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High-throughput method for the analysis of ethylenethiourea with direct injection of hydrolysed urine using online on-column extraction liquid chromatography and triple quadrupole mass spectrometry

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ABSTRACT

Ethylenethiourea (ETU) is of major toxicological concern, since in experimental animal studies, ETU has shown a large spectrum of adverse effects. High occupational exposure can be found among agricultural workers or during manufacturing of ethylenbisdithiocarbamates (EBDC). For the general public, sources of environmental exposure may be residues of ETU in commercial products, food and beverages. For the determination of ETU in human urine we present a high-throughput online on-column extraction liquid chromatography triple quadrupole mass spectrometry method using direct injection of hydrolysed urine samples. This method is simple, user- and environmentally friendly and all sample preparation is performed in 96-well plates. A labelled ETU internal standard was used for quantification. The method showed a good sensitivity with a limit of quantification (LOQ) of 0.5 ng ETU/mL urine and the calibration curve was linear in the range 0.25–200 ng ETU/mL urine. The within-run, between-run and between-batch precision was between 6% and 13%. Alkaline hydrolysis considerably increased the levels of ETU indicating a potential conjugate. The method was applied in an experimental dermal exposure study in humans, with sample concentrations ranging from 0.4 to 5.0 ng ETU/mL urine. The excretion in urine was 10% of the applied dose. The elimination profile seemed to differ between the two individuals. The results show an estimated half-life of ETU between 34 and 72 h. Although the experiment is limited to two individuals, the data provide valuable and new information regarding the toxicokinetics of ETU after dermal exposure.

1. Introduction

Ethylenbisdithiocarbamates (EBDCs) is a group of fungicides of which ethylenethiourea (ETU) is an environmental degradation product as well as a metabolite and impurity. Potential sources of ETU exposure to humans may be occupational or environmental. Occupational exposure may be high among agricultural workers and among workers manufacturing EBDCs [1–9]. Further, ETU is used as a vulcanization agent in the production of polychloroprene (neoprene) and polyacrylate rubbers and in several other products such as dyes. In the general population, residues of ETU in products can be one of many sources of environmental exposure to ETU. Associations have been observed between ETU, and smoking, wine drinking and consumption of fruit and vegetables [4,10]. ETU is of major toxicological concern. In animal studies, ETU has caused a large spectrum of adverse effects, mainly concerning mutagenic, teratogenic, carcinogenic and hepatogenic effects [11–13]. However, the evidence for such effects in humans is less well founded. ETU has been classified to be "reasonably anticipated to be a human carcinogen" based on sufficient evidence of carcinogenicity from animal experiments [14]. Some data suggest that ETU affects the lymphocyte genome and the thyroid gland among heavily exposed workers [1,2,15]. Both for EBDCs and ETU, there is a need for large scale epidemiological studies of exposure-effect relationships. In such studies, an accurate exposure assessment is required. Biomarkers have many advantages in comparison with other methods of exposure assessment, but reliable analytical methods are needed. Many analytical methods for measurement of ETU in biological samples have been presented [16]. Several mass spectrometry (MS) based analytical methods for quantification of the low levels of ETU present in human urine after occupational or environmental exposures, using gas chromatography/mass

Abbreviations: ADI, acceptable daily intake; b.w., body weight; CID, collision induced dissociation; EBDC, ethylenbisdithiocarbamates; ETU, ethylenethiourea; IS, internal standard; LC/MS/MS, liquid chromatography triple quadrupole mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; QC, quality control.

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spectrometry (GC/MS) [17], and liquid chromatography/tandem mass spectrometry (LC/MS/MS) have been developed [18–21]. However, these methods are all laborious, time-consuming and not suitable for epidemiological studies.

Dermal exposure is thought to play an important role in the risk assessment of pesticides [22]. However, the knowledge of dermal exposure to pesticides is limited. Moreover, there is only little knowledge of exposure assessment techniques for quantification of dermal exposure. For data of biomarker levels to be useful, supporting toxicokinetic data are needed. Knowledge of the parent compound and the major metabolites excreted in urine, including the excretion half-life, is important [23]. In guinea pigs, dermally exposed to ETU, 14% of the applied dose was absorbed after 24 h [24]. There are several occupational studies addressing dermal EBDC and ETU exposure. Dermal exposure to ETU is related to the presence of ETU as contaminant in EBDC-based formulation, or in re-entry workers that come in contact with treated crops where EBDC degradation products may be present. In studies of agricultural workers exposed to EBDC, dermal exposure to ETU was measured using filter pads [8,25]. A significant correlation was found between end-shift ETU levels in urine and the measured levels on pads [25]. This result is in agreement with a study of workers at a production plant where ETU levels in urine correlated with EBDC contamination on the hands [5]. Earlier, no studies of experimental dermal exposure to ETU have been performed in humans. On the other hand very few pesticide dermal exposure studies have been performed in humans [22,26–29].

