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SHORT COMMUNICATION

3,4-Dichloroaniline–haemoglobin adducts in humans: preliminary data on agricultural workers exposed to propanil

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Propanil is one of the major herbicides used on rice-paddies and is thought to produce adverse health effects through the action of its metabolite 3,4-dichloroaniline (3,4-DCA). The feasibility of monitoring human exposure to propanil on the basis of 3,4-DCA adducts to haemoglobin (Hb) was investigated. We developed a method based on gas chromatography negative ion chemical ionization–mass spectrometry (NICI–GC–MS) to quantify 3,4-DCA released from human Hb after alkaline hydrolysis of the protein. 3,4-DCA–Hb adducts were identified in agricultural workers exposed to propanil and were detectable even 4 months after the last herbicide application. Urine samples collected at the same time had no measurable level of 3,4-DCA. 3,4-DCA–Hb adducts might be useful for monitoring human exposure to 3,4-DCA from agricultural sources.

Keywords: haemoglobin adducts, propanil, biomonitoring.

Introduction

Propanil is a widely used herbicide in rice production and has been shown to produce methaemoglobinaemia in animals (McMillan *et al.* 1990a, 1991) and in occupationally exposed humans (Kimbrough 1980).

Propanil-induced methaemoglobinaemia is mediated by oxidized metabolites formed following enzymatic hydrolysis of propanil to 3,4-dichloroaniline (3,4-DCA) (McMillan *et al.* 1990b). 3,4-DCA was found to be N-hydroxylated. The N-hydroxy metabolite can undergo rapid redox cycling within the erythrocytes, resulting in the oxidation of oxyhaemoglobin to methaemoglobin (McMillan *et al.* 1990b).

Consistent with the mechanism described above, the formation of adducts between 3,4-DCA and haemoglobin (Hb) was found in rats exposed to propanil, although quantification was not attempted (McClure *et al.* 1996).

Despite the growing importance in biomonitoring studies of exposure biomarkers such as protein adducts (Bryant *et al.* 1988, Day *et al.* 1990, Sabbioni and Beyerbach 1995, Pastorelli *et al.* 1996), there are few reports on their use in the assessment of human exposure to agricultural chemicals (Sabbioni and Neumann

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1990, Pastorelli *et al.* 1995). The need to establish whether the identification of 3,4-DCA–Hb adducts is a useful or even a realistic approach to the molecular dosimetry of aromatic amine herbicides prompted us to set up a highly selective procedure for the quantification of these adducts in humans.

This is a preliminary investigation to verify the formation of 3,4-DCA–Hb adducts in agricultural workers occupationally exposed to propanil and to validate the use of this biomarker in comparison with the commonly used monitoring of urinary 3,4-DCA.

Materials and methods

Chemicals

3,4-Dichloroaniline (3,4-DCA) and 3,5-dichloroaniline (3,5-DCA) were obtained from Aldrich Chimica (Milan, Italy). Pentafluoropropionic anhydride and pyridine were purchased from Fluka (Switzerland). Extrelut 20 pre-filled columns and all solvents, of analytical grade, were from Merck (Bracco, Italy). Propanil was a kind gift from Rohm and Haas, Italy.

Analysis of 3,4-DCA adducts by GC–MS

Gas chromatographic and mass spectrometric data were obtained on a Finnigan 4000 instrument, equipped with a PPNICI unit for positive and negative ions and a DANI 86·10 gas chromatograph (DANI S.p.A., Monza, Italy) with a PTV (programmed temperature vaporizer) injector operated in the solvent split mode. The instrument was operated in the negative ion mode, using ammonia as reagent gas, and was controlled by a Vector/Two (Tecnivent Corp., St Louis, MO, USA) data system for data acquisition and processing. The ion source pressure was 0·25 torr and temperature 150 °C. The gas chromatographic column was a capillary column CP Sil19 CB 25 m × 0·32 mm i.d., film thickness 0·2 μm (Chrompack, Cernusco S/N, Italy). Oven temperature was kept at 100 °C for 1 min, then raised to 200 °C at 15 °C min^{-1}, followed by a second temperature ramp to 295 °C at 20 °C min^{-1}. The PTV temperature was set at 60 °C for 6 s then raised to 280 °C in 30–45 s. The carrier gas (He) head pressure was 0·25 bar.

