Haemoglobin adducts of aromatic amines: diamines and polyaromatic amines

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Abstract

Aromatic amines and nitroarenes are important antioxidants and intermediates in the synthesis of dyes, pesticides and plastics. In the present paper we introduce methods for the synthesis of deuterated standards: 3-[^{2}H$_8$]aminofluoranthene, 3,3′-dimethyl-[^{2}H$_4$]benzidine, [^{2}H$_4$]benzidine, N'-acetyl-[^{2}H$_4$]benzidine, 2,4-[^{2}H$_6$]toluenediamine, 2,6-[^{2}H$_6$]toluenediamine. These standards have been used for the quantification of haemoglobin adducts of diamines and polyaromatic amines. Haemoglobin was hydrolysed in 0.1 M sodium hydroxide and the hydrolysate extracted with dichloromethane. The extracts were derivatised with heptafluorobutyric anhydride and analysed by GC–MS with negative chemical ionisation. In one run up to 15 aromatic amines can be determined: 6-aminochrysene, 3-aminofluoranthene, 2-aminofluorene, 1-aminopyrene, benzidine, 3,3′-dichlorobenzidine, 3,3′-dimethoxybenzidine, 3,3′-dimethylbenzidine, 3,3′-methylenedianiline, 4,4′-methylenedianiline, N'-acetyl-benzidine, N'-acetyl-4,4′-methylenedianiline, 4,4′-methylene bis(2-chloroaniline), 2,4-toluenediamine and 2,6-toluenediamine. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Aromatic amines and nitroarenes are important antioxidants and important intermediates in the synthesis of dyes, pesticides and plastics. They are environmental pollutants and are found in lakes, rivers [1] and in soil [2]. Nitroarenes are air pollutants as they are byproducts of diesel engines [3]. Aromatic amines and nitroarenes are considerable carcinogenic components in cigarette smoke [4].

It is essential in occupational health and for risk assessment to have fast and reliable methods to monitor exposure of humans. Haemoglobin adducts are suitable biomarkers for the exposure to aromatic amines. They reflect exposure over the last 4 months. Haemoglobin adducts are used as marker for internal exposure, as markers for the metabolic activation of the amino group and with carcinogenic amines as markers for the dose in the target organ. The presence of haemoglobin adducts indicates that the potential toxic or genotoxic metabolite — the N-hydroxy arylamine — is bioavailable in the erythrocytes and in tissues.

Aromatic amines and nitroarenes are metabolised and their metabolites, N-hydroxy arylamines and nitrosoarenes, react with DNA, proteins and gluta-

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thione. The resulting damage to the biomolecules is often responsible for the toxic or genotoxic effects of aromatic amines. The haemoglobin adduct formation involves the reaction of the metabolite nitrosoarene with cysteine residues of haemoglobin to form a sulfinic acid amide [5]. At neutral pH the sulfinic acid amide adducts are stable, but they are readily hydrolysed under basic conditions, releasing the parent aromatic amine [6,7]. This characteristic was used in the presented study. The structure of the resulting sulfinamide adduct between 4-nitrosobiphenyl and human haemoglobin in vitro, was examined by X-ray analysis. The nitrosoarene reacted with the thiol group of cysteine 93β [8].

We have published a method for the determination of haemoglobin adducts of monocyclic arylamines [9]. There are various diamines and polyaromatic amines, which are important in the workplace or in the environment. They form haemoglobin adducts, but could not be detected by the published method. Therefore, we developed a method to analyse haemoglobin adducts of diamines and polyaromatic amines in humans. This included the synthesis of some internal standards.

2. Materials and methods

2.1. Chemicals

6-Aminochrysene (6Chry) (A4705-2), 3-aminofluoranthene (Afl) (A5540-3), 2-aminofluorene (2AF) (A5550-0), 1-aminopyrene (1AP) (A7790-3), 65% $^2HNO_3$ (17673-7), 98% $^2H_2SO_4$ (17679-6), 3,3′-methylenedianiline (3,3′MDA) (37826-7), [$^2H_8$]toluene (43438-8), 2,4-toluenediamine (24TDA) (10191-5), 2,6-toluenediamine (26TDA) (14811-3) were purchased from Aldrich (Steinheim, Germany); ammonium formate (09739), benzidine (Bz) (12125), 3,3′-dimethylbenzidine (DmBz) (89580), 3,3′-dimethoxybenzidine (DmeBz) (33430), 4-methylaniline (89630), anhydrous sodium sulphate (71959), 4,4′-methylenedianiline (MDA) (32950) recrystallised from water, bovine haemoglobin (51290), and heptafluorobutyric anhydride (HFBA), 10% Pd on carbon (75990) from Fluka (Neu-Ulm, Germany); sodium dihydrogenphosphate

monohydrate (6346), dichloromethane (SupraSolv, 106054), ethyl acetate (SupraSolv, 110972), methanol (SupraSolv, 106011), sodium hydroxide (6498), and water for the HPLC (15333) from Merck (Darmstadt, Germany); n-heptane from Malinckrodt Baker (Griesheim, Germany), Celite Hyflo Super Cel® by Johns Manville (0005) from Roth (Karlsruhe, Germany); 4,4′-methylene bis(2-chloroaniline) (MOCA) from Dr. Ehrenstorfer (Augsburg, Germany). The numbers in parentheses are the catalogue numbers.

The internal standard (I.S.) 2-[$^2H_9$]aminofluorene ([$^2H_9$]2AF) (D-3804) was purchased from ABCR, CDN-Isotopes (Karlsruhe, Germany) and 3,3′-dichloro-[$^2H_6$]benzidine ([$^2H_6$]DcBz) from Cambridge Isotope Labs. (Andover, MA, USA).

