Determination of Ethylene Thiourea in Urine by HPLC–DAD

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Abstract

Ethylene thiourea (ETU) is a metabolite of ethylenebisdithiocarbamates (EBDCs); it is the best indicator of exposure to these fungicides. Therefore, high-performance liquid chromatography with photodiode-array detection (HPLC–DAD) was optimized and validated for the determination of ETU in human urines. Urine samples were extracted by solid-phase extraction using Extrelut® and analyzed using HPLC–DAD set at 231 nm. The analyses were carried out using a mobile phase of 0.01M phosphate buffer (pH 4.5) on a C18 Uptisphere NEC-5-20, 250- × 4.6-mm × 5-µm column. The internal standard used was 4-pyridinecarboxylic acid hydrazide. The method was successfully validated in compliance with requirements set by the International Committee on Harmonization 1996. The lower limit of quantitation was at 1 µg/L, and the linearity was studied from 1 to 100 µg/L. There were 272 urine samples collected from farmers exposed to EBDCs in different regions in France analyzed in this study.

Introduction

Ethylene thiourea (ETU) is a metabolite of ethylenebisdithiocarbamates (EBDC), the fungicides which are most widely used in agriculture. Besides ETU, other degradation products of EBDCs are formed in vivo including ethylene bisthiuram disulfide (ETD), ethylene urea (EU), 5,6-dihydro-3 H-imidazo(2,1-C)-1,2,4-dithiazole-3-thione (DIDT), and ethylene diamine (EDA) (Figure 1). However, in urine, ETU is the best indicator of an exposure to these fungicides (1,2). The world-wild consumption of dithiocarbamates is between 25,000 and 35,000 metric tons per year (3). Exposure occurs mainly by inhalation and by percutaneous absorption under occupational conditions (e.g., farmers). Ingestion of treated food, however, remains a common cause of exposure to residues of fungicides in the general population (4). The target organ, in subchronic and chronic exposure to ETU-generating compounds, is the thyroid (5). Several studies showed that ETU produce thyroid carcinomas at low doses in rodents (3). Therefore, in order to build an epidemiological study and focus on prevention guidelines, it was necessary to develop a method of analysis to determine ETU in human biological fluids.

Many chromatographic methods have been described to determine ETU in food crops (6), human biological fluids (7), and in animal's biological fluids (8). None of these methods could be adapted to be used in our laboratory, due essentially to bad separations of ETU from interfering peaks.

In this paper, we describe a simple, inexpensive, and adaptable method of determination of ETU using a high-performance liquid chromatograph (HPLC) with a photodiode-array detector (DAD). Two-hundred seventy-two urine samples were analyzed in this study. Samples were collected from farmers in different regions in France, during the period when the farmers applied various trademarks of EBDCs (Zineb®, Mancozeb®, or Maneb®).

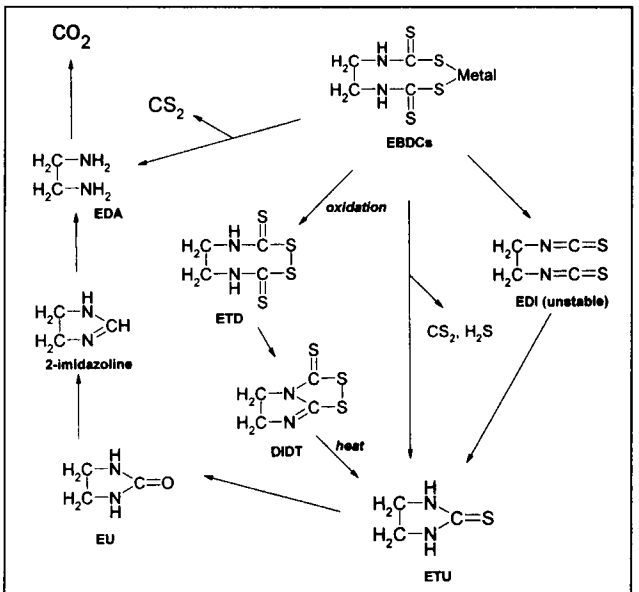

Figure 1. Metabolic pathways for the decomposition of EBDCs and reaction leading to ethylene thiourea. Abbreviations: ETU, ethylene thiourea; ETD, ethylene bisthiuram disulfide; EU, ethylene urea; EDI, ethylene diisothiocyanate; EDA, ethylene diamine; EBDCs, ethylene bisdithiocarbamates; and DIDT, 5,6-dihydro-3 H-imidazo(2,1-C)-1,2,4-dithiazole-3-thione. Figure adapted from reference 3.

