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Immunoassay of haemoglobin–acrylonitrile adduct in rat as a biomarker of exposure

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Acrylonitrile (AN) is a rat carcinogen. Human exposure may come from chemical industries and smoking. A haemoglobin adduct of acrylonitrile (Hb–AN) has been used as a biomarker of exposure by means of gas chromatography–mass spectrometry (GC–MS) analysis. We have developed specific monoclonal antibodies (Mab) to human Hb–AN and wish to report evaluation of an immunoassay in rats using an Mab that cross-reacts with rat Hb–AN. A dose response study of LD 0, 10, 50, and 90 in Sprague–Dawley rats was undertaken, with each rat receiving [2,3-^{14}C]AN at 50 μCi kg^{-1} sc, and Hb from an aliquot of blood was taken for covalent binding analysis by liquid scintillation spectrometry and fluorescence ELISA. The dose responses of rats at 0·25, 0·5, 1·0, and 2·0 h after AN doses of 20, 50, 80, 115 mg kg^{-1} were compared by both methods with Hb and globin samples. Regression analysis showed a linear relationship between immunoassay and ^{14}C-AN binding. This indicates that an antigenic form of Hb–AN may be used as a surrogate of Hb–AN adduct. The sensitivity of ELISA was tested in rats exposed for 1 h to sub-toxic doses of AN (10–1·1 mg kg^{-1}). Quantification of Hb–AN by immunoassay was achieved by calibration with a synthetic adduct HbAN4h, a reference adduct prepared by treating rat Hb with excess AN for 4 h. ELISA and GC–MS analysis of N-terminal valine–AN in the Hb–AN adduct were compared and similar detection levels were found. This rat study appears to have validated the new immunoassay method for biomonitoring of AN exposure.

Keywords: immunoassay, haemoglobin, acrylonitrile, adduct, biomarker.

Introduction

Acrylonitrile (AN), a commodity vinyl monomer, has been classified by the International Agency for Research on Cancer (1987) to be a rat carcinogen and a Group 2A agent that is probably carcinogenic to humans. A high risk level of E-4 (1 in 10000) was estimated for respiratory cancer in workers exposed to an air concentration of 1 μg of AN per cubic metre (0·47 ppb) by the US Environmental Protection Agency (1983). However, more recent epidemiological studies (Swaen *et al.* 1992) of workers exposed to AN have found no indications that AN has a carcinogenic effect. This serious dispute should be resolved in future studies with an internal dosimeter of AN such as the haemoglobin–acrylonitrile (Hb–AN) adduct. The established method for quantifying Hb–AN involves removing haem from Hb followed by a modified Edman degradation of the globin to cleave off the N-terminal valine for GC–MS analysis of *N*-(2-cyanoethyl)valine (Osterman-Golkar *et al.* 1994, Tavares *et al.* 1996). Another analysis of rat Hb–AN made use of electrospray ionization mass spectrometry for detecting AN adduction to the globin chains (Stevens *et al.* 1994). For a large scale cohort study, a quantitative

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immunoassay of Hb–AN would be more practical. We have previously developed specific monoclonal antibodies (Mab) to human Hb–AN for ELISA (Wong *et al.* 1996). This report aims to evaluate the new immunoassay in rats using an Mab that cross-reacts with a rat Hb–AN adduct. The Hb–AN immunoassay of rat exposure to acrylonitrile has shown dose response, a linear relationship with ^{14}C-AN binding, and congruence with GC–MS analysis of the N-terminal valine–AN adduct.

Methods

Chemicals

Chemical reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI), including AN which contained 35–45 ppm of hydroquinone monomethyl ether as a polymerization inhibitor. The radiotracer [2,3-^{14}C]–AN at 0·3 mCi ml^{-1} in ethanol:water (1:1) with a specific activity of 5·3 or 5·5 mCi mmol^{-1} was obtained from Sigma Chemical Co. (St Louis, MO), as were the other biochemicals. Pentafluorophenylisothiocyanate (PFPITC) was obtained from Fluka Chemical Corp. (Ronkonkoma, NY) and L-valine (d8, 98 %) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA).

Animals

Balb/c mice, male, 6–8 weeks old, were purchased from Charles River Breeding Laboratories (Raleigh, NC). Male Sprague–Dawley rats, weighing between 225 and 249 g, were obtained from Harlan (Indianapolis, IN).

