SHORT COMMUNICATION

Haemoglobin adducts as biomarkers of exposure to the herbicides propanil and fluometuron

Gail Y. H. McClure, James P. Freeman, Jackson O. Lay and Jack A. Hinson

Aromatic amine herbicides, including propanil, fluometuron, alachlor, trifluralin, and pendimethalin, were examined for their ability to form haemoglobin adducts in rats as potential biomarkers of exposure. Many aromatic amines are known to form haemoglobin adducts via conversion to the nitroso metabolite and binding of this metabolite to cysteinyl groups on haemoglobin. Since red blood cells are long lived, adducts formed with these cells may be reliable biomarkers of exposure with the potential for showing progressive accumulation. Gas chromatographic–mass spectrometric analyses of haemoglobin revealed that adducts were formed in rats treated with the rice herbicide propanil and the cotton herbicide fluometuron. Adducts were not detected with the herbicides alachlor, trifluralin, or pendamethalin.

Keywords: *haemoglobin adducts, herbicides, propanil, fluometuron, agricultural biomonitoring.*

Introduction

Agricultural workers are inherently exposed to various pesticides (herbicides, insecticides, fungicides) on a chronic basis. Many of these workers are inadequately trained in the safe handling of pesticides and may suffer adverse consequences such as occupational cancers, neurotoxic disorders, reproductive problems, immunological diseases, and dermatologic conditions (Anonymous 1988, Weisenburger 1993). Improved assays for assessing exposure to agricultural chemicals are needed to better correlate health effects with exposure levels (Dubelman and Cowell 1989), and biomonitoring of reversible biological effects has been suggested to be an effective way of assessing irreversible toxic effects (Lewalter and Korallus 1986). For example, Sabbioni and Neumann (1990) have reported haemoglobin-binding for a number of aromatic amines which are metabolic products of urea and carbamate pesticides. Additionally, Sabbioni (1992) has shown specific structural relationships between the possible metabolic products of many herbicides and intermediates of industrial manufacturing and the binding-index to tissue and blood proteins. The development of sensitive, reliable methods that can quantify recent worker exposures as well as indicate cumulative exposures during an agricultural season would be extremely useful. Such biomarkers would target those workers with greatest need for remediation in safe handling procedures, as well as provide a tool for epidemiology studies of adverse health effects.

A variety of aromatic amines have been shown to be metabolized to derivatives which become adducted to haemoglobin (Figure 1) and these adducts have proven to be reliable biomonitors of exposure (Albrecht and Neumann, 1985, Binner and Neumann 1988, Skipper and Tannenbaum 1990, Hinson and Roberts 1992). For example, a number of carcinogenic aromatic amines occur in very small amounts in cigarette smoke and these adducts have been utilized to estimate carcinogen exposure. Additionally, there are a number of heavily used aromatic amine herbicides (anilines or aromatic nitro compounds which may be metabolically reduced to aromatic amines). Therefore, these herbicides may form haemoglobin adducts and may be used as biomarkers of exposure.

In this study we have used gas chromatographic–mass spectrometric procedures to determine if a number of commonly used aromatic amine (aniline) herbicides form haemoglobin adducts. Five commonly used aniline derived herbicides (United States Department of Agriculture 1991) were tested to determine if they would form haemoglobin adducts in Sprague–Dawley female rats: fluometuron, alachlor, trifluralin, pendamethalin, and propanil. The chemical structures of the herbicides are shown in Figure 2.

MATERIALS AND METHODS

Chemicals

Propanil was synthesized by treatment of 3,4-dichloroaniline (Aldrich Chem. Co.) with propionyl chloride (Fluka Chem. Co.) as previously described (McMillan *et al.* 1990a). The herbicides trifluralin, pendimethalin, fluometuron and alachlor, were purchased from Chem. Services, West Chester, PA. The purity of all herbicides was greater than 99%. Pentafluoropropionic anhydride was a product of Fluka Chemical Co. Solvents were pesticide grade C (Fisher Chemical Co.). Drabkin haemoglobin assay materials were purchased from Sigma Chemical Co. (St Louis, MO). All other chemicals were from routine sources and were of the highest purity available.