The aim of this study was to develop a high-throughput online on-column extraction LC/MS/MS method for analysis of ETU in human urine. The method was applied in a human experimental dermal exposure study of ETU.

2. Materials and methods

2.1. Chemicals and materials

The internal standard (IS) [$^2H_4$]-ETU was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Hexane was from Lab-Scan (Dublin, Ireland). Sodium hydroxide (NaOH), hydrochloric acid (HCl) and methanol (hyper grade for LC–MS) were from Merck (Darmstadt, Germany). ETU was a PESTANAL® analytical standard, formic acid (FA), pentafluorobencyl bromide (PFBr) and tetrabutylammonium hydrogen sulphate (TBA) were from Sigma–Aldrich Inc. (St. Louis, MO, USA). Purified water from a Millie-Q Integral 5 system (Millipore, Billerica, MA, USA) was used.

Plastic 96-well-plates SQW block with clear glass insert vials 1.5 mL, SQW 45 × 7.7 mm, sealed with a welled sealmat block cover, was from La-Pha-Pack®GmbH (Langerwehe, Germany) and 96-well-aluminium plates with1.5 mL aluminium block cover, was from J.G. Finneran Associates, Ltd (Surrey, United Kingdom).

2.2. Instrumentation

Quantitative analysis was conducted using a triple quadrupole linear ion trap mass spectrometer, equipped with TurboIonSpray source (QTRAP 5500; AB Sciex, Foster City, CA, USA) coupled to a liquid chromatography system with four pumps (UFLC^{RX}, Shimadzu Corporation, Kyoto, Japan). The MS/MS analyses were carried out using selected reaction monitoring (SRM) in positive atmospheric pressure chemical ionization (APCI) mode. Air was used as nebulizer spraying gas. Pure nitrogen was used as curtain gas and collision gas. The APCI temperature was set at 450 °C. The instrument was tuned to a peak resolution of 0.5 ± 0.1 Da at half the peak height in high resolution mode. To establish the appropriate SRM conditions, standard solutions were infused into the MS/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 application software (Applied Biosystems, Foster City, CA, USA).

2.3. Preparation of calibration standards, quality control and samples

Stock solutions were prepared in duplicates by dissolving accurately weighed amounts of [$^2H_4$] ETU (IS) and ETU in methanol. The IS and ETU standard stock solutions were diluted further in methanol and stored at −20 °C. Urine samples for the calibration standards and for quality control samples were obtained from healthy volunteers at our laboratory.

For the calibration curve, 475 μL blank urine was spiked with 25 μL of the standard solutions and 25 μL of the IS solution, giving a urinary concentration between 0.25 and 200 ng ETU/mL and 5 ng [$^2H_4$] ETU/mL urine. The calibration curve was corrected with the amount found in the urine. As quality control (QC) urine samples naturally containing 2 and 7 ng ETU/mL and the 7 ng/mL QC spiked to 32 ng/mL were used. The low, medium and high QC-samples were divided into several aliquots and stored at −20 °C. The chemical blank was prepared from Millie-Q water and thereafter treated like the other samples. The urine samples and QC-samples were vortex-mixed after thawing and aliquots of 500 μL were transferred into 1.5 mL glass vials and placed in an aluminium 96-well plate, and 25 μL of IS solution was added.

For the hydrolysis, 20 μL of 2.5 M NaOH was added to the samples, standards, QC-samples and chemical blanks, giving a final concentration of 0.09 M NaOH. To prevent evaporation during hydrolysis the glass-vials were sealed with a sealmat and a cover was screwed on. After sealing, the samples were mixed thoroughly for 1 min and then transferred to a heating oven. Hydrolysis was performed for 1 h at 100 °C. The aluminium 96-well-plates was used in the hydrolysis step, because of the possibility to secure the cover of the vials with four screws and the capacity of fast temperature transfer throughout the plate. After hydrolysis, the samples were cooled to room temperature and the glass vials were moved to plastic 96-well-plates compatible with the autosampler. Aliquots of 15 μL of 5 M HCl were added to acidify the samples. The samples were mixed thoroughly and centrifuged for 10 min at 2600 × *g* before analysis.

