Molecular Dosimetry of 2,4-Difluoroaniline in Humans and Rats by Determination of Hemoglobin Adducts

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Exposure to 2,4-difluoroaniline (DFA) was monitored by GC–MS of DFA adducts bound to hemoglobin (Hb). In two studies, involving 20 and 16 workers potentially exposed to low concentrations of DFA, median concentrations of 10 (range 1–83) and 20 (range 4–322) pmole/g Hb were found, respectively. For better interpretation of these results, the in vivo binding of DFA to Hb was investigated. DFA was administered orally at doses of 0, 0.078, 0.775, 7.75, and 77.5 μmole/kg/day, to 10 male and 10 female Fischer 344 rats for 10 consecutive days (2 rats/sex/dose group). A linear relation between dose and adduct concentration was observed. At the two lowest doses (0.078 and 0.775 μmole/kg/day) no methemoglobinemia was observed, but adducts could easily be measured. At these doses, the mean adduct levels were in the same range as found in the human studies. As yet, no occupational exposure limit for DFA has been established. The German biological tolerance value (BAT-value) for aniline was set at 7.2 nmole/g Hb. This BAT-value is based on the relation between methemoglobinemia and adduct formation. The amount of Hb binding by aniline and DFA was found to be similar in the rat. Assuming that this is also the case in humans, the BAT-value for aniline may tentatively be used for DFA as well. In both studies of occupationally exposed workers, the adduct levels were well below this BAT-value. — Environ Health Perspect 102(Suppl 6): 27–29 (1994)

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Introduction

2,4-Difluoroaniline (DFA) is produced as a fine chemical for use in pharmaceutical industries. The metabolic activation (Figure 1) of DFA starts with N-oxidation to the corresponding hydroxylamine which may be further oxidized in an autocatalytic cooxidation process with hemoglobin (Hb) yielding the nitrosoarene and methemoglobin (met-Hb) (1). Exposure to DFA may be monitored by assessment of the extent of methemoglobinemia. However, this method is rather insensitive and only suitable to detect very recent exposure. The nitrosoarene can react with the β-93 cysteine residue of Hb to a sulfenic acid amide. After isolation of Hb, the sulfenic acid amide will yield the original DFA upon mild alkaline hydrolysis (2). Determination of the amount of DFA adduct bound to Hb is a promising tool for biologic monitoring of potentially exposed workers (3,4). We measured the Hb adducts in workers potentially exposed to DFA by a modification of the gas chromatography–mass spectrometry (GC–MS) method developed by Bryant et al. (5,6). To gain more insight into the meaning of the results, the in vivo binding of DFA to Hb also was investigated in the rat.

Materials and Methods

Chemicals

DFA was obtained from Shell Chemicals (Stanlow, UK) and was >99% pure. All other reagents were purchased commercially and of the highest purity.

Study Population

Hb adducts of DFA in human blood were determined in two studies. In the first study, blood samples were obtained from 20 male workers potentially exposed to low DFA concentrations. In the second study, blood was collected from 16 male workers involved in the demolition of a plant that had produced DFA. Blood samples were collected by venapuncture from the antecubital vein in sterile 10 ml heparinized vacuum tubes and centrifuged. The plasma anduffy coat were removed and the erythrocytes were resuspended three times in 10 ml physiologic saline. The washed erythrocytes were resuspended with purified
water and lysed by subsequent freezing and thawing. Following sedimentation of membranes the solution was dialyzed during 48 hr against purified water. The amount of Hb was assessed spectrophotometrically using a Syxms K-1000 blood analyzer.

After addition of the internal standards 4-chloroaniline (pCA) and 25-aniline (d5A), aliquots of the dialysate were hydrolyzed by incubation with 0.1 M NaOH (final concentration) during 2 hr at room temperature and protected from light and analyzed by GC-MS (5,6).

Animal Experiments
SPF Fischer 344 rats were obtained from Charles River (Manso, UK). The rats had free access to a commercial diet ( Biosor, Manea, UK) and tap water and were kept on a 12 hr light and dark cycle. DFA was dissolved in a 1:1 (v/v) PEG 200:water mixture and administered by oral gavage (3 ml/kg) to 10 male and 10 female rats for 10 consecutive days. The doses used were 0, 0.078, 0.775, 7.75 and 77.5 μmole DFA/kg bw/day (2 rats/sex/dose group). On day 11, the animals were anesthetized by a lethal dose of sodium pentobarbitone, and blood samples were collected by cardiac puncture. Methemoglobinemia was assessed as reported previously (7) in a small portion of the blood. From the remainder, Hb was isolated and hydrolyzed, after addition of the internal standards according to Sambion and Neumann (8), and the amount of bound DFA was determined by GC-MS.

