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Determination of hemoglobin adducts following oral administration of 1-nitropyrene to rats using gas chromatography–tandem mass spectrometry

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Abstract

1-Nitropyrene (1-NP) has successfully been used as a marker for environmental monitoring of exposure to diesel exhaust. This study presents a sensitive and selective method for detection of Hb adducts after oral administration of a single dose 1-NP to rats, by measuring 1-aminopyrene (1-AP) after in vitro hydrolysis of the adducts. Released 1-AP was extracted with hexane and derivatized with heptafluorobutyric acid anhydride prior to GC–MS–MS analysis. Optimal conditions for the release of 1-AP were hydrolysis under nitrogen, in 1 *M* NaOH at 70°C for 60 min. Analysis of a stock solution of Hb adducts of 1-NP utilizing these conditions showed to be reproducible over a period of several weeks with a coefficient of variance of 9.5%. The determination limit was 10–20 pg 1-AP per 70–90 mg globin. A study of the time course of Hb adduct formation showed a fast absorption and an early peak concentration of released 1-AP, approximately 39 pg 1-AP/mg globin at 3 h after exposure. After the maximum was reached, 1-AP concentrations decreased bi-phasically. Initially a fast decline was observed, followed by a slow decrease to 5.9±1.9 pg 1-AP/mg globin at 24 h after administration.

Keywords: Hemoglobin adducts; 1-Nitropyrene

1. Introduction

Diesel exhaust (DE), a major source of environmental pollution, is classified as a probable human carcinogen [1]. Several classes of carcinogens have been identified in DE, such as polycyclic aromatic hydrocarbons (PAHs) and its nitrated derivatives. 1-Nitropyrene (1-NP), a weak carcinogenic nitroarene, has been proposed as a specific marker for exposure to mutagens associated with DE [2]. It has been stressed, that the lack of exposure data complicates risk assessment in epidemiological studies investigating the relationship between occupational exposure to DE and (lung) cancer [3]. Therefore, sensitive and selective methods assessing the actual exposure to DE are needed. Determination of hemoglobin (Hb) adducts gives an indication of the integrated biologically active dose over the last 4 months, assuming that the Hb adducts are stable and do not alter the half-life of erythrocytes. Determination of Hb adduct formation has been applied for exposure assessment to several types of compounds,

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such as ethylene oxide [4], butadiene [5], nitrotoluenes [6,7], arylamines [8] and nitroarenes [7,9]. 1-NP is extensively metabolized both in vivo and in vitro. Oxidative as well as reductive metabolites may arise [10,11]. Several reactive metabolites of 1-NP have been shown to bind covalently to DNA [12,13]. Covalent binding of 1-NP metabolites to blood proteins has been investigated to a lesser extent. The structurally related arylamines and nitroarenes have been suggested to bind covalently to cysteine in hemoglobin via their N-hydroxylamine and/or nitroso metabolites [7,9,14]. For several PAHs, such as benzo[*a*]pyrene [15] and fluoranthene [16], Hb binding via epoxide metabolites has been reported. Although involvement of 1-NP epoxides in Hb binding has not been described previously, it was tentatively suggested that 1-NP metabolites can bind to albumin via an epoxide metabolite [17]. Determination of protein adducts of 1-NP may be a valuable biomarker for measuring nitro-PAH exposure and development of sensitive and specific methods for the determination of protein adducts are needed [18–20]. Hb adducts of 1-NP have previously been detected in humans by GC–NCI-MS [7] and in rats by high-performance liquid chromatography (HPLC) with fluorescence detection [21] and by scintillation counting in case of administration of radioactive labeled 1-NP [12]. Since the development of a reliable analytical method is a requisite in the process of studying the suitability of Hb adducts of 1-NP as a potential biomarker for exposure to DE, we here provide a detailed description of such a method. In addition, levels of Hb adducts at different time points following administration of 1-NP to rats are evaluated and the sensitivity and selectivity of gas chromatography–tandem mass spectrometry (GC–MS–MS) for the analysis of the released 1-AP after hydrolysis of Hb adducts of 1-NP is demonstrated.