Animals

Fifteen, female, 10-month old Sprague–Dawley rats weighing 400–550 g were obtained from the National Center for Toxicological Research (Jefferson, AR). Animals were individually housed in standard Plexiglas rat cages in a colony room maintained at 70–72°F with a 12 h light/dark cycle (lights on at 0600–1800) with food and water available at all times.

Isolation of haemoglobin adducts

To determine if adducts would form with the selected herbicides, rats were administered herbicides ($n = 3$) in separate studies. Each of the five herbicides (25 mg ml^{-1} in corn oil) was administered at a dose of 100 mg kg^{-1} by i.p. injection. This dose was chosen based upon preliminary data with 3,4-dichloroaniline, a metabolic product from propanil, which indicated significant

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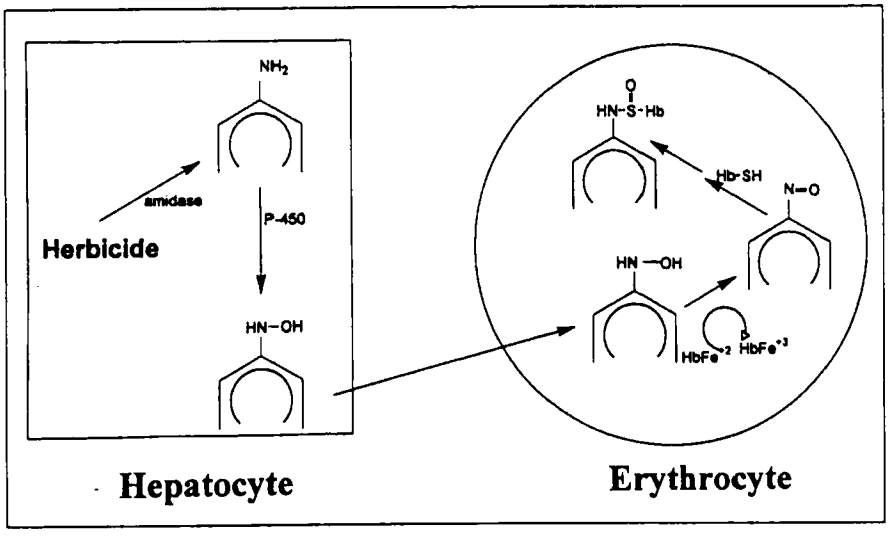

Figure 1. Postulated mechanism of aromatic amine haemoglobin adduct formation.

haemoglobin adducts were formed with this dose. Reported literature values for all these herbicides indicated that this dose was at a minimum 10-fold less than the LD_{50} (Thomson 1983). It was thought that this high dose gave the best possible chance for detection of any haemoglobin adducts which may be formed. The sacrifice time at 24 h ensured that more than 80–90% of the parent herbicides were metabolized (Emmerson and Anderson 1966, Sharp 1988, McMillan *et al.* 1990b). Therefore, rats were anaesthetized 24 h after dosing in a carbon dioxide atmosphere and 5–8 ml of blood were subsequently removed which yielded 2–3.5 ml of erythrocytes.