N-Acetyl-4,4′-methylenedianiline (AcMDA), 4,4′-[$^2H_4$]methylenedianiline ([$^2H_4$]MDA) and N-acetyl-4,4′-[$^2H_4$]methylenedianiline ([$^2H_4$]AcMDA) were synthesised according to [10].

N′-Acetyl-benzidine (AcBz), 3-[$^2H_8$]aminofluoranthene ([$^2H_8$]Afl), 3,3′-dimethyl-[$^2H_4$]benzidine ([$^2H_4$]DmBz), [$^2H_4$]benzidine ([$^2H_4$]Bz), N′-acetyl-[$^2H_4$]benzidine ([$^2H_4$]AcBz), 2,4-[$^2H_6$]toluene diamine ([$^2H_6$]24TDA) and 2,6-[$^2H_6$]toluene diamine ([$^2H_6$]26TDA) were synthesised as described below.

All other chemicals were bought from Aldrich and Fluka. 3,3′-Dichlorobenzidine (DcBz) is now available as a very dilute solution from Promochem (Wesel, Germany).

2.2. Syntheses of the deuterated standards

2.2.1. [$^2H_4$]Benzidine ([$^2H_4$]Bz)

Bz (2.0 g, 10.86 mmol) was dissolved in ^2H_2O (2.4 g) and ^2HCl (5.1 g) and sealed in an ampoule. The mixture was heated in an autoclave at 200°C for 17 h. After removal of the solvents the residue was taken up in ^2H_2O (2 ml) and ^2HCl (4 ml) and heated in an autoclave at 200°C for 18 h. After evaporation to dryness the remaining substance was dissolved in ^2H_2O (2 ml) and ^2HCl (4 ml) and heated in an autoclave at 200°C for 66 h. The residue was made basic with a sodium hydroxide solution and extracted with ethyl acetate. The organic layer was dried over sodium sulfate and evaporated yielding [$^2H_4$]-benz-

idine ($[^2H_4]$Bz) (1.8 g, 9.56 mmol, 88%) as a powder.

MS (EI, 70 eV), m/z (%): 192(14), 191(14), 190(21), 189(46), 188(100, $[^2H_4]$Bz), 187(20), 186(5), 185(2), 184(1, $[^2H_0]$Bz). HFBA-derivative MS (NCI/methane), m/z (%): 565(3), 564(16), 563(17), 562(24), 561(53), 560(100, $[^2H_4]$Bz-HFBA – HF]), 558(0.4), 556(0.15, $[^2H_0]$Bz-HFBA – HF]). ^{1}H-NMR (250 MHz, C^2HCl_3): [ppm]=3.68 (br. s, 4 H, NH_2), 6.70 (s, 4 H, *ortho*-Hs), 7.33 (s, 4 H, *meta*-Hs). ^{13}C-NMR (63 MHz, C^2HCl_3): [ppm]= 115.41 (*ortho* to NH_2, C-2), 127.25 (*meta* to NH_2, C-3), 131.77 (s, C-4), 144.91 (s, C-1).

2.2.2. 3,3′-$[^2H_4]$Dimethylbenzidine ($[^2H_4]$DMBz)

DMBz (1 g, 4.71 mmol) was dissolved in ^2H_2O (4 ml) and ^2HCl (6 ml) and sealed in an ampoule. The mixture was heated in an autoclave at 250°C for 19 h. After removal of the solvents the residue was taken up in ^2H_2O (4 ml) and ^2HCl (4 ml) and heated in an autoclave at 250°C for 20 h. After evaporation to dryness the remaining substance was dissolved in ^2H_2O (4 ml) and ^2HCl (4 ml) and heated in an autoclave at 250°C for 21 h. The residue was made basic with a sodium hydroxide solution and extracted with ethyl acetate. The organic layer was dried over sodium sulfate and evaporated. The product was purified by a Kugelrohr distillation at 180°C yielding $[^2H_4]$DMBz (891 mg) as a white powder. MS (EI, 70 eV), m/z (%): 219(16), 218(74), 217(100), 216(56), 215(10), 201(10), 200(10), 185(10). No signal visible at 212 for $[^2H_0]$DmBz. HFBA-derivative: MS (NCI/methane), m/z (%): 592(4), 591(21), 590(78), 589(100), 588(41). No signal visible for $[^2H_0]$DmBz at 584.

2.2.3. 3-$[^2H_8]$Aminofluoranthene ($[^2H_8]$Afl)

Afl (610 mg, 2.81 mmol) was deuterated twice in ^2H_2O (2 ml) and ^2HCl (2 ml) at 200°C for 22 h. The residue was made basic with a sodium hydroxide solution and extracted with ethyl acetate. The organic layer was dried over sodium sulfate and evaporated. The residue was recrystallised from benzene yielding $[^2H_8]$Afl (444 mg, 70%) as a yellow powder.

MS (EI, 70 eV), m/z (%): 227(11), 226(70), 225(100, $[^2H_8]$Afl), 224(91), 223(70), 22(37),

221(16), 220(9), 219(6), 218(4), 217(2, $[^2H_0]$Afl). HFBA-derivative: MS (NCI/methane), m/z (%): 403(15), 402(76), 401(100), 400(93), 399(70), 398(36), 397(13), 396(8), 395(6), 394(6), 393(2, $[^2H_0]$Afl), 382(3), 381(21), 380(22), 379(17), 378(14), 377(5).

