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Highlights

- Hair terbuthylazine (TBA) was investigated as a potential biomarker of exposure
- TBA was measured in both rat and human hair
- After the application season TBA in hair follows the trend: agriculture workers>rural residents> urban residents
- These evidences encourage further investigations on the use of hair TBA as a biomarker of exposure

Terbuthylazine in hair as a biomarker of exposure

Rosa Mercadante[a], Elisa Polledri[a], Erminio Giavini[b], Elena Menegola[b], Pier Alberto Bertazzi[a], Silvia Fustinoni[a§]

[a]Department of Occupational and Environmental Medicine, University of Milan and Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Via S. Barnaba, 8 – 20122 Milan, Italy

[b]Department of Biology, University of Milan, Via Celoria, 26 – 20133 Milan, Italy

[§]Authors' correspondence address:

Via S. Barnaba, 8 – 20122 Milan, Italy

phone: +39 02 503 20116

fax: + 39 02 503 20111

e-mail: silvia.fustinoni@unimi.it

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Abstract

Terbuthylazine (TBA) is an herbicide widely used in corn cultivation. Herein we evaluate the measurement of hair TBA as biomarkers of exposure. Five Sprague Dawley rats were gavaged with TBA for three days, and then the back hair was shaved and analyzed for TBA. In addition, head hair samples from 10 corn farmers, 9 rural residents, and 6 urban residents were collected at the end of the application season. Hair TBA was detected by liquid chromatography triple quadrupole mass spectrometry after solvent extraction. TBA was quantifiable in all rat samples with a mean concentration of 0.92 (± 0.26) ng/mg, which corresponds to a 0.12% incorporation rate. TBA was quantifiable in all farmer samples (median: 0.67 ng/mg), in 75% of rural resident samples (0.01 ng/mg) and in none of the urban resident samples (< 0.01 ng/mg), with a statistical difference among groups ($P < 0.01$). Our results suggest that TBA is incorporated in hair and prompt further investigation on the use of hair TBA as a potential biomarker of cumulative exposure.

1. Introduction

Terbuthylazine (TBA; Figure 1) belongs to the cloro-triazine family of powerful herbicides that act as inhibitors of plant photosynthesis and is the active ingredient of many different formulations on the market. TBA is applied to corn, sorghum, potatoes, peas, sugar cane, vines, fruit trees, citrus, coffee, palm oil, cocoa, olives, rubber, and other trees in tree nurseries and new plantings. After the application, TBA is absorbed by roots and leaves and is distributed throughout the plant. It can be used in both pre-and post-emergency treatments and is particularly suitable for weed control of annual dicots (WHO, 1998). In Italy, TBA is largely applied to corn, which is cultivated over an area of about 1.1 millions of hectares, largely in the North, including 365,000 hectares in Lombardy (ISTAT, 2009). The sowing period starts in early February and lasts until early May (Rapparini, 2009).

Human exposure studies have shown that TBA is mildly to moderately irritating to the eyes, and slightly irritating to the skin, but it is not a skin sensitizer (Health and Safety Database, 2010). The US Environmental Protection Agency classifies TBA in Group D as "not classifiable as to human carcinogenicity" (EPA, 2006). Ecotoxicology studies shown that TBA has strong soil sorption (WHO 1998), so it may be transported to both ground and surface waters and can reach the atmosphere (Otto et al., 2007, Irace-Guigand et al., 2003). TBA is slowly degraded in the soil so, after repeated treatments, it is enriched in the top soil and can exert direct toxic effects on different soil animals (Salminem et al., 1996).

Human exposure to TBA may occur in agriculture settings during mixing and/or loading of herbicide formulation, crop application, re-entry activities, and cleaning and maintenance of the equipment (Arbuckle et al, 2002). Additionally, individuals in the general population residing in a rural area may be exposed (EPA, 2006). Exposure assessment in agriculture includes several critical issues, including the use of plant protective products follows a calendar based on atmospheric conditions and crop needs, rather than predefined time tables; there are different routes of exposure, different application techniques and equipment, and variable use of personal protective devices.