2. Experimental

2.1. Chemicals and reagents

1-NP (>99%) was purchased from Aldrich (Milwaukee, WI, USA). d^9-1-NP (>99%) was obtained from Chemsyn Science (Lenexa, MO, USA). Tris-HCl was purchased from USB (Cleveland, OH, USA). Heptafluorobutyric acid anhydride (HFBA) and zinc (granular, 30 mesh, analytical-reagent grade) were supplied by Acros (Geel, Belgium). Sodium dodecyl sulfate (SDS), ammonium chloride, potassium phosphate, dimethylsulfoxide (DMSO), sodium hydroxide (NaOH) and hydrochloric acid (HCl, 37%) were obtained from Merck (Darmstadt, Germany). Glass beads (160–250 μm) were obtained from Tamson (Zoetermeer, The Netherlands). Coomassie Brilliant Blue was supplied by Sigma (St. Louis, MO, USA). All organic solvents were purchased from LabScan Analytical Sciences (Dublin, Ireland) and were of HPLC grade. Pure water was obtained by treatment of demineralized water in a Nanopure system (Barnstead, Boston, MA, USA). Only disposables or new glassware were used to avoid contamination.

2.2. HPLC analysis with fluorescence detection after post-column on-line zinc reduction

HPLC analyses were performed using a Varian Star 9010 solvent delivery system with a Varian Star 9095 autosampler, with on-line zinc reduction and fluorescence detection (Jasco 820-FP, Jasco, Tokyo, Japan) at an excitation wavelength of 360 nm and an emission wavelength of 430 nm. In this system, a column (50 mm×4.6 mm I.D.) filled with zinc–glass beads (3:1) was placed behind a Merck reversed-phase C_{18} column (15 cm×4 mm I.D.), in order to reduce nitro groups to amino groups and achieve a sensitive and selective detection of both amino and nitro compounds in a single run. Exclusion of the zinc column resulted in the detection of metabolites with an amino group only. Samples (50 μl) were analyzed using a linear gradient of 100% 10 m*M* Tris-HCl buffer to 100% methanol in 60 min at 1.0 ml/min.

2.3. GC–MS–MS analysis

Quantitation of adduct levels was achieved using a Varian 3400 CX gas chromatograph, equipped with a Varian Saturn 4D Ion Trap MS detector and a Varian 8200 CX autosampler. A 4 μl volume (in toluene) was injected on column at 0.5 μl/s with a post-injection "hot needle" time of 0.1 min. A 5 m×0.53

mm I.D. deactivated fused-silica "retention gap" and a 30 m×0.25 mm I.D. capillary fused-silica DB-5MS column with a film thickness of 0.25 μm (J&W Scientific, Folsom, CA, USA) were applied. Helium was used as carrier gas at 14 Pa column head pressure. The initial oven and injector temperature was 110°C. The oven temperature was kept at 110°C for 1 min, followed by an increase at a rate of 10°C/min to 300°C. The transfer line was set at 290°C. The mass spectrometer was operating in EI mode at 70 eV. For MS–MS analysis, the parent ion of 1-AP derivatized using HFBA with mass *m/z* 413 was stored in the ion trap and further fragmented to a daughter ion with *m/z* 216, at an excitation RF of *m/z* 120 and an excitation voltage of 94 V (non-resonant CID). For the internal standard, HFB-d^9-1-AP, the parent ion with mass *m/z* 422 was trapped and fragmented to daughter ion *m/z* 225 using the same conditions. The daughter ions were used for quantitation. A mass range of *m/z* 180–430 was scanned at 0.6 s/scan. The background mass was set at *m/z* 99 to reduce the background noise and thus increase the sensitivity. Data acquisition and analysis was performed with Varian Saturn software.

2.4. Internal standard

The internal standard (I.S.) for analyses using GC–MS, d^9-1-AP, was prepared by reduction of d^9-1-NP. d^9-1-NP was dissolved in 1 ml of methanol–Tris-HCl (9:1, v/v). Zinc (100 mg) and ammonium chloride (100 mg) were added. The mixture was kept shaking gently overnight at 37°C. After addition of 1 ml of pure water, the mixture was extracted twice with ethyl acetate. The solvent layers were combined and evaporated under a gentle stream of nitrogen at 30°C. The residue was dissolved in methanol. HPLC–fluorescence detection (see Section 2.2) showed that the reduction was complete and that only one compound was produced. This compound appeared to be d^9-1-AP upon GC–MS analysis (EI mode), with total mass of *m/z* 422 after derivatization and a major fragment of *m/z* 225. The I.S. was used for GC–MS–MS analysis of Hb hydrolysates (addition before extraction with hexane) and for GC–MS–MS analysis of calibration curves of 1-AP in Hb hydrolysate.