Haemoglobin adducts were isolated using a modification of the method of Bryant *et al.* (1987). The whole rat blood was collected in heparinized test tubes and packed in ice. Serum was removed after centrifugation at 5000 × *g* for 15 min. The erythrocytes were washed with freshly prepared solution of 0.9% NaCl in distilled water (X3) and then frozen at –20°C in polypropylene tubes. Subsequently, frozen red cells were thawed, 15 ml of distilled water added, and transferred to 50 ml glass-stoppered centrifuge tubes. The samples were then vortexed and allowed to stand for 1 h at room temperature. Solutions were then transferred to high speed centrifuge tubes and spun at 10 000 × *g* for 10 min. The clear haemoglobin solution was transferred to dialysis tubing and dialysed at 4°C against 4 l of water with three water changes during a 24 h period to remove any possible metabolites or parent compound, as previously described by Bryant *et al.* (1987). Samples were stored at –80°C in polypropylene tubes before further workup. Before sodium hydroxide hydrolysis of haemoglobin adducts, 100 µl of the dialysed haemoglobin solution was removed and assayed for haemoglobin content using the Drabkin Assay (Sigma Chem. Co.). Subsequently, the internal standard 2-fluoro-5-methylaniline was added and sufficient 10 M NaOH was added to make the solution 0.1 M. The NaOH–haemoglobin solution was allowed to stand for 3–4 h at room temperature. To the NaOH–haemoglobin solution (roughly 13–15 ml total volume), 20 ml of hexane were added and the hydrolysed anilines were extracted in a tightly capped tube by a gentle rocking motion for about 5 min. Emulsions were broken by sonication and subsequent freezing. The clear hexane phase was removed and dried by passing through a column of $MgSO_4/Na_2SO_4$ and collected in a 50 ml pear-shaped flask. The process was repeated with an additional 10 ml of hexane yielding a total of 25–30 ml of hexane–aromatic amine solution. The aromatic amines were subsequently derivatized by addition of 2 µl of triethylamine and 2 µl of pentafluoropropionic anhydride. The reaction was allowed to proceed at room temperature for 20 min. The 30 ml solution was then concentrated to approximately 1 ml using a rotary evaporator. Finally the 1 ml solution was carried to complete dryness in a mini-vial under a gentle stream of nitrogen. The sample was subsequently dissolved in 20 µl of hexane and stored at –20°C until GC–MS analysis. Because of differential volatility of the internal standard and the adduct, quantification was not attempted.

Mass spectral analyses

A Finnigan Mass Spectrometer Model 4023 (Finnigan MAT Corp., San Jose, CA); a GC: Varian Model 3400 (Varian Associates, Sunnyvale, CA); a Temperature Programmable Transfer Line: Lab Systems Services (San Jose, CA); and an Injector: Varian Septum Programmable Injector (SPI) were used for analysis. The GC column (J & W Scientific, Folsom, CA) was a DB-5, 30 m; 0.25 mm i.d. × 0.25 mm film thickness. A carrier/flow of helium at 2- psi was used with the SPI injector. The SPI program was as follows: 60°C × 0.1 min to 250°C × 25 min at 200°C min^{-1}. The GC column program was set initially for 1.0 min at 75°C with a ramp of 10°C min^{-1} to 175°C and immediately followed by a second ramp to 280°C at 20°C min^{-1} and held for 20 min. The transfer program was set at 225°C for 5.0 min with a ramp of 20°C min^{-1} to 250°C and held for 20 min. One µl of the sample was injected. The MS scans were performed in the Electron Ionization (EI) mode at 70 eV, nominal. Full scans from 100 daltons to 500 daltons (1 s scan time) were used initially to determine the ions to be monitored in the MID (Multiple Ion Detection) scans. The MID scans of propanil and fluometuron adduct samples used 152, 188, 271, 307 (± 0.25) daltons for 0.21 s each with a total scan time of 1.0 s.

