Journal of Chromatography B, 878 (2010) 2563–2566

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

# Determination of ethylenethiourea in urine by liquid chromatography–atmospheric pressure chemical ionisation–mass spectrometry for monitoring background levels in the general population☆

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ARTICLE INFO

*Article history:*
Received 21 September 2009
Accepted 26 October 2009
Available online 31 October 2009

*Keywords:*
Biological monitoring
Liquid chromatography
Mass spectrometry
Urine
Pesticide residues
Ethylenethiourea
Dithiocarbamates

ABSTRACT

This study reports a sensitive analytical method suitable for the quantitative analysis of ethylenethiourea (ETU) in human urine and its application to samples from the general population. Sample preparation involved the use of diatomaceous earth extraction columns to remove matrix interferences. Quantification was achieved by liquid chromatography–mass spectrometry using positive ion atmospheric pressure chemical ionisation. Within-day and between-day variability of 14% ($n = 10$) and 11% ($n = 6$), respectively, were obtained at 98 nmol/l (10 μg $l^{-1}$). The assay was linear over the investigated range 2.5–245 nmol/l, with a limit of detection of 2.5 nmol/l. The method was applied to monitoring background levels of ETU in urine samples from the general population in the UK. Results obtained from 361 spot samples contained ETU levels ranging from less than the detection limit (54% of samples) to a maximum of 15.8 μmol/mol creatinine (14.3 μg/g creatinine). The 95th percentile was 5.7 μmol/mol creatinine (5.2 μg/g creatinine).



## 1. Introduction

Ethylenethiourea (ETU) is the primary degradation product of ethylenebisdithiocarbamates (EBDCs) [1,2], which include some of the most widely used fungicides in agriculture. Although EBDCs are characterised by their low toxicity, ETU is of much greater toxicological concern due to is suspected causation of pathological effects including goitrogenic and potential carcinogenic effects [3–6].

Exposure to EBDCs and/or ETU in the general population is likely to arise from a number of environmental sources. It has been shown from food and urinary analysis that wine, fruit and vegetables, on which these fungicides are predominantly used, can represent a feasible route of exposure [7,8]. In addition, ETU can be formed when foods containing EBDC pesticides are cooked. The cooking process can convert EBDCs to ETU and therefore ETU is of the greatest significance in contaminated processed foods [9]. On the other hand bystander exposure might also contribute to levels in the general population from people either living nearby areas that are treated with EBDCs or through recreational activities in areas where drift can be possible [10].

ETU is a specific metabolite of EBDCs found in urine and has been suggested as a good biomarker of occupational and environmental exposure to ETU or EBDCs. Studies have reported ETU values in both these scenarios [8,11,12] and some provided reference values of ETU in the general population in Italy [11,12] as an aid to interpreting results from monitoring studies. Fustinoni et al. published results from other European countries (Bulgaria, Finland and the Netherlands) as well as Italy [13].

Highly sensitive and specific analytical methods are required for ETU analysis due to the low levels found in the environment. Recent improvements in analytical methods for the determination of ETU describe the use of diatomaceous earth columns for clean-up of urine samples [14] and the use of liquid chromatography–mass spectrometry [15–17].

In this study we describe the combination of the use of diatomaceous earth columns for a simplified sample clean-up procedure of urine samples and with atmospheric pressure chemical ionisation–liquid chromatography–mass spectrometry (APCI–LC–MS) to give a sensitive and specific method suitable for biological monitoring of ETU. To our knowledge the combination has not been previously reported. The method is applied to a large number of urine samples from the general population to provide an idea of background levels of ETU in the UK.

☆ This paper is part of the special issue "Biological Monitoring and Analytical Toxicology in Occupational and Environmental Medicine", Michael Bader and Thomas Göen (Guest Editors).