Preparation of reference adduct HbAN4h

Hb was prepared from blood according to a standard procedure described by Tornqvist *et al.* (1986): blood was centrifuged to pellet RBC, washed in 0·9 % NaCl, lysed in water, and centrifuged at 12 000 × *g* to obtain Hb in the supernatant. A 1 ml solution of 0·5 M Tris–HCl at pH 7·5 containing the freshly-isolated Hb (0·6 mM) and AN (150 mM) was incubated at 37°C for 4 h. The Hb reaction mixture was chromatographed on a Sephadex G25 column to isolate the adduct HbAN4h whose concentration in the eluent was determined by optical density at 307 nm. The extent of S-cyanoethylation was measured by titrating the mercapto group in Hb and HbAN4h with 4,4′-dipyridinedisulphide (Ampulski *et al.* 1969). In the preparation of rat HbAN4h, the titratable SH decreased from 6·18 in Hb to 0·44 in the adduct, yielding a 93 % reaction. Under the same reaction conditions, about 90 % of the mercapto groups in human and mouse HbAN4h were reacted. Analysis of the adduct HbAN4h by electrophoresis was carried out on a vertical polyacrylamide gel according to a procedure by Berstein and Bowman (1976): 10 % T resolving gel in Tris–HCl buffer (pH 9·15) and 4 % T stacking gel in Tris–HCl buffer (pH 7·4) in 5 mM Tris–glycine running buffer (pH 8·3) for 6 h at a constant current of 15 mA through the stack and 20 mA through the resolving gel. Each sample well was loaded with about 25 μg of protein containing 0·002 % bromophenol blue tracking dye. The PAGE showed a residual Hb band lagging behind a more-negatively charged and more intense band assigned to the adduct. The synthetic HbAN4h prepared from human, rat and mouse blood were used as reference adducts in ELISA.

Preparation of Mab

The human Hb–AN adducts including HbAN4h were used to immunize Balb/c mice followed by raising hybridoma, subcloning, and screening of monoclonal antibodies by ELISA according to standard procedures (Reik *et al.* 1987). Specific Mab were obtained as reported previously (Wong *et al.* 1996). To prepare Mab to Hb–AN adducts for the present study, hybridoma clones were selected for culture, and 5×10^5 cells of each cell line in 0·5 ml of serum-free DMEM medium were injected ip to pristane-primed Balb/c mouse. After 8–10 days, the ascites fluid was removed, centrifuged, and the supernatant in pH 4·8 acetate buffer was purified by precipitation of contaminating proteins with caprylic acid. The supernatant was dialysed against PBS at 4 °C for 6 h followed by precipitation of the Mab immunoglobulin with saturated ammonium sulphate. Further purification of Mab was done on a protein A/G–Sepharose 4B column which was eluted with 0·1 M glycine at pH 2·7, the eluent neutralized and quantitated by optical density at 280 nm.

ELISA

The ELISA methodology was adapted after Moscoso *et al.* (1989) for optical and Kitamura *et al.* (1989) for fluorescence measurement. Briefly, a polystyrene microplate was coated with a Hb antigen, washed, blocked with 10 % calf serum, and the wash repeated. The primary Mab was added to the microwells and incubated at 37 °C. For optical measurement at 410 nm, goat anti-mouse IgG-alkaline phosphatase and substrate *p*-nitrophenyl phosphate were added, incubated for 30 min at 25 °C, and colour development was stopped by adding 3 N NaOH. For fluorescence reading at 450 nm, the indicating system of biotinylated anti-mouse IgG and streptavidin–galactosidase was used with 4-methylumbelliferyl β-D-galactoside as the substrate. Quantitative calibration of ELISA of the Hb–AN adduct made use of the synthetic HbAN4h. The intra-assay variatio

given by six replicates on the same microplate was within 5 %, whereas five interday assays showed variation within 10 %. The sensitivity of detection of the Hb–AN adduct was found to be 0·1 nmol g^{-1} Hb.

