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SHORT COMMUNICATION

Biomonitoring of arylamines: haemoglobin adducts of aniline derivatives

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Aromatic amines are important intermediates in industrial manufacturing. They are used in a large number of products, such as pesticides, dyes, plastics and pharmaceuticals. The parent arylamines can be metabolically released from these arylamine-based compounds and form DNA and protein adducts after *N*-oxidation to *N*-hydroxy arylamines. Aromatic amine derivatives, including the industrial intermediates acetoacetanilide, acetoacet-m-xylidide and *N*-ethylaniline, were examined for their ability to form Hb adducts in rats as potential biomarkers of exposure. The haemoglobin binding indices (HBI = binding [mmol mol^{-1} Hb]/dose [mmol kg^{-1} body weight]) of the arylamines were determined 24 h after oral administration to female Wistar rats. The precipitated haemoglobin was dissolved in 0.1 M sodium hydroxide in the presence of internal standards. After hexane extraction the released arylamines were analysed by gas chromatography-mass spectrometry (GC-MS). For aniline released from acetoacetanilide an HBI of 15 and for 2,4-dimethylaniline released from acetoacet-m-xylidide an HBI of 0.129 were determined. The HBI of aniline released from *N*-ethylaniline was 45.

Keywords: haemoglobin adducts, arylamines, GC-MS, biomonitoring

Abbreviations: A, aniline; [D_5]A, 2,3,4,5,6-[D_5]-aniline; 24DMA, 2,4-dimethylaniline; [D_3]24DMA, 2,4-dimethyl-3,5,6-[D_3]-aniline; 245TMA, 2,4,5-trimethylaniline; HBI, haemoglobin binding index; GC-MS, gas chromatography-mass spectrometry; Hb, haemoglobin; SDS, sodium dodecyl sulphate; PFPA, pentafluoropropionic anhydride

Introduction

Aromatic amines are important intermediates in industrial manufacturing. The arylamines are used in a large number of products, such as pesticides, dyes, plastics and pharmaceuticals. Due to the broad range of applications there is ample evidence of human exposure to arylamines and amides (Searle and Teale 1990). Arylamines are activated through cytochrome P450-mediated oxidation to the *N*-hydroxy derivative in the liver (Beland and Kadlubar 1990). Microsomal flavin-containing monooxygenases (FMO) and prostaglandin H synthase (PHS) (Smith *et al.* 1991) are two other classes of enzymes which can *N*-oxidize arylamines and might be important in organs with small amounts of cytochrome P450, e.g. PHS in the bladder. Ziegler *et al.* (1988) reported the FMO-catalysed formation of *N*-hydroxy-4-aminobiphenyl from *N*-methyl-4-aminobiphenyl. The *N*-hydroxy arylamine may be further metabolized to *N*-sulfonyloxyarylamines, *N*-

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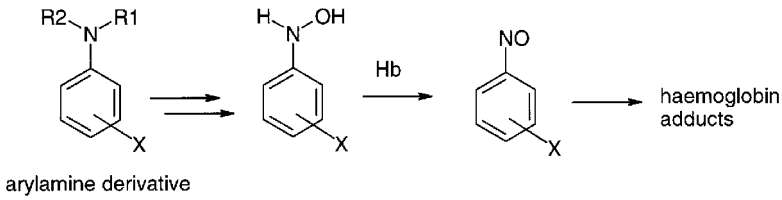

Figure 1. Metabolic activation of arylamine derivatives.