2.4. Analysis

The two mobile phases used consisted of 0.1% (v/v) FA in water (mobile phase A) and 0.1% (v/v) FA in methanol (mobile phase B). The two dimensional separation was carried out, using two identical analytical columns Genesis® Lightn AQ (C18, 4.6 × 100 mm, 4 μm, Grace Vydac, Hesperia, CA, USA) and two sets of LC pumps, each set containing two pumps. The columns and LC pumps were connected through a diverter valve. An aliquot of 20 μL of the sample was injected on the first column and the separation was carried out by isocratic elution, using 100% mobile phase A and a flow rate of 0.7 mL/min. After 2.5 min, the diverter valve switched over and the effluent was diverted into the second column during 1 min and thereafter the diverter valve switched over again. The second set of pumps continued the isocratic elution of the analytes on the second column, using 100% mobile phase A. A diverter valve on the MS diverted the column effluent to the MS between 4.6–6.9 min. The first column was cleaned with 95% mobile phase B at a flow rate of 1.2 mL/min for 1 min, followed by equilibration with 100% mobile phase A for 2.5 min, during the time ETU was eluting on the second column. The second column was reconditioned with 95% mobile phase B for 0.5 min in the end of the analytical run and then

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

Compound	Transitions (Da)	Collision energy (V)
	Quantifier ions	
ETU	103.1 → 60.0	42
[$^2H_4$]ETU	107.1 → 48.0	25
	Qualifier ions	
ETU	103.1 → 44.0	20
[$^2H_4$]ETU	107.1 → 64.0	42

equilibrated with 100% mobile phase A for 2.5 min in the beginning of the next analytical run. The columns were maintained at 40 °C. The LC/MS/MS analysis was performed using SRM transitions and collision energies for ETU and IS quantifier ion and qualifier ions as tabulated in Table 1. All samples were prepared in duplicates and analyzed by single injections. Concentrations were determined by peak area ratios between analyte and the IS. All values were corrected for the chemical blank.

2.5. Sample preparation for cross method comparisons

Sample preparation and analysis were performed according to a previously described method using an extractive derivatisation [18]. In this method, there was an incomplete hydrolysis, thus we included hydrolysis at 0.09 M and 100 °C as a minor modification. The samples were prepared in 13 mL glass tubes. Samples and calibration standards were prepared as in Section 2.3. The sample was hydrolysed for 1 h at 100 °C after an addition of 20 μL of 2.5 M NaOH. After cooling, the derivatisation reagents 0.4 M TBA, 10 M NaOH, dichloromethane and PFBr were added. The samples were derivatised for 90 min in an ultrasonic bath and analyzed by LC/MS/MS.

2.6. The impact of hydrolysis

Determination of the optimal time for hydrolysis was performed using an authentic urine sample, previously quantified to contain 7 ng ETU/mL urine. Samples were prepared in triplicates for nine time points between 0 and 240 min. The test was performed at a constant amount of 0.09 M NaOH and a temperature of 100 °C, except for the non-hydrolysed sample.

To test the impact of hydrolysis on real samples, ten urine samples were selected from an experimental oral exposure to an EBDC (mancozeb) [18]. Five samples were from a male volunteer (A) and five samples from a female volunteer (B). All samples were prepared in duplicates and prepared with and without hydrolysis according to the presented method. In the samples without hydrolysis, NaOH and HCl were mixed just before addition to the samples to avoid hydrolysis.

2.7. Validation of the analytical method

Limit of detection (LOD) and limit of quantification (LOQ) were determined as the mean level of the peak, within 0.1 min of the expected retention time of ETU, plus three and ten times the standard deviation, respectively [30]. LOD and LOQ were determined in chemical blank samples ($n = 14$). To assess the LOD and LOQ in urine matrix, 11 samples with known low ETU levels were selected and then analyzed.

The linear range of the calibration curve was determined from ten concentration levels between 0.25 and 200 ng ETU/mL urine. The equation of the curve was calculated by linear regression and the correlation coefficient (r) was used as a measure of the fit of the curve.

The precision of the method was determined using three different approaches, within-run, between-run and between-batch precision. The precision was calculated as the coefficient of variation (CV) of repeated measurements. The within-run precision was obtained from spiked urine at three concentration levels, 1, 10 and 100 ng ETU/mL urine. The samples were divided into ten aliquots and the sample preparation was performed in one sample batch, during one day.

The between-run precision was determined by including the three QC urine samples, containing 2, 7 and 32 ng/mL of ETU, in eleven analytical sample batches. The QC-samples were prepared in duplicates in each different analytical sample batch. The batches were prepared and analyzed on separate days, during a period of six months. Thus, the standard deviation was calculated from 11 individual mean results.