Dose–time response of rat to AN

Rats were injected sc in the scruff of the neck with AN in distilled water spiked with [2,3-^{14}C]AN so that each rat received 50 μCi kg^{-1} while control animals received distilled water only. All injection volumes were 5 ml kg^{-1}. Doses used in mg kg^{-1} and the LD responses expected were: 1·2, 3·3, and 10 (sub-LD0), 20 (LD0), 50 (LD10), 80 (LD50), and 115 (LD90). Three animals were treated at each dose, and blood was obtained by cardiac puncture with a syringe coated with heparin (1000 U ml^{-1}) at 0·25, 0·5, 1·0, and 2·0 h after AN administration unless otherwise noted. The blood was centrifuged, the packed erythrocytes washed with 0·9 % saline, and lysed in distilled water to yield Hb or globin (Gb) by precipitation with 6 mM HCl in acetone at –20 °C. The globin pellet was washed twice with ethanol and then once with acetone, ethyl acetate and diethyl ether to remove unbound radioactivity. For scintillation counting, about 10 mg of dried globin was dissolved in 1·5 ml of 50 mM sodium acetate buffer, pH 4·8. A 1·0 ml aliquot was removed, mixed with Atomlight (Dupont NEN, Boston, MA) and counted (Packard liquid scintillation spectrometer 2000CA, Downers Grove, IL). The unused blood and Gb samples were kept at –70 °C for subsequent ELISA assay.

GC–MS analysis

The N-terminal valine adducts were quantitated according to Tornqvist *et al.* (1986) using a modified Edman degradation procedure. Briefly, the internal standard used was *N*-(2-cyanoethyl)–d7-valine which was synthesized by incubating d8-valine and AN in water at 80 °C for 24 h. One deuterium atom of the d8-valine was lost to solvolysis during the reaction, yielding a 2-cyanoethyl–d7-valine adduct. For each analysis of the N-terminal valine adducts, 5 mg of globin was weighed out and dissolved in a solution of 1-propanol (0·5 ml) and 0·5 M $KHCO_3$ (1·0 ml). The internal standard (20 ng CE–d7-valine) and PFPITC (7 ml) were added, and the mixture was mixed overnight, heated at 70 °C for 2 h, extracted with hexane (2 × 2 ml), and the extracts combined and evaporated under nitrogen. The residue was redissolved in 1 ml of toluene, washed with 2 ml of 0·1 M K_2CO_3, evaporated under nitrogen, 30 ml of toluene was added to dissolve, and 1 ml of this solution containing the analytical derivative, 1-cyanoethyl-5-isopropyl-3-phenyl-2-thiohydantoin, was analysed by gas chromatography–mass spectrometry (Hewlett Packard 5890-5971 system and capillary GC column DB-1) with selective ion monitoring. Positive mass spectral ions formed by electron impact were monitored with dwell times of 75 ms each; these included m/z = 377 (analyte) and m/z = 384 (internal standard). The ratio of integrated peak areas (sample area/internal standard peak area) was linear with increasing analyte. Quantitation was based on a response factor of 1·10 determined from 0·2 to 100 ng of standard *N*-(2-cyanoethyl)valine. The complete instrumentation was described previously by one of us (Benz *et al.* 1997).

Results and discussion

Mab to rat Hb–AN adduct

Acrylonitrile binds extensively to haemoglobin in rodents (Fennell *et al.* 1991) and humans (Bergmark 1997). Although substantial sequence homology is conserved in Hb of these species, rat has a highly reactive β-125 cysteine in addition to the reactive β-93 found in human (Hughes *et al.* 1981). The total number of cysteine residues in rat and human Hb are 10 and 6, respectively. Whether the antigenic sites in these Hb–AN adducts share the same structural features would be of interest to determine. Thus, the cross-species activities of five hybridoma cell lines raised to human Hb–AN adduct are examined in table 1. The Mab binding of HbAN4h, an *in vitro* reference adduct formed by treating human, Sprague–Dawley rat, or Balb/c mouse haemoglobin with excess AN for 4 h, was screened by optical ELISA. Mab A5G4 was active across species, indicating the presence of a common antigenic site in Hb–AN of the three species. On the other hand, G5F12 showed binding only to the human adduct which may possess an unique determinant. Although G6A1 favoured the rodent adducts over the human, its binding activity was moderate. E3A5 and A2D1 were highest in binding the

Table 1. Cross species binding activity of Mab raised to human Hb–AN immunogen[a].

Ag: HbAN4h	A5G4	A2D1	E3A5	G5F12	G6A1
Human	1·10	1·40	1·52	1·24	0·44
Rat, SD	1·20	1·29	0·24	0·02	0·95
Mouse, Balb/c	1·10	0·22	0·15	0	0·86

[a] Measured in optical density in ELISA; Ag plated at 11 ng per well.

human and rat Hb adducts respectively, hence the latter Mab was used in monitoring Hb adduct formation in rat. While this cross-species activity lends itself to verification of the human immunoassays by rodent exposure studies, it also suggests that care should be taken in preparing Hb samples from the red blood cells. The latter should be washed free of serum so that other globin–AN adducts which may potentially binds to the Mab applied will not contribute to the ELISA of Hb.