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Materials and Methods

Reagents

Dichloromethane and acetonitrile were HPLC grade and were purchased respectively from Merck (VWR™ International) and Carlo Erba. ETU and 4-pyridinecarboxylic acid hydrazide (internal standard) were purchased from Merck. Extrelut NT (kieselguhr) refill pack for column filling, used as a solid phase in the extraction, was purchased from Merck. Distilled water was obtained by using a Milli-Q academic A 10 purification system (Millipore).

A phosphate buffer solution (0.01M, pH 4.5) was prepared by dissolving 1.56 g of sodium dihydrogen phosphate monohydrate GR ($NaH_2PO_4.H_2O$) (Merck) in 1 L of distilled water and then filtering using nylon membranes (47-mm diameter, 0.4-µm pores, Osmonics, Inc., Sigma-Aldrich) under vacuum.

Ethylene urea (EU) (another EBCDs metabolite) was purchased from Merck.

Standard solutions

The internal standard (IS) used was 4-pyridinecarboxylic acid hydrazide; 100 mg was dissolved in 200 mL of distilled water (i.e., a stock solution at 500 mg/L). A stock solution of ETU was prepared by dissolving 100 mg in 100 mL distilled water to obtain a final concentration at 1000 mg/L. This stock solution was diluted to have working solutions at 100, 50, 40, 20, 10, 5, 2, and 1 mg/L. Each of these working solutions was diluted to 1:1000 by adding 10 µL of each to 10 mL of free ETU-IS urine. The following concentrations of loaded urine were obtained: 100, 50, 40, 20, 10, 5.0, 2.0, and 1 µg/L. Five-hundred microliters of IS was added to each standard urine before extraction. All were vortex mixed for about 15 s and extracted.

Quality controls in urine, at three levels, 5, 30, and 80 µg/L, were prepared using the same procedure.

Samples

Subject urines were collected and immediately frozen at –18°C in opaque 75-mL bottles. Each subject supplied two or more samples: one collected before application of EBDCs and the other(s) afterwards. These samples were transferred to laboratory in dry ice, immediately thawed over 12 h at 4°C, and then left at room temperature for 2 h before analysis.

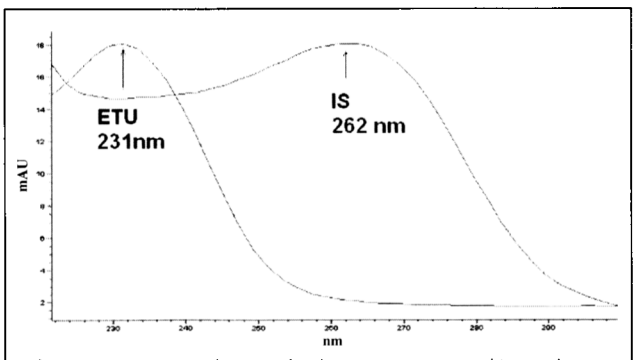

Figure 2. UV spectra of ETU with a λ max at 231 nm and internal standard (IS) with a λ max at 262 nm.

Extraction procedure

The extraction was performed using glass columns (23-cm long × 1.5-cm diameter) filled with 9 g of Extrelut. Ten milliliters of urine was loaded on the solid phase and left for 15 min (as suggested by the manufacturer; no pretreatment was necessary before loading urine). Twenty milliliters of dichloromethane was added afterwards in two steps (10 mL each time). The eluted solutions were gathered in 20-mL glass tubes and then evaporated at 36°C under an air flux. The elution and evaporation processes were repeated three times on the same 20-mL glass tubes because of the limited tube volume (which made a total volume of 60 mL of dichloromethane). Dry residues were dissolved with 250 µL mobile phase, transferred to 350-µL autosampler vials, and injected.

HPLC

The LC analysis was performed using a Hewlett Packard 1050 series HPLC with a DAD spectrophotometer (Agilent). The column used is a 250- × 4.6-mm (5-µm particle size) C18 Uptisphere NEC-5-20, (a un-endcapped column that has a high selectivity for polar and very polar compounds) with a pre-column of the same phase, from Interchim, (Montluçon, France). The spectrophotometer was set at 231 nm from zero to 8.0 min and then at 262 nm from 8.01 min until the end of run (Figure 2). A column thermostat T-6300 (Merck) was used for holding the temperature column at 30°C during the analysis.