2.2.4. Acetylation of $[^2H_4]$Bz and Bz

$[^2H_4]$Bz or Bz (4 mmol) was dissolved in ethyl acetate (30 ml) and triethylamine (4.8 mmol). At 0°C, acetic acid anhydride (6 mmol) was added over a period of 30 min. When no benzidine could be detected by TLC (CH_2Cl_2–MeOH, 20:1) the reaction was quenched by addition of 1 M sodium hydroxide (25 ml). The mixture was filtered over a glass frit and the organic layer was concentrated. The resulting white solid was purified by flash chromatography on silica gel with CH_2Cl_2–MeOH (20:1) yielding $[^2H_4]$AcBz or AcBz. HFBA-derivative: MS (NCI/methane), m/z (%): 411(3), 410(14), 409(15), 408(22), 407(50), 406[100, ($[^2H_4]$AcBz-HFBA – HF)], 405(8), 404(4), 403(2), 402(0.8, $[^2H_4]$AcBz), 364(21), 350(30), 349(60), 348(18).

2.2.5. 2,4-$[^2H_6]$Dinitrotoluene ($[^2H_6]$24DNT) and 2,6-$[^2H_6]$dinitrotoluene ($[^2H_6]$26DNT)

$^2H_8$-toluene (1 g, 9.98 mmol) was added to a mixture of concentrated $^2HNO_3$ (65%, 3.2 ml) and 2.25 ml $^2H_2SO_4$ (98%, 2.25 ml) in 15 min to keep the temperature below 30°C. After 90 min the temperature was raised to 70°C. The reaction was monitored by tlc (silica-gel, heptane–ethyl acetate, 85/15, R_f values: 0.48 for 2-nitrotoluene, 0.45 for 4-nitrotoluene, 0.23 for 2,4-dinitrotoluene, 0.3 for 2,6-dinitrotoluene, 0.34 for 2,4,6-trinitrotoluene). After 5 h the reaction mixture was stirred for further 43 h at room temperature, and then poured on ice water (10 ml) and dichloromethane (10 ml). The organic phase was collected from a separating funnel. The water phase was extracted another time with dichloromethane (10 ml). The collected organic phases were then washed twice with water (10 ml), evaporated to dryness under reduced pressure. The residue was recrystallised from hot methanol. 2,4-$[^2H_6]$Dinitrotoluene was obtained with 50% (990 mg) yield. The supernatant was evaporated and taken up in 5 ml heptane–ethyl acetate (50:50) and

purified by flash chromatography (2.5×25 cm) on silica-gel (silica-gel 60, 40–63 μm). The compounds eluted with a pressure of 0.3 bar with heptane–ethyl acetate (85:15). Fractions of 2 ml each were collected. Up to fraction 90 only mononitrotoluenes eluted. From fraction 90 to 105 [$^2H_6$]26DNT was collected. After evaporation of the solvent 117 mg (5.9%) [$^2H_6$]26DNT were obtained. Fractions 106–164 contained a mixture of [$^2H_6$]24DNT and [$^2H_6$]26DNT. From fractions 165–195 pure [$^2H_6$]24DNT could be collected. After evaporation of the solvents, the residue was recrystallised from methanol: [$^2H_6$]24DNT (144.5 mg, 7.3%).

[$^2H_6$]24DNT: MS (EI, 70 eV), *m/z* (%): 188(5), 170(77), 94(100), 66(65). MS (NCI/methane): 189(8), 188(100), 172(13), 158(8).

[$^2H_6$]26DNT: MS (EI, 70 eV), *m/z* (%): 188(2), 170(75), 94(100), 66(72). MS (NCI/methane): 189(8), 188(100), 172(7), 158(10).

2.2.6. 2,4-[$^2H_6$]Toluenediamine ([$^2H_6$]24TDA) and 2,6-[$^2H_6$]toluenediamine ([$^2H_6$]26TDA)

[$^2H_6$]Dinitrotoluene (0.24 mmol) in dry methanol (Fluka, No. 65542), 10% Pd/C (30 mg) and ammonium formate (146 mg) was reacted for 5 min at room temperature under nitrogen. The reaction mixture was then filtered over celite. After washing of the celite with methanol (1 ml) the residue was evaporated, taken up in water (3 ml) and extracted with CHCl$_3$ (3×3 ml). The combined organic phases were dried over Na$_2$SO$_4$ and evaporated to dryness.

[$^2H_6$]24TDA and [$^2H_6$]26TDA were obtained with a yield of 83 and 87%, respectively.

[$^2H_6$]24TDA: MS (EI, 70 eV), *m/z* (%): 128(7), 127(12), 126(100), 125(38), 124(53), 123(3), 122(1, [$^2H_0$]24TDA), 121(0.6). [$^2H_6$]26TDA: MS (EI, 70 eV), *m/z* (%): 129(3), 128(21), 127(68), 126(100), 125(34), 124(14), 123(1), 122(0.8, [$^2H_0$]26TDA), 109(15), 108(20), 98(18), 97(17), 81(14), 82(11), 80(15). HFBA-derivatives: MS (NCI/methane), *m/z* (%): [$^2H_6$]24TDA: 518(1), 517(3), 516(1), 499(19), 498(100), 479(12), 478(73), 458(16). [$^2H_6$]26TDA: 519(2), 518(4), 517(4), 501(6), 500(45), 499(100), 498(97), 481(3), 480(6), 479(12), 478(14).

2.3. Stock solutions and spiking solutions

Stock solutions of the aromatic amines at concentrations of 10 g/l were prepared by dissolving 100 mg of each aromatic amine (2AF, Afl, 1AP, 6Chry, AcBz, AcMDA, Bz, DcBz, DmBz, DmeBz, 3,3′MDA, MDA, MOCA, 24TDA and 26TDA) in 10 ml ethyl acetate. The solutions were stable at −35°C for more than 1 year.

AcMDA (240 μl) of the stock solution and 100 μl of each stock solution of AcBz, 2AF, Afl, 1AP, Bz, 6Chry, DcBz, DmBz, DmeBz, 3,3′MDA, MDA, MOCA, 24TDA, and 26TDA were transferred into a 10-ml volumetric flask and the volume was made up with ethyl acetate. The concentration of this solution were 240 ng/μl for AcMDA and 100 ng/μl for AcBz, 2AF, Afl, 1AP, 6Chry, Bz, DcBz, DmBz, DmeBz, 3,3′MDA, MDA, MOCA, 24TDA, and 26TDA.

