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Analysis of chlormequat in human urine as a biomarker of exposure using liquid chromatography triple quadrupole mass spectrometry

Christian H. Lindh*, Margareta Littorin, Gunvor Johannesson, Bo A.G. Jönsson

Division of Occupational and Environmental Medicine, Department of Laboratory Medicine, Lund University, SE-221 85 Lund, Sweden

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ABSTRACT

In this study, a method using liquid chromatography triple quadrupole mass spectrometry (LC/MS/MS) is described for the analysis of the plant growth regulator chlormequat (CCC) in human urine. Analysis was carried out using selected reaction monitoring (SRM) in the positive ion mode. [$^2H_4$] labeled CCC as internal standard (IS) was used for quantification of CCC. The limit of detection (LOD) was determined to 0.1 ng/mL. The method was linear in the range 0.3–800 ng/mL urine and had a within-run precision of 4–9%. The between-run precision was determined at urine levels of 7.0 and 31 ng/mL and found to be 5 and 6% respectively. The reproducibility was 3–6%. To validate CCC as a biomarker of exposure, the method was applied in a human experimental oral exposure to CCC. Two healthy volunteers received 25 μg/kg b.w. CCC in a single oral dose followed by urine sampling for 46 h post-exposure. The CCC was estimated to follow a first order kinetic and a two compartment model with an elimination half-life of 2–3 h and 10–14 h respectively. One hundred 24 h urine samples were collected from non-occupationally exposed individuals in the general population in southern Sweden. All samples had detectable levels above the LOD 0.1 ng/mL urine. The median levels were 4 ng/mL of CCC in unadjusted urine. The levels found in the population samples are several magnitudes lower than those found in the experimental exposure, which corresponds to an oral exposure of 50% of the ADI for CCC.

1. Introduction

Plant growth regulators are widely used in agricultural food production. In the production of cereals they are used to shorten and strengthen the stem. In the European Union it is anticipated that 70% of the wheat is treated with plant growth regulators. They are also used in some fruit and vegetable productions, e.g. to improve the fruit set and yield in fruit trees. Another area of use is in the production of ornamentals where they enhance flowering. In Sweden, growth regulators are allowed for the latter purpose as well as for the cultivation of rye and grass seed for sowing. The most commonly used plant growth regulator is chlormequat, also known as chlorocholine chloride (CCC).

The toxicity of CCC has been studied in animals. High-level exposures have been found to be toxic for reproduction [1,2]. Further, it has also been suggested that CCC may have such effects on animal reproduction at doses below the acceptable daily intake (ADI) for humans [3,4] but a recent study did not support this [5]. The suggested effects of CCC on mammalian health and fertility call for accurate methods to determine the exposure. Biomarkers have several advantages in comparison with other methods of exposure assessment since it determines the internal dose.

LC/MS/MS is a powerful method for determination of organic substances in biological samples. CCC is a highly hydrophilic compound, weakly retained in reversed-phase chromatographic systems, which makes separation and sample preparation difficult. In water and food samples, CCC has been analyzed by ion-pair liquid chromatography and by cation exchange coupled to LC/MS [6]. A method using hydrophilic interaction liquid chromatography (HILIC) coupled to MS/MS was described for the analysis of CCC in food samples [6]. A method for the determination of CCC in sow milk and pig serum has also been described using LC/MS/MS [7]. However, no method has been presented for the analysis of CCC in human biological samples.

Knowledge of pesticide metabolism in humans, including basic elimination kinetics is needed for the interpretation of biomarkers in the population. Thus, data from controlled human experimental exposures are essential to validate such biomarkers. Regarding CCC, no human experiments have been performed. Evidently, there is a lack of data on human toxicokinetics. In animal experiments with ^{14}C-labeled CCC in rats, absorption was rapid and elimination was

Abbreviations: ADI, acceptable daily intake; b.w., body weight; CCC, chlormequat chlorid; CID, collision induced dissociation; HILIC, hydrophilic interaction liquid chromatography; IS, internal standard; LC/MS/MS, liquid chromatography triple quadrupole mass spectrometry; LOD, limit of detection; LOQ, limit of quantification.

* Corresponding author. Tel.: +46 46 173819; fax: +46 46 143702.
E-mail address: Christian.lindh@med.lu.se (C.H. Lindh).

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essentially complete within 24 h and occurred mainly via urine as unmetabolized CCC [2].

