Reference values for ethylenethiourea in urine in Northern Italy: Results of a pilot study

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Abstract

This study was carried out to define reference values for urinary ethylenethiourea (ETU) in the Northern Italy population and to identify the sources of exposure. Ninety-five healthy subjects were selected. A spot urine sample was collected in the morning, and analyzed using GC/MS in the EI/SIM mode. Thirty-nine subjects showed urinary ETU concentrations lower than the limit of detection (LOD, 0.4 μg/g creatinine), and the remainders ETU concentrations ranging from 0.5 to 11.6 μg/g creatinine. No correlation was shown between smoke or alcohol intake and urinary ETU concentrations. Based on data on ethylene-bis-dithiocarbamate (EBDC) concentrations in food, we estimated a total EBDCs intake of 31.7–50.1 μg/day. These values are largely below the ADIs, but explain the presence of small amounts of ETU in the urine samples we have analyzed. Finally, it was estimated that the mean ETU in urine in the Italian general population is 0.6–0.8 μg/g creatinine, with a 95th percentile of 4.5–5.0 μg/g creatinine. These values can be used as reference, to compare the results of biological monitoring activities carried out on EBDCs occupationally and environmentally exposed populations.

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1. Introduction

Due to their low acute toxicity and short environmental persistence, ethylene-bis-dithiocarbamate (EBDC) fungicides are widely used worldwide (Maroni et al., 2000), and a significant number of workers are exposed to these compounds in the industrial production and formulation as well as in agricultural application. Ethylenethiourea (ETU) is a major product of the metabolic and environmental degradation of these compounds, and it is also present as impurity in several EBDCs formulations (Blazquez, 1973; Bontoyan et al., 1972). Therefore, ETU can be measured in human urine samples collected after EBDC occupational exposure.
Since ETU is a specific EBDCs metabolite, its urine concentration has been suggested as an indicator for biological monitoring of occupational and environmental exposure to ETU and/or EBDCs (Aprea et al., 1997; Colosio et al., 2002). This context, concentrations ranging from <1 to some hundreds micrograms of ETU/g of urine were detected (Fustinoni et al., 2005).

The main limit to the routine use of ETU in biological monitoring activities is represented by the lack of reference values. This study has been carried out to define reference values for ETU concentration in urine, and to investigate the influence of smoking habit and alcohol consumption in the Northern Italy adult population.

2. Material and methods

2.1. Subjects

The subjects under study were selected from a larger group involved in a project carried out in the period 2000–2003, addressed to monitoring pesticide exposure in agricultural workers and in the general population. Ninety-five healthy subjects (29 females and 66 males), resident in the north of Italy and non-occupationally exposed to EBDCs or ETU, were selected. For each of these subjects, personal data on smoking habits, alcohol consumption and drug intake were collected by questionnaire.

2.2. Sampling and analytical methods

A spot urine sample was collected in the morning from all subjects under study (second urine of the day), in 15 ml polyethylene tubes shielded from light with an aluminum foil. Samples were chilled and delivered to the laboratory within 24 h from collection. In the laboratory, they were frozen and kept at –20 °C until analysis.

ETU was extracted on a diatomaceous earth column using dichloromethane and derivatized with the mixture of N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide and tert-butyl(dimethyl)silyl chloride. The derivative was analyzed using GC/MS in the EI/SIM mode. The whole procedure was carried out in the presence of ethylenethiourea-d₆, as internal standard. The limit of detection (LOD) of the assay was 0.5 µg/g creatinine (Fustinoni et al., 2005).

2.3. Statistical analysis

Statistical analysis was carried out with the statistical package SPSS for Windows 10.0.

Since 40% of the values were below the LOD, data distribution was truncated at its left (“left censored”). Therefore, the distribution of the data was checked for the 56 points above LOD with histogram of the raw points and the Quantile–Quantile (Q–Q) plot. Finally a log-normal distribution was assumed. Since log, data follow a normal distribution, geometric mean (GM) and geometric standard deviation (GSD) were calculated. GM and GSD are in the original measurement units and characterize the lognormal distribution.

Values below the LOD were replaced by single value substitution methods (typically LOD and 1/2 LOD) and by multiple imputations with the log–probability plot method, in which the regression of log of concentration versus normal score is used to extrapolate “fill-in” values >LOD (Helsel and Hirsch, 1992). These “fill-in values” are retransformed back to original units, and combined with data ≥ LOD to compute estimates of summary statistics. Another estimation method used for values below LOD is the maximum likelihood estimation (MLE). In general, ML estimates are more precise than probability plotting, and both methods are unbiased, when observations fit the assumed distribution exactly and when the sample size is large. The likelihood function assumes that each detected data (log-transformed) follows a normal distribution, while for data below LOD, the cumulative probability of the detection limit is used in lieu of the likelihood. We entered the log-likelihood function in the spreadsheet Excel, and its maximum was computed with the “solver” utility. GM, GSD, Median, the 90th and 95th quantiles were used for distribution-free prediction intervals.