Results

The initial herbicide tested for adduct formation in rats was propanil. Blood was drawn 24 h after administration. The blood was processed as described in Materials and Methods for the possible presence of a 3,4-dichloroaniline adduct of haemoglobin. A small sample of the pentafluoropropionic anhydride derivatized extract was analysed by GC–MS using a Finnegan 4023 mass spectrometer (Figure 3). GC–MS analysis indicated the presence of 3,4-dichloroaniline. As shown in Figure 3(B) the retention time was 7 min 20 s (7: 20), which was the same as the authentic compound. The fragmentation

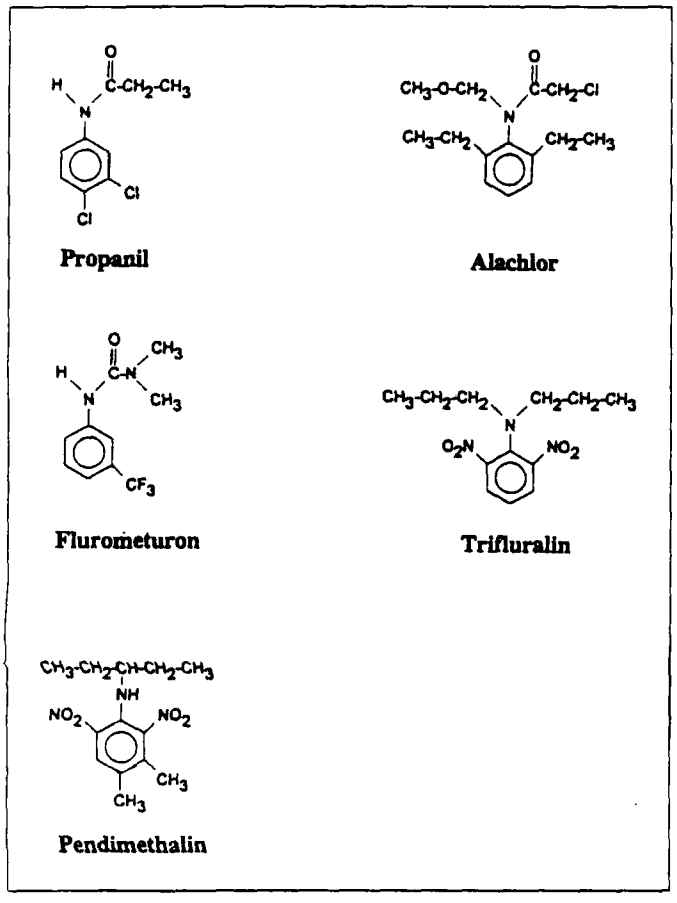

Figure 2. Chemical structures of aromatic amine herbicides.

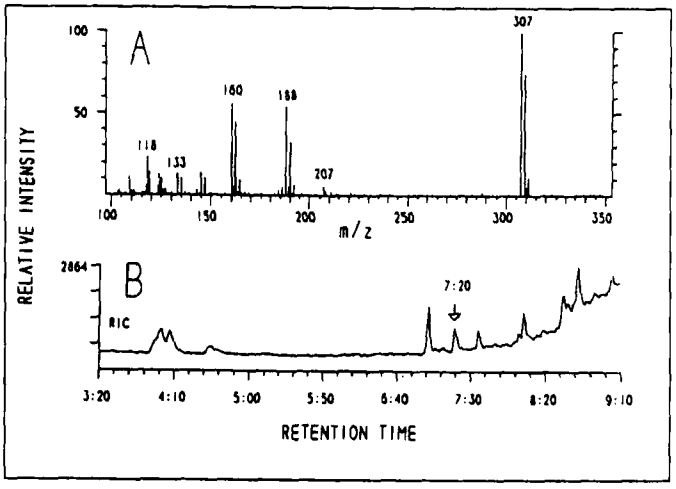

Figure 3. Mass spectral analysis of derivatized haemoglobin adduct from rats treated with propanil. Rats were treated with propanil and the adduct was analysed by GC–MS analysis as described in Materials and Methods. The upper panel (A) is a full scan mass spectrum of the peak eluting at 7 : 20. It shows a characteristic isotopic pattern associated with two chlorine atoms in its molecular ions (*m/z* 307–311) and in two of the fragments (*m/z* 160–164 and 188–202). The lower panel (B) is a chromatogram of the derivatized 3,4-dichloroaniline adduct.