The aim of the present study was to develop a sensitive LC/MS/MS method for the analysis of CCC in human urine. CCC in urine was also evaluated as a biomarker of exposure after experimental human exposures. Further, the method was also applied in urine samples from the general population in southern Sweden.

2. Material and methods

2.1. Chemicals and materials

CCC (97%) was from Fluka/SigmaAldrich (St. Louis, MO) and the internal standard (IS) [$^2H_4$]-CCC was purchased from Dr. Ehrenstorfer (Augsburg, Germany). The chemical structure of CCC is shown in Fig. 1a. Acetonitrile and methanol were from Lab-Scan (Dublin, Ireland) and ammonium acetate, acetic acid and formic acid from Merck (Darmstadt, Germany). Water was produced by an USF Elga Maxima system (USF Elga Ltd., High Wycombe, UK). Isolute HCX-Q (100 mg, 1 mL) columns from Biotage (Uppsala, Sweden) was used.

2.2. Instrumentation

Quantitative analysis was conducted using a triple quadrupole linear ion trap mass spectrometry equipped with a TurboIonSpray source (QTRAP 5500; AB Sciex, Foster City, CA, USA) coupled to a liquid chromatography system (UFLCXR, Shimadzu Corporation, Kyoto, Japan; LC/MS/MS). Air was used as nebulizer and auxiliary gas. Pure nitrogen was used as curtain gas and collision gas. The temperature of the auxiliary gas was set at 550 °C and the ion spray voltage was 4500 V. The MS analyses were carried out using selected reaction monitoring (SRM) in the positive ion mode. To establish the appropriate SRM conditions, standard solutions were infused into the MS for optimization. Collision-induced dissociation (CID) of each $[M+H]^+$ was performed and the product ions giving the best signal to noise ratio were selected for the SRM analysis. All data acquisition and processing was performed using the Analyst 1.5.1 software (Applied Biosystems). An ASPEC XL4 sample preparation robot (Gilson, Inc., Middleton, WI) was used for the sample preparation. A Sigma 3E-1 centrifuge (Sigma, Deisenhofen, Germany) was used for centrifugation of sample vials prior to analysis.

2.3. Preparation of standards

Stock solutions were prepared in duplicates by dissolving accurately weighed amounts of [$^2H_4$]-CCC, and CCC in 10 mL water. Standard solutions were prepared by further dilution of the stock solutions in water and the final calibration standards by spiking urine with the standard solutions. Urine samples for the calibration standards and for quality control samples were obtained from healthy volunteers at our laboratory. The levels were quantified by standard addition methodology. Urine with a low amount of CCC was selected for the calibration standards. The urine was spiked with 0.1–800 ng/mL CCC and then corrected with the amount found in the urine. Two quality control urine samples with quantified levels of 7.0 ng and 31 ng of CCC were selected. These samples were then used in all analytical batches. Each analytical batch also included a calibration curve including 7 standard levels, a chemical blank and a urinary blank.