The 90th and 95th quantiles were used to define the 90th and 95th non-parametric one-sided prediction intervals (PI) of our distribution. Prediction intervals were used to determine whether a new observation is likely to come from a different distribution than previously collected data. The non-parametric interval has the advantage to be distribution-free.

Since the log-transformed data can be assumed normally distributed, a multiple linear regression model was applied to see the effect of age (years), gender (0, male; 1, female), residence (0, Pavia; 1, Trento), smoking habits (0, non-smoker; 1, current smoker), alcohol consumption (0, no; 1, yes). Three regression runs were made, one for each of the following substitution models, namely: LOD, 1/2 LOD imputation and probability plot method. In the literature, the recommended regression method, when the dependent variable is left-censored, is the Tobit model. It is a regression method based on maximum likelihood estimates and on the conditional likelihood distribution of ETU concentrations given certain values of independent variables. Unfortunately, SPSS does not run this method and there are not many validated software performing this regression, and we figure out results would not change much.

3. Results

In total, 95 healthy subjects were involved in the study: 66 males and 29 females, aged 24–66 years (mean 41.5 years; standard deviation 8.9 years). Among the
subjects, 68 were not smokers, and 72 reported wine or beer consumption during meals.

Among the subjects investigated, 39 (41%) showed ETU urine concentrations less than LOD, while the remainders showed measurable ETU concentrations, ranging from 0.5 to 11.6 μg/g creatinine.

In Fig. 1 is depicted the frequency distribution of the values above the LOD; it is evident a non-normal distribution, with more than half of the observations included in the range between 0.5 and 2.5 μg/g creatinine. Only two observations exceeded the value of 7.5 μg/g creatinine. Since about 40% of the observations were under the LOD, the distribution was typically truncated on the left side. The transformation in natural loge values brought about the normalization of the distribution, as shown in Fig. 2 (Quantile–Quantile plot).

Therefore, we assume the data to follow a log-normal distribution. Table 1 shows values for the GM, GSD, quantiles obtained after the application of different substitution methods of non-detects and MLE.

Results from different methods are quite similar. As expected, the LOD substitution overestimates GM. We might say the 95% CI of GM is between 0.5 and 0.8. The Q90 is about 3 μg/g creatinine, and the Q95 is about 5 μg/g creatinine. In other words, concentrations greater than 3 μg/g creatinine will occur approximately 10% of the times, while values above 5 μg/g creatinine can be considered coming from a different distribution at a 5% level.

Since literature data suggests that ETU levels in urine can be affected by tobacco smoke and wine and beer intake, we have investigated, with the multiple linear regression model, the relationship between these variables. Regression suggested that the only variable affecting ETU concentrations in urine was age, while neither tobacco smoke nor alcohol intake exerted any appreciable effect on urinary ETU concentration. The diagnostic showed that the effect observed by regression model was attributable to an influence exerted by the two highest measured values (respectively, 11.6 and 8.1 μg/g creatinine). A second run of the regression carried out without the two outliers did not show any significant effect of age on ETU urine concentration.

### 4. Discussion and conclusions

The most interesting finding of our study is that about 60% of the subjects under study showed detectable amounts of ETU in urine, with values ranging from 0.5 μg/g creatinine to 11.6 μg/g creatinine. Since it is