2.5. Animal treatment

Rats were given intragastrically either 10 mg of 1-NP in trioctanoin each and sacrificed at 6 or 12 h after exposure, or 1 mg of 1-NP in trioctanoin/kg body weight and sacrificed at 1.5 or 3 h after exposure. Different dosing and various time points of sacrifice were used because the time course and dose–response relationships of Hb adduct formation by 1-NP metabolites in vivo had not been studied extensively yet. The blood of these rats was used for the development of the method. The time course of Hb adduct formation was studied with blood of rats intragastrically administered 1 mg of 1-NP in trioctanoin/kg body weight and sacrificed at 1.5, 3, 6, 9, 12 and 24 h after exposure. Control rats were given trioctanoin only. In all animal experiments three male Sprague–Dawley rats (264±12 g) were used per time point of sacrifice. The rats were housed separately in metabolism cages and had free access to water and a standard diet (RHM, Hope Farms, Woerden, The Netherlands).

2.6. Collection and preparation of Hb

Blood was collected at several time points after exposure via a heart puncture using a syringe with heparin and processed immediately. Collection of the erythrocytes was achieved by centrifugation at 900 *g* for 20 min at 4°C. The erythrocytes were washed 3 times with an equal volume of PBS before the cells were hydrolyzed in ice-cold water. After addition of 0.67 *M* potassium phosphate (pH 6.5), the lysate was centrifuged at 10 000 *g* for 20 min at 4°C. The supernatant contained Hb. Aliquots of Hb were treated with 5 volumes of methanol to remove free, non-covalently bound metabolites. After addition of the methanol, the mixture was kept on ice for 5 min, followed by centrifugation at 2000 *g* for 3 min. The supernatant was discarded and the pellet resuspended in methanol. This procedure was repeated 3 times. Subsequently, the pellet was resuspended in 200 μl of DMSO and 500 μl of 0.5% SDS, sonicated for 15 min and shaken gently. From this suspension, aliquots were taken for hydrolysis and protein determination. For the protein determination, aliquots of the Hb suspension were treated with 1% of HCl–

acetone, precipitating the globin (Glb). The pellet was washed with acetone twice, before dissolving the Glb in pure water. The protein content was determined using Coomassie Brilliant Blue according to Bradford [22].

2.7. Hydrolysis of Hb adducts of 1-NP

To study the yield of 1-AP after hydrolysis, Hb of rats exposed to 10 mg of 1-NP and sacrificed at 6 and 12 h was pooled with Hb of rats exposed to 1 mg of 1-NP/kg body weight and sacrificed at 1.5 or 3 h after administration. Blood obtained from rats given different amounts of 1-NP and sacrificed at various time points was pooled to obtain a mixture representing Hb adduct levels at different exposure levels and time points after administration. The pooled Hb was treated as described in Section 2.6 and was used as a stock solution. To study the optimization of the hydrolysis, several factors potentially affecting the hydrolysis were varied. Aliquots were hydrolyzed by addition of NaOH (0.1 *M*, 0.5 *M*, 1 *M* or 5 *M* final volume) or HCl (1 *M* final volume). The duration of the hydrolysis varied from 30 to 240 min, at temperatures ranging from 22°C to 90°C. The influence of the presence of oxygen was studied by hydrolyzing under nitrogen (allowing the nitrogen to flow through the sample during 5 s immediately followed by sealing the tubes). For the determination of the time course of Hb adduct formation after administration of 1-NP, aliquots of Hb were hydrolyzed under nitrogen atmosphere, in 1 *M* NaOH (final volume) for 60 min at 70°C.