glucuronides or *N*-acetoxyarylamines. These highly reactive intermediates are responsible for the cytotoxic and genotoxic effects of this class of compounds. The metabolites can form adducts with DNA, tissue proteins and with the blood proteins albumin and haemoglobin in a dose-dependent manner (Kadlubar and Beland 1985, Beland and Kadlubar 1990). The *N*-hydroxy arylamine migrates into the blood stream and, in the erythrocyte, is cooxidized with the haem iron to generate methaemoglobin and a nitrosoarene. The nitrosoarene may react with the SH-group of a cysteine residue from the beta chain of the globin to form a sulfinic acid amide adduct (Ringe *et al.* 1988, Kazanis and McClelland 1992). These adducts are stable at physiological pH, but they are labile under mild basic and acidic conditions *in vitro*. In performing these hydrolyses the parent arylamine is released (Skipper and Tannenbaum 1990). Stable haemoglobin adducts tend to accumulate in a dose-related manner and persist for the lifetime of the protein. Haemoglobin adducts have been used in several studies to biomonitor exposed populations (Ward *et al.* 1996, Schütze *et al.* 1994, Sepai *et al.* 1995b, Skipper and Tannenbaum 1994).

Many consumer products are based on arylamines. Therefore, it is important to investigate the metabolic release of arylamines from such compounds. Rinde and Troll (1975) observed the reductive cleavage of azo dyes into the arylamine components during *in vivo* experiments. Haemoglobin binding of arylamines in humans after pesticide exposure was reported by Lewalter and Korallus (1986). The parent arylamines were metabolically released and covalently bound to haemoglobin, after oral application of urea and carbamate pesticides to female Wistar rats (Sabbioni and Neumann 1990, McMillan *et al.* 1990b). Haemoglobin adducts of 2,6-dimethylaniline were detected in rats and humans after administration of lidocaine (Bryant *et al.* 1994). Lidocaine, which is one of the more commonly used drugs in the United States, is a derivative of 2,6-dimethylaniline (26DMA). Toluenediamines were found in blood, urine, and redon drainage after implantation of polyurethane-covered breast prostheses. The toluenediamines are degradation products from the polyurethane foam which is used as coating for implants (Sepai *et al.* 1995a).

For the present study we investigated the haemoglobin binding of further important products and intermediates. We determined the haemoglobin binding of acetoaceticanilide, acetoacet-m-xylidide (*N*-(2,4-dimethylphenyl)-3-oxo-butyric acid amide), and *N*-ethylaniline (figure 2). Acetoaceticanilide and acetoacet-m-xylidide are intermediates in dye production. They are acetoacetic acid amides. The release and subsequent protein binding of arylamines from acetoacetic amides has not been studied yet. The influence of these amino substituents on the formation of Hb adducts was examined.

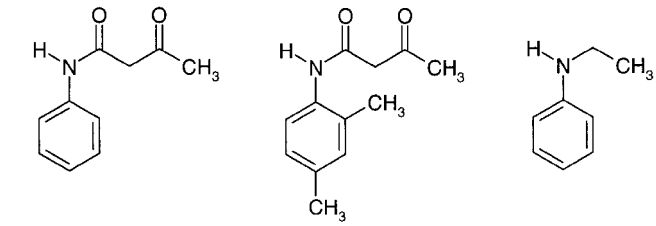

Figure 2. Acetoaceticanilide, acetoacet-m-xylidide, and *N*-ethylaniline.

Materials and methods

Chemicals

Aniline (33029) and *n*-hexane (34484) were purchased from Riedel-de Haën (Seelze, Germany), tricaprillin (7249) from Roth (Karlsruhe, Germany) and 2,4,5-trimethylaniline (P28660) from Pfaltz and Bauer (Waterbury, CT, USA), 2,3,4,5,6-[D_5]-aniline (17,569-2), 2,4-dimethylaniline (24,091-5), acetoacetanilide [*N*-phenyl-3-oxo-butyric acid amide] (A873-2), acetoacet-*m*-xylidide [*N*-(2,4-dimethylphenyl)-3-oxo-butyric acid amide] (36,862-8), and *N*-ethylaniline (42,638-5) from Aldrich (Steinheim, Germany), anhydrous sodium sulphate (71959) from Fluka (Neu-Ulm, Germany), sodium dodecyl sulphate (13760), sodium dihydrogen phosphate monohydrate (6346), water (15333), sodium hydroxide (6498) from Merck (Darmstadt, Germany) and pentafluoropropionic anhydride (PFPA) from Pierce (Oud-Beijerland, Netherlands). 2,4-[D_3]-Dimethylaniline ([D_3]24DMA) was synthesized according to Sabbioni and Beyerbach (1995). The numbers in parentheses are catalogue numbers.