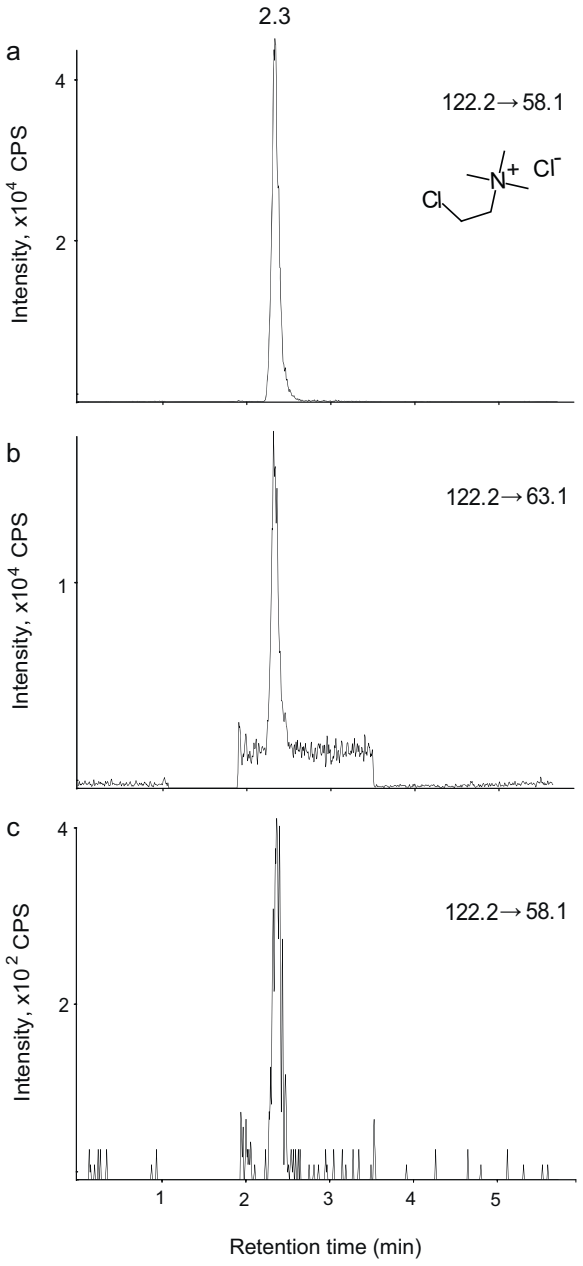

Fig. 1. LC/MS/MS SRM chromatograms showing (a) a urine sample spiked with 25 ng/mL of CCC for the quantifier transition 122.2–58.1. (b) A urine sample spiked with 25 ng/mL of CCC for the qualifier transition 122.2–63.1. (c) A urine sample from a subject non occupationally exposed to CCC and the quantifier transition 122.2–58.1. The urinary level was quantified to 0.17 ng/mL. The chemical structure of CCC is included in the figure.

2.4. Sample preparation

Urine (0.1 mL) was transferred to 8 mL test tubes and added with 0.9 mL of 0.01 M ammonium acetate containing 1 ng [$^2H_4$]-CCC as IS. The test tubes were then vortex-mixed. The samples were extracted using HCX-Q columns and a sample preparation robot. The columns were conditioned with 1 mL of methanol followed by 1 mL of 0.01 M ammonium acetate. The samples were then applied on the columns followed by washing steps with 1 mL 0.01 M ammonium acetate. The analytes were then eluted from the columns with 1 mL methanol containing 1% formic acid and transferred to analytical vials. The samples were kept frozen at −20 °C until analysis. The sample vials were centrifuged at 3000 × *g* immediately before the

Table 1
Summary of the SRM transitions for CCC and [$^2H_4$]-CCC used in the LC/MS/MS analysis.

Analyte	Transitions		Ce[a] (V)	Ion ratio[b]
CCC	122.2 → 58.1	Quantifier ion	40	1:2.8
CCC	122.2 → 63.1	Qualifier ion	30	
[$^2H_4$]-CCC	126.2 → 58.1	Quantifier ion	40	1:2.6
[$^2H_4$]-CCC	126.2 → 67.1	Qualifier ion	30	

[a] The collision energies (Ce) in volt (V) for each transition are tabulated.
[b] The ion ratio between 122.2 → 58.1/122.2 → 63.1 and 126.2 → 58.1/126.2 → 67.1

analysis. Samples quantified to more than 800 ng/mL of CCC were further diluted ten times and reanalyzed using the entire analytical procedure.

2.5. Analysis

Aliquots of 3 μL were injected on an Atlantis HILIC column (150 mm × 2.1 mm, 3 μm; Waters, Milford, MA, USA). The mobile phases consisted of 0.05 M acetic acid/ammonium acetate buffer (pH 3.75) in water and acetonitrile. The separation started with a 1.0 min isocratic step at 30% acetonitrile, followed by a linear gradient of acetonitrile to 95% in 3 min. The column was then equilibrated in during 2 min at 30% acetonitrile. A diverter valve was used and the column effluent was diverted to the MS between 1.8 and 3.3 min. The flow rate was 0.3 mL/min and the column was maintained at 50 °C. The LC/MS/MS analyses were performed using SRM transitions and collision energies as described in Table 1. Concentrations were determined by peak area ratios between the analytes and the IS.

2.6. Analytical method validation

The limit of detection (LOD) was determined as three times the standard deviation of the ratio of the peak at the same retention time as CCC and the IS determined in 22 water blanks. The ratio was then used to calculate the LOD using the average slope from 11 calibration curves. The limit of quantification (LOQ) was similarly determined as ten times the standard deviation of the ratios. To further asses the LOD in urine matrix, a sample with a low CCC levels was selected. The sample was divided into ten aliquots and analyzed using the entire analytical procedure.