Aliquots of this solution were further diluted in ethyl acetate to prepare the spiking solution I (240 pg/μl for AcMDA and 100 pg/μl for AcBz, 2AF, Afl, 1AP, 6Chry, Bz, DcBz, DmBz, DmeBz, 3,3′MDA, MDA, MOCA, 24TDA, and 26TDA), the spiking solution II (48 pg/μl for AcMDA and 20 pg/μl for AcBz, 2AF, Afl, 1AP, 6Chry, Bz, DcBz, DmBz, DmeBz, 3,3′MDA, MDA, MOCA, 24TDA, and 26TDA) and the spiking solution III (12 pg/μl for AcMDA and 5 pg/μl for AcBz, 2AF, Afl, 1AP, 6Chry, Bz, DcBz, DmBz, DmeBz, 3,3′MDA, MDA, MOCA, 24TDA, and 26TDA).

2.4. Internal standard stock solutions

Stock solutions of the deuterated aromatic amines at concentrations of 1 g/l were prepared by dissolving 10 mg of each standard ([$^2H_4$]AcMDA, [$^2H_4$]AcBz, [$^2H_9$]2AF, [$^2H_8$]Afl, [$^2H_4$]Bz, [$^2H_6$]DcBz, [$^2H_4$]DmBz, [$^2H_4$]MDA, [$^2H_6$]24TDA and [$^2H_6$]26TDA) in 10 ml ethyl acetate.

[$^2H_4$]AcMDA (240 μl) of the stock solution, and 100 μl each of [$^2H_4$]AcBz, [$^2H_9$]2AF, [$^2H_8$]Afl, [$^2H_4$]Bz, [$^2H_6$]DcBz, [$^2H_4$]DmBz, [$^2H_4$]MDA, [$^2H_6$]24TDA and [$^2H_6$]26TDA were transferred into 1-ml volumetric flasks and the volume was made up with ethyl acetate. The concentration of this solution were 240 ng/μl for [$^2H_4$]AcMDA and 100 ng/μl for [$^2H_4$]AcBz, [$^2H_9$]2AF, [$^2H_8$]Afl, [$^2H_4$]Bz, [$^2H_6$]DcBz, [$^2H_4$]DmBz, [$^2H_4$]MDA, [$^2H_6$]24TDA and [$^2H_6$]26TDA.

Aliquots of this solution were further diluted in ethyl acetate to prepare the I.S. solution with a

concentration of 240 pg/μl for [^{2}H$_4$]AcMDA and 100 pg/μl for [^{2}H$_4$]AcBz, [^{2}H$_9$]2AF, [^{2}H$_8$]Afl, [^{2}H$_4$]Bz, [^{2}H$_6$]DcBz, [^{2}H$_4$]DmBz, [^{2}H$_4$]MDA, [^{2}H$_6$]24TDA and [^{2}H$_6$]26TDA).

2.5. Isolation of haemoglobin

Haemoglobin was isolated from freshly drawn, heparinised blood (in 10 ml EDTA–Monovetten from Sarstaedt) following a procedure published recently [9].

2.6. Hydrolysis and extraction of haemoglobin

Dried haemoglobin (200 mg) was dissolved in 4 ml 0.1 *M* sodium hydroxide solution in the presence of 24 μg 4-methylaniline and 10 μl of the I.S. solution. The solution was shaken in a water bath for 1 h at room temperature. The mixture was extracted with dichloromethane (6 ml) using a vortex mixer for 3 min. The samples were centrifuged for 5 min at 3000 *g*, frozen in liquid nitrogen and thawed to obtain a better phase separation. The organic layer was passed through a Pasteur pipette filled with anhydrous sodium sulphate. The sodium sulphate was rinsed with dichloromethane (1 ml). The combined organic extracts were derivatised with HFBA (2 μl). After 10 min at room temperature 4-methylaniline (20 μg) in 50 μl methanol were added and then the solutions were dried at 40°C using a speed vacuum centrifuge (Speed-Vac, Uniequip). The residue was transferred into microinserts with 3×50 μl ethyl acetate and dried again. For analysis the final extracts were taken up in 15 μl ethyl acetate.

2.7. GC–MS analysis

GC–MS analyses were performed on a Hewlett-Packard (Waldbronn, Germany) gas chromatograph (HP 5890A) equipped with an autosampler (HP 7276) interfaced to a mass spectrometer (HP 5989A). The arylamines were analysed by splitless injection (2 μl) onto a fused-silica column Rtx5-MS (Restek) 15 m×0.25 mm I.D. with a film thickness of 0.25 μm equipped with a retention gap (1 m×0.25 mm, methyl-silyl deactivated) from Analyt (Mühlheim, Germany). The initial oven temperature, the injector temperature and the transfer line temperature were

set at 80, 320 and 280°C, respectively. The oven temperature was held at 80°C for 1 min, increased at a rate of 50°C/min to 200°C, held for 1 min, then increased at 30°C/min to 260°C and held for 1.5 min, and then increased at a rate of 50°C/min to 300°C and held at this temperature for 1.5 min. Helium was used as carrier gas with 90 kPa column inlet pressure at 80°C. In the negative chemical ionization mode (NCI) the electron energy was 240 eV and the ion source temperature was 250°C. Methane was used as reactant gas at typically 1.5 Torr.

The mass spectrometer was operated in the selected ion monitoring (SIM) detection mode. The fragments with *m/z* 357, 366, 474, 494, 498, 479 were recorded between 2.50 and 5.05 min with a dwell time of 50 ms, the fragments with *m/z* 373, 393, 401, 556, 560, 570, 574, 584, 589, 616, 624, 630, and 638 were recorded between 5.05 and 6.80 min with a dwell time of 30 ms and the fragments with *m/z* 402, 406, 416, 419 and 420 were recorded between 6.80 and 8.00 min with a dwell time of 80 ms.

