tested, and somewhat marked effects on pharmaceutical concentrations have been discovered,[28–30] while complete removal has not been observed. In a modern wastewater treatment system, urine is flushed to WWTPs along with the pharmaceuticals, where conjugated pharmaceuticals can transform back into their active forms by microbial metabolism and be transported to watersheds; the transformation can take place already in the sewer network (pressurized sewer in anaerobic conditions, retention time 21 h [31]) where CIP was slightly degraded while SMX, TRI and CBZ had negative removal implying a microbial or

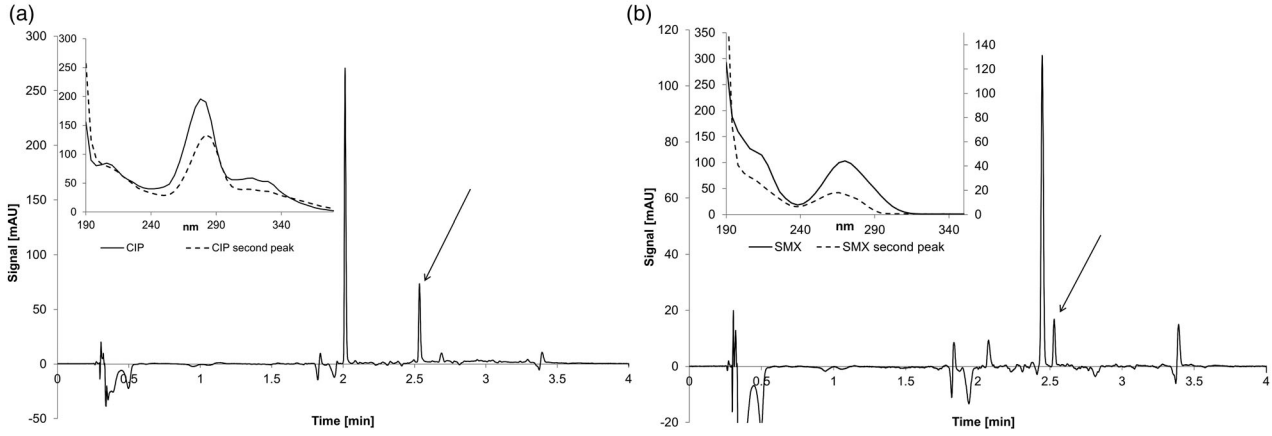

Figure 3. Chromatograms and spectra of CIP (a) and SMX (b) after five months of storage of urine-amended individual pharmaceutical. The additional peaks are indicated with arrows. The spectra of the additional peaks have resemblance to the original compound. Note that the signal of SMX second peak is on the secondary axis.

chemical breakdown of conjugated compounds.[31] Source separation of urine removes this route of pollutants. Yet, urine storage might not be enough to reduce pharmaceutical risks in fertilizer use in which case, other feasible treatment practices to enhance pharmaceutical removal should be studied.

The complete removal of RMP, also with the two studied amendments, was strikingly opposite to the other seven studied pharmaceuticals. The main mechanism for the removal of RMP from the liquid phase could be biodegradation, which would be a novel finding as no information of RMP biodegradation could be found. Biological removal was proposed, but not confirmed, by the disappearance of the RMP parent compound and appearance of transformation products in the LC-ESI-MS/MS analysis. Chemical degradation also produces transformation products which cannot be ruled out. However, as RMP is a very large molecule (823 g/mol) compared with the other studied pharmaceuticals (a range from 224 to 331 g/mol [27]), this is opposite to the assumption that the smaller the molecule, the easier it would be to degrade it. It might be, that although pH with RMP alone rose >9, the different bacteria present in urine thrive at different stages and are able to break down the compound gradually. While RMP was similarly removed also in the presence of CIP, and where pH remained 6.5–7.9 in all assays, the explanation could be that urine contained bacteria that are resistant to RMP/CIP but might not have ureases thus causing lower pH. The disappearance of RMP in the presence of all amendments suggested that it is readily biodegradable.

The concentrations of the seven other pharmaceuticals reduced somewhat less indicating that neither biological nor chemical processes were sufficient in enabling the complete removal of the compounds. The effect of antivirals and antibiotics can be seen in the pH of the different urine assays: with antivirals, pH rose close to or over 9 despite the urease inhibitor addition whereas with antibiotics pH rise remained lower (except for TRI/SMX and SMX individually, pH > 9.2). As the pH of samples amended with faeces were similar with urine amended with the urease inhibitor, it is possible that in the urease inhibitor assays the main affecting factor in the pH was the urease inhibitor together with pharmaceuticals. Therefore, in assays with faeces the combination of two antibiotics and anti-tuberculotics was probably still sufficient in preventing bacterial growth. The reason for the low or no (bio)degradation could therefore also be the concentrations of antibiotic compounds or the inhibitory effects on microbes present in urine, as antibiotics are designed to prevent bacterial growth. The inhibitory effect of therapeutic groups on bacterial growth was seen, for example, as lower pH rise with CIP and RMP, of which CIP is, in addition to tuberculosis treatment, effective against common bacteria found in urine, such as *Proteus mirabilis*, *Escherichia coli* and *Klebsiella* sp.[32] The aforementioned bacteria are also susceptible to TRI and SMX.[33] In the case of antivirals, the high concentration could lead to toxicity, but the antivirals ought not to affect the bacteria as they are designed to stop virus infections.