The recovery of the method was determined by analyzing five aliquots of urine spiked with 5, 50 and 500 ng/mL of CCC. The samples were prepared using the described procedure. Five standard solutions were prepared by spiking elution buffer (methanol with 1% formic acid) with 5, 50 and 500 ng/mL of CCC.

The within-run precision of the method was determined by analyzing the two quality control urine samples containing 7.0 and 31 ng/mL of CCC. The samples were divided into twenty aliquots and the sample preparation was performed in one sample batch, during one day. The standard deviation was calculated in 20 individual results. The within-run precision was also determined in a sample spiked with 5000 ng/mL. The sample was divided into ten aliquots. These were diluted ten times prior to the sample preparation using the described procedure. The standard deviation was calculated from 10 individual results.

The between-run precision was determined by including two quality control urine samples containing 7.0 and 31 ng/mL CCC in 16 different analytical batches. The samples were prepared and analyzed in different analytical sample batches, during a period of 4 months. The standard deviation was calculated from 16 individual results.

The reproducibility of the method was determined by analysis of 100 urine samples from a non-occupationally exposed Swedish population (see below). Each urine sample was divided into two aliquots which were then subjected to the entire analytical procedure. The samples were prepared and analyzed in separate analytical batches and on different days. The analysis of the samples on the LC/MS/MS was also performed on different days. The formula below was used to calculate the standard deviation s, where x_1 and x_2 are the results of the different aliquots analyzed and n the number of samples.

$$s = \sqrt{\frac{1}{2n} \cdot \sum_{i=1}^{i=n} (x_1 - x_2)_n^2}$$

A qualitative assessment of matrix effects was studied using a post-column infusion with a constant amount of 100 ng/mL of CCC or [$^2H_4$]-CCC at a rate of 6 μl/min into the LC flow prior to the ion source of the MS. The MS was run in SRM mode while 6 different urine samples were injected and separated on the HILIC column using the same conditions as the method.

The stability of CCC in urine was determined by comparing quantified results in 27 urine samples from the experimentally exposed volunteers. The samples were analyzed at two occasions, before and after storage for 7 months at −20 °C.

2.7. Human experimental exposure

Two healthy volunteers, one female (age 64; weight 56 kg) and one male (age 40; weight 75 kg), participated in the study. They had given their written informed consent to participate. The study was approved by the ethics committee at Lund University, Sweden (721-1395-05Mm).

The two subjects received one single oral dose of CCC. An accurately weighed amount of CCC was dissolved in 10 mL of water. An aliquot containing 1.40 mg for the female and 1.87 mg for the male, respectively, was added to 200 mL of water. Thus, the dose was 25 μg CCC/kg of bodyweight (b.w.) for both subjects corresponding to 50% of the ADI. The ADI for CCC is 0.05 mg/kg/day [1].

The first urine sample was collected immediately prior to the dosing and then in intervals *ad libitum* during 46 h for the subjects post exposure. All urine voided was collected. The volume and creatinine levels were determined in all urine samples. Creatinine was evaluated for the adjustment for urinary dilution. The creatinine levels were analyzed using an enzymatic method [8] by an accreditated laboratory (Clinical Chemistry, Lund University Hospital, Sweden). The urine samples were stored at −20 °C until sample preparation and analysis. All samples were divided into two aliquots and analyzed in duplicate sample batches. The mean values of the two determinations were used in further calculations.

The half-life of elimination in urine was estimated from the slope of the curves in the natural logarithm–linear (ln–linear) concentration versus time plot. The urinary concentration was given at the mid point time between two sample collection times. The concentrations adjusted for urinary dilution using creatinine were compared with the unadjusted values.

2.8. Environmentally exposed human population

One hundred 24 h urine samples were collected from individuals in the general population in southern Sweden. The subjects had given their informed consent before participating in the study. Ethical approval was granted by the ethics committee at Lund University, Sweden (721-1395-05Mm). The median age of the participants was 48 years (range 23–64) with 42 male and 57 female participants (1 unknown). All samples were divided into two aliquots and analyzed in duplicate sample batches.

Table 2
The precisions of the method determined in urine samples.