In Table 1 the retention times and the monitored mass fragments for the HFBA derivatised amines and their I.S.s are listed. The separation of the amines can be seen in a representative chromatogram (Fig. 1).

3. Results

3.1. Synthesis of the deuterated standards

The amine was dissolved in a mixture of ^{2}H$_2$O and 37% ^{2}HCl, sealed in an ampoule and placed in an autoclave for 18 h at 200°C (for Bz and Afl) or 250°C (for DmBz). After removal of the solvents the same procedure was repeated at least once in the same manner. The resulting solution was made basic with aqueous sodium hydroxide and extracted with ethyl acetate. The organic layer was dried over sodium sulfate and evaporated yielding [^{2}H$_4$]Bz, [^{2}H$_8$]Afl and [^{2}H$_4$]DmBz as a powder. [^{2}H$_4$]DmBz was purified by a Kugelrohr distillation at 180°C.

The degree of deuteration was determined in C^{2}HCl$_3$ by ^{1}H NMR spectroscopy on a Bruker AC 250 (250 MHz) instrument. The signals of the methyl and amine protons were used as reference for

Table 1
Retention times, main mass fragments of analysed HFBA–amines and their I.S.s in the electron impact ionisation and negative chemical ionisation mode

| Amine	Retention time (min)	NCI (amu)	EI (amu)	Internal standard NCI (amu)	Recovery (%) 1 ng	RSD[a] (%)	Recovery (%) 100 pg	RSD[a] (%)
26TDA	2.99	494, 474	514 (23), 345 (100), 317 (55)	[²H₆]26TDA (498, 479)	108	9	104	7
24TDA	3.09	494, 474	514 (34), 345 (100), 317 (21)	[²H₆]24TDA (498, 479)	96	7	98	11
2AF	4.81	357	377 (94), 180 (100), 151 (61)	[²H₉]2AF (366)	109	4	95	5
3,3′MDA	5.25	570, 530	590 (19), 302 (61), 132 (100)	[²H₄]MDA (574)	87	4	103	4
Bz	5.72	556	576 (62), 379 (100)	[²H₄]Bz (560)	92	6	103	4
MDA	5.87	570	590 (35), 397 (25), 382 (19), 134 (100)	[²H₄]MDA (574)	94	3	108	5
DcBz	6.05	624, 626, 628	646 (14), 644 (20), 609 (36), 449 (61), 447 (91), 252 (100), 250 (42), 227 (54), 225 (90)	[²H₆]DcBz (630)	106	5	107	8
DmBz	6.09	584	604 (22), 435 (19), 408 (21), 407 (100)	[²H₄]DmBz (588)	103	6	93	4
MOCA	6.20	638, 640, 642	658 (8), 623 (24), 300 (100)	[²H₄]MDA (574)	99	7	94	9
Afl	6.25	393	413 (86), 216 (100), 189 (92)	[²H₈]Afl (401)	109	4	111	8
1AP	6.49	393	413 (21), 216 (100), 189 (74)	[²H₈]Afl (401)	87	10	89	8
DmeBz	6.61	616	636 (56), 452 (53), 318 (39), 225 (42), 214 (100)	[²H₄]DmBz (588)	96	8	108	9
AcBz	7.06	402	422 (42), 380 (90), 183 (100)	[²H₄]AcBz (406)	101	7	102	12
AcMDA	7.21	416	436 (65), 394 (100), 359 (19)	[²H₄]AcMDA (420)	97[b]	3	98[c]	14
6Chry	7.69	419	439 (66), 242 (25), 215 (100)	[²H₈]Afl (401)	116	10	120	7

[a] RSD = relative standard deviation.
[b] 2.4 ng.
[c] 240 pg.
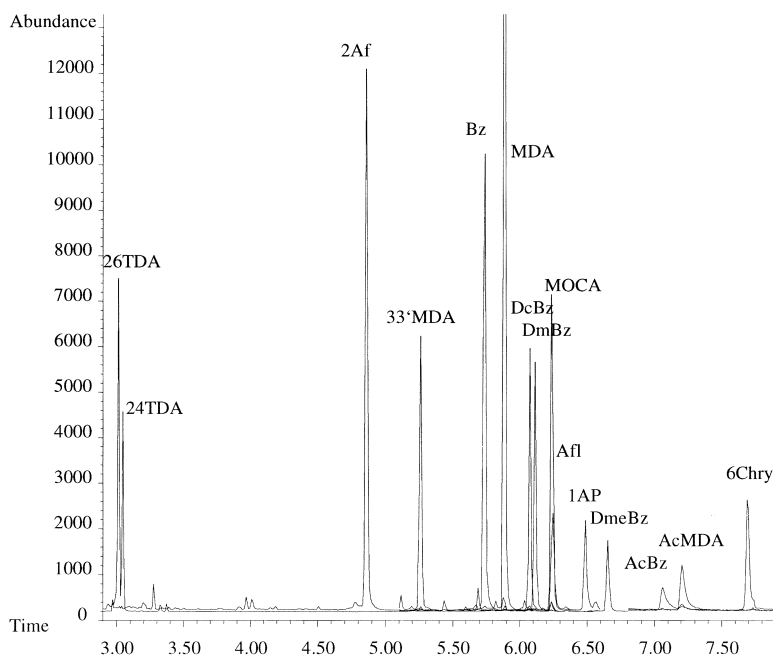
Fig. 1. Representative overlayed single ion chromatograms of an extract from a spiked haemoglobin sample; 500 pg AcBz, 2AF, Afl, 1AP, 6Chry, AcBz, AcMDA, Bz, DcBz, DmBz, MDA, 24TDA, and 26TDA, 1.2 ng AcMDA in 200 mg haemoglobin.

the integration of the residual ring protons. In [^{2}H$_4$]Bz the positions *ortho* to the amino group were deuterated with 97.4% and the positions *meta* to the amino group were completely undeuterated. In [^{2}H$_4$]DmBz no signal for the protons *meta* to amino group could be detected, which is due to quantitative deuteration in the *meta* positions. The position *ortho* to the amino group was deuterated with 51%. Deuteration of DmBz at 200°C resulted in incomplete deuteration of the *meta* positions.

