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# Solid-phase extraction and gas chromatographic–mass spectrometric determination after hydrolysis of 2-aminofluorene haemoglobin adducts in blood of rats

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ABSTRACT

2-Nitrofluorene is an environmental pollutant that binds covalently to haemoglobin after nitroreduction and successive N-hydroxylation. These haemoglobin adducts can be cleaved *in vitro* by mild base-catalysed hydrolysis. For the enrichment of arylamines from the aqueous hydrolysate, an extraction procedure with an organic solvent is widely used. Because of the formation of a thick emulsion layer between the aqueous and organic solvent layers, the extraction is laborious and inefficient. The use of Amberlite $XAD_2$ provides a simple extraction procedure yielding a recovery of *ca.* 70%. Calibration curves in haemoglobin solution were prepared with a correlation coefficient of 0.998 ($n = 12$). The inter-day coefficient of variation amounted to 14%.

## INTRODUCTION

Xu *et al.* [1] were among the first to report the identification of 2-nitrofluorene (NF) in diesel exhaust. From that moment several investigators have reported this compound to be present in environmental samples, such as heavy-duty and light-duty diesel engine exhaust [2–11] and gasoline engine exhaust [5]. It was found to be present in outdoor air samples [12–14] and in indoor air samples in emissions from kerosene heaters and fuel gas and liquefied petroleum burners [15]. NF can be converted into 2-aminofluorene (AF) by bacterial nitroreductases present in the intestinal microflora. The liver may also contribute to the reduction of nitroarenes, but is considered to be of minor importance in the *in vivo* metabolism of nitroarenes. In the liver, AF can be N-hydroxylated by microsomal P-450 to N-hydroxy-2-aminofluorene (N-OH-AF). This activation was also found to take place in the human red blood cell cytosol [16,17]. The reactive nitroso analogue formed in the presence of oxyhaemoglobin can bind covalently to the free thiol (SH) groups of haemoglobin (Hb).

Protein adducts are used for the molecular dosimetry of chemical carcinogens. Arylamines that are known as potential human carcinogens may bind covalently to Hb. This interaction is identified as a sulphinic acid amide binding of the acylnitroso intermediate to an SH group of a cysteine residue in Hb. This type of protein binding reflects the biotransformation of nitroarenes and arylamines into genotoxic intermediates. It would also represent the systemic bioavailability of these reactive intermediates. For several arylamines, a correlation between binding to Hb and

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DNA was observed in laboratory animals [18], and bladder cancer patients were found to have statistically significant elevated 4-aminobiphenyl (ABP) Hb adduct levels [19]. ABP is a known human bladder carcinogen.

It is possible to identify the parent arylamine after hydrolysis of the Hb adduct. This can be done by mild base-catalysed hydrolysis of a purified Hb solution [20].

The fluid–fluid extraction of the arylamines from the hydrolysate is laborious [19]. Birner *et al.* [21] described the use of $C_{18}$ solid-phase extraction for the determination of benzidine and some benzidine congeners. We have developed a simple method for the extraction of AF from the hydrolysate using $XAD_2$ as a solid sorbent. This modification of the extraction procedure provides simplification and improvement of the present determination methods of Hb adducts of arylamines.

## EXPERIMENTAL

### *Reagents, chemicals and solvents*

Toluene and isooctane (HPLC grade) were obtained from Lab-Scan Analytical Sciences (Dublin, Eire). Ethanol (absolute), sodium chloride and sodium hydroxide were supplied by Merck (Darmstadt, Germany). Tris(hydroxymethyl)-aminomethane was purchased from United States Biochemical Corp. (Cleveland, OH, USA). Glycine hydrochloride was obtained from Sigma (St. Louis, MO, USA). Aqua pure was prepared treating demineralized water in a Nanopure system (Barnstead, Boston, MA, USA). NF (98%) and heptafluorobutyric anhydride (HFBA) were obtained from Janssen Chimica (Geel, Belgium). AF (98%) was supplied by Aldrich Europe (Bornem, Belgium). 2-Fluoro-7-nitrofluorene (99.8%) was purchased from Aldrich (Milwaukee, WI, USA). 2-Amino-7-fluorofluorene (AFF) was prepared from 2-fluoro-7-nitrofluorene by nitroreduction with hydrazine monohydrate and Raney Nickel [22]. N-OH-AF (98%) was obtained from Chemsyn Science Labs. (Lenexa, KA, USA). Sunflower oil was obtained from OPG (Utrecht, Netherlands).

