Determination Method for Anti-malarial Drugs in Agricultural Soil Fertilized with Human Urine

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ABSTRACT
Several researches have been conducted to show that human urine is available to crops. On the other hand, pharmaceuticals contained in the urine might be widely spread in environment when urine is directly reused. Antimalarial drugs are of common use in a malaria-prone area as Sub-sahel region and potentially present in urine; therefore, the monitoring for determining the possible accumulation in agricultural fields is needed. However, the determination method of anti-malarial drugs in soil has not been developed so far. In this study the determination method using LC/MS and solid phase extraction (SPE) were investigated. Recovery rate of CQ, DX, Q and AS extraction from water were 87±0.8%, 139±3.0%, 96±1.0%, 64±1.2%, respectively. Recovery rate of target compounds in methanol +KCl extraction from soil was relatively higher than water extraction. The values of CQ, DX, Q and AS in methanol +KCl extraction from soil were 16±1.3%, 65±2.7%, 80±5.1% and 88±2.7%, respectively. The values of CQ, DX, Q and AS in methanol extraction from plant were 16±1.3%, 65±2.7%, 80±5.1% and 88±2.7%, respectively. This study showed that KCl has a high potential to achieve a high recovery rate from soil.

Keywords
urine reuse, anti-malarial drugs, agricultural soil, determination method, LC/MS

INTRODUCTION
Currently, 2.4 billion people don’t have access to sanitary facilities (UN, 2011). The

Millennium Development Goals were set to reduce these numbers to half by 2015, however, it is difficult to achieve in sub-Sahel region in Africa. Resource recycling based and small foot printed sanitation system such as concept of Ecological Sanitation (EcoSan) (EcoSanRes, 2012) or Onsite Wastewater Differentiable Treatment System (OWDTS) (Lopez Zavala *et al*., 2002) is one of solution to acheave The Millennium Development Goals. This concept is to collect yellow water, black water and gray water, respectively. Then they are treated or untreated to use aguricultural water and nutrients.

It is effective to use urine as fertilizer because it contains 12.3 g/L-N, 0.9 g/L-P, 2.0 g/L-K (Wilsenach *et al*., 2007). There are some reports that using urine is possible to grow up vegetables (Pradhan *et al*., 2010; Sene *et al*., 2012). However, it also contains residues of pharmaceuticals consumed by human (Winker *et al*., 2009). Therefore, there is potential risk that these compounds might be spread in environment when urine is directly reused. Some reports indicate that bacterial resistance to drugs could have been increased because of pharmaceutical spread in environment (Harnisz *et al*., 2011). It has been known that germination and young plant growth was inhibited by pharmaseutical (Jjemba, 2002) and a pharmaceutical compond, which was pottensialy contaminated in source-separated urine, was taken up into plant shoot (Winker *et al*., 2010). Therefore, if urine was directly used as fertilizer, judicious care would be taken.

In order to monitor these micllo-pollutants in environment and agricultural field, development of determination method is essential. Many determination method for pharmaceuticals has been already developed, however, the method about reaginal-specific pharmaceuticals such as anti-malarial drug was not foucused, so far. 3.3 billion people were at risk of malaria in 2010 all over the world (WHO, 2011) and most of them lived in sub-Sahel region in Africa. The case of Burkina Faso, approximately 50% of intervention was used for anti-malarial drug in 2010 (WHO, 2010). Therefore, it is necessary to establish the determination method of anti-malarial drugs for permeation of urine reuse. However, determination method of anti-malarial drugs in soil has not been developed so far. In this study, the determination method using LC/MS and solid phase extraction (SPE) were investigated.

MATERIALS AND METHODS

Selection of pharmaceuticals

This study focused on four anti-malarial drugs: Chloroquine diphosphate salt (CQ, CAS-N. 50-63-5), Doxycycline hyclate (DX, CAS-N. 24390-14-5), Quinine hydrochloride Dihydrate Chinin-hydrochlorid dihydrat (Q, CAS-N. 6119-47-7) and Artesunate (AS, CAS-N. 83507-69-1) since they are highly excreted in urine as

unchanged form and commonly distributed in sub-Sahel region. They also have different characteristics and vary in their LogKow and pKa. CQ, DX and Q were dissolved in pure water and AS was dissolved in methanol to a concentration of 100 mg/L and stored in -30°C. They were used with several dilutions in this study. CQ, DX and Q were purchased from Sigma-Aldrich, and AS was purchased from Tokyo Chemical Industry.