The deuterated toluenediamines were synthesised from [^{2}H$_8$]toluene. After nitration of the toluene, the resulting main products 2,4- and 2,6-dinitrotoluene were purified by silica-gel chromatography. The products were reduced quantitatively with ammonium formate and Pd/C. All deuterated products were characterised by GC–MS and electron impact ionisation and after derivatisation with HFBA by GC–MS with negative chemical ionisation. For the HFBA derivatives of [^{2}H$_4$]Bz, [^{2}H$_4$]AcBz, [^{2}H$_4$]DmBz, [^{2}H$_8$]Afl, [^{2}H$_6$]24TDA and [^{2}H$_6$]26TDA, the following relative abundance were observed for the *m/z* fragment of the ^{2}H$_0$-amine compared to most abundant fragment of the ring deuterated amine: 0.3, 0.3, 0.01, 2, 0.3 and 0.3%, respectively.

3.2. Calibration and linearity

The linearity of the assay in spiked haemoglobin samples was established by dissolving portions of haemoglobin (200 mg) in 0.1 *M* sodium hydroxide (4 ml) in the presence of the I.S. solution and different amounts of spiking solutions (as for the sodium hydroxide solutions). For the first level 10 µl I.S. solution, for the second level 10 µl of I.S. solution and 10 µl of spiking solution I, for the third level 10 µl I.S. solution and 10 µl spiking solution II and for the fourth level 10 µl I.S. solution and 10 µl spiking solution III were used. The haemoglobin samples were stirred, extracted, derivatised and then analysed. For each concentration of arylamine, the mean of triplicate samples was calculated. The ratios of the measured peak areas arylamine/I.S. were plotted against the amount of arylamine. A linear regression analysis was performed resulting in calibration lines for each amine. Regression coefficients

of all calibration curves were ≥0.99, except for DmBz, DmeBz, and MOCA with r=0.98, 0.94, and 0.96, respectively

3.3. Calculation of results

For the determination of hemoglobin adducts from human samples, the amounts of arylamines were quantified via the deuterated I.S.s. The measured peak area of the arylamine was divided by the measured peak area of the I.S. The resulting ratio and the calibration line obtained from spiked haemoglobin samples (see above) was used to determine the concentration of the amine.

3.4. Standardisation and quality control

Control samples should be included for each batch analysed. It is recommended to perform at least three control samples per day with sodium hydroxide and I.S. solution only, to check background levels of chemicals and glassware used. Quality control samples with 10 µl spiking solution I and 10 µl I.S. solution should be run with each batch. Control samples should be analysed in triplicate.

Furthermore, it is recommended to run a calibration curve for each population examined, which should be checked or re-evaluated during the course of the study. Analysing samples from large collectives of exposed humans, a sample with high adduct levels should be analysed with each batch as quality control sample.

The robustness of the method was tested for MDA, Bz, AcMDA and AcBz by working up the same haemoglobin samples from exposed humans over a period of 3 months. The values varied with less than ±15% within this period. The robustness for the other compounds was not determined.

3.5. Precision and accuracy

The precision and accuracy from spiked hemoglobin samples were determined in relation to spiked water samples. The accuracy cannot be determined in comparison to already derivatized samples (e.g. HFBA–AcMDA, or HFBA–AcBz), since the chromatography is very poor without any matrix resulting from haemoglobin extracts.

Triplicate samples of haemoglobin were spiked with 10 μl spiking solution I and 10 μl spiking solution II in the presence of 10 μl I.S. solution. In Table 1, the listed recoveries of aromatic amines from haemoglobin solutions are relative to the recoveries from spiked sodium hydroxide solutions treated as the hemoglobin samples. The presence of more matrix appears to increase the recovery of 6Chry in comparison to the I.S. [^{2}H$_8$]Afl.

3.6. Limit of detection

The LOD for the derivatised standards is in the 100-fg range. The LOQ for the presented method is ≈5 pg per sample for the bicyclic diamines, ≈30 pg for AcBz and AcMDA and ≈20 pg for the poly-aromatic amines, using splitless injection. The presented method uses 200 mg of haemoglobin. For samples from environmental exposures 1 g of haemoglobin must be used, as the LOQ of 5–30 pg/200 mg is not sufficient.

3.7. Covalent binding of arylamines to hemoglobin

In order to test if, with the given procedure for the isolation of hemoglobin, non-covalently bound arylamines were eliminated, hemoglobin was precipitated with and without the presence of the I.S. mixture and washed with the sequence of organic solvents presented in Section 2.5. This experiment was performed in duplicate. The precipitated hemoglobin was hydrolyzed in the presence of the standard amine mixture. In the spiked sample and in the control samples no deuterated compounds could be detected.

The same experiment was performed by precipitating hemoglobin from lysed erythrocytes with and without the presence of the standard amine mixture (spiking solution I) and washing with the sequence of organic solvents presented in Section 2.5. The precipitated hemoglobin of the spiked sample and of the control were hydrolyzed in the presence of the I.S. mixture. The levels of amines present in the control and spiked sample were the same. Therefore, the work up procedure described for the isolation of hemoglobin eliminates non-covalently bound amines.