The gradient used for solvent A (phosphate buffer) and solvent B (acetonitrile) was as follows: 0–7.5 min, A 100%; 7.5–15.0 min, B linearly from 0 to 14%; 15.1–20.0 min, B 90%; 20.1 min, A 100%. Post-time A was 100% for 15 min. The flow rate was 1.5 mL/min.

Validation

For this method, a full validation was established, following International Committee on Harmonization recommendations (9).

Selectivity. To assure the method selectivity, blank samples of urine obtained from six different sources were tested for interference. Selectivity was ensured at lower limit of quantification (LLOQ). Automated comparison of spectra of ETU or IS with an archived spectra library was performed to obtain a qualitative identification by calculating a match factor (Agilent ChemStation®). The match factor represents the degree of similarity (0–1000) between the reference and the unknown peak spectra that fall within a certain retention time window. Values above 990 indicate that the spectra are similar.

Additionally, ratios of chromatographic signals of ETU and IS were automatically calculated to determine a peak purity check to discover potential non-baseline separated peaks (Agilent ChemStation).

Linearity of the standard curve. The calibration curve consisted of a blank, a zero, and seven non-zero urine samples (100, 50, 20, 10, 5, 2, and 1 µg/L). Each calibration standard was tested on six replicates. The linearity was evaluated by the correlation coefficient of the peak-area ratios (ETU/IS), the slope and the intercepts of a linear regression. The equation of the calibration curve was $y = bx + a$ where the independent variable (x) represents the concentration of ETU in the sample, the

dependent variable (y) represents the peak-area ratios, b is the slope, and a is the y-intercept.

LLOQ. The LLOQ is the lowest standard on the calibration curve that could be determined with an accuracy of 80–120% and a precision better than 20%.

Accuracy. Accuracy is determined by replicate analysis of urine samples containing known amounts of ETU. Quality controls (QCs) at 3 concentration levels (5, 30, and 80 µg/L) were analyzed 15 times. The QCs should be within a range of ± 15% of the theoretical value to obtain acceptable accuracy.

Precision. Precision of the method was tested for within-run and between-run reproducibility. It was expressed as the coefficient of variation [CV % = standard deviation (SD)/mean × 100] of replicate measurements. This should not exceed 15% at all concentrations to obtain acceptable precision. Within-run precision was evaluated using six determinations for the seven concentration levels of the standard curve (1, 2, 5, 10, 20, 50. and 100 µg/L). Between-run precision was evaluated using the QCs injected once every run for 15 runs.

Stability. A partial stability study of ETU in urine was performed on the three QC concentration levels and six subject samples in different storage conditions: freeze (–18°C) and thaw was performed for three cycles. Subject samples and QCs were thawed for 12 h and refrozen for another 12 h for each cycle. Stability was also tested on 9 QCs during 20 h at 37°C after only one thawing; this partial stability study of ETU reflected the situation likely to be encountered during sample handling before arrival at the laboratory.

Recovery. The extraction recovery for ETU and IS was determined four times at three different concentrations 5, 30, and 80 µg/L of ETU and 12 times at the concentration used during the assay for IS. The peak areas obtained after extraction for both of them were compared with those resulting from the simple injection of working solutions at the same concentrations.

Results

Chromatography and detection

Under the chromatographic conditions described, ETU and 4-pyridinecarboxylic acid hydrazide (IS) had retention times of 4.5 ± 0.5 min and 11.7 ± 0.5 min, respectively. The quantitation of signals was based on the peak area for both components. Each peak spectrum was matched with the reference spectrums and the match factor was always above 990 for the compounds of interests. Figure 3 shows a representative chromatogram for a urine standard at 100 µg/L.

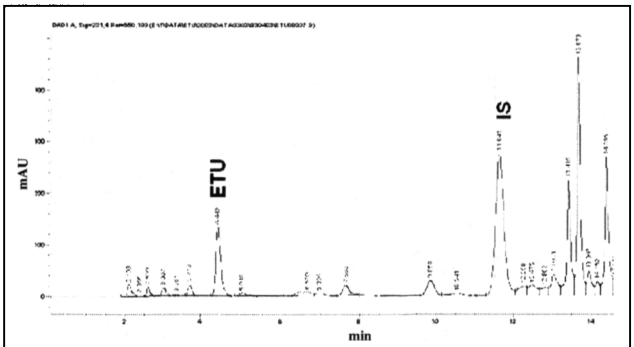

Figure 3. Chromatogram of a urine spiked at 100 µg/L. Retention times of ETU and IS were 4.4 and 11.6 min, respectively.