Dose and time dependence of Hb–AN adduct formation in rat by ELISA and correlation with ^{14}C binding

Covalent binding of AN to the globin of red blood cells in rat given single acute AN injection was recently reported by one of us (Benz *et al.* 1997). A fairly linear function of AN dose was found over a 100-fold range from 1·1 (sub-toxic) to 115 mg kg^{-1} (LD90). The ELISA samples were representative haemoglobin and

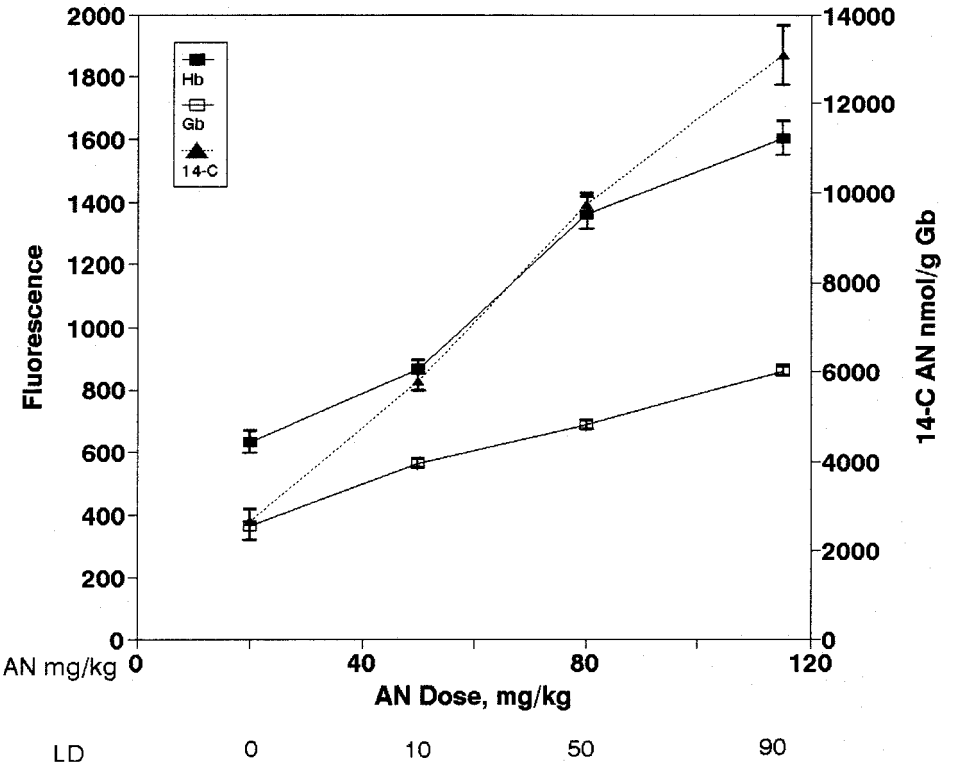

Figure 1. Plot of ^{14}C-AN covalently bound to haemoglobin and fluorescence ELISA of haemoglobin (Hb) and globin (Gb) of rat blood obtained 1 h after sc administration of AN. Doses of AN are LD0–20 mg kg^{-1}, LD10–50 mg kg^{-1}, LD50–80 mg kg^{-1}, and LD90–115 mg kg^{-1}. Each point represents the mean ± SD.