	Number of samples	CCC concentration (ng/mL)	CV (%)
Within run precision	$n = 10$	0.17	9
	$n = 20$	7.0	8
	$n = 20$	31	6
	$n = 10$	5000[b]	4
Between run precision	$n = 16$[a]	7.0	6
	$n = 16$[a]	31	5

[a] Quality control sample included in 16 different analytical batches.
[b] The urine was spiked to 5000 ng/mL and the level was quantified to a mean of 4910 ng/mL.

The mean values of the two determinations were then presented.

3. Results and discussion

3.1. Mass spectrometry and chromatography

The quaternary ammonium compound CCC is already charged in solution and gave a strong response in positive electrospray ionisation MS. With the molecular ion $[M+H]^+ = 122.2$ as precursor ion, the likely product ion $[M-ClC_2H_5]^+ = 58.1$ was formed. This transition was chosen as the quantifier ion since it gave the best signal to noise ratio. A second SRM was chosen as a qualifier ion and used to strengthen the identity of the analyzed CCC in urine. Due to a high noise level in the qualifier transition, it could not be used if CCC levels were below 5 ng/mL. The transitions with optimum collision energies and ion ratios are listed in Table 1.

The CCC could easily be separated with good peak shapes on the HILIC column. However, a gradient elution using acetonitrile/ammonium acetate buffer from 30 to 95% of aqueous buffer with a relatively high ionic strength (0.05 M, pH 3.75) was necessary to obtain good peak shapes and to minimize matrix suppression. Also, to overcome matrix effects in the analysis, the urine samples had to be diluted ten times. The cycle time for an analytical run including equilibration time was 5.3 min. Thousands of injections could be performed on the analytical column before signs of degradation could be observed.

3.2. Method validation

The LOD for CCC was 0.1 ng/mL urine and the LOQ 0.4 ng/mL, determined from analysis of 22 water blanks. It has not been possible to obtain urine samples free of CCC. To asses the LOD in urine matrix, a sample with a low CCC levels was selected. The CCC level was determined with standard addition methodology to 0.17 ng/mL. The coefficient of variation (CV) of 10 determinations was 9%. Thus, performance of the method in urine matrix close to the LOD and below LOQ is excellent, indicating that the LOD determination from water blanks were valid.

A chromatogram showing a urine sample spiked with 25 ng/mL of CCC for both the quantifier and qualifier transitions is shown in Fig. 1a and b. In Fig. 1c, a urine sample quantified to 0.17 ng/mL illustrates the signal/noise level close to the LOD.

For the calibration curves urine with a low amount of CCC were selected and then corrected with the amount found in the urine. The obtained calibration curves in the range 0.3–800 ng/mL urine show excellent linearity with $r^2 > 0.9979$ and a slope of 0.11 ± 0.0066 ($n = 10$).

The within-run and between-run precisions are shown in Table 2. The mean quantified level of the urine samples spiked with 5000 ng/mL was 4910 ng/mL. The reproducibility of the method determined in 100 urine samples from a Swedish population by repeated analyses is shown in Table 3.The data indicate that the reproducibility of the method is excellent even close to the LOD. Also, the robustness of the method is demonstrated since the precision in the determinations is good even if the urine samples vary widely in dilution. Samples containing levels above 800 ng/mL had to be further diluted prior to analysis.

Table 3
The reproducibility of the method was determined in 100 urine samples from a Swedish population without any known occupational exposures. Each sample was divided into two aliquots which were then subjected to the entire analytical procedure.

Sample concentration range (ng/mL)	Number of samples	Mean concentration (ng/mL)	CV (%)
0.3–2.2	$n = 25$	1.3	6
2.2–4.1	$n = 25$	3.1	4
4.1–7.1	$n = 25$	5.2	6
7.1–33	$n = 25$	13	3

The recoveries of the method determined in urine spiked with 5, 50 and 500 ng/mL CCC were 84, 70, and 73% respectively. The recovery was corrected for the small amount present in the urine.

The qualitative assessment of matrix effects showed a minor influence at the same retention time as CCC for both the quantifier transition and the transition for the IS. Data for six different urine samples are shown in Supplementary data.

The stability of CCC in urine was determined after storage. The urinary levels in 27 samples from the experimentally exposed volunteers were analyzed using the described method before and after 7 months of storage at −20 °C. The urinary levels in the samples were in the range 7–400 ng/mL. The results are shown in Fig. 2. Using linear regression, an equation of $y = -0.69 + 1.06x$ ($r = 0.98$) was obtained. Thus, CCC seems to be stable at least for a storage during half a year at −20 °C.