pattern of this peak (Figure 3(A)) showed an isotopic molecular ion pattern consistent with two chlorine atoms in the molecule. It matched the library spectrum of the pentafluoropropionic amide of 3,4-dichloroaniline. The 3,4-dichloroaniline derivatized with pentafluoropropionic anhydride produced ions at *m/z* 307 and 188. The *m/z* 307 ion is a molecular ion and the major mass fragment at *m/z* 188 ion is consistent with a product produced via a loss of —CF2—CF3 from the molecular ion. The retention time and mass spectrum identify the molecule adducted to haemoglobin as 3,4-dichloroaniline. These data extend our previous work which indicated that following administration of radiolabelled propanil, radiolabel became associated with haemoglobin and was not released following solvent extraction (McMillan *et al.* 1991).

The herbicide fluometuron was also injected into rats and a blood sample was removed at 24 h. This blood sample was processed for GC–MS analysis as described in Materials and Methods. Analysis of a sample of the pentafluoropropionic anhydride derivatized extract (Figure 4) indicated that 3-trifluoromethylaniline was a haemoglobin adduct of fluometuron in the rat. The retention time of 3 min 11 sec (3:11 in Figure 4(B)) and fragmentation of this adduct (Figure 4(A)) were identical to authentic 3-trifluromethylaniline, and the spectrum was identical to that found in the computer library. Mass spectral analysis of the fluometuron-adduct derivatized with pentafluoropropionic anhydride produced a molecular ion at 307 and a major fragment ion at *m/z* 188 consistent with a product produced via a loss of —CF2—CF3 from the molecular ion. Coincidentally, these are the same nominal weight ions as that for 3,4-dichloroaniline, but there are no intense chlorine isotope ion patterns for this compound. These data indicate that 3-trifluoromethylaniline is a haemoglobin adduct of fluometuron, and are consistent with previous reports that the aniline itself will bind to haemoglobin (Sabbioni 1992).

Haemoglobin from Alachlor-treated rats was also analysed for the presence of haemoglobin adducts using similar approaches. It had been previously reported that radiolabelled Alachlor covalently bound to haemoglobin (Brown *et al.* 1988). No evidence for the formation of haemoglobin adducts was found with Alachlor. This herbicide was monitored for the presence of a peak at *m/z* 295 (2,6-diethylaniline). Also, no GC–MS peak was observed at *m/z* 339, a peak which may be observed if a direct alkylation of haemoglobin by alachlor occurred (alkaline hydrolysis of protein would yield *N*-methoxymethyl-2,6-diethylaniline which presumably would have been detected as the pentafluoropropionic acid amide).

Haemoglobin from rats treated with two other very commonly used herbicides was analysed for the presence of haemoglobin adducts. These herbicides were trifluralin and pendimethalin. Again herbicides were injected into rats and after 24 h blood samples drawn. Both of these herbicides were analysed for the presence of dinitroaniline adducts and other adducts which would have been consistent with formation via mononitro reduction and dinitro reduction products. No evidence was found for the formation of haemoglobin adducts with either herbicide.