3,4-DCA was analysed by high resolution gas chromatography–negative ion chemical ionization mass spectrometry with selected ion recording (HRGC–NICI-SIR). 3,4-DCA was quantitated as its pentafluoropropionic amide (PFPA) derivative. The sample (100 μl hexane) was derivatized by addition of pentafluoropropionic anhydride (10 μl) and anhydrous pyridine (5 μl). The reaction mixture was incubated at 60 °C for 15 min, then 0·5 ml of 5% NH_4OH and 1 ml of hexane were added. The solvent extract was transferred to a glass minivial and concentrated to 100 μl under a gentle stream of nitrogen. The evaporation step was carefully controlled as it was crucial to avoid loss of the volatile derivatives.

The samples were analysed by monitoring the ions *m/z* 287 and 289 for 3,4-DCA and the external standard 3,5 dichloroaniline (3,5-DCA), which was added before derivatization. A calibration curve was obtained by analysing mixtures containing different amounts of 3,4-DCA (10–20 fmol injected) plus a constant amount of external standard 3,5-DCA (120 fmol injected) as their PFPA derivatives. The identity of the analyte was confirmed by comparing its retention time and ion ratios with those of the 3,4-DCA standards. 3,4-DCA in the samples was quantified by comparing the peaks area ratio of 3,4-DCA/3,5-DCA with those obtained from the calibration curve.

Animals' treatment and isolation of haemoglobin adducts

Male CD rats (200 ± 10 g bw) were purchased from Charles River (Calco, Como, Italy). Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 Febbraio 1992, Circolare No. 8, G.U., 14 Luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, US Research Council, 1996).

Upon arrival, the rats were housed at 22 °C temperature, 50% relative humidity, with a 12-hour light/dark cycle, for a week before use.

One group of rats ($n = 3$) was given propanil in corn oil at a dose of 100 mg kg^{-1} bw by ip injection. Control rats ($n = 3$) were given the vehicle alone.

The rats were sacrificed 24 h after dosing and each animal's blood was collected in heparinized tubes. Fresh blood was separated into plasma and red blood cells (RBC) by centrifugation (5 min at 3000 *g*). The RBC were rinsed three times with equal volumes of 0·9% NaCl solution and then lysed with four volumes of 0·7 M phosphate buffer, pH 6·5. The haemolysate was centrifuged at 10000 *g* for 20 min to remove cellular debris and the volume was reduced to about 5 ml by filteri

YM-10 (10 kDa cutoff) Amicon membrane in a 50 ml Amicon stirring cell under N_2 pressure. The Hb solution was cooled at 4 °C and added dropwise to 20 ml of acid acetone (0·1% HCl) maintained at less than –10 °C, with stirring. The precipitated globin was pelleted by centrifugation, washed with cold acetone, dried and weighed.

The adducts were isolated by alkaline treatment of 100 mg of globin dissolved in 10 ml of 1 N NaOH in a glass tube. The hydrolysis was carried at 37 °C for 2 h. The volume of the hydrolysate was then adjusted to 20 ml with 1 N NaOH and loaded onto an Extrelut-20 prepacked extraction column. 3,4-DCA was eluted with methylene chloride (50 ml). The eluate was concentrated to 100 µl under a gentle stream of nitrogen. Subsequently the external standard 3,5-DCA was added and the sample derivatized as described.

Volunteers and sampling

Paddy-fields were sprayed with Stam formulation (35% propanil) using a tractor-pulled tank with a pump-operated spraying device. The spraying lasted on average 5 hours a day. There were 10 field applications (few days between the sprayings) for the first subject and 2 days in succession for the second. The workers weighed and diluted the herbicide with water before application.

Exposure to propanil was studied in only two male agricultural workers from the province of Pavia, who gave their informed consent to give blood and urine samples for the study. The subjects were monitored at different times. Blood and urine samples were taken a few days before the herbicide field applications, in order to assess basal values of 3,4-DCA–Hb adducts and urinary 3,4-DCA; then samples were collected within 2 days and 4 months after the last herbicide spraying.