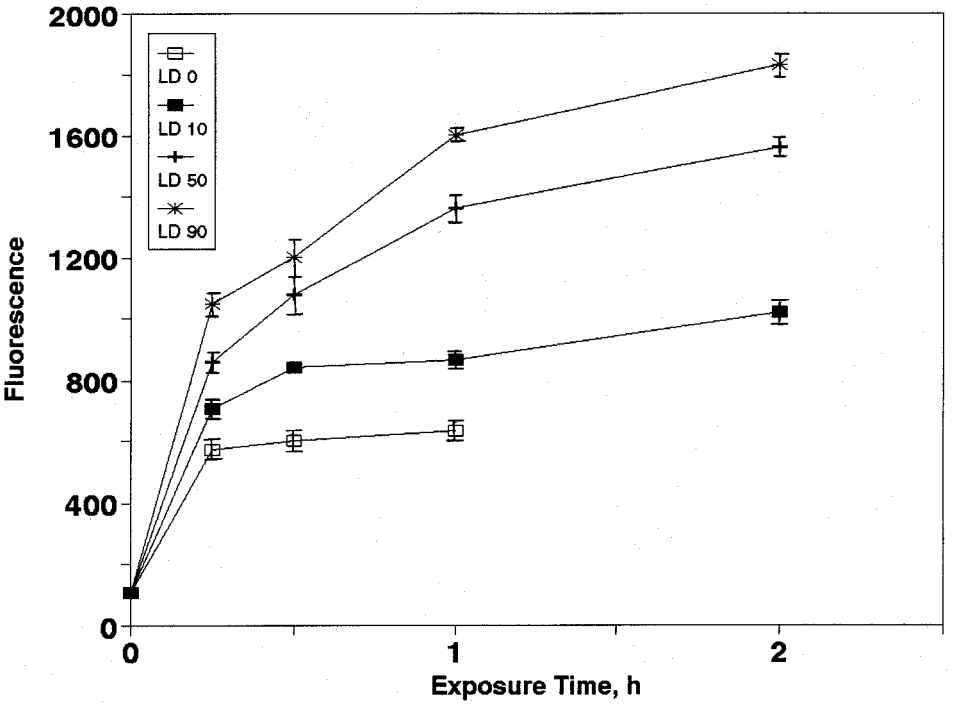

Figure 2. Dose–time–response curves for fluorescence ELISA of Hb–AN adduct in haemoglobin (Hb) of stored rat blood obtained at various times following sc administration of AN. Doses of AN are LD 0–20 mg kg^{-1}, LD10–50 mg kg^{-1}, LD50–80 mg kg^{-1}, and LD90–115 mg kg^{-1}. Each point represents the mean ± SD.

globin samples prepared from the red blood cells of the above study. In figure 1, fluorescence ELISA of the haemoglobin and globin samples isolated from rats after 1 h of exposure are plotted with ^{14}C-AN binding to globin for the following doses (mg kg^{-1} and LD responses expected, cf. Benz *et al.* 1997): 20-LD0, 50-LD10, 80-LD50, 115-LD90. There appears to be a fairly linear dose relationship in the ELISA of globin samples ($r = 0{\cdot}996$) as well as in ^{14}C scintillation counting ($r = 0{\cdot}998$). The haemoglobin ELISA dose response is less linear ($r = 0{\cdot}985$) but it gives a higher gradient of fluorescence than globin. In figure 2 is shown the time course of adduct formation at each AN dose as monitored by fluorescence ELISA of the haemoglobin samples. At each time period, the higher doses have elicited higher fluorescence. These dose–time–fluorescence relationships are equivalent to those of ^{14}C-AN covalent binding to rat globin. The time dependence is apparent at the two higher doses of 80 and 115 mg kg^{-1} where Hb–AN formation continues to increase after 1 h of exposure, albeit at a slower rate. However, at the low dose of 20 mg kg^{-1}, ELISA of Hb–AN has already reached a plateau by 0·25 h, the first point of sampling. Apparently, the cyanoethylation of Hb *in vivo* to yield an antigenic Hb–AN is a favourable reaction, one that may be used to advantage in monitoring low level exposures to AN. Thus, rats exposed for 1 h to sub-toxic doses of AN: 1·2, 3·3, 10, and 20 mg kg^{-1} were analysed by ELISA and ^{14}C counting to evaluate the lower limit of applicability of this immunoassay. Table 2 shows the correlation of AN doses with the ratio of fluorescence of globin from exposed rats to that of control. Even at the lowest dose of 1·2 mg kg^{-1}, the relative fluorescence of 1·155 stands out from the control background. Also shown is the

Table 2. Sensitivity of ELISA of Gb–AN adduct in rat injected with sub-toxic doses of AN.