Amberlite $XAD_2$ was obtained from Serva (Heidelberg, Germany) and precleaned according to ref. 23. Lymphoprep was supplied by Nycomed Diagnostica (Oslo, Norway). Other chemicals used were of the highest purity available.

### *Apparatus*

The Hb concentrations and free SH groups were determined using a Pye Unicam 1750 UV–Vis spectrophotometer (Philips Analytical, Eindhoven, Netherlands). GC–MS analysis of the arylamines was performed on a Varian 3400 GC system equipped with a Varian 8100 autosampler and a Varian Saturn ion trap MS detector (Walnut Creek, CA, USA).

### *Animals*

Female homebred Cpb:WU (Wistar) rats of 200–300 g body weight received AF by intraperitonial injection (0.03 mmol/kg) and NF or AF by gavage (1.0 mmol/kg). Fresh blood samples for calibration purposes were obtained from male homebred Cpb:WU/Kun (Wistar) rats of 200–400 g body weight.

### *Blood sample collection*

Blood was collected from diethyl ether-anesthetized rats by cardiac puncture 48 h after administration. The samples were collected with a syringe that contained heparin to prevent blood clotting, and immediately transferred to a vacutainer.

### *Preparation of Hb solution*

Aliquots of 3 ml of blood were diluted (1:1) with Tris–Gly–HCl buffer (5.0 m*M* tris(hydroxymethyl)aminomethane, 5.0 m*M* glycine hydrochloride, 0.15 *M* sodium chloride, pH 7.4), carefully mixed and layered over 4.0 ml of Lymphoprep. Blood cells were separated by centrifugation at 2000 *g* for 20 min. The lymphocytes were collected from the intermediate layer and kept for further analysis (not presented in this paper). After two washings with 6.0 ml of Tris–Gly–HCl buffer (pH 7.4), the erythrocytes were lysed by adding 12 ml of ice-cold aqua pure and keeping the solution on ice. After 15 min, 1.2 ml of 750

m*M* NaCl Tris–Gly–HCl buffer (pH 7.4) was added followed by careful mixing. Cellular debris was removed by centrifugation at 10 000 *g* at 4°C for 30 min. The arylamines not covalently bound to proteins were captured from the supernatant (10.0 ml) by overnight vigorous horizontal shaking with 100 mg of $XAD_2$. After centrifugation (2000 *g* for 5 min) 8.0 ml of the supernatant were collected, and 200 μl of Hb solution were kept for determination of the Hb concentration (in duplicate).

*Determination of Hb concentration*

The concentration of Hb in the solution was determined using the hemiglobincyanide method described by Van Kampen and Zijlstra [24]. The absorbance was determined at 540 nm.

*Hydrolysis of Hb adducts and extraction of AF*

An aliquot of 100 μl of a 0.05 m*M* solution of AFF (internal standard) in ethanol was added to the Hb solution. Hydrolysis of the adducts was established by adding sodium hydroxide solution until a pH of *ca.* 12 was reached. Per aliquot, 100 mg of $XAD_2$ were added, and the hydrolysate was extracted by overnight vigorous shaking on a horizontal shaker. The hydrolysate was removed with a vacuum syringe equipped with a needle of 0.4 mm I.D. Next, the $XAD_2$ was washed three times with aqua pure and dried at 70°C.

*Desorption and derivatization*

The arylamines were desorbed from the solid sorbent with 4.0 ml of toluene by sonication for 20 min. The toluene solution was transferred and evaporated under a gentle stream of nitrogen at 60°C, until a volume of *ca.* 0.3 ml remained. The amines were derivatized by adding 20 μl of HFBA, followed by 30 min of heating at 60°C in a closed glass test-tube. Isooctane (2 ml) was added to the reaction mixture. The remaining derivatization reagent was neutralized by the addition of 1.0 ml of aqua pure. The organic phase was collected and dried under nitrogen at 60°C. The residue was redissolved in 70 μl of isooctane and sonicated for 5 min. This solution was transferred to a vial with insert, septum and screw-cap for GC–MS analysis.

*GC–MS analysis*

Aliquots of 1 μl followed by 0.5 μl of solvent plug (separated by an air gap) were injected on-column in a septum-equipped programmable injector. The autosampler was set at 3 μl/s with a 0.1-min post-injection hot needle time. The column system consisted of a 2.5 m × 0.53 mm I.D. deactivated fused-silica retention gap (different brands) and a 30 m × 0.25 mm I.D. DB-5MS-coated ($d_f$ = 0.25 μm) fused-silica capillary column (J & W Scientific, Folsom, CA, USA). The carrier-gas used was helium at a column head-pressure of 97 kPa.