Selectivity

No interfering peak was noticed in the blank samples at the same retention time for ETU and IS. The presence of these was confirmed in every sample by their UV spectra, as mentioned, and the purity of every signal was tested. Another EBCDs metabolite, EU, susceptible to interfering with ETU because of their similar chemical structures and metabolic pathways (Figure 1), was tested in the same conditions. EU was highly retained on the column, and no interference was noticed (100% acetonitrile mobile phase was used to elute EU). Spectral library searches and peak-purity checks were also performed along with the chromatographic analysis of the unknown samples before quantitation.

Linearity

The ETU/IS peak-area ratio varied linearly with the corresponding concentrations of ETU (x) over the 1–100 µg/L range. Mean equation was $y = 0.0033\,x - 0.0017$. The correlation coefficient of standard curve by least squares linear regression analysis was > 0.999.

Table I. Accuracy, Within-Run, and Between-Run Precision of HPLC Determination of ETU Concentrations using Quality Controls and Calibration Curve Standards

	Accuracy		
	Theoretical values (µg/L) range ± 15%	*n*	Mean (µg/L) ± SD
QC 1	4.25–5.75	15	4.72 ± 0.65
QC 2	25.5–34.5	15	27.95 ± 3.1
QC 3	78–92	15	75.49 ± 9.13

	Within-Run Precision			
	Theoretical values (µg/L)	*n*	Mean ratio ETU/IS ± SD	CV %
Calibration curve standards	1	6	0.0110 ± 0.0015	13.2
	2	6	0.0187 ± 0.002	10.6
	5	6	0.0383 ± 0.0043	11.1
	10	6	0.0645 ± 0.0092	13.8
	20	6	0.119 ± 0.015	12.6
	50	6	0.322 ± 0.037	11.4
	100	6	0.649 ± 0.031	4.7

	Between-Run Precision			
	Theoretical values (µg/L)	*n*	Mean (µg/L) ± SD	CV %
QC1	5	15	4.72 ± 0.65	13.9
QC2	30	15	27.95 ± 3.1	11.2
QC 3	80	15	75.49 ± 9.13	12.1

Accuracy

The method yields acceptable results for accuracy for the QC samples at three levels (5, 30, and 80 µg/L). Accuracy was within 15% for all concentrations (Table I).

Precision

The CV evaluating within-run precision did not exceed 15% for all concentrations. Similarly, the CV evaluating between-run precision did not exceed 15% for any of the three QC levels. (Table I).

Limit of quantitation

LLOQ of ETU, based on acceptable accuracy and precision, was at 1 µg/L with a CV = 13.2% for the mean assay results (n = 6). The analyte peak was identifiable, discrete, and reproducible, and the UV spectrum and its purity were verified for all replicates. According to FDA guidance for industry (10), it was not necessary to determine the limit of detection in a bioanalytical method.

Recovery

ETU recovery varied from 50.6 to 74%, and internal standard recovery varied from 42.5 to 50.2% (Table II).

Table II. Mean Recovery for Each Concentration Level Calculated using the Quality Controls

	Concentrations	n	Recovery
ETU	5 µg/L	4	66.2–74%
	30 µg/L	4	52.1–57.7%
	80 µg/L	4	50.6–60.5%
IS	500 µg/L	12	42.5–50.2%

Table III. Study of ETU Stability on Quality Controls and Subject Samples

		Theoretical Values (µg/L)	n	Concentrations ± SD (µg/L)	Variation %
Three cycles of freeze and thaw	QC 1	5	5	2.72 ± 0.43	–36.9 to –7.4
	QC 2	30	5	27.83 ± 2.19	–4.7 to +14.1
	QC 3	80	5	76.35 ± 3.25	–1.5 to +8.6
		Initial Concentrations (µg/L)	n	**Concentrations after Stability Test (µg/L)**	**Variation %**
	subject 1	13.19	1	14.94	+13.3
	subject 2	10.89	1	9.733	–10.6
	subject 3	10.21	1	10	–2.0
	subject 4	51.36	1	57.67	+12.3
	subject 5	40.79	1	41.45	+1.6
	subject 6	26.49	1	24.47	–7.6
		Theoretical Values (µg/L)	n	**Concentrations after Stability Test (µg/L)**	**Variation %**
After 20 h at 37°C	QC 1	5	3	4.11 ± 0.218	+6.2 to +16.4
	QC 2	30	3	27.83 ± 1.95	–2.8 to +11.1
	QC 3	80	3	75.15 ± 8.39	–5.3 to +13.8

Stability

When tested on subject samples, stability variations were included between –10.6% and +13.3% after three cycles of freeze and thaw, which is less than the variability of the analytical method. But, ETU QCs at low concentration (5 µg/L) seems to degrade (–36.9 to –7.4% variation) after three freeze-and-thaw cycles. (Table III). Otherwise, after 20 h at 37°C (a sample left about one day at a summer temperature), ETU shows no significant degradation (Table III).