2.8. Identification of released products

After hydrolysis of aliquots of pooled Hb, samples were extracted with 3 volumes of hexane followed by an extraction with 3 volumes of ethyl acetate. The solvent layers were combined and evaporated under a gentle stream of nitrogen at 30°C, after addition of a few drops of 1 *M* HCl to increase the recovery. The residue was dissolved in methanol–water (1:1, v/v) and analyzed by HPLC with fluorescence detection and post-column on-line reduction.

2.9. Extraction, derivatization and quantitation of released compounds

If acidic conditions were used for hydrolysis, 10 *M* NaOH was added to the hydrolysate until pH>10. Next, extraction was conducted by addition of 3 volumes of hexane, mixing thoroughly for 30 s and centrifugation at 2000 *g* for 3 min. In some samples the separation between the layers was not clear. The separation was improved by freezing (in liquid nitrogen) and thawing (in tap water) followed by centrifugation. The extraction was repeated and the hexane layers were combined. HFBA (50 μl) was added and samples were kept at 70°C for 30 min for an optimal derivatization. Excess HFBA was removed by extraction with 1 ml of pure water. Hexane was evaporated under a gentle stream of nitrogen at 30°C. The residue was dissolved in 10–25 μl of toluene and analyzed using GC–MS–MS.

2.10. Calibration curves and recovery

Several concentrations (10 or 25 μl of 0–50 ng/ml) of 1-AP were spiked (in duplicate) in hydrolysates of Hb of control rats to obtain calibration curves. These samples were extracted as described in Section 2.9. Calibration curves were prepared for each series of samples analyzed. Moreover, in every series involving hydrolysis of Hb adducts of 1-NP, aliquots of the stock solution of Hb adducts of 1-NP used for the validation of the hydrolysis were analyzed along, as a control for the conditions of hydrolysis. Recoveries were calculated using the slope of a linear regression of peak areas of 1-AP versus the concentration. The same concentrations of 1-AP in toluene derivatized and analyzed in the same way were set at 100%.

3. Results and discussion

3.1. Identification of released compounds

Extracts of Hb hydrolysates were analyzed by HPLC with fluorescence detection and post-column on-line reduction to study the compounds released from Hb of rats following administration of a single

oral dose of 1-NP. The only detectable product (after mild as well as strong alkaline hydrolysis) was eluted at the same retention time as 1-AP (Fig. 1). This product was positively identified as 1-AP by GC–MS analysis in EI mode. The other peaks observed in the HPLC chromatogram were also found in HPLC chromatograms of sham-exposed rats. El-Bayoumy et al. [12] reported the occurrence of a heme adduct in rats sacrificed 24 h after oral administration of 1-NP. However, since in our study 1-AP was released upon hydrolysis, it seems more likely that 1-AP is the cleavage product of binding of 1-nitrosopyrene to cysteine present in the globin moiety. This mechanism regarding Hb binding has been suggested for related compounds, such as arylamines and nitroarenes [7,9,14]. Although 1-NP is known to be metabolized to reactive 1-NP-epoxides in vivo [23], no compounds were released using the described conditions for hydrolysis which could indicate the involvement of such metabolites in Hb adduct formation.

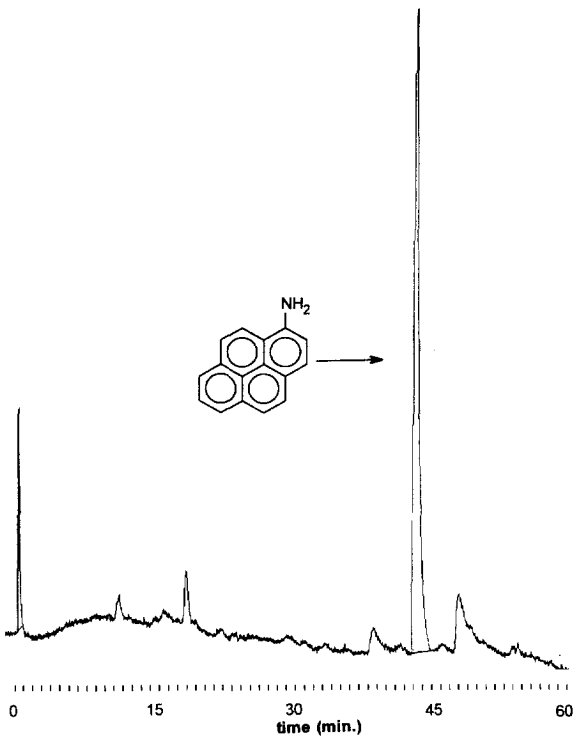

Fig. 1. HPLC analysis with fluorescence detection after post-column on-line zinc reduction of compounds released upon hydrolysis of pooled Hb of rats exposed to a single dose of 1-NP. For chromatographic and experimental conditions see Section 2.