Blood samples were collected into heparinized tubes, stored in a thermal container and sent immediately to the Mario Negri Institute, where they were processed. RBC were immediately separated from plasma by centrifugation. The red cells were washed four times with 0·9% NaCl solution and lysed with 2 vol. distilled water. Samples were processed as described for rat blood samples.

Urine samples were analysed at the 'Clinica del Lavoro' in Pavia, following the method of El-Bayoumy *et al.* (1986), with some modifications. Briefly, 5 ml of urine were acidified with 0·5 ml of concentrated HCl. After 30 min, 1 ml of 10 N NaOH was added. The solution was then extracted twice with one volume of diethyl ether. The solvent extracts were combined and dried under a stream of nitrogen. The residue was redissolved in 0·2 ml of methanol for gas chromatographic analysis with an NPD detector, as reported in Catenacci *et al.* (1990).

Results

3,4-DCA-Hb adducts in rats and agricultural workers

NICI mass spectra of derivatized 3,4-DCA and 3,5-DCA standards showed major fragments ions at *m/z* 287 and *m/z* 289 corresponding to loss of hydrofluoride from the molecular ion $[M]^-$ and from $[M + 2]^-$, the latter being due to the natural isotopic abundance of chlorine. This fragmentation pattern is in agreement with previous data (Sabbioni and Beyerbach 1995).

Figure 1 shows typical GC-SIR chromatograms obtained by injecting 3,4-DCA standard solution and human Hb samples. Calibration curves obtained by analysing increasing amounts of 3,4-DCA (10–120 fmol injected) and a constant amount of 3,5-DCA (120 fmol injected) showed a linear relationship between the ratio of the peak areas of 3,4-DCA and of the external standard. The correlation coefficient was 0·99. Recovery experiments of 3,4-DCA and 3,5-DCA showed that 3,5-DCA was not recovered with the same efficiency of 3,4-DCA, thus preventing the use of 3,5-DCA as internal standard. 3,5-DCA was therefore added to the samples just before the derivatization step. Appropriate recovery controls were run for every set of globin samples.

Recovery, determined in human Hb spiked with 1 ng of 3,4-DCA and worked up like the *in vivo* samples, was 89% (SD 2·85). The detection limit for 3,4-DCA in SIR analysis was < 0·5 fmol 3,4-DCA per mg Hb.

We detected 3,4-DCA released from alkaline hydrolysis treatment of the globin samples. Precipitation of the globin was efficient at separating the protein from