3.8. Derivatisation: trans acylation of AcBz and AcMDA to the corresponding diamines

A comparison of the sensitivity of pentafluoro-propionic anhydride (PFPA) with HFBA derivatives gave a better sensitivity for the HFBA derivatives. Pre-experiments for the derivatisation of MDA and AcMDA with perfluoro organic acid anhydrides gave contradictory results for AcMDA. Therefore, the derivatisation step was further investigated with HFBA. Samples with different concentrations of AcMDA were derivatised with HFBA with and without triethylamine. Samples were analysed by HPLC–UV and/or GC–MS/EI. The expected derivative HFBA–AcMDA, but additionally the by-product DiHFBA–MDA and the peak of an unknown compound could be detected. The acetyl group of these diamines was cleaved off and replaced by the derivatising reagent. This phenomenon is known as *trans* acylation.

For the appearance of additional, probably via *trans* acylation formed products, the following conclusions could be drawn:

- In all samples with a molar ratio AcMDA/HFBA of 1:5 only HFBA–AcMDA was detected as derivatisation products using HPLC analysis as well as GC–MS analysis.
- In samples with a molar ratio AcMDA/HFBA of 1:500 and without the addition of triethylamine, only HFBA–AcMDA was detected in the HPLC and GC–MS analysis. In samples with triethylamine, additional small amounts of DiHFBA–MDA were observed.
- In all samples with a molar ratio AcMDA/HFBA of 1:5000 (only analysed by GC–MS) predominantly the *trans* acylation product was detected. In samples without triethylamine no *trans* acylation product could be found, when excessive derivatisation reagent was removed by shaking with ammonia solution prior to the GC–MS analysis.

These experiments show that the formation of *trans* acylation products happens only with huge excesses of derivatisation reagent and the formation is promoted in the presence of triethylamine or at

higher temperatures (e.g. as during a GC–MS analysis). The addition of triethylamine for the derivatisation step is therefore not recommended. The complete removal of acidanhydride prior to analysis or the addition of another aromatic amine before the derivatisation step is possible to avoid the unwanted reaction products. The last method was finally applied to avoid the excess of derivatisation reagent. Huge excesses of remaining HFBA during the derivatisation of samples can be avoided by the addition of μg-amounts of another aromatic amine (4-methylaniline) in methanol.

The best derivatisation reagent was HFBA. The HFBA derivatives eluted only slightly later than the PFPA derivatives. The limit of detection for the HFBA derivatives is better, which is especially advantageous for the detection of AcMDA. In contrast to DiHFBA–MDA, HFBA–AcMDA contains only one electron acceptor group.

3.9. Column chromatography

The chromatography was normally performed on a capillary column Rtx-5MS (15 m×0.25 mm, 0.25 μm layer) from Restek. The separation of substances can obviously be carried out with similar columns from other suppliers (e.g. Optima-5 MS from Macherey-Nagel). For analysis of MDA and AcMDA a column with 0.5-μm film thickness was used, too. Advantages and disadvantages of a 0.5-μm compared to a 0.25-μm film thickness were not examined systematically.

The baseline separation of DiHFBA–24TDA and DiHFBA–26TDA was only possible on a column with a 0.5-μm film thickness [11] (e.g. XTI 15 m×0.25 mm, 0.5 μm, from Restek).

The derivatised diamines have good chromatographic properties and are relatively insensitive to impurities in the samples. The chromatography of the derivatised monoacetylated compounds HFBA–AcBz and HFBA–AcMDA is not without problems. The peak form of HFBA–AcMDA and HFBA–AcBz are dependent on sample matrix and column conditions and are not always optimal. Peak tailing or slight peak doubling could not be avoided in some instances. Therefore, the LOQ for HFBA–AcMDA is approximately five times higher than the LOQ for DiHFBA–MDA. Using splitless injection, the pre-

column (retention gap) must be changed after 20 injections, because increasing peak tailing makes it impossible to quantify HFBA–AcBz and HFBA–AcMDA. The GC column must be relatively new. Using on-column injection results in better peak shapes, but the precolumn (retention gap) must be cut every five injections. Under less than optimal separation conditions the HFBA–AcMDA peak interferes with the integration of HFBA–AcBz. For an interference-free determination of HFBA–AcBz it is better to take a longer column or to abandon the HFBA–AcMDA analysis. However a long column tends to yield more tailing peaks than a shorter column.

The same GC temperature program was used for on column injection as for splitless injection. The extract was injected with a capillary syringe at 80°C. The column inlet pressure remained unchanged at 68 kPa.

The yields for the polyaromatic amines are about a factor of 5 better with on column injection in comparison to HFBA–MDA, for which there is no difference in sensitivity between the two injection methods.

The following points should be followed to prevent contaminations and interferences of the analyses. Always use disposable glassware from the same supplier. Only solvents with the best specifications should be used; also for the work-up of haemoglobin. Aliquot freshly opened derivatisation reagent and sodium sulfate immediately. Important sources of contamination are glassware, sodium hydroxide, sodium sulfate, the derivatisation reagent and the apparatus for the concentration of samples.

4. Discussion

Per day, haemoglobin can be precipitated from at least 24 blood samples. Thirty precipitated haemoglobin samples can be prepared for GC–MS analysis per day. One GC run takes approximately 15 min. The analysis of 30 samples takes about 15 h (e.g. 1 night) when the samples are analysed in duplicate. With the analysis of monoacetylated diamines it is often not possible to inject so many samples without any interruption.

The present method was applied for the analysis of

benzidine and azo dye exposed workers (Fig. 2). Only the corresponding deuterated I.S.s were used for the analysis of these collectives. Benzidine (range: 0–2.6 ng/100 mg Hb) and AcBz (range 0.25–71.2 ng/100 mg Hb) could be released from haemoglobin of 33 exposed workers. In 15 controls no adducts were present. For these experiments the calibration curves for AcBz and Bz were established at five concentration levels (range 1–25 ng/100 mg

Hb). For both substances the calibration curves were linear over the chosen range. The correlation co-efficients of the calibration curves were 0.99 for Bz, and AcBz.