3.3. Human experimental exposure

In urine sampled before the experimental exposure, the CCC concentrations were 1.6 ng/mL for the female and 0.3 ng/mL for the male subject. After the oral ingestion of CCC, corresponding to a dose of 25 μg/kg of b.w. for CCC, the urinary levels rapidly increased. The maximum CCC levels reached after 2 h was 2.8 μg/mL (3.4 μmol/mmol creatinine) for the female subject. For the male subject the maximum levels was 8.1 μg/mL (5.0 μmol/mmol creatinine) after 1 h. CCC was thereafter rapidly excreted, and was essentially complete within 24 h. After 24 h the

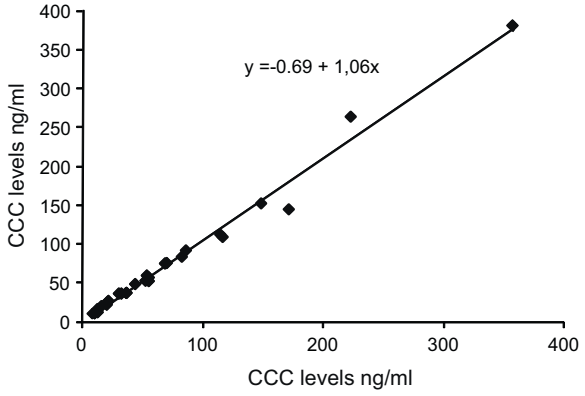

Fig. 2. Urinary levels of CCC in 27 urine samples analyzed two times before and after a 7 months storage time at −20 °C. The urinary levels in the samples were in the range 7–400 ng/mL. Using linear regression, an equation of $y = -0.69 + 1.06x$ ($r = 0.98$) was obtained when plotting the urinary concentrations before and after storage.

Table 4
Summary of the urinary elimination for the female and male volunteers after a single oral dose of 25 μg/kg b.w. of CCC.

Subject	The mid point time intervals	$t_{½}$[a] creatinine adjusted urinary levels (h)	r[b]	$t_{½}$[a] unadjusted urinary levels (h)	r[b]
Female	2–13	2.4	0.99	3.0	0.92
Female	18–46	14	0.96	11	0.93
Male	2–12	2.2	0.95	2.1	0.83
Male	16–45	10	0.94	11	0.93

[a] The half-life of elimination ($t_{½}$) in urine is estimated from the slope of the curves in the log–linear concentration versus time plot.
[b] Correlation coefficient (r) for the correlation line.

total recovery of the doses was 92% and 105% for the female and male subject, respectively. After 46 h the total recovery of the doses was 97% and 109% for the female and male subject, respectively. The results indicate a complete recovery of CCC in the urine from the two subjects and this also suggests a high accuracy of the developed method.

The half-life of elimination in urine was estimated from the slope of the curve in the ln–linear concentration versus time plot. The data suggest that CCC is excreted in urine following first order kinetics and a two compartment model for both subjects. The results are shown in Table 4. The ln–linear creatinine adjusted concentrations versus time plots for CCC for both the female and the male subjects are shown in Fig. 3a and b. The toxicokinetic data in this study indicate that similar results were obtained with both creatinine-adjusted and unadjusted data. The creatinine adjusted results were only slightly better than the unadjusted ones. Since this study was limited to two individuals only, a definite conclusion on whether adjustment for urinary dilution should be performed or not cannot be drawn. Additional human experimental studies are necessary to clarify the absorption and metabolism of CCC. However, the data presented provide valuable and new information regarding the toxicokinetics of CCC in humans. No controlled human volunteer study has been performed for CCC previously.