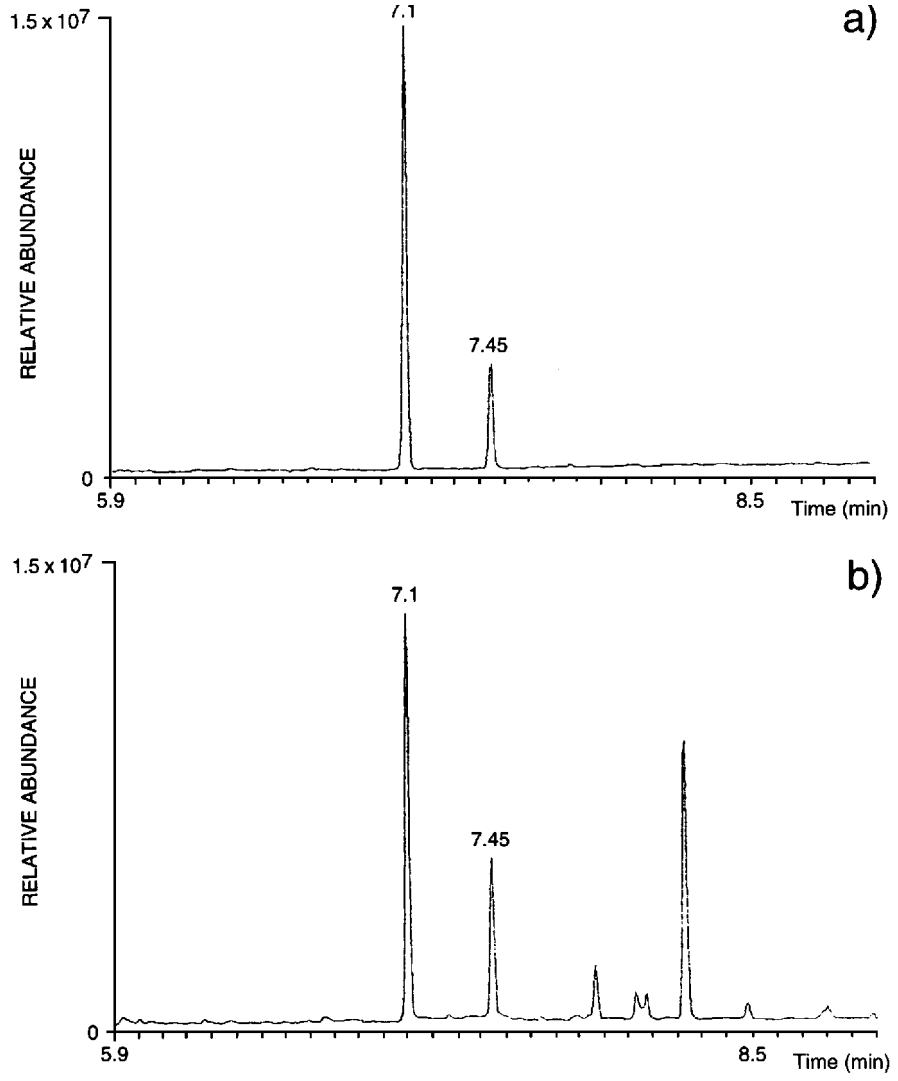

Figure 1. Typical HRGC-SIR analysis of PFPA derivative of 3,4-DCA and 3,5-DCA. The trace shown was produced by monitoring at *m/z* 287. The peak at retention time 7·1 min is the external standard 3,5-DCA. The peak at retention time 7·45 min is 3,4-DCA. (a) Standard mixture of 3,4-DCA and 3,5-DCA (10 and 20 pg injected). (b) 3,4-DCA released from a positive human Hb sample.

unbound 3,4-DCA, which was not recovered along with the globin fraction when aliquots of control Hb were spiked with 3,4-DCA. Therefore the 3,4-DCA measured after hydrolysis of the protein derives from covalently bound 3,4-DCA adducts, sensitive to alkaline treatment.

The 3,4-DCA adduct formed with Hb of rats 24 h after a single ip propanil dose of 100 mg kg^{-1} amounted to 48·66 pmol 3,4-DCA per mg Hb (SD 13·32).

Alkaline-releasable 3,4-DCA was positively identified in the Hb of the agricultural workers exposed to propanil. No adducts were detected in the Hb derived from unexposed controls (laboratory staff, data not shown). Individual 3,4-DCA adduct levels are shown in table 1, as fmol 3,4-DCA released per mg Hb hydrolysed.

Table 1. 3,4-DCA–Hb adducts and urinary excreted 3,4-DCA in two Italian agricultural workers exposed to propanil.

	Sampling time in relation to propanil application	3,4-DCA–Hb (fmol mg^{-1} Hb)[a]	Urinary 3,4-DCA (nmol ml^{-1} urine)[b]
Worker 1	Before	17	< 0·01
	Within 2 days	621	1·07
	4 months after	65	< 0·01
Worker 2	Before	15	< 0·01
	Within 2 days	31	3·5
	4 months after	57	< 0·01

[a] The limit of detection was < 0·5 fmol per mg Hb.
[b] The limit of detection was < 1 nmol 3,4-DCA per ml urine.

Both volunteers had measurable basal adduct levels before field applications of the herbicide. After spraying, in the first worker 3,4-DCCA–Hb adducts attained a level (621 fmol 3,4-DCA per mg Hb) 36 times higher than the background (17 fmol 3,4-DCA per mg Hb). The second worker showed a slightly higher amount of 3,4-DCA-Hb (31 fmol 3,4-DCA per mg Hb) than the basal level (15 fmol 3,4-DCA per mg Hb).