	AN dose, mg kg ×1h			
	1·2	3·3	10	20 (LD0)
Relative fluorescence[1]	1·155	1·392	1·608	3·660
Covalent ^{14}C binding AN nmol g $^{-1}$ Gb	102	300	1010	2200

[a] Ratio of fluorescence of Gb sample to that of control; fluorescence of control = 97 counts.

binding of ^{14}C-AN to globin, a measure of total alkylation of Hb. In comparing these responses to the AN doses of 20 and 1·2 mg kg^{-1}, a difference of 16·7 fold, ^{14}C binding has increased 21·6 times vs only a 3·2-fold increase of ELISA. This suggests that specific alkylation of Hb to form HbAN antigen as detected by ELISA is a limiting but earlier event compared with alkylation at the other nucleophilic sites in Hb which are more numerous. Hence, further cyanoethylation of Hb at higher AN doses may not yield correspondingly higher antigenic adduct. In order to obtain an overview of how fluorescence ELISA is related to ^{14}C-AN binding, the fluorescence of Hb and Gb samples are paired with the ^{14}C data and plotted in figure 3. A linear relationship is revealed by regression analysis with a correlation coefficient of about 0·96 for both the Hb and Gb samples. This indicates that fluorescence ELISA of an antigenic form of Hb–AN may be used as a surrogate measurement for the total Hb–AN adduct.

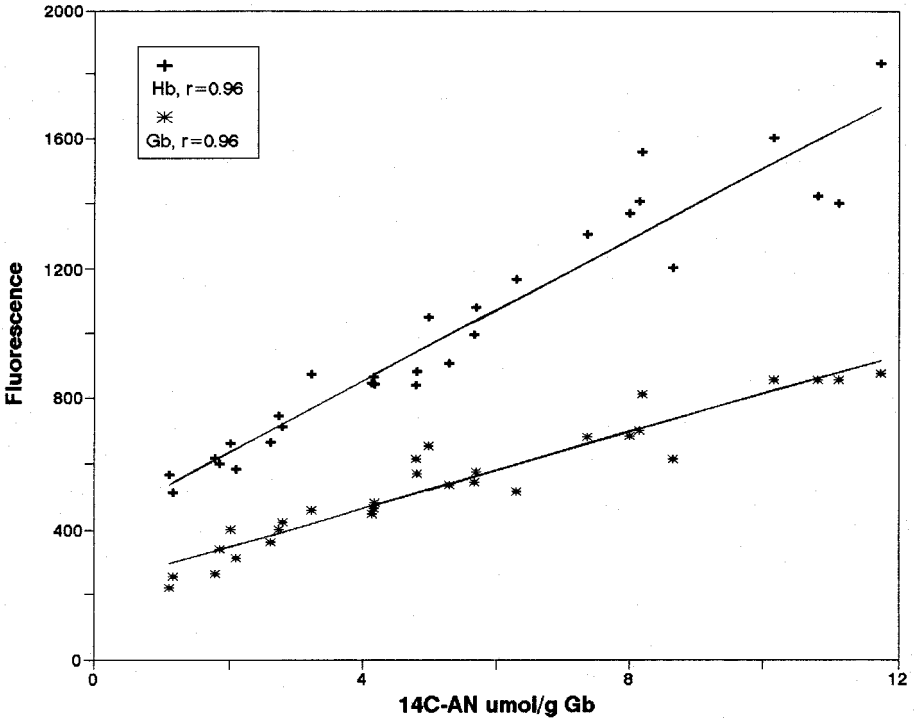

Figure 3. Correlation of fluorescence ELISA of Hb–AN/Gb–AN adduct in Hb/Gb of rat blood with ^{14}C-AN covalently bound to globin of red blood cells.

Quantification of Hb–AN adduct

It is desirable to convert fluorescence units of ELISA to molar quantities of Hb–AN adduct. Although it is possible to project total Hb–AN adduct from the fluorescence-^{14}C-AN correlation in figure 3, this conversion technique would be limited to rat exposure but unavailable for human studies. We have attempted a calibration of ELISA of the rat adduct with synthetic Hb–AN mixtures: a microplate well was coated with 5 μg ml^{-1} of control rat Hb containing varying amounts of rat HbAN4h or coated with the Gb/GbAN4h mixture to simulate an *in vivo* sample. The synthetic adduct was prepared by treating rat Hb with excess AN for 4 h which resulted in 5·7 less titratable mercapto groups than in rat Hb. In figure 4 are shown the calibration curves of rat HbAN4h and its globin derivative in fluorescence ELISA, where the AN adducts are expressed in nmol g^{-1} Hb or Gb. It is likely that a common antigenic site is shared by HbAN4h and Hb–AN formed *in vivo*, since both have shown specific binding with the same Mab. Thus, it is justified to use the synthetic HbAN4h for calibration, i.e. converting the fluorescence of an *in vivo* Hb sample to nmol HbAN4h g^{-1} Hb. The time response in terms of HbAN4h g^{-1} Hb and GbAN4h g^{-1} Gb for the 80 mg kg^{-1} dose are shown in figure 5. Similarly, the Hb–AN dose response is illustrated in figure 6 for the adduct assayed in the plateau region of the time plot. These plots are reminiscent of those shown in figures 1 and 2 using the fluorescence and ^{14}C data.