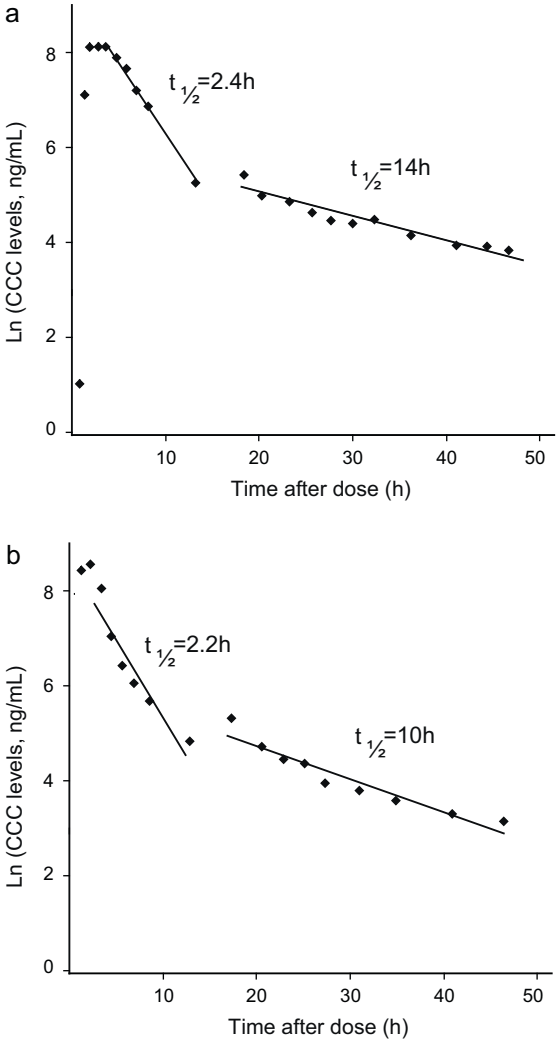

Fig. 3. Urinary elimination of CCC for the female and male volunteers after exposure to 25 μg/kg b.w. of CCC. The half-life of elimination in urine is estimated from the slope of the curves in the log–linear concentration versus time plot. (a) Urinary elimination for the female volunteer with the midpoint time in the intervals 2–13 h and 18–46 h were plotted against the logarithm of the creatinine adjusted urinary levels (ng/mL). (b) Urinary elimination for the male volunteer with the midpoint time in the intervals 2–12 h and 16–45 h were plotted against the logarithm of the creatinine adjusted urinary levels (ng/mL).

3.4. Environmentally exposed human population

Earlier, no study on CCC in biological fluids from humans has been presented. In this study, one hundred 24 h individual urine samples were collected from the general population in southern Sweden. The unadjusted levels were 0.3–33 ng/mL urine and the creatinine adjusted 0.4–28 nmol/mmol (Table 5). All samples had detectable levels above the LOD of 0.1 ng/mL urine. These levels are several magnitudes lower than those found after the experimental exposure to 50% of ADI.

3.5. Ethics

The use of controlled experimental exposure to pesticides in humans can be questioned from an ethical perspective. It has been discussed if such experiments can be ethically justifiable at all [9]. Usually, assumptions about absorbed dose and metabolite excretion are made from animal data [10,11]. However, knowledge of human pesticide metabolism including basic elimination kinetics is a prerequisite to be able to interpret biomarker data in humans. Thus, there is a need for controlled experimental exposures to validate biomarkers of human exposure. In recent years, very few studies have been conducted, mainly hindered by ethical issues. However, with the rapid development of advanced LC/MS/MS techniques, studies where volunteers are exposed to pesticides can be conducted with exposure doses below the recommended ADIs. In the present study the exposure levels were 50% of the ADI for CCC. In this study, we also show that 100% of a tested Swedish population has detectable levels of CCC in urine, albeit in low concentrations. Thus, the ethical problem of experimental exposure to CCC should be minor.

Table 5
Concentrations of CCC in 100 urine samples from an environmentally exposed Swedish population.

Sample concentration	Range	Mean	5 percentile	33 percentile	50 percentile	66 percentile	95 percentile
Unadjusted (ng/mL)	0.3–33	5.6	0.7	3.0	4.0	5.4	15
Creatinine adjusted (nmol/mmol)	0.4–28	5.7	1.0	3.0	4.3	5.6	16

4. Conclusions

A method using LC/MS/MS for the analysis of CCC in human urine has been developed. The method has excellent reproducibility, within-run, and between-run precisions. The method has a sufficiently low LOD to enable detection of CCC in low exposed populations. The use of small sample volumes enables analysis of biobanked samples.

The method was applied in a human experimental exposure study where CCC was estimated to follow first order kinetics and a two compartment model with an elimination half-life of 2–3 h and 10–14 h respectively. Although this study is limited to two individuals, the data provide valuable and new information regarding the toxicokinetics of CCC in humans. The study also shows that there is a non-occupational exposure to CCC in a Swedish general population. The source of the assumed environmental exposure is supposedly mainly alimentary. In complementary studies we will try to elucidate this matter further.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.03.046.

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