Animal experiments

Female Wistar rats (190–225 g) were obtained from the Zentralinstitut für Versuchstierkunde (Hannover, Germany). They had free access to food (Altromin 1324) and water. Each of the test compounds (in tricaprillin) was given by gavage to groups of two animals. The administered dose was 0.5 mmol kg^{-1}. The control animals were given tricaprillin only. After 24 h the rats were anesthetized with diethyl ether and blood was taken by heart puncture with a heparinized syringe. The haemoglobin was isolated following a procedure published recently (Sabbioni and Beyerbach 1995).

Hydrolysis of haemoglobin and extraction of aniline

The hydrolyses were performed according to a published procedure (Sabbioni 1992). Forty milligrams of dried haemoglobin were dissolved in 3 ml of a 0.1 M sodium hydroxide solution containing 0.05 % SDS (alkaline hydrolysis). The surrogate internal standard was added to the haemoglobin solutions. [D_5]A (1 µg in 10 µl of hexane) was added to the hydrolysates from acetoacetanilide-exposed animals, [D_5]A (100 ng in 10 µl of hexane) to the hydrolysates from *N*-ethylaniline-exposed animals.

After 1 h of stirring at room temperature hexane (3 ml) was added and the mixture was vigorously stirred for 10 min. The samples were centrifuged for 5 min at 3000 g, frozen in liquid nitrogen and thawed to obtain a better phase separation. The hexane phase was collected and the hexane extraction was repeated. The combined organic extracts were concentrated under a gentle stream of nitrogen to a volume of 1 ml. Then 245TMA (1 µg in 10 µl of hexane) was added as volumetric internal standard (Sabbioni 1992).

All samples were extracted with hexane at neutral pH (3 ml of 0.1 M phosphate buffer pH 7.4 containing 0.05 % SDS (neutral hydrolysis)) and worked up as described above to establish whether the aromatic amines detected were covalently bound.

Hydrolysis of haemoglobin and extraction of 2,4-dimethylaniline

The hydrolyses were performed according to a published procedure (Sabbioni and Beyerbach 1995). Forty milligrams of dried haemoglobin were dissolved in 3 ml of a 0.1 M sodium hydroxide solution containing 0.05 % SDS (alkaline hydrolysis). The surrogate internal standard was added to the haemoglobin solutions: 10 ng [D_3]24DMA in 10 µl of hexane. After 1 h of stirring at room temperature hexane (3 ml) was added and the mixture was vigorously stirred for 10 min. The samples were centrifuged for 5 min at 3000 g, frozen in liquid nitrogen and thawed to obtain a better phase separation. The hexane phase was collected and the hexane extraction was repeated. The combined organic extracts were passed through pipettes filled with anhydrous sodium sulphate. The sodium sulphate was rinsed with hexane (2 ×1 ml). The arylamines in the dried organic phase were derivatized with PFPA (10 µl).

After 15 min at room temperature the solution was concentrated to dryness under a gentle stream of nitrogen and the residue was taken up in hexane (15 µl).

All samples were extracted with hexane at neutral pH (3 ml of 0.1 M phosphate buffer pH 7.4 containing 0.05 % SDS (neutral hydrolysis)) and worked up as described above to establish whether the aromatic amines detected were covalently bound.