The injector was programmed after a 1-min hold at 100°C to a final temperature of 280°C at 100°C/min, with a 12-min hold at 280°C. Separation from the matrix was achieved by programming the GC oven temperature after a 1-min hold at 100°C to a final temperature of 300°C at 20°C/min, with a 9-min hold at 300°C. The transfer-line and ion-source temperatures were 290°C and 220°C, respectively. The mass spectrometer was operated in the EI mode at an electron ionization energy of 70 eV. A mass range of $m/z$ 150–500 was scanned at a rate of 1 s/scan (comprising 4 μscans) between 8 and 9 min after injection when AF and the internal standard coeluted from the column. System automation and data handling were performed using the Varian Saturn software.

*Standard preparation*

Solutions of AF and AFF in ethanol were prepared and used as stock solutions. They were stored in the dark at 4°C. Aliquots of 8.0 ml of Hb solution were spiked with AF in the range 1–33.5 nmol. To each standard, 20 nmol of AFF were added. The final concentration of ethanol was kept below 2% to prevent precipitation of Hb. The standards were treated as if they were samples.

*Preparation of AF–Hb adducts and determination of free cysteine residues*

Fresh blood from eight non-exposed male Wistar rats (70 ml) was pooled and diluted 1:5 with Tris–Gly–HCl buffer (pH 8.8) with 150 m*M* sodium chloride. From this solution two equal volumes of 130 ml were taken. One of these portions was incubated for 5 h with 1.0 ml of 4.2 m*M* N-OH-AF in ethanol at 37°C. The other portion was kept as a reference. Amberlite $XAD_2$ was added (20 mg/ml) to extract free arylamines by overnight vigorous shaking at room temperature. Next, Hb solutions were prepared and the Hb concentrations were determined. The number of free SH groups per residue was determined using the thiopyridone method [25]. From the number of free SH groups and the Hb concentration, the reduction of free SH groups per Hb molecule was calculated. This number reflects the number of adducts formed by covalent binding of N-OH-AF to the SH groups of the Hb. The adduct concentration can thus be calculated.

*Validation procedures*

The method was calibrated by analysing a series of Hb solutions with known amounts of added AF. The incubated blood was diluted with non-treated blood in the range from 1:5 to 1:500. These dilutions were divided in equal portions and analysed in duplicate. The reproducibility was studied by performing duplicate analysis on a series of fourteen fresh blood samples that were collected from rats receiving NF or AF by gavage, and three blood samples that were collected from rats receiving AF by intraperitoneal injection.

*Calculations*

A calibration curve was constructed by plotting the ratio of the peak areas of AF and the internal standard *versus* the AF amount in the Hb solution. The linearity was determined by linear regression analysis. The detection limit was established by comparing the peak area of the lowest standard with the extracted Hb solution blank. Recoveries were calculated from the slope of a linear regression of peak areas of AF *versus* the concentration. In this case, the corresponding solutions of AF in toluene were assumed to represent 100% recovery.

## RESULTS

The number of free SH groups per molecule Hb amounted to 2.9 ± 0.3 in the non-treated Hb solution. After incubation with N-OH-AF this number decreased by 1.1 ± 0.5 (38 ± 24%). This decrease represents the average number of SH groups per Hb molecule that formed sulphinamide bonds. Based on this estimated adduct formation, the concentration of AF–Hb adducts in the stock corresponded to *ca.* 0.2 μmol adduct per ml blood. This stock was further diluted in non-treated blood, resulting in a series of standards with estimated adduct levels ranging from *ca.* 0.4 to 40 nmol adduct per ml blood. These dilutions were used for the calibration curve. Based on the peak area of AF *versus* the concentration of AF–Hb adducts, a calibration plot was calculated resulting in a correlation coefficient of 0.988. The recovery of AF from the blood-diluted adduct standard was calculated to be 10.0 ± 0.5%. However, because of the apparent inaccuracy of the calculated adduct level in the stock, it would not be adequate to use this relation for calibration purposes. In order to establish a reliable calibration curve, we plotted the ratio of the peak areas of AF and the internal standard *versus* the amount of AF in a series of Hb solutions. This resulted in a calibration curve with a correlation coefficient of 0.998 and a coefficient of variation of 9.2%. The recovery of AF (mean ± S.D.) extracted from a Hb hydrolysate spiked with AF was calculated to be 73.8 ± 10.4% in a concentration range of 0.021–8.2 μg/ml. The detection limit was estimated to be *ca.* 0.5 nmol AF per g Hb.