A between-batch precision of the method was determined by analysis of 88 urine samples collected in the dermal exposure study (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 CV was calculated as previously described [18].

The mean obtained concentration was measured at three concentration levels for the spiked samples used to determine the within-run precision method.

The stability of the ETU standard solutions in methanol was investigated by reanalysis after storage in −20 °C for 7 months.

A cross-method comparison was conducted. The ten urine samples described in Section 2.6 were prepared using the method in Section 2.5 and compared with the here presented method. The results were compared using linear regression.

2.8. Human experimental study of dermal exposure

Two healthy volunteers, one male (age 42; weight 75 kg) and one female (age 65; weight 56 kg) participated in the study. They had given their written informed consent and the study was ethically approved by The Regional Ethical Review Board in Lund, Lund University, Sweden (721-1395-05 mM and Dnr2013/6). The participants minimized the intake of conventionally grown food a few days before as well as during the study. The two subjects received one single dose, administrated topically on the inner forearm on an area of 75 cm^2. The dose to the male volunteer consisted of an accurately weighed amount of ETU, dissolved to a concentration of 3 mg/mL of ethanol/water 50% (v/v), giving 0.30 mg in 100 μL. The female received an aliquot, diluted with ethanol/water 50% (v/v) to a final concentration of 0.22 mg in 100 μL. The doses corresponded to the accepted daily intake (ADI) for ETU, 4 μg/kg b.w./day [31] assuming 100% absorbtion. After administration, the vehicle was allowed to evaporate to dryness before the skin was covered with aluminium foil. After 8 h of exposure, the cover was removed. The remainder of the dose was wiped off the exposed area four times using ethanol/water 50% (v/v) and cotton swabs. Thereafter, the subjects cleaned their forearms with soap and water. The aluminium foil and cotton swabs were stored at −20 °C until analysis. Before analysis, ETU on the aluminium foil and cotton swabs was recovered by extraction into 1 L of Millie-Q water.

The first urine sample was collected immediately prior to the exposure and then in intervals ad libitum during 144 h for the male subject and 170 h for the female. All urine voided was collected. All samples were stored at −20 °C until analysis. Sample volume, creatinine and density were determined in all the collected samples to adjust for the urinary dilution. Creatinine was analyzed with an enzymatic method [32] and density with a hand refractometer. The concentration adjusted for urinary density, C_d, was calculated according to the equation $C_d = C(\text{observed}) \times (1.016 - 1)/(\rho - 1)$, where C(observed) is the obtained concentration in the urine sample, ρ is the measured specific density and 1.016 was used as the average urine density [33]. The half-life ($t_{½}$) of the elimination in

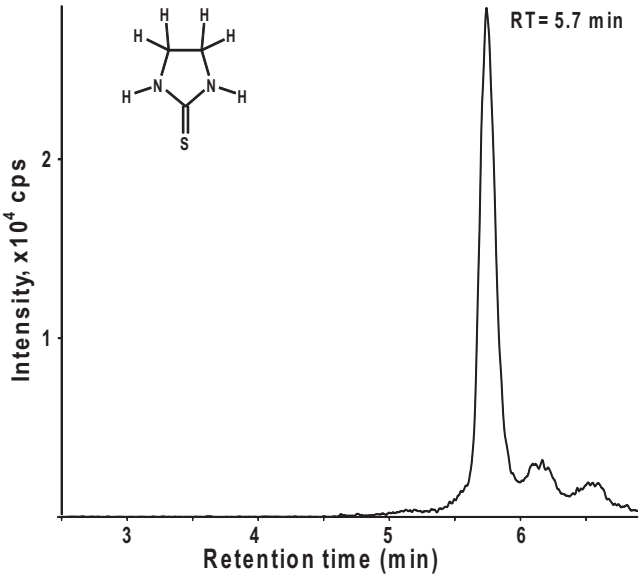

Fig. 1. LC/MS/MS SRM chromatogram showing a urine sample naturally containing ETU and quantified to a concentration of 7 ng/mL, for the quantifier transition 103.1–60.0 Da.

urine was estimated from the slope of the curve in the natural logarithm-linear (ln-linear) concentration versus time plot, where time is given as the mid time points between two sample collection time points.