Table 1 Characteristics of anti-malarial drugs

	Unchanged urine excretion	Morecular weight	LogKow	pKa
Chloroquine	44%	320	4.3	10.1
Doxycyxline	40%	444	-0.2	3.09
Quinine	13%	324	2.6	8.7
Artesunate	-	384	2.5	3.74

Extraction from water

At first, extraction from water was conducted because the best conditions of solid phase extraction and LC/MS should be decided. 1.00 mg of anti-malarial drugs were disolved with 1 L pure water with adjusted pH 2.00±0.05 with HCl.

Solid phase extraction

20 ml of them were loaded to a previously conditioned SPE cartridge (Oasis HLB, 6 cc/ 500 mg) and washed with 5 mL of pure water after 5 ml methanol and pure water were loaded. Target compounds in the cartridge were eluted with 5 ml of acetonitrile and 5 ml of mixture of acetonitrile/pure water 1:1 (v/v).

Analyze by LC/MS

The eluate was injected into LC/MS system. Conditions for LC/MS were as follows : column : SunFire C18 (2.1 × 50 mm, 3 μm). Column temperature : 40°C. Moblie phase : 10 mM ammonium formate in ultrapure water containing 0.3% formic acid (buffer A) and acetonitrile (buffer B). Ionization : ESI positive. Source temperature : 140°C. Disolvetion temperature : 350°C. Gas flow rate : 650 L/hr. Corn desolvation : 40 L/hr. The mobile phase was deliverd using a stepwise gradient elution program : 2% of acetonitrile (buffer B) at 0 min, 30% of B at 2 min, 60% of B at 10 min, 70% of B at 17 min and 100% of B at 17.5 min with flow rate of 0.3 ml/min. The second part of run included 3.5 min of rinsing (100% B with 0.3 ml/min) and a re-equilibration step to the

initial solvent up to 27 min. The selected *m/z* transitions for CQ, DX, Q, AS are 320.2±0.2, 445.2±0.2, 325.1±0.1, 221.1±0.4, and collision energy (v) are 40, 30, 50, 35, respectively.

Extraction from soil

Water, methanol and mixture of methanol /1 M KCl at the ratio of 1:1 (v/v) were used as extraction solution from soil and compared in the present study. 0.03 mg of anti-malarial drugs were spiked in 10 g of soil mixture (river sand and compost at the ratio of 4:1) and extracted with 30 mL of extraction solution by shaking and sonication. Shaking and sonication was performed for 10 min; then, the suspension was centrifuged (3000rpm, 5min) and the supernatant was collected. These procedures were repeated. The collected supernatants were filtered through glass filter (GA-100 pore size 1.0 μm, ADVANTEC) and volume was adjusted to 90 ml. Then 30 ml of them were transported in 2000 mL of pure water with adjusted pH 2.00±0.05 with HCl. The liquid was loaded to a previously conditioned SPE cartridge (Oasis HLB, 6 cc/ 500 mg) by 50 ml/min flow of concentrator (SepPak Concentrator Uni, Waters). The condition of washing, loading and elution were same as extraction from water. The eluate was filtered (DISMIC, PTFE 0.45 μm, ADVANTEC) and injected into LC/MS system. The condition of LC/MS system was same as extraction from water.

Extraction from plant

The plants (Komatsuna) were purchased from store and milled by mortar after freeze dry. Then 0.03 mg of anti-malarial drugs were spiked in 1 g of plant and extracted with 30 mL of methanol by same method of soil extraction.

RESULTS AND DISCUSSION

Extraction from water

Recovery rate of CQ, DX, Q and AS were 87±0.8%, 139±3.0%, 96±1.0%, 64±1.2%, respectively. Why recovery rate of AS was low than others because its value of water solubility was most low. On the other hands, recovery rate of other 3 anti-malarial drugs were more than 80%. It was showed that condition of solid phase extraction and LC/MS were effective to analyze them.