Pharmaceuticals are excreted in urine in tens to hundreds of mg/L (see Table 1), while in WWTPs the influent typically contains ng/L to µg/L concentration of pharmaceuticals. Although these concentrations are orders of magnitude lower than in urine, the bacteria in WWTPs are not able to break down all of the pharmaceutical compounds and addition to that, transformation products are formed, which together with pharmaceuticals end up in the environment.[12] As inhibition is concentration dependent, it is likely that the expected lower pharmaceutical concentration in real-life urine storage systems might enhance biological degradation; however, no information is available on the toxicity response of the studied pharmaceuticals. On the other hand, the emergence of transformation products in the LC-analysis suggested the (bio)transformation of the pharmaceuticals and the emergence of breakdown products in the LC-ESI-MS/MS analysis implied the formation of possibly more harmful products from paired compounds. The environmental relevance of transformation products is yet quite a new field of study.[13] The degradation products and their formation are currently studied). Further determination and identification of the formed compounds will give indication regarding the possibility of the compounds having more harmful characteristics than the main compound. The removal of the antibiotic compounds could be expected to some extent in large-scale source-separated urine, but the behaviour of the antiviral pharmaceuticals may prove more problematic.

The current study showed that at least some of the pharmaceuticals selected in this study underwent degradation although only slightly, and the degradation could either be chemically or biologically induced. Earlier studies of pharmaceutical biodegradation using closed bottle tests and lower pharmaceutical concentrations have demonstrated that CIP (test concentration 5.95 mg/L [34]), SMX (3.8 mg/L [35]) and TRI (0.5 mg/L [36]) are not readily biodegradable. ZDV and NVP are also recorded as not readily biodegradable (degraded 3–4%), the same as 3TC, which have been found to pose toxic effects on activated sludge (degradation −3%) (all compounds 50 mg/L [37]). However, closed bottle tests use low bacterial density: source-separated urine can

support the growth of bacteria up to about 10^8 cfu/mL,[38] while in closed bottle tests the recommended bacterial concentration ranges from 10^4 to 10^6 cfu/L.[39] The current study used pharmaceutical concentrations of 10–80 mg/L which are similar or higher than in previous tests, yet the bacterial density in urine was probably quite high. Thus, the biodegradability rate of these compounds may have increased with higher bacterial densities and the diversity of microorganisms in urine could have increasingly affected the biodegradability, as it was probably with SMX, which has been shown to be degradable by bacteria in WWTPs (89% removal [12]). Biological activity was proposed by pH increase, but on the other hand, similar removals during storage were observed without pH change and the confirmation of the affecting mechanisms (biological and chemical) require more research.

The study proposed that chemical (precipitation/sorption) of the studied pharmaceuticals was low or insignificant in all studied storage conditions, which was supported by the fact that, for example, in the groups of antibiotics and anti-tuberculotics in most assays the pH did not rise above 8, which is considered as a minimum pH prerequisite for the formation of struvite precipitates, with a pH-optimum of 9.4–9.7.[40] Previously, when the coprecipitation of CBZ and other pharmaceuticals (CBZ was the only same pharmaceutical as in the current study) with struvite in urine was investigated, it was proved using mass balances that the studied pharmaceuticals remained in the solution (> 96% of CBZ).[41] RMP was completely removed when pH was 9.4 (individually) as well as in pH 7.4 (urease inhibitor addition), suggesting that the precipitation with struvite was not the major removal mechanism. Adsorption on particulate matter, which is abundant in source-separated urine and was removed when samples were filtrated, was also regarded as a possibility. In practice, the role of adsorption could differ depending on adsorption sites in different conditions. Stability studies for the pharmaceuticals, for example, with purified water were chosen not to be conducted, since although they might have given some indication of the compound stability, ionic strength and other properties of human urine (see Table 2) are quite different from plain water, making comparison unequal.

Hypothesis was that the addition of faeces would supplement urine with additional bacteria thus improving compound removal. However, the results clearly showed that faeces did not enhance pharmaceutical removal even though they were expected to increase microbial content in assay jars. Faeces are practically always present in source-separated urine,[15] and it was hypothesized that the bacteria derived from faeces would enhance the biological removal of the compounds. Thus, the results imply that pharmaceutical removal may in fact be reduced in the presence of faecal contamination, but the mechanism is yet to be discovered.

It appeared that the used urease inhibitor resulted in a low pH increase as anticipated, but it did not, however, affect positively on pharmaceutical reduction. This indicates that the mechanism in pharmaceutical concentration reduction could well be biological and was inhibited by the urease inhibitor. Originally, the hypothesis was that lower pH generally enables better microorganism growth thus enhancing the biological removal of pharmaceuticals as bacterial extracellular enzymes can break down bonds in pharmaceutical molecules. In addition, the HPLC analysis showed that 59% of the urease inhibitor was still present in the sample after six months (data not shown). To our knowledge, no information is available regarding the urease inhibitor's effect on bacterial enzyme activity besides ureases. The addition of the urease inhibitor might have co-affected the removal of compounds by preventing such activity thus explaining the poor concentration reductions.

4. Conclusions

From the present study, we conclude that during the six-month storage of source-separated urine

- the pharmaceutical concentration reductions ranged from less than 1% to more than 99%: without amendments reductions were for anti-tuberculotics 41.9–99%; for antivirals <52% (except with 3TC 75.6%) and for antibiotic compounds <50%.
- in assays with amendments, the reductions were all <50% (except with RMP >99%). Transformation products were detected for 3TC, CIP and SMX, but the parent compounds were still present in urine after six months.
- RMP concentration reduced to below LOD in all assays and four unidentified transformation products were detected.
- faeces and urease inhibitor amendments resulted in similar or lower reduction than without them which was contradictory to the anticipation that the bacterial activity should increase and therefore improve concentrations reductions. The urease inhibitor prevented ureolysis and subsequent pH rise, but did not enhance pharmaceutical concentration reduction.
- biological activity was proposed by increased pH during storage, but similar removal during storage was observed without pH change. Thus, the

confirmation of the affecting mechanisms (biological and chemical) requires more research.

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