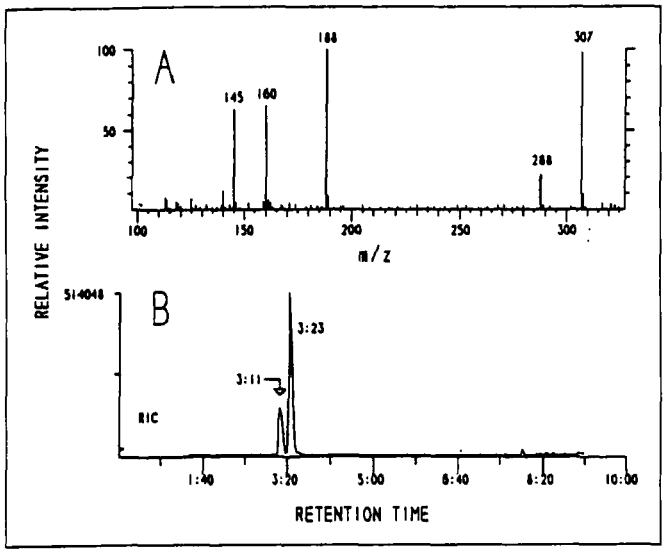

Figure 4. Mass spectral analysis of derivatized haemoglobin adduct from rats treated with fluometuron. Rats were treated with fluometuron and the adduct was analysed by GC–MS analysis as described in Materials and Methods. (A) is a full scan mass spectrum of the fluometuron adduct. The mass spectrum shows a molecular ion at *m/z* 307 and fragment ions at *m/z* 288, 188, 160, and 145. There is no indication of any chlorine in the mass spectrum. In the lower panel (B) the peak at 3 : 11 is the recombined ion chromatograph (RIC) of *m/z* 307 and 188. The peak at 3 : 23 is an internal standard.

Discussion

We have shown that propanil (Stam), a very heavily used herbicide in rice production, forms haemoglobin adducts in rats. Additionally, we have shown that an extensively used herbicide for cotton production, fluometuron (Cotoran), also forms haemoglobin adducts. No evidence was found for haemoglobin adducts from alachlor used on corn and soy beans (Lasso), trifluralin used on soy beans (Treflan), or pendamethalin used on soy beans (Prowl). The use of haemoglobin adducts to monitor propanil and fluometuron exposure offers several potential advantages. Foremost is the fact that these adducts will accumulate within the erythrocytes, whereas other protein adducts such as those which may occur with albumin are removed from the circulation and do not accumulate. Since human erythrocytes have a life span of 120 days, haemoglobin adducts may give a cumulative account of past exposures and record recent incidence of continuing exposures.

The utilization of haemoglobin adducts as biomarkers of exposure to herbicides is potentially feasible and effective. With the carcinogenic aromatic amine, 4-aminobiphenyl, Green *et al.* (1984) showed that approximately 5% of a single dose was bound to haemoglobin in rats and that with chronic administration to rats, adducts accumulated to a level 30 times greater than a single dose. Upon cessation of dosing the level of adducts dropped as ageing erythrocytes were cleared from the blood stream. Skipper and Tannenbaum (1990) have suggested that the formation of a stable type of adduct that persists in circulation, and the linearity throughout a five-fold log dose range make 4-aminobiphenyl an effective haemoglobin dosimeter of exposure for this aromatic amine. Moreover, Bryant *et al.* (1988) showed that, in humans, 4-aminobiphenyl adducts occurred in cigarette smokers and that upon cessation of smoking these adducts decreased to low levels (Maclure *et al.* 1990).

In this paper we have shown that haemoglobin adducts are formed from propanil and fluometuron. A second step will be the development of a sensitive quantifiable assay. For 4-aminobiphenyl haemoglobin adducts Bryant *et al.* (1988) developed a quantitative assay using capillary gas chromatography coupled with negative-ion chemical-ionization mass spectrometry. This assay was capable of quantifying pmoles of adduct per gram of haemoglobin. Using an appropriate internal standard such as a ^{13}C derivative or a deuterated derivative it should be possible to develop an equally sensitive assay for propanil haemoglobin adducts as well as fluometuron haemoglobin adducts. These assays may accurately quantify human exposures of less than a milligram of herbicide. This level of precision coupled with the fact that erythrocytes (haemoglobin) slowly turnover, may allow for an accurate estimate of past and continuing exposures.

Acknowledgements

This work was partially supported by a grant from the Kellogg Foundation of Battle Creek, MI. This work was submitted in partial fulfillment of the Master of Science in Occupational and Environmental Health for G. Y. H. McClure, Ph.D.

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Received 12 October 1995, revised form accepted 9 November 1995