Fig. 1 shows the total ion chromatogram and the mass spectrum of a representative blood sample. Characteristic *m/z* values found in mass spectra of HFB derivatives of AF and AFF are the molecular ions 377 and 395, respectively. These ions have been used for quantification. The ions *m/z* 180 and 198 represent $[M - 197]^+$ through

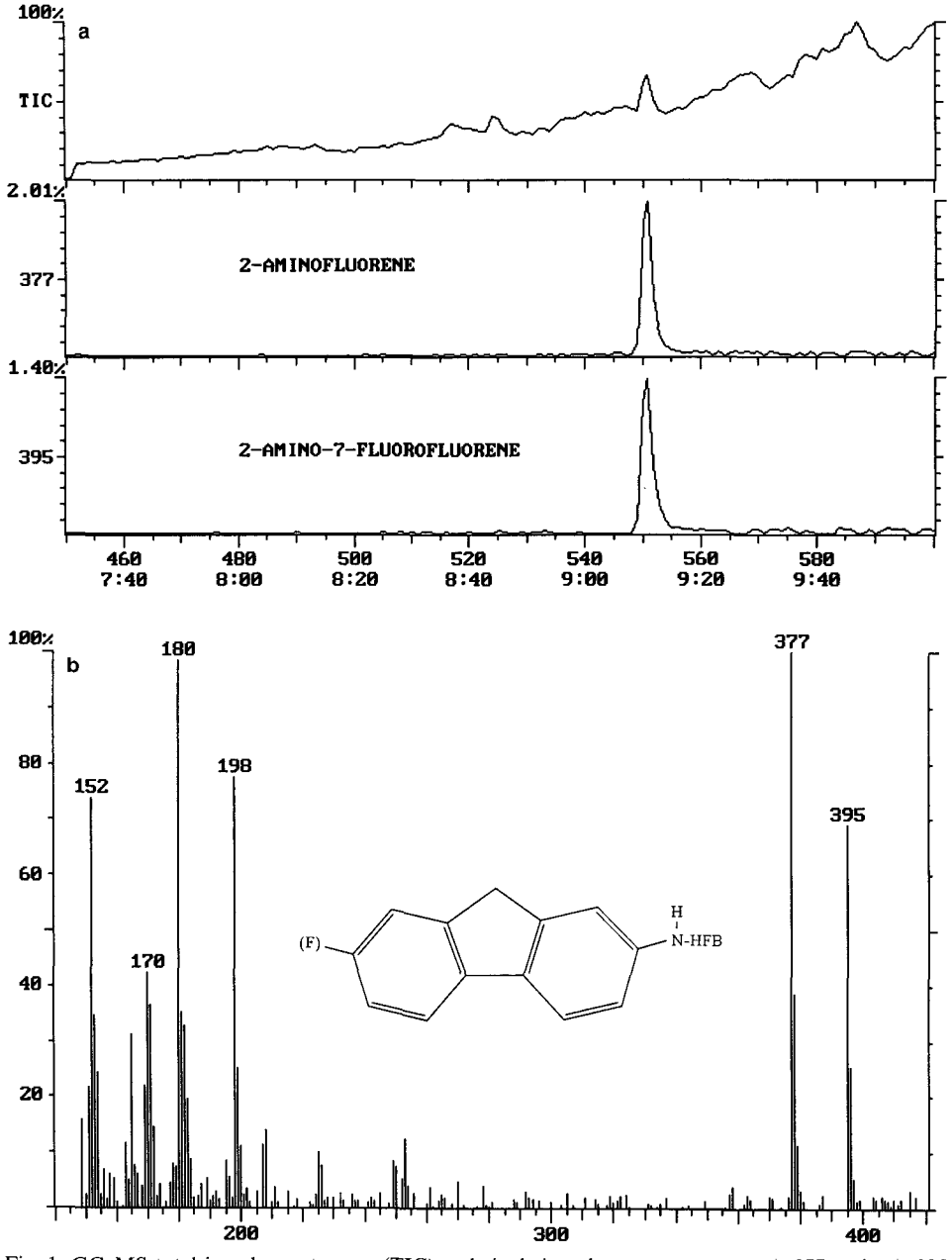


Fig. 1. GC–MS total ion chromatogram (TIC) and single-ion chromatograms at $m/z$ 377 and $m/z$ 395 (a) and the electron impact ionization mass spectrum (b) of a hydrolysed blood sample of a rat that received an intragastric dose of NF of 1 mmol/kg.

loss of $COC_3F_7$. The ions $m/z$ 165 and 183 ($[M - NHCOC_3F_7]^+$) are also found, *ca.* 50% less abundant than the molecular ions and $[M - 197]^+$. Also expected are $m/z$ 153 and 171 ($[M - 224]^+$), possibly with formation of a tropylium ion through loss of $CNHCOC_3F_7$ [26], but $m/z$ 152 and 170 ($[M - 225]^+$) are more abundant. These ions are probably formed through loss of $H_2CNCOC_3F_7$. The ion $m/z$ 152 is also found (more abundant than $m/z$ 153) in a mass spectrum of the underivatized AF in the National Institute of Standards and Technology library.