Biomonitoring is a valid tool to perform exposure assessment as it integrates all sources and routes; however, very often conventional matrices such as blood and urine only allow the

investigation of recent exposures (Maroni et al., 2000). To overcome this limitation and assess cumulative exposure to toxicants, the use of head hair has been proposed as a matrix for biological monitoring. The use of hair began in the 1960s to assess exposure to heavy metals; over the years, improved analytical methods, both in terms of sensitivity and specificity, have allowed the detection of organic substances. Currently, hair analysis is applied to the routine measurements of drugs of abuse in forensic sciences, and has other research applications in clinical medicine, occupational and environmental toxicology (Villain et al., 2004; Tsatsakis et al., 2008).

Mechanisms of incorporation of chemicals in hair are not completely understood, but it is believed that adsorbed chemicals are transported by blood to capillaries located in the proximity of the hair follicle where they enter hair growing cells by passive diffusion. Other mechanisms to explain the presence of chemicals in hair include incorporation from deep skin compartment during hair shaft formation, deposition by diffusion from sweat or sebum secretions into the completed hair shaft, and environmental contamination after the hair has emerged from skin (Prasgt and Balikova, 2006). In deep skin incorporation and, to a lesser extent, in deposition by diffusion from sweat or sebum secretions, chemicals are into hair shaft, but not into hair cells. In both cases they were associated to cumulative exposure and are believed to be stronger linked to hair in comparison with chemicals deposited by environmental contamination (Prasgt and Balikova, 2006; Boumba et al., 2006).

The aim of the present work was to evaluate the use of TBA in head hair as a potential biomarker for assessing cumulative TBA exposure. In this work, we verified the presence of TBA in the keratin matrix of rats after parenteral administration of the herbicide. We also evaluated the level of TBA in the hair of individuals exposed in their work and/or living environments.

2. Materials and methods

2.1. Animals

Five Sprague Dawley male rats (Crl:CD Charles River Laboratories, Calco, Italy), 7-8 weeks old, were used for the experiment. Before starting treatment, a pre-treatment hair sample (T0) from the dorsal region (about 10 cm^2 of area) between shoulder blades was obtained by shaving. This hair sample served as negative control. The animals were kept in single cages in 12 h

dark/light cycles and were fed with food pellets (Italiana Mangimi) *ad libitum*. Animals received TBA dissolved in ethanol (5 mg/kg body weight) by gavage once a day for three days, for a total administered TBA of 2.25 mg/rat. The administered dose was chosen as a reasonable compromise between low toxicity [no observable effects level (NOEL): 2.1 mg/kg body weight per day; WHO, 1998], and sufficient levels to observe a significant uptake in rat hair. On the fourth day, when the hair regrowth was apparently almost complete, but the shaved area was still identifiable, the rats were sacrificed and post-treatment hair samples were collected both from the previously shaved area (T1) and from a proximal unshaved dorsal area (T2). For each hair sample, the weight and shaved area were registered.

2.2. Study population

The field survey was conducted in 2009 and involved 25 subjects belonging to three groups with different potential exposures: 10 corn farmers (agricultural workers; AW) living in a village in Cremona province, Lombardy, Italy involved in applying TBA in the pre-emergency treatment of crops; 9 rural residents (RR) from the same village, without family ties with the AW; and 6 subjects living and working in the urban area of Milan (urban residents; UR), the Lombardy capital, located 40 km from the rural area. From March to May, the farmers treat the sown fields. Hair samples were collected at the end of the treatment season (June) by cutting a lock of hair as close as possible to the root in the occipital region of the head. A typical sample weighed 100 mg, and was 5 cm long (ranging from 2 to10 cm). Each sample was attached with paper masking tape on a paper sheet with a mark to indicate the direction of the root and was stored at room temperature in the dark.

A questionnaire administrated by an industrial hygienist was used to collect information about personal characteristics, including hair color, smoking habit, proximity of residence to corn fields, and consumption of green garden vegetables produced in a personal garden. Farmers provided additional information about the use of plant protection products, i.e. name and trademark of the formulation, the concentration of TBA in the formulation, mixing and loading operations, treated area, amount of formulation applied per area, application techniques and equipment, machinery maintenance activities, and use of personal protective devices. All subjects were informed about the aim of the study and gave their written informed consent.