Comparison of Hb–AN by ELISA with valine–AN by GC–MS

The established method for the determination of Hb–AN is by GC–MS analysis of *N*-(2-cyanoethyl)valine, the N-terminal valine–AN in the Hb adduct. In a pilot study of 41 chemical workers by Bergmark *et al.* (1993), the valine–AN

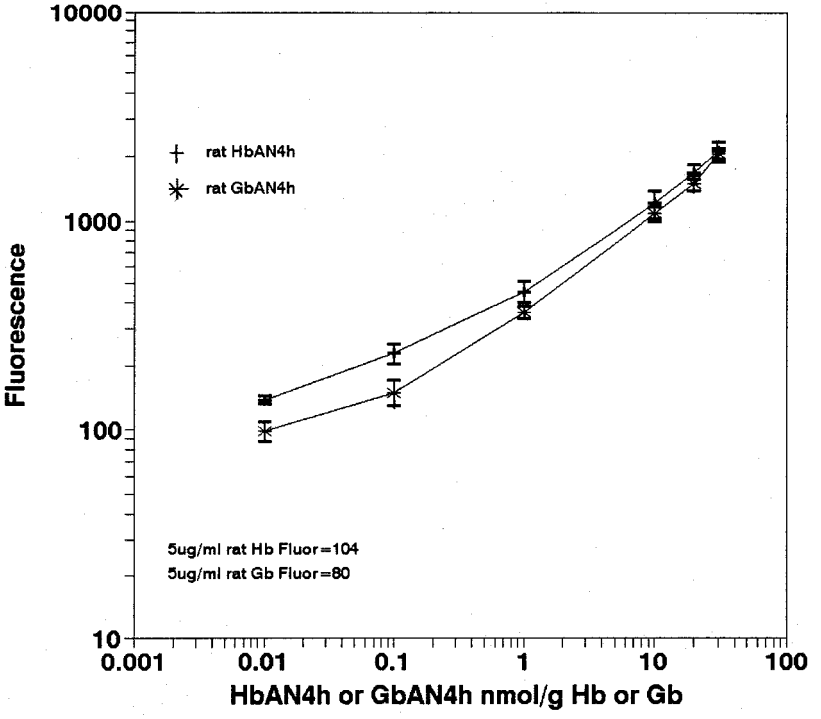

Figure 4. Calibration of fluorescence ELISA of rat Hb–AN/Gb–AN adduct with synthetic rat HbAN4h/GbAN4h added to 5 μg ml^{-1} of rat Hb/Gb. Each point represe

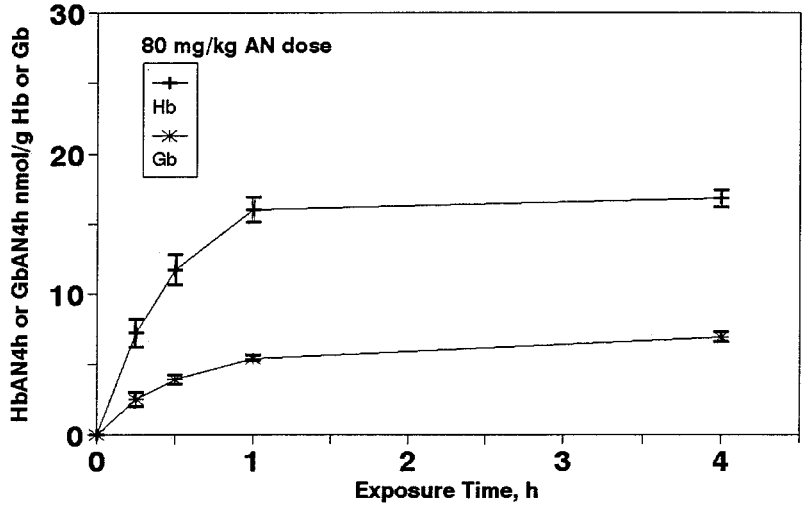

Figure 5. Quantitation of fluorescence ELISA of Hb–AN adduct as HbAN4h in rat blood in a time response to sc administration of 80 mg kg^{-1} of AN. Each point represents the mean ± SD.