3. Results and discussion

3.1. Mass spectrometry and chromatography

Previously, published methods for the analysis of ETU in biological samples are too time-consuming for large scale epidemiological studies. We here present a high-throughput method for the analysis of ETU with direct injection of hydrolysed urine using 2 dimension-LC set up as an online on-column extraction. This method is simple and all sample preparation is performed in 96-well plates. Thus, the method is environmental friendly with a significant reduction in solvent consumption. Also, we omitted dichloromethane commonly used for sample preparation [9,17–21,34].

The chosen column provided consistent and reproducible chromatography and retained ETU with stable retention times even in 100% aqueous eluent. The direct injection of urine was possible since an online on-column extraction LC separation and two separate switching valves were used. Thus, most of the urine matrix was discarded, which reduced the chemical background and kept the ion source clean. The cycle time for an analytical run, including equilibration time, was 7.0 min. Thousands of injections were performed on the analytical columns before signs of degradation were observed.

ETU showed an excellent sensitivity in APCI and positive ion mode. The transition 103.1/60 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 ETU in urine (Supplementary data Figs. S1a and S1b). The transitions and optimum collision energies are shown in Table 1. When the analysis was performed with the instrument in high resolution mode, a better signal to noise and a reduction of interfering peaks was obtained. A typical chromatogram of a urine sample is shown in Fig. 1 (Supplementary data Fig. S1c).

Table 2
Increase in ETU levels after hydrolysis in ten urine samples, five samples collected from two individuals [27].

Sample	Subject	Non hydrolysed ETU concentration (ng/mL)	Hydrolysed ETU concentration (ng/mL)
1	A	2.4	9.7
2	A	3.8	18
3	A	4.0	21
4	A	0.4	7.7
5	A	0.2	1.2
6	B	0.2	2.7
7	B	2.3	16
8	B	1.9	13
9	B	2.9	12
10	B	1.9	3.5

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2013.06.035.

3.2. Hydrolysis

To evaluate the effect of hydrolysis, a urine sample was treated with 0.09 M NaOH at 9 different time point during 240 min. The level in the non-hydrolysed urine was 0.1 ng ETU/mL. The levels increased rapidly, and the highest ETU levels were found after 45 min. However, we chose 60 min hydrolysis time in the method (Supplementary data Fig. S2).

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To further evaluate the effect of hydrolysis, ten urine samples from an earlier oral experimental exposure of two subjects was analyzed with and without hydrolysis [18]. It was found that the levels of ETU increased considerably in all samples after hydrolysis. The levels are shown in Table 2. The levels of hydrolysis and non-hydrolysed urine samples were also compared using linear regression. The curve shows a linearity with $r = 0.88$ and a slope of $y = 4.7x$. Previously published methods for urine analysis have not addressed hydrolysis or conjugation of ETU. Interestingly, it has been shown that ETU can form protein adducts that can be released with mild acid hydrolysis [35,36].

3.3. Validation of the analytical method

The LOD and LOQ for ETU determined from analysis of 14 chemical blanks were 0.08 ng/mL and 0.16 ng/mL respectively. Since there seems to be a ubiquitous exposure to ETU, it is difficult to obtain urine samples with low ETU levels and this has an influence on the value of the LOD. After selecting 11 urine samples with low levels of ETU, the LOD was 0.2 ng/mL and the LOQ 0.5 ng/mL (Supplementary data Figs. S3a–S3c). The LOD was in the same range as reported in previous methods [9,17–21,34] and sufficient for the measurement of environmental human exposure. The CV was 19% in urine samples spiked with 0.25 ng/mL ($n = 6$) and 10% at LOQ, 0.5 ng/mL urine.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2013.06.035.

For the calibration curves, urine with a low amount of ETU were selected and then corrected with the amount found in the urine. The obtained calibration curves in the range 0.25–200 ng/mL urine showed excellent linearity with $r = 0.999$ and a slope of 0.0248 ± 0.0016 ($n = 6$).

The within-run, between-run and between-batch precisions are presented in Table 3. The between-run precision was determined

Table 3
Precision of the method at different concentration levels. The within-run precision was calculated from spiked urine samples and the between-run and batch precision from authentic urine samples.

Precision	ETU concentration (ng/mL)	*n*	Mean obtained ETU concentration (ng/mL)	CV (%)
Within-run	1	10	1.2	9.9
	10	10	11	13
	100	10	98	12
Between-run	2	11	1.9	6.2
	7	11	6.5	4.0
	32	11	28	6.1
Between batch	Range 0.3–5.0	88	1.7	6.2

over a period of six months. In Table 3, the obtained concentrations at three concentration levels for spiked samples are presented.