<table>
<thead>
<tr>
<th>Method</th>
<th>GM</th>
<th>GSD</th>
<th>GM₉₀</th>
<th>GM₉₅</th>
<th>Q₅₀</th>
<th>Q₉₀</th>
<th>Q₉₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD substitution</td>
<td>0.82</td>
<td>2.39</td>
<td>0.69</td>
<td>0.98</td>
<td>0.50</td>
<td>2.92</td>
<td>4.53</td>
</tr>
<tr>
<td>1/2 LOD substitution</td>
<td>0.62</td>
<td>3.11</td>
<td>0.49</td>
<td>0.78</td>
<td>0.50</td>
<td>2.92</td>
<td>4.53</td>
</tr>
<tr>
<td>Robust log–probability extrapolation</td>
<td>0.68</td>
<td>2.99</td>
<td>0.53</td>
<td>0.83</td>
<td>0.50</td>
<td>2.92</td>
<td>4.53</td>
</tr>
<tr>
<td>Maximum likelihood estimates (MLE)</td>
<td>0.56</td>
<td>3.74</td>
<td>0.43</td>
<td>0.73</td>
<td>0.56</td>
<td>3.01</td>
<td>4.96</td>
</tr>
</tbody>
</table>
reasonable to assume that the presence of this metabolite is the consequence of low-level environmental exposures to EBDCs and ETU, some authors (Aprea et al., 1996, 1997) suggested that the source of exposure for the general population is represented by consumption of contaminated food, mainly vegetables, and drinks, such as wine and spirits. Under experimental conditions, EBDC residues show, after application on tomatoes, a decrease of 50–70% in 20 days, while a sharp reduction in ETU contents occur during the first 24 h after treatment, followed by a slow decline in the following 5 days. A reduction of about 80% is observed 20 days after the fungicide application; 70% of the applied ETU is removed by washing and further losses of ETU occur by boiling the vegetables (6%), and during storage of the tomato paste (3%) (Knio et al., 2000). It can be concluded, based on these data, that small of EBDCs and ETU may be present in treated food.

As for wine and other alcohol drinks, some authors did not observe any ETU presence in wine, but in grapes (Chovancova et al., 1985), while other authors observed, in several samples of commercial beers and wines, a proportion of samples containing ETU at concentrations exceeding the limit of detection of the analytical method (0.01 ppm) of 22.6% and 7.3%, respectively, but the number of samples containing >0.1 ppm of ETU was practically negligible (Casanova and Guichon, 1988). On the other hand, studies are available showing ETU concentrations in wine up to 8.8 μg/l (Aprea et al., 1997). Due to this high variability, it is very difficult to evaluate a “mean” contribution of wine and beer intake to ETU human exposure. These findings are consistent with those of our study, which did not show any significant correlation between alcohol intake and ETU concentration in urine. As for the amount of ETU intake with food, data collected in the frame of the food quality program carried out in several European countries are not adequate to clarify the problem, since only dithiocarbamates are usually measured, and not ETU. Since ETU concentrations in food are usually measured in the USA (WHO, 2003), it is possible to estimate, based on data collected by the Food and Drug Administration (FDA, 2003), a mean ETU daily intake in the USA in the order of 0.2–0.4 μg/(kg bw day). These estimates bring about the possibility of presence of small amounts of ETU in urine samples collected from the USA general population. Of course, due to differences either in diet or in pesticide application patterns and modalities, USA estimates can be hardly extrapolated to European countries. On the other hand, data available on Italy are consistent with these estimates. Guidano and Fabbriini (2000) calculated, based on the results obtained from June 1994 to June 1997 in a non-official monitoring carried out by the National Observatory on Pesticide Residues (NOPR), a medium daily intake of dithiocarbamates from fruit and vegetables, extrapolated from the 1997 estimates of 0.4531 μg/(kg bw day), that is, for a 70 kg adult, 31.7 μg/day. Similar results were obtained by other Italian authors, who, based on the official data provided by the Italian Monitoring Program, coordinated by the Italian Ministry of Health, estimated a 90th percentile intake of 0.716 μg/(kg bw day) that is, for a 70 kg adult, of 50.1 μg dithiocarbamate/day (Camoni et al., 2001). If we take into account that the Italian estimates include the whole group of dithiocarbamate, and not only EBDC, and that only part of the EBDC absorbed via diet are transformed in ETU, we can conclude that Italian data largely explain the presence of small amounts of ETU in the urine of the general population.

These intake estimates are largely below the ADI for ETU established by the Joint Meeting on Pesticide Residues on Food (JMPR) on 1993, that is, 0.004 mg/kg bw (IPCS, 2001); therefore it seems very unlikely that they may pose any health risk to humans. In any case, we think that our results on ETU concentration in urine of the general population might suggest the need of addressing food monitoring programmes not only at dithiocarbamates but also at ETU.

Since >60% of our samples showed measurable ETU urine concentrations, we have statistically elaborated these data in order to obtain reference values adequate for the area under study (northern Italy). We have applied different substitution methods to include in the elaboration the values under the limit of detection. Different substitution methods of the values under the LOD provides estimates of ETU mean concentrations in the general population quite similar, ranging from a minimum value of 0.6 μg/g creatinine to a maximum value of 0.8 μg/g creatinine, while the 95th percentile varies among 4.5 and 5.0 μg/g creatinine. Values of 3 and 5 μg/g creatinine have a probability of belonging to this group of values, of, respectively, 10% and 5%.

These values can be used as reference to compare the results of biological monitoring activities carried out on EBDCs occupationally and environmentally exposed populations.

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References