2.3. Detection of TBA in hair

TBA in hair was detected using a liquid chromatography-triple quadrupole mass detector in the presence of terbuthylazine-D5 (d_5-TBA, Dr. Ehrenstorfer, LabService, Anzola Emila, Italy) as isotopically labeled standard. Approximately 50 mg human hair, obtained by selecting 3 cm of hair starting from the root, or 14 - 47 mg rat hair, were rinsed with 2.5 mL of deionized H_2O, vortexed for a few seconds in a glass vial, and then the water was removed and transferred to a separate vial. Methanol (MeOH, 2.5 mL) was added to the washed hair sample, which was then extracted in an ultrasound bath at 59 KHz for 5 h at 50°C. Aliquots (0.5 mL) of both the rinsing solutions (RS) and the extracts (E) were added to 25 µL isotopically labeled standard (IS) solution and submitted to analysis. Analysis was performed on a Surveyor high performance liquid chromatography system (Thermo Scientific, Rodano, Italy) equipped with a Betasil C18 column (150 mm length, 2.1 mm internal diameter and 5 µm particle size; Thermo Scientific) kept at room temperature, using an isocratic mixture of aqueous formic acid (0.5%) and MeOH (30:70) at 0.3 ml/min as eluent. The liquid chromatography instrument was interfaced with a triple quadrupole mass spectrometer equipped with a hot-electro spray ionization source (TSQ Quantum Access with H-ESI; Thermo Scientific). TBA and the IS were detected in the positive ion mode and quantification was based on single reaction monitoring (SRM) following the transition m/z 230 → 174 for TBA and m/z 235 → 179 for d_5-TBA,. The TBA retention times was 3.91 min (coefficient of variation <0.3%). The method had a linearity up to 25 ng/mg hair, precision of less than 10%, evaluated as the coefficient of variation, with accuracy between 91 and 107% and a limit of quantification (LOQ) of 0.01 ng/mg hair.

2.4. Statistical analysis

Statistical analysis was performed using the SPSS 17.0 package for Windows (SPSS Inc., Chicago, IL, USA). Information from questionnaires were analyzed using descriptive statistics (for continues variables) or frequency analysis (for categorized variables). Differences in frequency distributions were evaluated using Fisher $\chi 2$ test. A value corresponding to one-half of the quantification limit (LOQ) was assigned to measurements of TBA below analytical quantification (Hornung and Reed, 1990). Non-parametric statistic was used to compare RR and

AW (Kruskal-Wallis test) and to correlate variables (Spearman's correlations). A P value of 0.05 was considered statistically significant.

3. Results

3.1. TBA absorption in rats

No TBA was found in pre-treatment samples, while TBA was found in both RS and E, in both the T1 and T2 samples (Table 1). Given the similarity of the T1 and T2 values, these data were pooled to estimate a mean TBA content of 0.92 (±0.27) ng/mg in post-treatment hair. On the basis of hair weight and the corresponding shaved area, a hair density of 10 mg/cm^2 was estimated. Calculating a total surface area of about 200 cm^2, according to Diack's formula: (7.47 × Body weight)$^{0.66}$ (Diack, 1930), a total of about 2000 mg hair/rat was estimated. Considering the average TBA level in the samples, it was calculated that, in the hair from the entire rat, there would be about 1843 (± 533) ng TBA, which corresponds to an incorporation rate of about 0.12% (± 0.036%).

3.2. Study population characteristics

The main characteristics of the study population are reported in Table 2. The groups differed in several characteristics: gender was not uniformly distribute among groups, with 100% AW males in and only 67% in both RR and UR; AW were older than UR, their body weight was higher and the percentage of participants with dark hair was lower than in the other groups. Smoking habits was similar in AW and RR, but cigarette consumption was much higher in RR than in AW; none of UR were smokers. Both AW and RR residence was very close to corn fields. AW and RR grew and consumed green vegetables from their own gardens.

All farmers applied TBA once to corn crops during the investigated season. A single subject also performed an additional TBA application. The amount of TBA applied ranged from 7 to 163 kg, with a mean value of 41 kg. The formulation was mixed and loaded mechanically for all but one AW, who performed it manually. The application technique and equipment was the same for all AW; no closed-cabin tractor was used in any application. No AW reported having performed machinery maintenance. During field activities, only 40% of AW used adequate personal protective devices (overalls, gloves and a dust mask).