levels were found to be 0·02–66 nmol g^{-1} Hb. Also, globin from smokers of 10–20 cigarettes per day contained valine–AN of about 90 pmol g^{-1}(Osterman-Golkar *et al.* 1994). We have compared the dose response given by fluorescence ELISA and by GC–MS in figure 6, showing nmol HbAN4h g^{-1} Hb and nmol valine–AN g^{-1} Gb for the four AN doses of 20, 50, 80, and 115 mg kg^{-1}. In both procedures, the blood samples were collected when AN binding had reached a plateau between 1 and 2 h

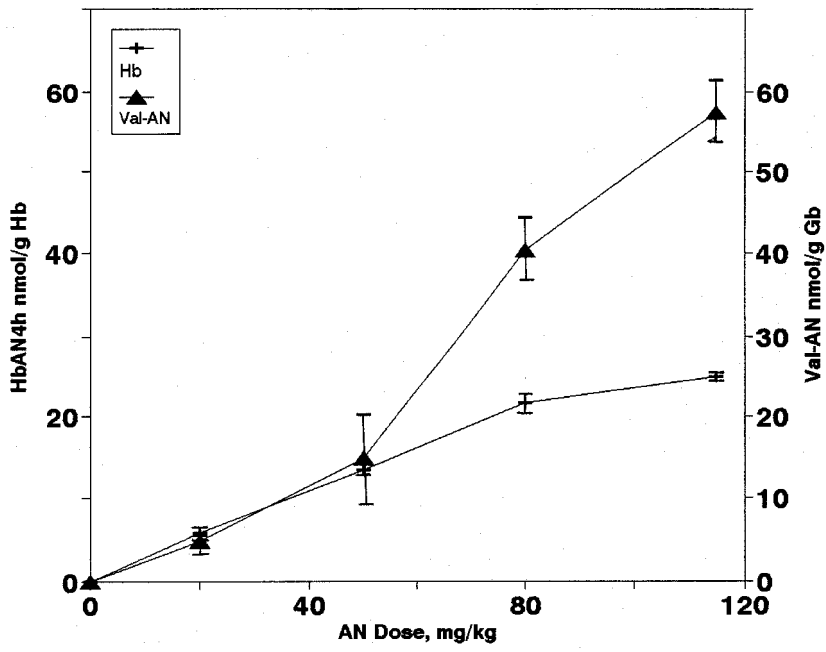

Figure 6. Comparison of Hb–AN adduct as HbAN4h by ELISA and valine–AN by GC–MS in rat blood obtained 1–2 h after sc administration of AN in a dose–response study. Each point represents the mean ± SD.

of exposure to AN. Since a rat given the LD90 dose had a maximum of about one AN bound per Hb, we assume a maximum of one valine–AN or one new antigenic site per Hb–AN may be formed *in vivo*. Thus, quantitation of Hb–AN by ELISA and of valine–AN by GC–MS are directly comparable. The ELISA response is linear up to the AN dose of 80 mg kg^{-1} as opposed to valine–AN where a break-point appears at 50 mg kg^{-1}. For lower AN doses up to 50 mg kg^{-1}, both ELISA and GC–MS show the same level of detection, but beyond that the valine–AN levels are two to three times higher than ELISA. This may be due to the presence of four terminal valines per Hb which may become increasingly alkylated at higher doses. It is also interesting to compare the valine–AN data of the present rat study using single injection with that reported by Tavares *et al.* (1996) for oral dosing of AN in the range of 1–10 mg kg^{-1}. From the linear regression equation given for their oral dose–response curve, we have calculated the expected level of valine–AN to be 0·70 and 2·36 nmol g^{-1} Gb for the AN doses of 3·3 and 10 mg kg^{-1}, respectively. The corresponding values for single injection in this study are 0·79 and 2·21 nmol g^{-1} Gb. Even the dosing routes and the internal standardization procedures for GC–MS analysis were different, the results are quite similar. Overall, the dose–time responses given by ELISA of an Hb–AN antigen and GC–MS analysis of valine–AN in rat blood are found to be appropriate surrogates of ^{14}C-counting. This rat study appears to have validated the new immunoassay of Hb–AN for biomonitoring of AN exposure.

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