GC-MS analysis

GC-MS analyses were performed on a Hewlett-Packard gas chromatograph (HP 5890A) interfaced to a mass spectrometer (HP 5988A). The arylamines were analysed by splitless injection (2 µl) onto a fused silica column DB 1701 (J & W Scientific, Folsom, CA, USA) 15 m ×0.25 mm internal diameter with a film thickness of 1 µm. The injector and transfer line temperature were set at 180 °C. Helium was used as carrier gas with a flow rate of 1.5 ml min^{-1}. In the electron-impact ionization (EI) mode the electron energy was 70 eV and the ion source temperature was 200 °C.

Aniline was analysed using the following conditions: The GC oven temperature was held at 50 °C for 1 min, then increased 50 °C min^{-1} to 160 °C. In the single-ion monitoring mode (SIM) the positive ions m/z = 65, 66 and 93 were monitored for A, m/z = 98 for $[D_5]$A, and m/z = 120 and 135 for 245TMA. Each of these ions was detected with a dwell time of 50 ms. Aniline eluted with a retention time of r_t = 2.94 min, $[D_5]$A with r_t = 2.94 min, and 245TMA with r_t = 4.20 min.

Each sample was analysed in duplicate with GC-MS. The released aniline was quantified with a qualibration curve obtained from standard solutions with $[D_5]$A and different amounts of aniline (0–1 µg). All peak areas were normalized against the volumetric internal standard 245TMA to monitor the recoveries. The recovery of $[D_5]$A was 38 ± 3 % and 36 ± 3 % from the basic and neutral hydrolysate.

The analyses of 24DMA were performed on a Hewlett Packard gas chromatograph (HP 5890II) equipped with an autosampler (HP 7276) and interfaced to a mass spectrometer (HP 5989A) (Sabbioni and Beyerbach 1995). The PFPA derivatives of the aromatic amines were analysed by splitless injection on to a fused silica capillary column (J & W; DB 1701; I.D. 0.25 mm; length 15 m, 1 µm film thickness) with a 0.25 mm ×1 m Methyl-Silyl retention gap (Analyt; Müllheim, Germany). The initial oven temperature, the injector temperature and the transfer line temperature were set at 50, 200 and 200 °C, respectively. The oven temperature was increased at a rate of 50 °C min^{-1} to 200 °C, held for 1.2 min, then heated at 50 °C min^{-1} to 240 °C and held for 3.2 min. Helium was used as carrier gas with a flow rate of 1.5 ml min^{-1}. For negative chemical ionization (NCI), with methane as the reagent gas, the source pressure was typically 160 Pa, the electron energy was 100 eV, the emission current was 300 µA and the source temperature was 200 °C. The retention time of 24DMA and $[D_3]$24DMA was 4.47 min.

Calculations

The partition coefficients log *P* were calculated online with the program ACD/Log*P* 1.0 offered by Advanced Chemistry Development, Inc., Toronto, Ontario, Canada.

Results

Haemoglobin adducts of acetoaceticanilide, acetoacet-*m*-xylidide, and *N*-ethylaniline in rats were determined. Blood was drawn 24 h after administration and was processed as described in the Materials and Methods section. GC-MS indicated the presence of the released parent aromatic amines for all three test compounds. In control animals no haemoglobin adducts of aniline and 2,4-dimethylaniline could be found.

Aniline from acetoaceticanilide and N-ethylaniline

$[D_5]$A was added as a surrogate internal standard prior to hydrolysis. The released aniline was quantified with a qualibration curve obtained from standard solutions with $[D_5]$A and different amounts of aniline. For verification whether the aniline found in the alkaline hydrolysis is covalently bound to haemoglobin, the haemoglobin is hydrolysed at physiological pH (neutral hydrolysis). The identity of the extracted aniline was determined by GC-MS. In the GC-MS analysis the retention time and the ratio of the positive ions m/z = 93, 66 and 65 of the standard aniline and of the aniline extracted from haemoglobin are identical.