3.2. Detection of 1-AP-HFB using GC–MS–MS

1-AP was derivatized with HFBA for GC–MS–MS analysis. Comparison of GC–MS analysis with GC–MS–MS (both in EI mode), showed an increase in sensitivity with one order of magnitude. d^9-1-AP was used as I.S. (Fig. 2).

3.3. Validation of hydrolysis

The hydrolysis of 1-NP Hb adducts was optimized by comparing the amount of released 1-AP after hydrolysis under various conditions, using aliquots of pooled Hb derived from exposed rats. The factors time, temperature, presence of oxygen and alkaline/acidic conditions during hydrolysis were studied. All samples hydrolyzed under nitrogen showed higher concentrations of released 1-AP compared with samples hydrolyzed in ambient air (Fig. 3). Apparently the hydrolysis reaction mixture is susceptible for oxidative influences. An increase in time of hydrolysis did not always result in higher concentrations of released 1-AP.

Hydrolysis with 0.5 *M* NaOH instead of 0.1 *M* NaOH resulted in a significant increase of released 1-AP (Fig. 4). Increasing the alkalinity above 0.5 *M* NaOH did not increase the 1-AP concentration any further.

In several studies, Hb adducts of aryl amines [8] and nitroarenes [7,21] are hydrolyzed under mild alkaline or acidic conditions at room temperature or at 37°C. However, Fig. 5 shows that hydrolysis of Hb adducts of 1-NP at 50 or 70°C greatly enhances the yield of released 1-AP when compared to lower temperatures. Although the yield of 1-AP after hydrolysis of Hb adducts of 1-NP was optimized, it remains unknown whether the hydrolysis was complete. In order to investigate whether the detected 1-AP was not resulting from the presence of non-covalently bound 1-AP, some samples were extracted directly after the addition of the NaOH. GC–MS–MS analysis of these extracts showed that 1-AP could not be detected. Hydrolysis of Hb adducts of

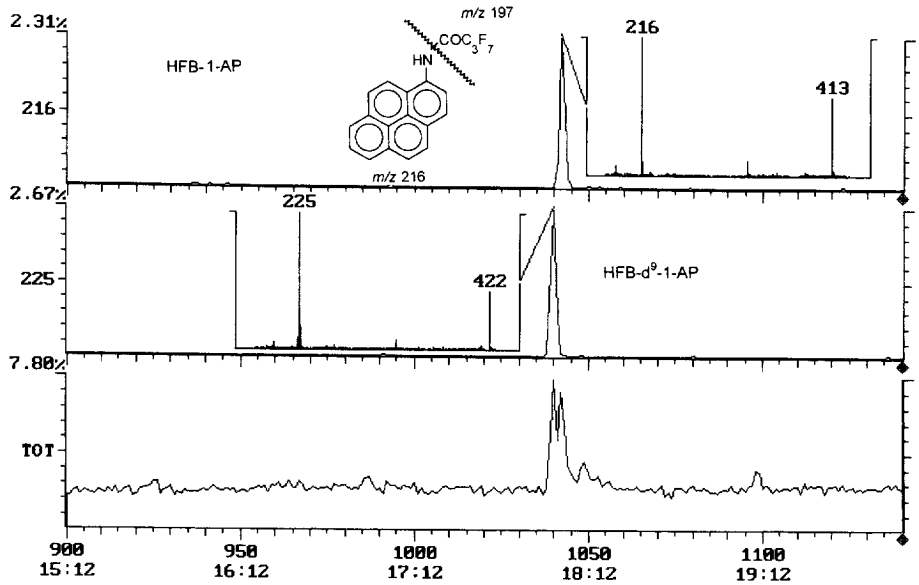

Fig. 2. GC–MS–MS single-ion chromatogram of the daughter ions of HFB-1-AP *m/z* 216 (upper panel) and HFB-d^9-AP *m/z* 225 (middle panel) with mass spectrum, and total ion chromatogram (lower panel) of hydrolyzed pooled Hb of rats exposed to a single dose of 1-NP. For chromatographic and experimental conditions see Section 2.