3.3 TBA in human hair

The results of the hair analysis are reported in Table 3 as percentage of quantifiable samples, mean, standard deviation, median and minimum and maximum of the distribution. In contrast to the animal samples, no TBA was quantifiable in the RS from human samples. In extracts, TBA was quantifiable in all AW, in 67% of RR and in none of the UR, with a statistical difference in the distributions of positive samples among groups ($P < 0.01$). The TBA concentration in hair was highest in AW and lowest in UR, with median levels of 0.61 (AW), 0.01 (RR), and <0.01 (UR) ng/mg hair (AW higher than UR, p<0.01). The results are also reported graphically in Figure 2, where, for convenience, to UR samples a value of ½ LOQ was assigned. The AW subject that reported manual mixing and loading had the highest TBA level (4.64 ng/mg hair).

Among all subjects hair TBA was not correlated with age, body weight, or smoking habit, while a marginally significant positive correlation with the proximity of residence to corn fields was found ($P = 0.081$). In AW, a negative correlation between the use of personal protective devices and age ($P = 0.035$) was found, while no correlation between hair TBA level and amount of TBA applied during the season was observed. In RR, the influence of hair color on the concentration of TBA was studied. All five subjects with dark hair had TBA levels above the LOQ, while only 1 out of 4 subjects with white or blond hair had TBA above the LOQ; nevertheless, the frequency distribution was not statistically different between the groups.

4. Discussion

In this study, we investigated the use of head hair TBA as a potential biomarker of cumulative exposure to TBA.

Parenteral TBA administration to rats was perform to investigate the keratin incorporation of TBA in controlled conditions. We were able to find TBA in rat hair, with mean concentration of 0.92 ng/mg hair, which corresponds to a 0.12% incorporation rate. Although this result suggests the absorption of TBA in hair, some aspects of our findings are controversial. Major issues are: a large amount of TBA (about one third of the total) detected in rinsing solutions, and the presence of similar levels of TBA in both pre-shaved (T1) and not pre-shaved (T2) area (Table 1). The first finding indicates a weak binding to hair, suggesting that part of TBA was not incorporated in cells and/or in shaft, but rather was deposited on hair surface, possibly for contamination with oral fluids and/or urine. Similarly the presence of similar levels of TBA in both pre-shaved (T1)

and not pre-shaved (T2) area can only in part reflect the concomitant presence in the hair of different growth phases of individual hair shafts. While not much is known about rat hair growth, on the healthy human head, 80 - 90% of individual hair is in the active growth phase, or the anagen phase, about 2% is in the transitional phase, or the catagen phase, and 10 - 18% is in the resting phase, or the telogen phase, in which the growth has stopped completely (reviewed in Boumba et al, 2006). Actually, given that the largest part of T2 samples corresponds to hair that grew before the three day-treatment, the concentration should be significantly lower than in T1 samples that grew during the treatment. Also in this case external contamination could have played a significant role in explaining results. However, although it cannot be excluded, contact with oral fluids and/or urine seems to be implausible as animals were kept in individual cages and therefore could not lick each other; also self-licking hardly explains our findings as hair samples were taken from the back, between shoulder blades, a position very difficult to be licked; finally the contact of hair with urine would have increased the content of urine metabolites, such as desethyterbuthylazine (WHO, 1998), whose presence was evaluated and found to be undetectable (LOQ 0.01 ng/mg hair), rather that of unmetabolized TBA itself. Another possibility is that different mechanisms may have mutually concurred to the presence of TBA in hair: in addition to those already mentioned in the introduction (i.e. incorporation in cells by passive diffusion and in hair shaft by deposition from deep skin compartment and/or sweat or sebum secretions before hair has emerged from skin, Prasgt and Balikova, 2006), also migration of chemicals through hair in the nonkeratinous region due to capillary forces has been described (Skopp et al, 1996). Again, although we did not observed TBA in the rinsing solution of human hair, the co-presence of different incorporation mechanisms may also have affected humans, but due to the lag time between exposure and hair sampling, hair washes may have removed all the weakly bonded TBA.