In the samples from acetoacetanilide treated rats, we found 550 ± 37 ng and 114.7 ± 7.14 ng aniline per 40 mg of Hb after alkaline and neutral hydrolysis, respectively. For the calculation of the HBI the values from the neutral hydrolysis were subtracted from the values from alkaline hydrolysis. This resulted in an HBI of 15 for aniline released from acetoacetanilide and an HBI of 45 for aniline released from *N*-ethylaniline. Expressed as percentage of the dose bound to Hb, the amount of aniline bound to haemoglobin was 0.22 % of the acetoacetanilide dose and 0.66 % of the *N*-ethylaniline dose.

2,4-Dimethylaniline from acetoacet-m-xylidide

[D_3]24DMA was chosen as internal standard for the analysis of 24DMA. In the Hb hydrolysates from acetoacet-*m*-xylidide treated rats we found 4.83 ± 1.3 ng and 0 ng 24DMA per 40 mg of Hb after alkaline and neutral hydrolysis respectively. The part of 24DMA bound to Hb was 0.018 % of the applied total dose. This resulted in an HBI of 0.129 for 24DMA released from acetoacet-*m*-xylidide. For structural confirmation, haemoglobin extracts after base hydrolyses were analysed without PFPA derivatization using GC-MS with electron impact ionization according to a procedure published previously (Sabbioni 1994). The retention time and the ratio of the positive ions m/z = 106, 120 and 121 of the standard 24DMA and of the 24DMA extracted from haemoglobin are identical.

Discussion

A wide variety of enzymes catalyse the *N*-dealkylation of *N*-alkylamines. The list includes flavoproteins, copper proteins, and non-haem iron proteins (Walsh 1979, Guengerich 1990). A number of possible mechanisms of *N*-dealkylation by haemoproteins have been postulated. Liver microsomes oxygenate *N*-alkylanilines to the *N*-hydroxy derivatives and *N*,*N*-dialkylanilines to the *N*-oxides which undergo subsequent *N*-dealkylation (Hlavica and Kiese 1969). Okazaki and Guengerich (1993) postulated that after enzymatic hydrolysis the *N*-alkyl group is cleaved as carbonyl compound. Hepatic acylamidases hydrolyse *N*-acyl arylamines to the parent arylamines. For example, the major metabolic pathway of the arylamine-based pesticide propanil in microsomal incubations was acylamidase-catalysed hydrolysis to 3,4-dichloroaniline (McMillan *et al.* 1990a, Singleton and Murphy 1973). The enzymes responsible for the catalysis of the deacetylation belong to the general class of arylacylamide amidohydrolases (EC 3.5.1.13) and are usually referred to as arylamidases or deacetylases (Weber 1985).

Haemoglobin binding of products based on aniline

The haemoglobin binding of aniline released from acetoacetanilide was compared with the haemoglobin binding from similar compounds (table 1). The highest binding was found after administration of nitrobenzene to rats (Sabbioni 1994, Suzuki *et al.* 1989). The amount of aniline bound to rat haemoglobin after the administration of acetoacetanilide is comparable with the binding after the administration of aniline. The Hb binding of acetoacetanilide is 1.5-fold lower than the Hb binding of aniline. After acetoacetanilide administration, the biologically available amount of *N*-hydroxyaniline in the erythrocytes has to be

Table 1. Comparison of HBI values after oral administration to rats.

Compound	AA[1)]	HBI[2)]	log *P* calculated
acetoacetanilide	A	15 ± 1	0.85 ± 0.29
aniline	A	22 ± 3 [a]	0.94 ± 0.19
nitrobenzene	A	79 ± 10 [c]	1.95 ± 0.20
N-methylaniline	A	16 ± 2 [a]	1.60 ± 0.21
N-ethylaniline	A	45 ± 5	2.13 ± 0.21
N,*N*-dimethylaniline	A	11 ± 2 [a]	2.33 ± 0.22
propham	A	2.4 ± 0.3 [b]	2.65 ± 0.28
acetoacet-*m*-xylidide	24DMA	0.129 ± 0.035	1.77 ± 0.29
2,4-dimethylaniline	24DMA	2.3 ± 1.0 [a]	1.86 ± 0.20
2,4-dimethylnitrobenzene	24DMA	0 [d]	2.87 ± 0.21