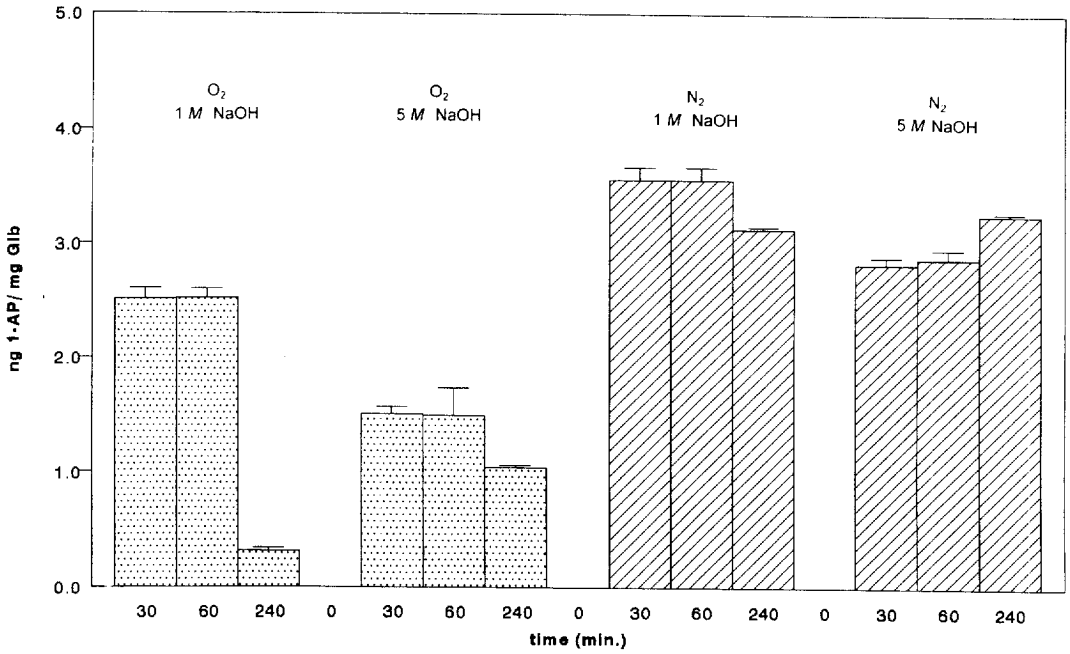

Fig. 3. Influence of time, alkaline conditions and the presence of oxygen on the hydrolysis of Hb adducts of 1-NP, yielding 1-AP. Samples were hydrolyzed either in ambient air (O_2) or under nitrogen (N_2) in 1 *M* or 5 *M* NaOH (final volume). The temperature during hydrolysis was set at 70°C. The experiments were conducted twice, on two different days and in duplicate. The results are presented as arithmetic means with standard deviations (error bars).