The presented method permits detection of several aromatic amines in one haemoglobin sample in one GC run. This is very important for the analysis of occupational samples, where it is often necessary to screen for multiple exposures to different chemicals.
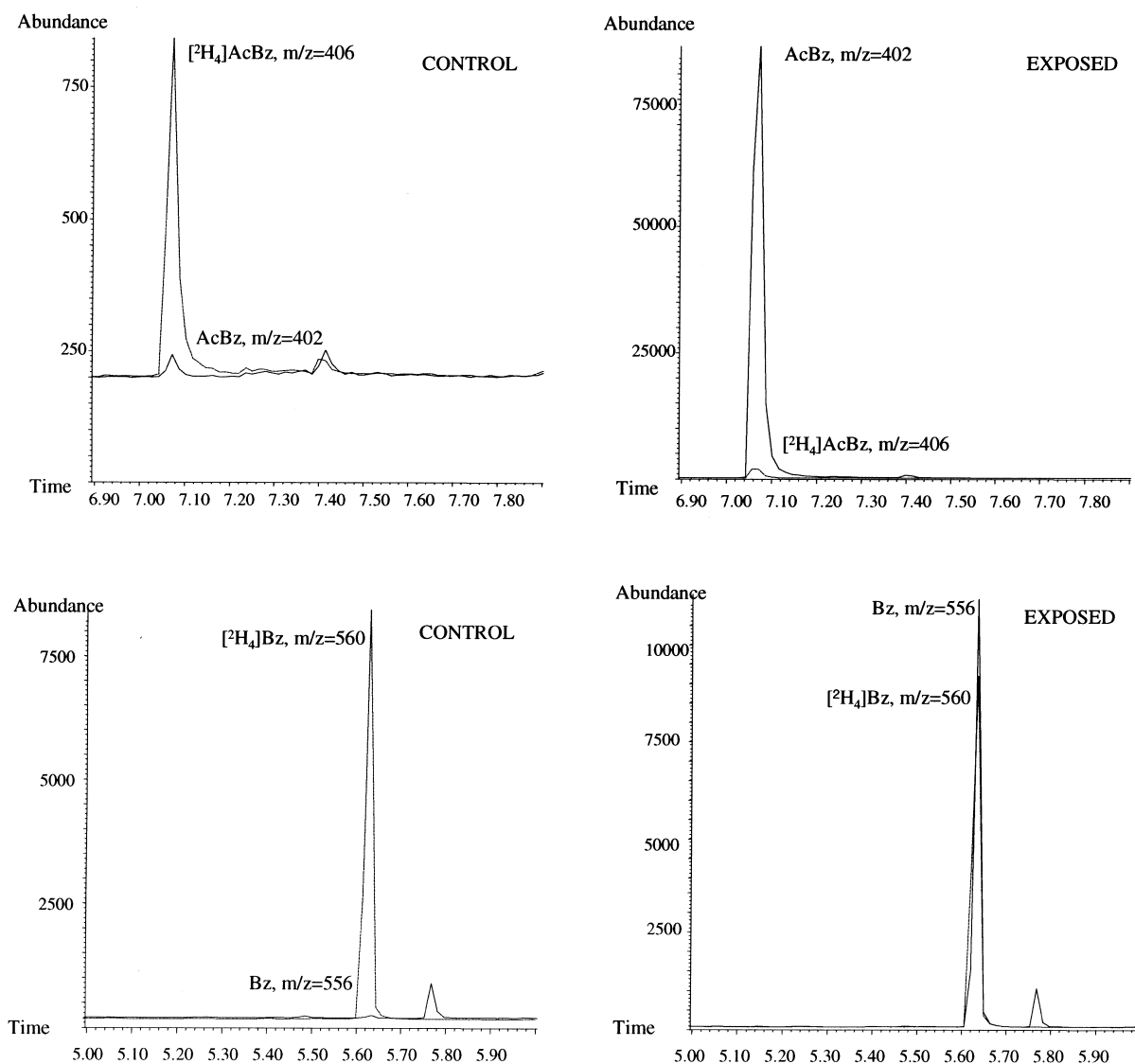

Fig. 2. Representative chromatograms of an extract (100 mg Hb) from an azo dye exposed worker (Bz=0.4 ng/100 mg Hb; AcBz=23.7 ng/100 mg Hb) and from an unexposed worker (control).

According to the objectives of the investigation, it is useful to select only the important amines for analysis and focus on these.

The advantage of measuring haemoglobin adducts instead of the parent aromatic amines are the following: haemoglobin adducts are usually stable over the lifetime of haemoglobin. Therefore, haemoglobin adducts are a dosimeter for the exposure of the last 4 months. Urine metabolites reflect only the exposure of the last 48 h. In addition, the presence of haemoglobin adducts indicates the biological availability of the potential genotoxic intermediates — *N*-hydroxy arylamines — of arylamines or nitroarenes. Haemoglobin adducts of aromatic amines can be formed after exposure to the corresponding nitro aromatic compounds or after exposure to isocyanates. In addition arylamines can be metabolically released for example from pesticides, azo dyes, and polyurethanes.

In particular with samples from people supposedly exposed to chemicals in the environment, it is necessary to take care that an additional detection mode is applied. It is recommended to confirm the structure of the allegedly found substances by electron impact ionisation. This might prove difficult with a quadrupole instrument due to the relatively low sensitivity. The haemoglobin hydrolysates should nevertheless be hydrolysed at basic and neutral pH. If the values under these conditions are the same or similar, no covalent bound adducts are present.

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