The stability of the standard solutions of ETU dissolved in methanol was examined and found to be stable for 7 months at −20 °C. Several studies have found that ETU is stable in urine samples: in room temperature and in darkness for 24 h, at −20 °C for 6 months [17], at 8 °C for 2 weeks, at −20 °C for 3 months [18], and at −18 °C for 12 months [4].

A cross-method comparison was conducted using ten urine samples from an oral experimental exposure of two subjects and analyzed after hydrolysis using the here presented method and a method using an extractive derivatisation and analysis using LC/MS/MS [18]. The obtained results showed an excellent correlation when the levels were compared using linear regression giving a linearity with $r = 0.975$ and the slope 1.00 (Fig. 2).

3.4. Human experimental study of dermal exposure to ETU

In urine sampled before the experimental exposure, the ETU levels were for the female subject 0.8 ng/mL and for the male subject 0.7 ng/mL, density adjusted. After the dermal exposure of ETU, corresponding to a dose of 4 μg/kg of b.w., the urinary levels increased slowly. After about 24 h, a plateau was reached which lasted for about 50 h for the female and 30 h for the male subject Fig. 3a and for the female subject, Fig. 3b. Similar plateaus have also been observed in human dermal exposure studies of other pesticides [27–29,37]. The maximum ETU level was reached after 39 h and was

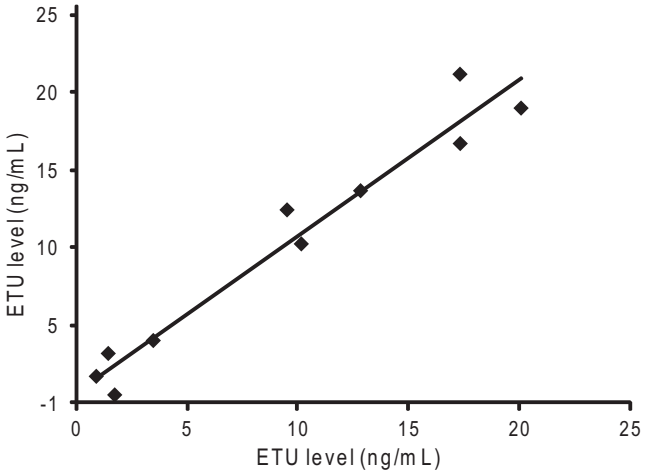

Fig. 2. Ten hydrolysed urine samples analyzed with two different analytical methods in the cross-method comparison. Urinary levels of ETU obtained by the modified method described by Lindh et al. [18] were plotted versus levels of ETU obtained by the method reported in this paper. The equation of the straight line was obtained by linear regression and found to be $y = 1.00x + 0.20$ ($r = 0.975$).

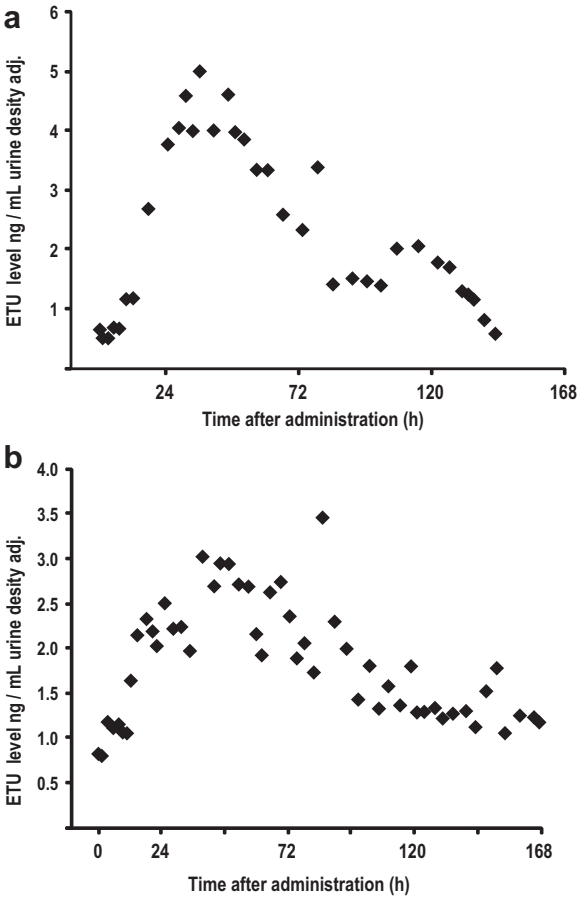

Fig. 3. (a) Urinary absorption and elimination for the male volunteer with the midpoint time in the interval 0–143 h was plotted against the density adjusted urinary levels (ng/mL). The volunteer was dermally exposed to a dose of 4 μg/kg b.w. on the inner forearm for 8 h. The first two urine samples were collected just before and 1 h after administration of the dose. (b) Urinary absorption and elimination for the female volunteer with the midpoint time in the interval 0–169 h was plotted against the density adjusted urinary levels (ng/mL). The volunteer was dermally exposed to a dose of 4 μg/kg b.w. on the inner forearm for 8 h. The first two urine sample was collected just before and 1 h after administration of the dose.