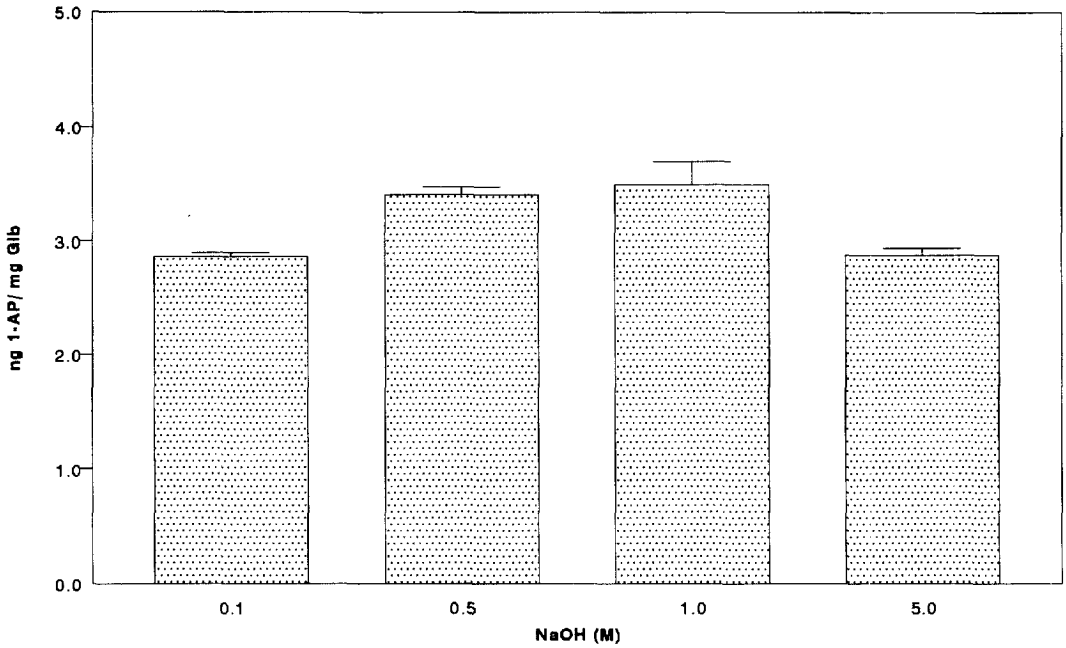

Fig. 4. Influence of alkaline conditions on the hydrolysis of Hb adducts of 1-NP, yielding 1-AP. Samples were hydrolyzed under nitrogen during 60 min at 70°C. The experiments were conducted twice, on two different days and in duplicate. The results are presented as arithmetic means with standard deviations (error bars).

1-NP under acidic conditions (in 1 *M* HCl, under nitrogen and with various temperatures) resulted not only in low amounts of released 1-AP, but also in poor recoveries (data not shown).

3.4. Calibration curves, recovery and detection and determination limit

A recovery, determined on three different days and in duplicate, of 72.2±5.4% was achieved when 1-AP was spiked in hydrolyzed Hb and extracted and derivatized as described. *R*-squares (r^2) of 0.997 or higher were obtained. The recovery was not influenced by addition of 1-AP prior to the hydrolysis instead of after the hydrolysis. The detection limit at a signal-to-noise ratio of 3 for 1-AP derivatized with HFBA was 350 fg 1-AP on column. A determination limit of 0.7–1.0 pg 1-AP/mg Glb was observed.

3.5. Inter-day variation

Aliquots of pooled Hb of rats exposed to 1-NP were analyzed in duplicate (hydrolyzing under nitrogen, in 1 *M* NaOH, at 70°C for 60 min) on eight different days, over 7 weeks. Over this period, the concentration of released 1-AP was 3.3±0.3 ng/mg Glb (coefficient of variation of 9.5%).

3.6. Time course of Hb adducts of 1-NP

Since the yield of 1-AP was optimal after hydrolysis of Hb adducts of 1-NP in 1 *M* NaOH at 70°C under nitrogen atmosphere during 60 min, these conditions were used for the determination of the time course of Hb adduct levels after oral administration of a single dose of 1-NP to rats. A maximum in released 1-AP was reached at 1.5–3 h after administration (Fig. 6). Subsequently, concentrations 1-AP decreased remarkably fast within 6 h after exposure. From this time point on, a significantly slower decrease of the amount released 1-AP was observed. Suzuki et al. [21] observed a peak level of released 1-AP at 12 h after oral administration of 1-NP, followed by a similar biphasic decline as was observed in this study. At 24 h