TABLE I

REPRODUCIBILITY OF THE DETERMINATION OF Hb ADDUCTS OF AF IN BLOOD SAMPLES FROM RATS

| Compound | Dose (mmol/kg) | Route[a] | $n$ | Hb adduct level (μmol/g Hb) | Coefficient of variation (%) |
|---|---|---|---|---|---|
| AF | 1.0 | i.g. | 8 | 4.6 ± 2.7 | 7.3 |
| AF | 0.03 | i.p. | 3 | 0.73 ± 0.04 | 14.0 |
| NF | 1.0 | i.g. | 6 | 0.03 ± 0.02 | 23.3 |

[a] i.g. = intragastric; i.p. = intraperitional.

AF–Hb adducts were determined in a series of rat blood samples collected 48 h after NF or AF administration. The inter-day coefficient of variation derived from seventeen blood samples was calculated to be 14% (see Table I). In the high-range adduct level, excellent reproducibility was achieved; in the low range (below 0.05 μmol/g Hb) the reproducibility exceeded 20%.

## DISCUSSION

The hydrolysis of the sulphamide bond is crucial for the determination of Hb adducts of arylamines. This is usually carried out by base-catalysed hydrolysis of the purified Hb solution. The presence of many fragments of bioorganic molecules (*e.g.* detergents, surfactants) may cause the appearance of an emulsion zone between the aqueous hydrolysate and the organic extraction solvent. This may cause a low recovery and a poor reproducibility in the arylamine extraction [19]. The emulsion is reported to be broken by repeated freezing and thawing. The organic solvents collected after repeated extraction steps are usually dried over anhydrous sodium sulphate and magnesium sulphate, and made alkaline with trimethylamine [20].

In this paper we have described the use of $XAD_2$ for the extraction of arylamines from hydrolysed Hb solutions. This modification comprises a simplification of the analysis of arylamine Hb adducts. There are no problems arising from the appearance of emulsions because a solid sorbent is added to the hydrolysate. At the same moment sodium hydroxide is added to start the hydrolysis. During the overnight extraction (*ca.* 16 h) the arylamines freed by hydrolysis can immediately be absorbed on the solid sorbent. The combination of prolonged hydrolysis and extraction results in favourable conditions for the extraction of AF. In such a way, a single extraction procedure gives a sufficient recovery (*ca.* 70%) of AF and the internal standard, and provides a good reproducibility. The solid sorbent can be very well dried, therefore there is no need to dry the toluene eluate over an anhydrous salt. We propose the use of Amberlite $XAD_2$ for the extraction of AF and other arylamines from Hb hydrolysate.

The calibration curve derived from dilutions of pure AF in Hb solutions shows excellent linearity and reproducibility. This curve can very well be used for calibration purposes. The dilutions of blood containing *in vitro* formed AF–Hb adducts in blood from non-treated rats result in a curve with a lower correlation coefficient ($r$ = 0.988). A more accurate estimate of the number of adducts, based on the determination of reduction of free SH groups per g Hb, could not be achieved. This is due to considerable deviations in the indirect determination of the number of SH groups, and to deviations in the determination of Hb concentrations caused by propagation of errors. Theoretically, the number of free SH groups in rat Hb would be 5–7. Crystallization of the Hb and possible formation of disulphide bonds could have contributed to the considerably lower number detected in this study [27]. Quantification of Hb adduct levels, using a series of dilutions of pure AF in Hb solution, tends to underestimate the

actual adduct level. In experimental animal studies, calibration based on the Hb standard solutions of 2-AF should be acceptable, because in most study designs the blood adduct levels in animals that received different treatments are usually compared. However, when using this method for dosimetry, it is important to determine the actual recovery of the hydrolysis to be able to correct for the unhydrolysed arylamines.

We are currently developing the solid-phase extraction procedure for the analysis of ABP and AF–Hb adducts from human blood samples. Efforts will be made to improve the sample clean-up and to enhance the sensitivity and reproducibility of the method.

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