3.0 ng/mL (density adjusted) in the female subject and 5.0 ng/mL (density adjusted) after 36 h in the male. The decline in elimination of ETU was estimated to begin at 39 h in the female and at 36 h in the male. For the calculation of the half-life of elimination, values after 143 h for the female and 101 h for the male were discarded due to low levels and a suspected interfering environmental exposure. The half-lives of ETU with correlation coefficients are shown in Table 4. The urinary elimination of ETU for the male volunteer is shown in Fig. 4a and for the female volunteer in Fig. 4b. Earlier, attempts have been made to estimate elimination half-lives in urine after occupational dermal and inhalation exposure to EBDC. The half-lives vary considerably between the studies and are in the

Table 4
Estimated half-life of ETU found in the two volunteers, dermally exposed to a dose of 4 μg/kg b.w. of ETU for 8 h on an area of 75 cm^2.

ETU urinary levels adjusted for	Female $t_{½}$[a] (h)	r[b]	Male $t_{½}$[a] (h)	r[b]
Density	72	0.89	34	0.94
Creatinine	75	0.75	29	0.89
Unadjusted	103	0.42	64	0.71

[a] The half-life of elimination ($t_{½}$) in urine estimated from the slope of the curve in the natural logarithm-linear (ln-linear) concentration versus mid time plot.
[b] Correlation coefficient (r) for the regression line.

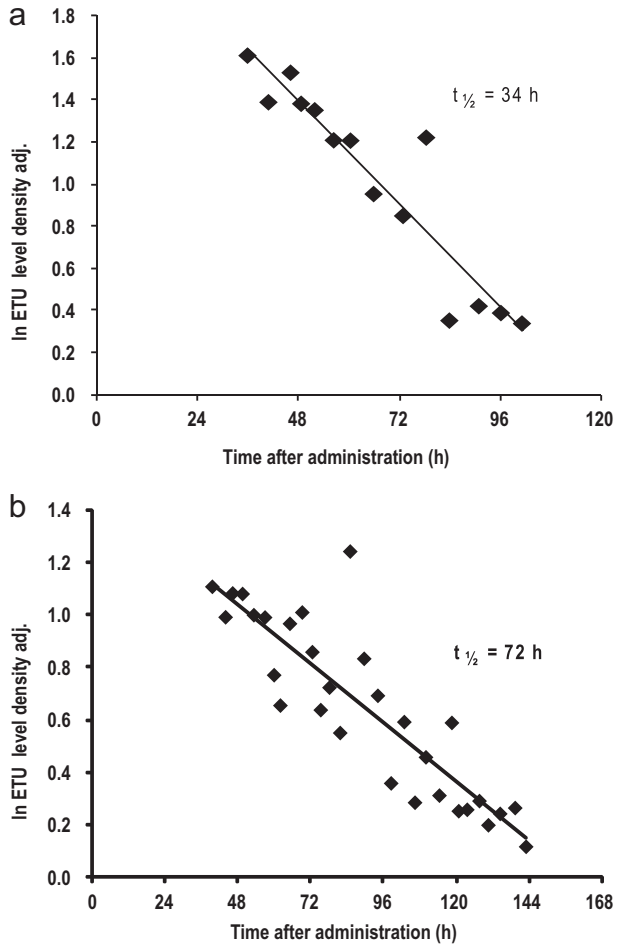

Fig. 4. (a) Urinary elimination of ETU for the male volunteer, after dermal exposure to a dose of 4 μg/kg b.w. of ETU for 8 h on an area of 75 cm^2 on the inner forearm. The half-life of elimination ($t_{½}$) in urine is estimated from the slope of the curve in the log-linear concentration versus time plot. Urinary elimination with the midpoint time in the interval 36–101 h was plotted against logarithm of the density adjusted urinary levels (ng/mL). (b) Urinary elimination of ETU for the female volunteer, after dermal exposure to a dose of 4 μg/kg b.w. of ETU for 8 h on an area of 75 cm^2 on the inner forearm. The half-life of elimination ($t_{½}$) in urine is estimated from the slope of the curve in the log-linear concentration versus time plot. Urinary elimination with the midpoint time in the interval 39–143 h was plotted against logarithm of the density adjusted urinary levels (ng/mL).