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1570-0232/$ – see front matter. 
doi:10.1016/j.jchromb.2009.10.028

## 2. Experimental

### 2.1. Chemicals and standard preparation

HPLC grade dichloromethane (DCM) and methanol were purchased from Rathburns (Walkerburn, UK). Formic acid (>96%, ACS reagent) was purchased from Aldrich (Gillingham, UK). ETU (99.5%) for use as the standard was purchased from $QM_x$ laboratories (Thaxted, UK). $^2H_4$-ETU (98% atom enrichment) was used as the internal standard and obtained from CDN isotopes (Quebec, Canada). Deionised water was purified using a Milli-Q plus system (Millipore, Billerica, MA).

A standard solution of ETU (9.8 μmol/l) was prepared in distilled water. The standard solutions of ETU for the calibration curve were prepared at the concentration of 0, 49, 98, 147, 196, and 245 nmol/l in urine. An unspiked sample of the same urine was kept as blank. An internal standard solution was prepared at a nominal concentration of 5 μmol/l.

### 2.2. High performance liquid chromatography

The HPLC system comprised of an Agilent Technologies (Palo Alto, CA, USA) 1100 binary series LC pump equipped with a vacuum degasser, and autosampler. The column was a Genesis® C18 250 cm, 4.6 mm id and 4 μm particle size (Jones Chromatography, Hengoed, UK). The mobile phase was water: methanol (80:20, v/v) with 0.1% formic acid. An isocratic separation was used with the column at a flow rate of 0.8 ml/min. An injection volume of 30 μl was used for LC–MS experiments.

### 2.3. Mass spectrometry

The outlet of the HPLC system was directly connected to an Agilent Technologies LC/MSD XCT ion trap mass spectrometer, equipped with an orthogonal ion source. ETU was detected in positive atmospheric pressure chemical ionisation mode using the following parameters: nebulizer pressure ($N_2$) 60 psi, drying gas flow rate ($N_2$) 5 l/min, dry gas temperature 350 °C, APCI temperature 350 °C, trap drive 21.2, capillary exit 98.7 V, skimmer 40.0 V, scan range 50–2200, dwell time 100 ms and an ion abundance target of 30,000. ETU (MW 102) was determined by monitoring $[M+H]^+$ ion at $m/z$ 103 and $^2H_4$-ETU (MW 106) $[M+H]^+$ ion at $m/z$ 107 in selected ion monitoring mode.

### 2.4. Extraction procedure and clean-up

Calibration standards were prepared by spiking an appropriate volume of an ETU standard solution into 2 ml urine. For estimation of recovery, pre-prepared frozen quality control material (pooled urine) spiked at the 98 nmol/l level was used; two quality control samples were run after every five samples. Samples (2 ml) were analysed in duplicate. Standards, quality controls and samples had 100 μl of internal standard added, were diluted with 1 ml of distilled water, applied to diatomaceous earth extraction columns (ChemElut® 1003, Varian, Poole, UK) and left to absorb for ~5 min. The analyte was eluted with DCM (6 ml), after an interval of ~10 min a second aliquot (6 ml) was applied. After a further ~10 min, the eluate was evaporated to dryness under a gentle stream of nitrogen and reconstituted in mobile phase (100 μl).

**Table 1**
Summary of linear regression parameters for calibration curve and limit of detection for determination of ETU in urine.

| Assay parameters | Values |
|---|---|
| Dynamic range (nmol/l) | 2.5–245 |
| Correlation coefficient ($r$) | 0.9969 |
| Determinations ($n$) | 6 |
| Slope ± S.E. | 0.0215 ± 0.0080 |
| Intercept ± S.E. | −0.0280 ± 0.0573 |
| LOD (nmol/l) | 2.5 |

**Table 2**
Within-day and between-day variability and system reproducibility.

| | ETU | | |
|---|---|---|---|
| | Within-day variability ($n$ = 10) | Between-day variability ($n$ = 6) | Overall system variability ($n$ = 59) |
| Mean (nmol/l) | 82 | 91 | 79 |
| SD | 11 | 10 | 11 |
| RSD (%) | 14 | 11 | 14 |

### 2.5. Method characterisation

Linearity was assessed by least squares linear regression using six calibration curves prepared on separate days from different urine samples. The variability of the method was evaluated by analysing, on 6 different days, 10 urine replicates spiked at 98 nmol/l (pooled urine). The limit of detection was defined as the amount injected (from an extracted urine sample) that gave a signal equivalent to three times the baseline noise.