Gas Chromatography–Mass Spectrometry Analysis
The hydrolysates were prepared for analysis according to Stillwell et al. (6) with some minor modifications. Briefly, the DFA was extracted into dichloromethane (DCM), derivatized with pentafluoropropionic anhydride (PFPA) without the addition of trimethylamine. Because the PFPA derivative of DFA is very volatile, the DCM was evaporated carefully (the last milliliter by hand) with a gentle stream of N2 at room temperature. d5-Aniline was added as a second internal standard to check whether losses had occurred during evaporation, because its PFPA derivative has a volatility comparable to DFA-PFPA. The residue was dissolved in ethyl acetate by sonification and, after centrifugation, the clear supernatant was analyzed on a Hewlett Packard 5890 series II gas chromatograph equipped with a 5971A mass selective detector in the electron impact mode (electron energy fixed at 70 eV; ion source at 180°C). The GC column was a 60 m x 0.25-mm fused silica capillary coated with a 0.1-μm film of Durabond-1. Helium was used as carrier gas at a flow of 1 ml/min. A 1-μl sample was injected using an HP7673 autosampler in the splitless mode with an injector temperature of 250°C. The initial oven temperature was 60°C for 1 min, followed by a temperature ramp (20°/min) to 170°C, another ramp (10°/min) to 300°C, and an isothermal phase at 300°C of 5 min. The following ions were monitored with dwell times of 50 msec each: m/z 275 and 128 (DFA), m/z 244 (d5A), and m/z 273 (pCA). Calibration graphs were constructed by plotting the ratio of the integrated area of the ions of DFA and pCA against the ratio of the amounts DFA and pCA in a series of Hb samples prepared from control blood (human or rat) spiked with varying amounts of DFA and prepared in the same way as the unknown samples. The concentration of DFA in the unknown sample was calculated from this graph and divided by the concentration of Hb in the sample.

Results
Industrial Workers
In the first study (20 workers), the median concentration was 10 (range 1–83) pmole DFA/g Hb. In the second study, a median adduct concentration of 20 (range 4–322) pmole/g Hb was found (Figure 2). From the three workers in the second study with the highest adduct concentrations (101, 286, and 322 pmole/g Hb) another blood sample was collected 55 days later. The concentrations of Hb adducts in these samples were 39, 46, and 89 pmole DFA/g Hb, respectively. Assuming first-order elimination kinetics and that no exposure occurred between the collection of the blood samples, half-lives of the Hb adducts of 40, 21, and 30 days, respectively, were calculated.

Rat Studies
In the two lowest dose groups (0.078 and 0.775 μmole/kg/day), no increase in met-Hb as compared to the controls was observed (Figure 3). However, adducts could easily be detected. In the dose groups of 0.078 μmole/kg/day, mean adduct concentrations of 44 (males) and 76 (females) pmole/g Hb and in the dose groups of 0.775 μmole/kg/day, mean concentrations of 297 (males) and 435 (females) pmole/g Hb were measured. In both male and female rats, the relationship between dose and adduct concentration was linear (Figure 4). Methemoglobinemia and adduct formation were higher in the female animals. The Hb binding index [HBI = binding (mmole/mole Hb) total dose (mmole/kg)] (1) was 53 ± 8 for female and 32 ± 4 for male rats.

Discussion
Exposure to DFA may be monitored by measurement of the met-Hb content in blood or the urinary concentration of 2-amino-3,5-difluorophenol sulfate, a major metabolite of DFA (7). Both methods, however, reflect only exposure during the last 24 hr. Hb adducts of aryamines are thought to be rather stable in vivo and, consequently, to accumulate in the body. Hence, the measurement of Hb adducts is a promising tool for the biologic monitoring of aryamines in the case of intermit-
tent exposure and chronic exposure to low concentrations. We adapted the method developed by Bryant et al. for 4-aminobiphenyl (5,6) and applied it to workers potentially exposed to low concentrations of DFA and to rats orally dosed with DFA.

The detection limit of the method was about 0.5 nM (<5 pmol/g Hb) and Hb adducts of DFA were readily detectable both in human and rat blood samples. In nonexposed persons, no Hb adducts of DFA could be detected. DFA formed less Hb adducts in male than in female rats. The HBIs were 32 ± 4 and 53 ± 8 for male and female rats, respectively. This agrees well with the results of Sabbioni (2) who found a HBI of 32 ± 6 in female Wistar rats after a single dose of 0.5 mmol DFA/kg.

It appeared that the adduct concentrations in the human samples corresponded with the levels found in the rats from the two lowest dose groups that had been administered 0.78 and 7.75 μmol DFA/kg (total dose). As yet, no occupational limit value has been established for DFA. Aniline is the only arylamine for which a biological tolerance value (BAT-value) has been established. This BAT-value is based on the relation between met-Hb and adduct formation (9). It is known that the formation of met-Hb and Hb adducts are linked both for DFA and aniline (1,2). In the rat, the HBIs of aniline and DFA are of the same order of magnitude (2). Assuming that the relative binding of DFA and aniline is equal in rats and humans, the BAT-value for aniline may be applied to DFA as well. The BAT-value for Hb adducts of aniline was set at 7.2 nmol/g Hb (i.e., 100 μg/l whole blood) (9). In both studies of workers occupationally exposed to DFA, the adduct levels were well below this tentative BAT-value.

An important advantage of the determination of Hb adducts instead of met-Hb or urinary metabolites is their longer availability in the organism. Theoretically, their life span could equal the life-span of the erythrocytes (126 days in men and 65 days in the rat). Sabbioni (2) found a much shorter half-life (6.4 days) for DFA in the rat. Even though the half-lives that were calculated in this study (21–40 days) may be overestimated because some exposure to DFA might have occurred to the three persons between the collections of the two blood samples, they are much shorter than expected from the life-span of the erythrocyte. This suggests either that Hb with DFA bound to it is more rapidly degraded than native Hb or that the DFA sulfanamide bond to the β-93 cysteine may be hydrolyzed in vivo. Nevertheless, the stability of the adduct is large enough to enable its detection several weeks following an exposure to DFA that would not induce methemoglobinemia.

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