range 32–100 h [7,8]. The half-life after an experimental oral exposure to EBDC contaminated with ETU was estimated to 19–23 h for one female and one male [18]. Our study also contains only two individuals of each sex, thus one should be careful to draw to many conclusions. The half-life reported here differed more between the male and female volunteer compared to that of the oral exposure. This may indicate that the differences between sex and age have larger impact on dermal absorption than on oral absorption. The longer plateau for the female subject may possibly be explained by the fact that skin properties change with age and differ between individuals and that may have an impact on the dermal absorption. This is not surprising, since it is well known that, xenobiotic metabolism and excretion kinetics varies greatly depending on age, sex and activity level [22].

Of the administrated dermal dose, 35% was recovered from the skin washings from the female and 24% from the male at the end of the 8 h exposure. After deduction of the amounts in the washings, the total recovery of ETU in the female urine was 10% of the dose after 143 h. Correspondingly, in the male, 10% of the dose was recovered after 101 h. The results indicate a high uptake of ETU after dermal exposure. After an oral exposure of EBDC contaminated with ETU, the recovery was 69–82% of the dose. [18].

The toxicokinetic data in this study indicate that an adjustment for the urinary dilution is recommended and both creatinine levels and urinary density may be applied. Density gave the best fit of the adjusted values. Most often, though, creatinine levels have been used to adjust for urinary dilution. However, creatinine levels may be affected by several factors such as gender, age, muscularity, and consumption of meat. Also urinary density may be affected by similar factors, but a recent study has shown that urinary cadmium levels adjusted for creatinine was more affected by gender, age, body size and meat intake than adjustment using urinary density [38]. When comparing individuals or populations with large differences in muscle mass, meat intake, or when both males and females of various ages are included, density adjustment may be the more applicable.

In our study, 0.1 mL of ethanol/water 50% (v/v) solution was chosen to get an even dispersal of ETU over the application area. The solution evaporated within a minute before the skin was occluded to protect the surface. We therefore assume that the use of an ethanol solution had minimal effect on the permeation of ETU. The use of ethanol as vehicle and washing solution has been used in other dermal absorption studies [27,28]. Occlusion of the exposed skin may also affect the dermal absorption by enhancing the hydration and temperature of the skin. Further, washing of the exposed skin may enhance the dermal absorption, especially if using soap. Surfactants have shown to alter the properties of the skin barrier [22].

We chose to expose the forearm of the subjects, the most commonly used anatomic site in dermal experimental exposure studies [22,26–29]. However, it has been shown that the forearm is less permeable compared to the forehead and the neck. This difference is due to skin thickness and amount of appendage like hair follicles, sweat- and sebaceous glands. Thus, the absorption may be higher, if other more permeable areas of the body are exposed [5,8].

3.5. Application of high-through put method in an epidemiological study

Spot urine samples (n = 1282) were collected from individuals environmentally exposed to EBDCs and ETU. Data from this study will be published elsewhere. All samples were divided into two aliquots and analyzed in duplicate sample batches. A total of 32 96-well plates were prepared with six QC samples per plate. Sample concentrations ranged from 0.3 to 210 ng ETU/mL. The between-run precision determined from QC samples were CV = 19% at 2.3 ng/mL (n = 63), CV = 11% at 7.1 ng/mL (n = 62) and CV = 9.3% at 30 ng/mL (n = 63). The samples were prepared and analyzed during about 2 months. Thus, the method is applicable in large epidemiology studies.

4. Conclusions

We present a high-throughput method for the analysis of ETU with direct injection of hydrolysed urine using online on-column extraction LC/MS/MS. This method is simple and user-friendly and all sample preparation is performed in 96-well plates. The method is environmentally friendly with a significant reduction in solvent consumption. The urinary levels of ETU increased considerably after alkaline hydrolysis at 0.09 M NaOH. The method has excellent within-run, between-run and between-batch precisions. The method has a sufficiently low LOD to enable detection of ETU in environmentally exposed populations.

The method was applied in a human experimental dermal exposure study where about 10% of the applied dose of ETU

was recovered in urine. The elimination profile seemed to differ between the two subjects. Although this study is limited to two individuals, the data provide valuable and new information regarding the toxicokinetics of ETU after dermal exposure. The method was also applied in an epidemiological study, showing that 1282 samples could be analyzed with a good precision during a short time.

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