The TBA level measured in rat hair was comparable with the 0.24-0.53 ng/mg hair diazinon measured in another study in which this plant protection product was administered orally to rats at doses of 6 and 3 mg/kg body weight per day for 1.5 months (Tutudaki and Tsatsakis, 2005). Given the similar daily dose, but the shorter duration of our experiment and therefore the lower amount of TBA administered, we argue that TBA has a higher affinity for the keratin matrix

compared to diazinon, and this may be explained by the higher affinity for lipids and limited TBA water solubility (log P octanol/water; 3.21; water solubility: 8.5 mg/l) compared to diazinon (log P octanol/water: 3.81; water solubility: 40 mg/l; ChemIDplus, 2011). On the other hand different procedures to rinse and extract rat hair were applied in the two experiments, and this may also explain the different findings.

Finally another peculiar observation in our rat experiment was the unexpectedly high hair growth rate. Before performing the experiment we planned to administer TBA for two weeks, but, after only three days, the pre-shaved area was almost completely regrown, so the samples were collected early. We argued that such high growth rate may be attributable to the young age of the treated animals (7 - 8 weeks), but further investigations to confirm this finding are needed.

In the study subjects, TBA was quantifiable in 100% of the farmers, 67% of the rural residents and in none of the urban residents, with median levels of 0.61, 0.01 and <0.01 ng/mg/hair, respectively. This trend was expected, and may be related to the cumulative exposure to TBA during the months before hair sampling. We analyzed a hair segment about 3 cm from the root, specifically to cover a temporal frame of about three months, corresponding to the period of TBA application in the fields, as hair grows at a rate of about 1 cm a month in human adults (reviewed in Boumba et al, 2006).

Farmers had variable hair TBA levels, with a minimum level of 0.07 ng/mg hair and a maximum value as high as 4.64 ng/mg hair. Any attempt to correlate these concentrations with the amount of TBA applied or with the treated area did not yield significant association, which may be related both to the small number of study subjects and also to the effective use of personal protective devices, care during handling operations, and other factors such as accidental exposures, for example, due to spilling. It is significant that, in our study, the subject with the highest level of TBA in hair was the one that applied a lower amount of pesticide, but also the only one that mixed and loaded the formulation manually. The appropriate use of personal protective device is difficult to assess, given that field operations were not followed by industrial hygienists. Our evaluation was based on worker reports, and was therefore influenced by personal factors such as risk perception. As a side result, we found that the use of personal protective device negatively correlated to the age of subjects, demonstrating the lax attitude of

older people to personal protective gear, probably as a result of familiarity with plant protection products, as well as less education and awareness on health and safety issues.

TBA is persistent in the environment, and reported to pollute the aquifers of North Italy area where corn crops are located (Otto et al, 2007). In addition to the farmers, most rural residents had quantifiable TBA levels, which may be explained by considering multisource exposure. Given the proximity of these subjects residences to the treated fields, inhalation exposure during the herbicide application is very likely (Health and Safety Database, 2011). On the other hand, all rural residents grow and consume vegetables from their own green garden, so the ingestion of contaminated food may represent a significant source of exposure. Often, these green gardens are watered using water from ditches; these ditches, surrounding the corn fields, may collect leaching water, and are themselves treated with herbicides. Finally also the ingestion of well water may represent a source of TBA exposure in these residents (Health and Safety Database, 2011). Samples from urban residents did not show traces of TBA. Any residual environmental pollution, including water and food contaminations, even if present, did not affect TBA content in hair at level above 0.01 ng/mg hair.

The concentration of TBA measured in rat hair was comparable to that found in agriculture workers. This was unexpected as we estimated a much higher dose in rats that received a total TBA dose of 2.25 mg/kg body weight, than in farmers, for whom dermal and inhalation exposure is hard to estimate, but based on previous studies on other plant protection products, may be estimated at tens of µg/kg body weight (Aprea et al, 2005). Possibly the reason for this evidence is related to the use of white rats for the experiment; dark hair accumulates some chemicals more efficiently than white or blond hair (reviewed in Prasgt and Balikova, 2006; Schramm, 2008) due to the higher capacity of melanocytes and pigmentation to incorporate chemicals into hair. Our results suggest that TBA may be among chemicals that show such behavior as rural residents with dark hair had a higher percentage of TBA positive hair samples (100%) compared to residents with white or blond hair (25%).