[1)] AA = haemoglobin bound arylamine, [2)]HBI = [(mmol AA mol^{-1} Hb)/(mmol AA kg^{-1} body weight)]
[a)] (Birner and Neumann 1988), [b)] (Sabbioni and Neumann 1990), [c)] (Suzuki *et al.* 1989), [d)] (Sabbioni 1994)

1.5-fold lower than after aniline administration. The haemoglobin binding is attributable to the metabolic activation of arylamine derivatives via *N*-hydroxy arylamines. The HBI allows a statement about the metabolic steps of the formation of *N*-hydroxy arylamines. However, it cannot be concluded from the HBI, that the main part of the acetoacetanilide is metabolized to aniline. A prediction of the total metabolism of chemicals cannot be made on the basis of haemoglobin binding indices. Only 0.22 % of the applied acetoacetanilide dose bound to Hb.

The haemoglobin binding of *N*-ethylaniline is 2-fold higher than the HBI of aniline and 3-fold higher than the HBI of *N*-methylaniline. *N*-Ethylaniline is the most lipophilic compound of the three (see log *P* values in table 1) and therefore better bioavailable. In addition, the *N*-hydroxyaniline can result from two possible metabolic pathways: (i) oxidative demethylation and oxidation with FMO as described by Ziegler *et al.* (1988) or (ii) demethylation and subsequent oxidation with CYP1A2. The relative velocity of the *N*-dealkylation is dependent on the lipid solubility and the basicity of the aniline derivatives (Schmidt *et al.* 1973). In general, haemoglobin binding does not correlate with the lipophilicity of the administered arylamines (Sabbioni 1992). For the present work the partition coefficients were calculated with the ACD/Log*P* program, since the experimental log *P* values (Hansch and Leo 1979) were not available for all compounds. The experimental log *P* values correspond well to the calculated values. For the present study, haemoglobin binding of the metabolically released arylamines did not correlate with the lipophilicity of the administered compounds (table 1).

In the case of the *N*,*N*-dimethylaniline two dealkylations have to proceed for the release of aniline. This reduces most likely the formation of aniline. Gorrod *et al.* (1979) observed in experiments with rabbit liver microsomes, that the methyl groups are cleaved sequentially and not simultaneously. In the carbamate pesticide propham, an amide bond has to be cleaved for the release of aniline.

Generally, the haemoglobin binding after administration of nitroarenes is lower compared to haemoglobin binding after administration of the corresponding arylamines (Sabbioni 1994). The nitrobenzene is an exception, additionally to 4-fluoro-, 2-chloro- and 3-chloro nitrobenzene. For these compounds more *N*-hydroxy arylamines is available through the reduction of nitroarenes as through the *N*-oxidation of arylamines.

Haemoglobin adducts of 2,4-dimethylaniline

The haemoglobin binding of 24DMA released from acetoacet-*m*-xylidide is 0.129 and about 20-fold lower than the parent arylamine (Birner and Neumann 1988). In contrast, after administration of the nitro compound 2,4-dimethylnitrobenzene, no Hb adducts of 24DMA could be detected (Sabbioni 1994). This can be explained by a preferred oxidation of the alkyl side chains as main metabolic pathway (Chism and Rickert 1989, DeBethizy and Rickert 1984, Rickert 1987).

Conclusions

In the present study we determined the haemoglobin binding of arylamine derivatives with *N*-alkyl and *N*-acyl groups. The metabolic release of arylamines and bioavailability of the corresponding *N*-hydroxy arylamine was shown. This study supports the significance of Hb adducts as biomarkers for exposure to arylamine products.

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