Four months after the latest field spraying, 3,4-DCA–Hb adducts were still detectable and were approximately four times the basal values in both workers.

Urinary excretion of 3,4-DCA in agricultural workers

Urinary 3,4-DCA levels are reported in table 1, as nmol 3,4-DCA excreted per ml urine. Basal and long term sampling values of excreted 3,4-DCA were below the limit of detection in both workers (< 1 nmol ml^{-1}) urine. 3,4-DCA was found in urine collected within 2 days from the latest herbicide application and ranged from 1·07 to 3·5 nmol 3,4-DCA per ml urine.

Discussion

This explorative investigation was undertaken to determine whether there is any relationship between occupational exposure to propanil by agricultural workers and the formation of 3,4-DCA–Hb adducts, as biomarker of the biologically effective dose.

We developed a highly sensitive method for the biomonitoring of low levels of 3,4-DCA in blood samples, based on gas chromatography coupled with negative ion chemical ionization–mass spectrometry. We found that 3,4-DCA binds to Hb of both rats and humans, to form an alkaline-sensitive adduct. The mechanism by which such adducts are formed *in vivo* is likely to be similar to the one for aromatic amines such as 4-aminobiphenyl (Green *et al.* 1984). It implies formation of a nitroso metabolite, and its binding to cysteinyl groups on Hb (Sabbioni 1994).

The adducts level measured in rat Hb 24h after propanil was consistent with previous findings obtained using a radiometric method (McMillan *et al.* 1990a). To our knowledge no other propanil animal dosing studies are available in the literature. Higher levels of 3,4-DCA adduct to rat haemoglobin have been reported, when 3,4-DCA itself was administered by gavage (Birner and Neumann 1988, Sabbioni 1992).

Our preliminary results with human Hb analysis showed 3,4-DCA

detectable in blood from agricultural workers exposed to propanil. To our knowledge this is the first evidence of 3,4-DCA–Hb adducts in human samples. The basal levels of adducts in both subjects comes as no surprise. The peculiarity of their job, requiring frequent handling of complex chemical mixtures, makes it practically impossible to set any biologically meaningful intervals between the last major exposure and the collection of the blood sample. We had limited information about their real exposure to propanil or any other source of 3,4-DCA in the past.

The higher level of 3,4-DCA adducts in the first volunteer than in the second one after the herbicide applications, might be explained by the more frequent propanil field sprayings by the first one, as reported in Materials and Methods. This was not observed in the urinary 3,4-DCA profiles, where the two workers excreted similar amounts. We hypothesize that the particularly rapid excretion of 3,4-DCA could be one of the reasons. From observations reported by our co-authors (Catenacci *et al.* 1990) 3,4-DCA excretion in humans reached a maximum peak within 6–10 h from exposure cessation and rapidly decreased to baseline levels. Because the collection of urine samples was performed within 2 days after the last herbicide spraying, it is likely that in this time-frame both workers had already reached a closely baseline level.

The lack of correlation between Hb adducts and urinary marker probably reflects differences in their kinetics, urinary 3,4-DCA being a metabolic compound and marker of recent exposure and Hb adducts a biomarker of cumulative exposure. Since human erythrocytes have a lifespan of 120 days, circulating levels of 3,4-DCA-modified Hb might reflect the integral of the 3,4-DCA dose over several months. This might also contribute to the measurable basal level and the level of adducts observed 4 months after the latest herbicide application, although urinary 3,4-DCA at the same time was below the limit of detection. This might be due to the method being less sensitive than the one used for Hb adducts, which is 1000 times more sensitive.

The significance of our adducts in terms of toxicity is unknown, so no extrapolation is possible in terms of risk assessment.

Our findings must be considered cautiously because only two individuals were analysed, so obviously no attempt can be made to describe a dose–response pattern in humans. However, it did prove feasible to measure 3,4-DCA–Hb adducts in humans exposed to propanil. In addition, the sensitive method described has more general applicability, because 3,4-DCA is not unique to propanil. Certain heavily used herbicides, such as linuron and diuron, may also be metabolized to 3,4-DCA (Sabbioni and Neumann 1990), resulting in the formation of 3,4-DCA–Hb adducts.

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