Discussion

Mobile phase was 100% aqueous from zero to 7.5 min to elute ETU and then acetonitrile was introduced gradually to elute internal standard and thus to reduce run time. After 15.1 min, acetonitrile was used at 90% until the end of the run (20 min). This allows to elute all non-polar compounds remaining on the column. Post-run for 15 min using 100% aqueous phase was necessary to equilibrate the column before the next injection. A new generation of ultrapure silica column (C18 Uptisphere NEC-5-20) was used. This stationary phase supports 100% of aqueous phase and keeps polar and non-polar molecules away from the solvent front. ETU seems to be more polar than EU, according to the high retention on the column of the latter. The presence of a sulfur atom in the ETU molecule could explain its polar nature. The column seems to support 100% of aqueous phase, according to the reproducibility of retention times for ETU and IS. On the other hand, it could affect the column lifetime.

After 8.01 min, the spectrophotometer was set at 262 nm, the λ max of the IS, allowing the elimination of interference compounds at the same retention time of the last. Creatinine was analyzed for each subject sample of urine and the final ETU concentration was corrected using urinary creatinine concentration (i.e. [ETU] µg/g creatinine).

Of the samples, 49% were free of ETU, 25% were between 1 and ≤ 5 µg/L, 19% were between > 5 and ≤ 15 µg/L, 2% were between >15 and ≤ 30 µg/L, and 5% were between > 30 and 100 µg/L (Figure 4). These concentration ranges were arbitrarily chosen and have no clinical signification. The large proportion of ETU-free samples could be explained by the presence of a control sample for each subject that was collected before application of EBCDs.

Figure 5 illustrates the evolution of ETU amount (corrected by urinary creatinine) for a farmer occupationally exposed by inhalation of a Maneb solution on days 1, 15, 29, 67, and 75. Urinary excretion of ETU indicates a significant absorption of EDBC after each exposition. On the other hand, the first part of the Figure 5 shows a plateau of excretion of ETU between 7 µg/g of creatinine and 10 µg/g of creatinine over seven days. This confirms the

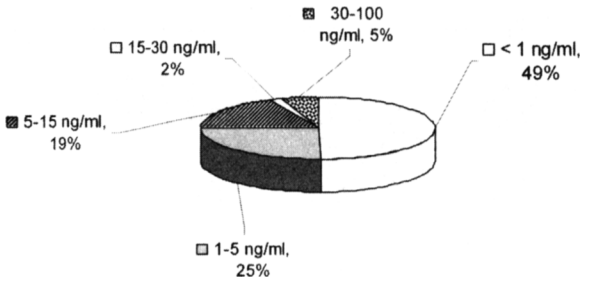

Figure 4. Proportions of each concentration range following urinary ETU analysis in 272 patients.

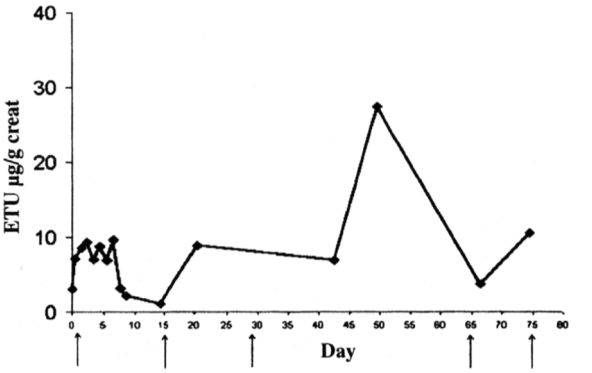

Figure 5. ETU amounts (µg/g creatinine) evolution during 75 days for a farmer exposed to Maneb. Arrows indicate exposure to Maneb.

deep tissue penetration of EDBCs (3), inducing a prolonged urinary excretion of ETU, a water soluble metabolite.

Conclusions

A selective, accurate, precise, linear, and reproducible method for determination of ETU in urine by HPLC was carried out. This method is easy to perform and appears to be reliable. The extraction procedure seems to be the critical step because it is time consuming. Nevertheless, to reach a low limit of ETU quantitation of 1 µg/L with a spectrophotometric detection, large urine sample volumes and adapted equipment for solid-phase extraction need to be used. The analytical method described in this paper would allow further studies on ETU pharmacokinetics.

Acknowledgments

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