In conclusion, our results indicate that TBA is incorporated into the hair of both rats and humans. In humans, TBA incorporation closely follows seasonal exposure, which demonstrates a need for further investigation on hair TBA as a potential biomarker of cumulative exposure.

Acknowledgments

We acknowledge the valuable contribution of Mr. Eugenio Polledri who helped in the recruitment of agriculture workers and rural residents, and all subjects who volunteered in the study.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

Legends for figures

Figure 1. Molecular structures of terbuthylazine (TBA).

Figure 2. TBA in the hair of the study subjects. Hair samples were collected and processed as described in the Materials and methods section.

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Table 1. Percentage of quantifiable samples and levels of TBA in hair of rats

		Pre-treatment hair T0		**Pre-shaved post treatment hair T1**			**Non-shaved post treatment hair T2**			**Mean T1+T2**
		Rinsing solution	**Extract**	**Rinsing solution**	**Extract**	**Sum**	**Rinsing solution**	**Extract**	**Sum**	**Rinsing solution + extract**
TBA (ng/mg hair)	% samples ≥ LOQ	0	0	100	100	100	100	100	100	100
	Mean (±SD)	<0.01	<0.01	0.36 (±0.04)	0.66 (±0.25)	1.02 (±0.29)	0.30 (±0.06)	0.53 (±0.19)	0.83 (±0.24)	0.92 (±0.27)
	Median	<0.01	<0.01	0.36	0.67	1.03	0.31	0.57	0.88	0.91
	Minimum-maximum	na	na	0.32-0.40	0.37-0.92	0.70-1.31	0.22-0.35	0.29-0.69	0.50-1.05	0.50-1.31

na not applicable

Table 2. Summary of selected characteristics of the study subjects

	Agriculture workers	Rural residents	Urban residents
No subjects	10	9	6
Male (%)	100	67	67
Age (years) *	57 (35-71)	52 (24-73)	50 (35-78)
Body weight (kg) *	82 (75-95)	73 (47-100)	72 (50-95)
No dark hair	4	5	4
Smokers (%) **No cigarette/day***	30 3 (2-5)	33 12 (6-20)	0 na
Proximity of residence to corn fields (m) *	51 (10-150)	73 (10-100)	>10.000
Green garden product consumers (%)	100	100	0
No TBA applications*	1 (1-2)	na	na
Amount of applied TBA (kg) *	41 (7-163)	na	na
Treated area (ha)*	45 (10-100)	na	na
Mechanical mixing and loading (%)	90	na	na
Cleaning and maintaining equipment (%)	0	na	na
Use of personal protective devices (%)	40	na	na

*mean (minimum-maximum)

na not applicable

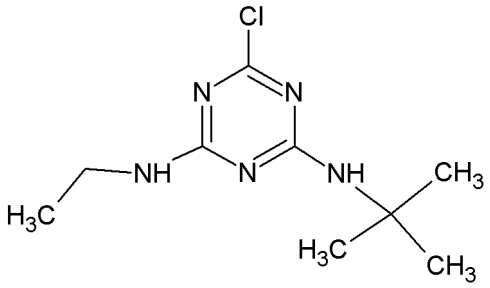
Table 3. Percentage of quantifiable samples and levels of TBA in hair of study subjects

		Agriculture workers	**Rural residents**	**Urban residents**
TBA (ng/mg hair)	% samples ≥ LOQ	100	67	0
	Mean (±SD)	0.96 (±1.36)[a]	0.02 (±0.01)	<0.01
	Median	0.61	0.01	<0.01
	Minimum-maximum	0.07-4.64	<0.01-0.04	na

[a] statistically higher than rural residents

na not applicable

Figure 1. Molecular structure of terbuthyazine (TBA)



TBA

Figure 2. TBA in hair of the investigated urban residents (UR), rural residents (RR), and agriculture workers (AW).