### 2.6. Human study

Three hundred and sixty one spot urine samples and questionnaire data were collected from individuals in the general population in the UK, who gave informed written consent. Ethical approval was granted by the Central Manchester Local Research Ethics Committee (REC Ref. 05/Q1407/93). The recruitment of participants and the study design have been described previously [18]. Samples were obtained from across the United Kingdom with roughly equal male and female participants. Samples with ETU results below the limit of detection were reported as half LOD (i.e. 1.25 nmol/l) and then corrected for creatinine.

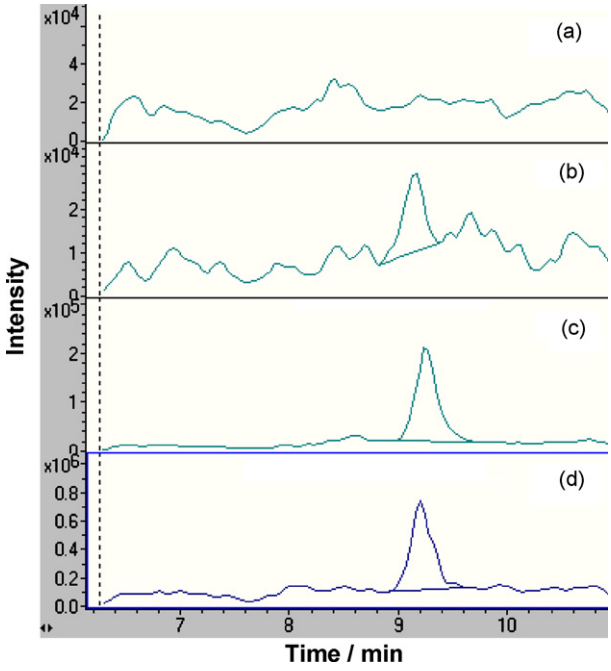


**Fig. 1.** Extracted ion chromatograms (a) blank urine extract (b) urine extract at the limit of detection of 2.5 nmol/l, (c) urine extract spiked at 98 nmol/l and (d) the internal standard. Retention time of ETU is 9.2 min.

**Table 3**
Comparison of ETU levels for the general UK population with other reported studies (all reported in μg/g creatinine).

| | *N* | %Positive | Mean | GM (GSD) | Range |
|---|---|---|---|---|---|
| This study | 361 | 46 | 1.3 | 0.5 (4.4) | <0.2–14.3 |
| Saieva et al. [20][a] | 69 | 22 | 0.4 | 0.3 | <0.5–3.3 |
| Colosio et al. [12] | 95 | 59 | – | 0.6 (3.1) | <0.5–11.6 |
| Aprea et al. [11][b] | 167 (urban) | 24 | 2.7 | 2.1 (2.0) | 0.8–8.3 |
| | 97 (rural) | 37 | 9.1 | 5.3 (2.6) | 0.9–61.4 |
| Fustinoni et al. [13] | 42 (Finland) | – | 0.9 | – | <0.5–11.6 |
| | 45 (Italy) | – | 1.3 | – | <0.5–8.1 |
| | 38 (Netherlands) | – | 1.2 | – | <0.5–4.8 |

[a] Conversion from nmol/day, assuming 1.6 l urine/day and 1.36 g creatinine per litre.
[b] Only positive samples in statistics.

### 2.7. Urinary creatinine

Creatinine was determined in all urine samples using a Cobas Mira (ABX France) and an automated alkaline picrate method [19]. The coefficient of variation for within-day analysis was 1.5% and for between-day analysis was 3% at 6 mM.