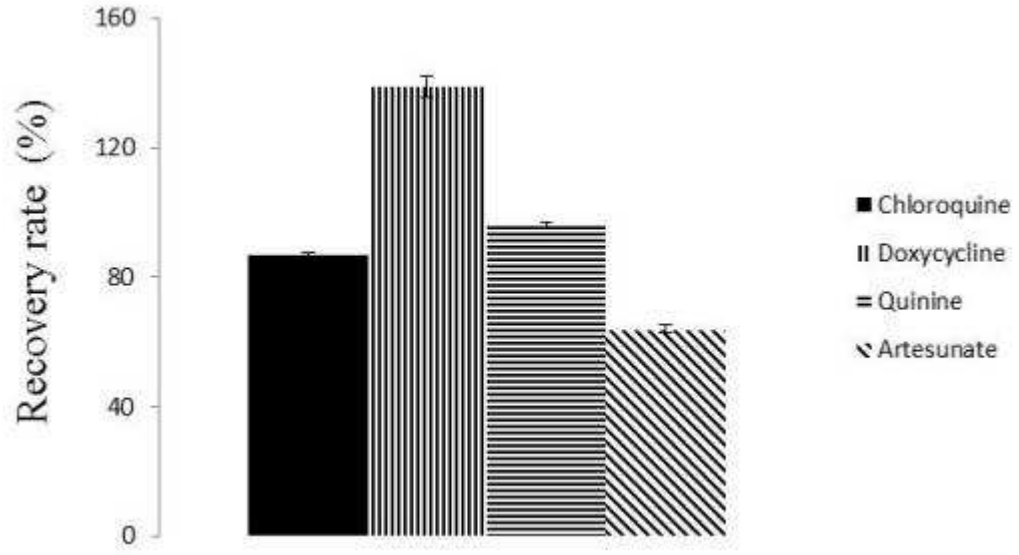

Figure 1 Recovery rate of CQ, DX, Q and AS that extracted from water. Mean value and standard deviation with 3 replications were shown in the figure. Black bar : recovery rate of CQ, Vertical stripes : recovery rate of DX, Horizontal stripes : recovery rate of Q, Slant stripes : recovery rate of AS.

Extraction from soil

Recovery rate of targets using methanol and methanol +KCl as extraction solutions were relatively higher than when using water. The values of CQ, DX, Q and AS in methanol +KCl extraction were 16±1.3%, 65±2.7%, 80±5.1% and 88±2.7%, respectively, and those in methanol extraction were 9±0.1%, 50±6.3%, 59±0.9% and 90±2.2%, respectively, and those in water extraction were 2±0.1%, 4±0.1%, 2±0.1%, 65±0.9%, respectively. Recovery rate of CQ, DX and Q in methanol +KCl extraction were significantly higher than that of methanol (*t*-test, $p < 0.05$). One reason of high recovery of DX and Q in methanol +KCl extraction might be amide radical, since positive charge of the amide radical would ionically bond with soil. Ionization tendency of potassium is the highest so it has potential to peel off ionically bond. On the other hands, recovery of CQ, which has a high LogKow, was not successful in the present study. Therefore, extraction with hydrophobic solvent (*e.g.* acetone, dichloromethane) should be further investigated.