## 3. Results and discussion

### 3.1. Sample preparation

The extraction of ETU from urine was investigated for ethyl acetate and dichloromethane extraction (without the ionic and pH adjustment previously presented by Fustinoni et al. [14]). Ethyl acetate showed reduced extraction efficiency to that of DCM and, at the 98 nmol/l spiking level, resulted in increased co-extracted interferences eluting closely to the ETU peak, affecting the limit of detection. Using the DCM extraction (see Section 2.4) produced results that were not significantly different to adjusting the urine prior to extraction with $NH_4Cl$ and KF adjustment. This reduced the time for sample preparation significantly.

### 3.2. Method characterisation

The calibration curves obtained in the range 2.5–245 nmol/l showed good linearity, Table 1 gives a summary of the regression parameters. The within-day and between-day precision parameters obtained from the analysis of spiked urine samples are reported in Table 2. Overall, the within-day and between-day variability is low demonstrating good reproducibility of the method.

The extracted ion chromatograms for blank urine samples show very few peaks originating from matrix making the peaks corresponding to target analytes clearly visible (Fig. 1). The LOD was 2.5 nmol/l (0.25 $\mu g\,l^{-1}$). This is in the same range as the methods of Saieva [20], Fustinoni [14], Sottani [16], Lindh [15] and Montesano [17] where the latter three methods use LC/MS/MS. The method reported here has the advantages of lower sample volume (2 ml as opposed to 10 ml, e.g. Sottani et al. [16], El Balkhi et al. [21]), a single extraction procedure (unlike Sottani et al. [16], Lindh et al. [15], Montesano et al. [17]) and no derivatisation step (unlike Fustinoni et al. [14], Lindh et al. [15]).

### 3.3. General population study

Results are presented as creatinine corrected (Fig. 2, Table 3) in agreement with the majority of previously published studies and in light of Lindh's observation [15] that creatinine adjusted values gave marginally better correlation coefficients than unadjusted levels in a volunteer study. Fifty four percent of the samples had levels of ETU below the limit of detection, which is similar to that found by Colosio et al. [12] in the Italian population. Table 3 shows that the levels of ETU found in the general UK population are similar to those reported elsewhere in Europe. No statistically significant associations were found between ETU levels in urine and UK region, fruit and vegetable consumption, wine consumption or smoking status. The findings on smoking and alcohol agree with those of Colosio et al. [12] and Saieva et al. [20] but are unlike those reported by Aprea et al. [11] where smoking and wine consumption did influence ETU levels in their urban population. Our study surprisingly found a statistically significant association between the frequent exposure to environmental tobacco smoke and lower levels of ETU however the assessment of exposure frequency was by self-reported questionnaire data.

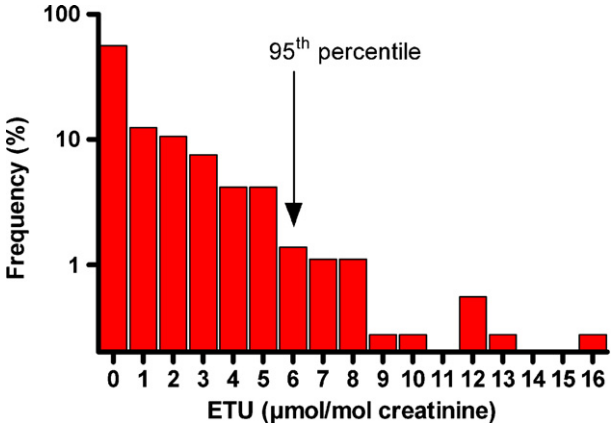


**Fig. 2.** Percentage frequency distribution ($n = 361$) for ETU in the UK general population (logarithmic scale).

## 4. Conclusion

This paper reports a straightforward, sensitive and specific method for the determination of ETU in urine. Detection limits and variability are comparable with other recently reported methods but the sample preparation is less onerous. A large study of the general UK population shows that ETU is detected in roughly half of participants with levels similar to those reported in other parts of Europe.

## Acknowledgements

The authors would like to thank Alex Capleton and Louise Assem for their initial involvement in the population study and Monica Rivas Casado (School of Applied Science, Cranfield University) for statistical analyses.

The method development work was funded by the Food Standards Agency in the UK and the population study was funded by the European Chemical Industry Council (CEFIC).

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