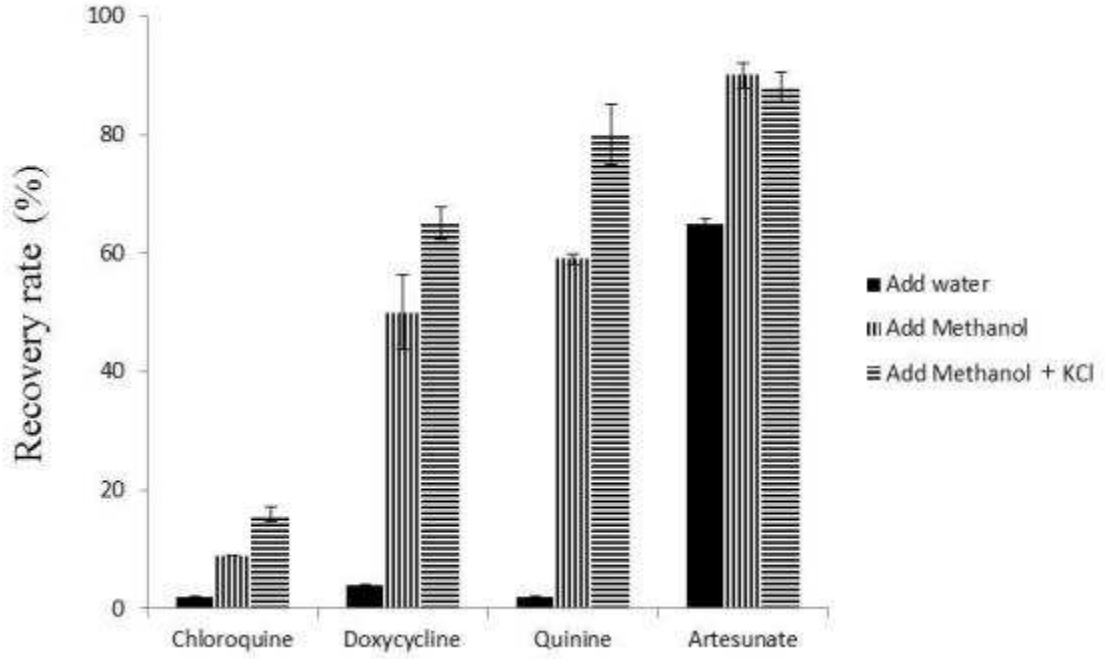

Figure 2 Recovery rate of water, methanol, and methanol＋KCl that extracted from soil. Mean value and standard deviation with 4 replications were shown in the figure. Black bar : recovery rate of water, Vertical stripes : recovery rate of methanol, Horizontal stripes : recovery rate of methanol＋KCl.

Extraction from plant

Recovery rate of CQ, DX, Q and AS in methanol extraction were 16±1.3%, 65±2.7%, 80±5.1% and 88±2.7%, respectively. There are some reports which used acetone or dichloromethane as extraction solutions (Boxall *et al*., 2006). Therefore, extraction with hydrophobic solvent (*e.g.* acetone, dichloromethane) should be further investigated.

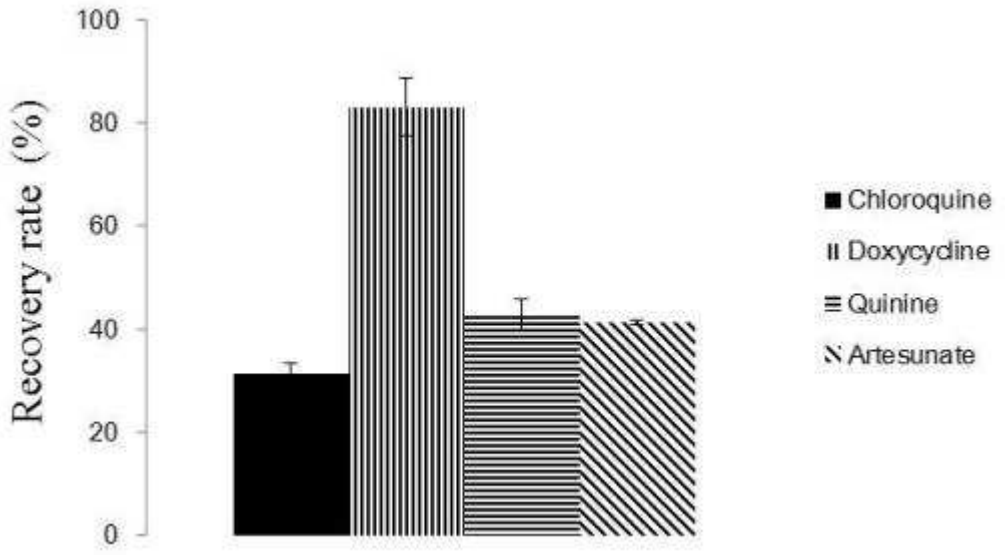

Figure 3 Recovery rate of CQ, DX, Q and AS that extracted from plant. Mean value and standard deviation with 4 replications were shown in the figure. Black bar : recovery rate of CQ, Vertical stripes : recovery rate of DX, Horizontal stripes : recovery rate of Q, Slant stripes : recovery rate of AS.

CONCLUSIONS

This study showed that KCl has a high potential to achieve a high recovery rate from soil. Conditions of solid phase extraction and LC/MS were found. Recovery rate of C, DX and Q were more than 80%. However, conditions of extraction from soil and plant were not enough so far. Improvement of recovery rate of them which extracted from soil and plant should be further investigated.

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