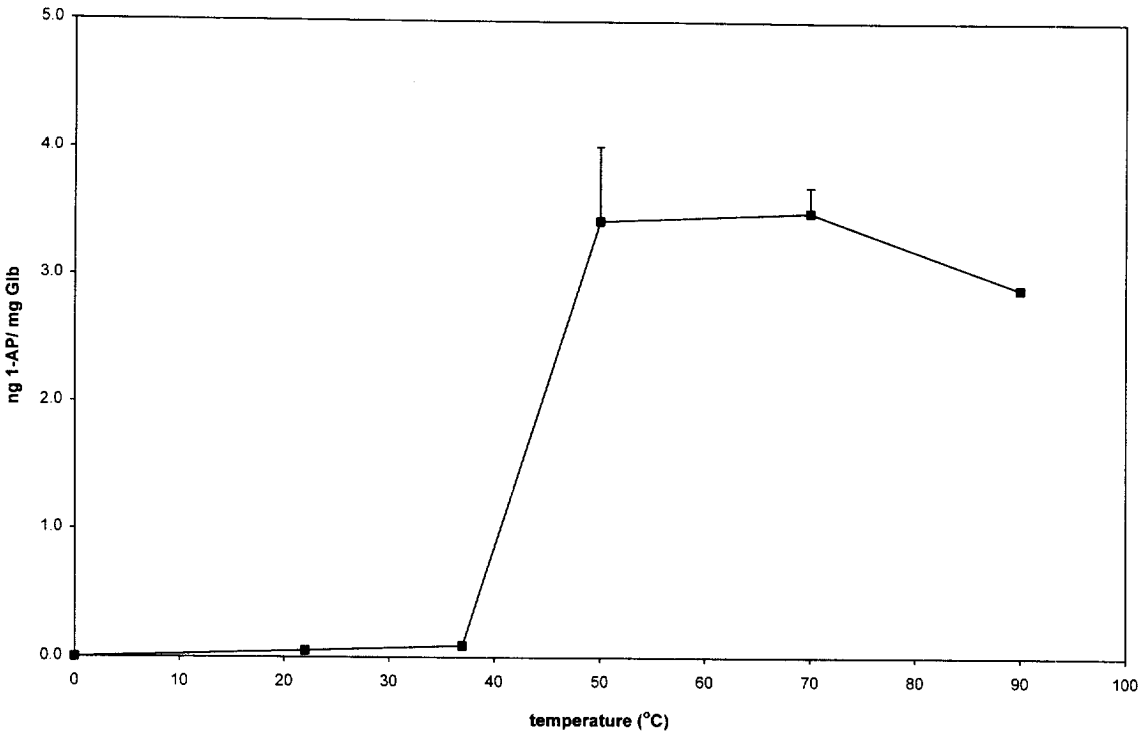

Fig. 5. Influence of the temperature on hydrolysis of Hb adducts of 1-NP, yielding 1-AP. Hydrolysis was conducted under nitrogen, in 1 *M* NaOH (final volume) for 60 min. The experiments were conducted twice, on two different days and in duplicate. The results are presented as arithmetic means with standard deviations (error bars).

after administration still 5 pg 1-AP/mg Glb was detected (Fig. 6). This is in the same order of magnitude as reported previously after administration of radioactive labeled 1-NP to rats [12]. The large deviation at 3 h after exposure was due to an outlier. In this rat 72.5±5.2 pg 1-AP/mg Glb was detected, whereas the average of the other two rats showed levels of 16.2±1.2 pg 1-AP/mg Glb.

4. Conclusions

This study presents a reproducible, selective and sensitive method for the determination of 1-NP Hb adducts by measuring released 1-AP, using GC–MS–MS. The method involves removal of free metabolites by treatment with methanol followed by hydrolysis of the Hb adducts of 1-NP. The yield of 1-AP was optimal when Hb adducts were hydrolyzed under nitrogen, in 1 *M* NaOH, at 70°C for 60 min. Released 1-AP was extracted efficiently with hexane, prior to derivatization with HFBA. In accordance with previous studies [12,21], low Hb adduct levels were observed 24 h after administration of a single oral dose of 1-NP (5 pg 1-AP/mg Glb). Chronic exposure to the compound will result in an accumulation of Hb adducts due to the long half life of the protein, assuming that the Hb adducts are stable. Recently, Hb adducts of 1-NP (pmol 1-AP/g Hb range) have been detected in humans occupationally exposed to DE [7]. In the case of these low 1-NP Hb adduct levels the method presented here could be adjusted by hydrolyzing larger quantities of Hb. This method will be validated further for its suitability in monitoring of exposure to DE.

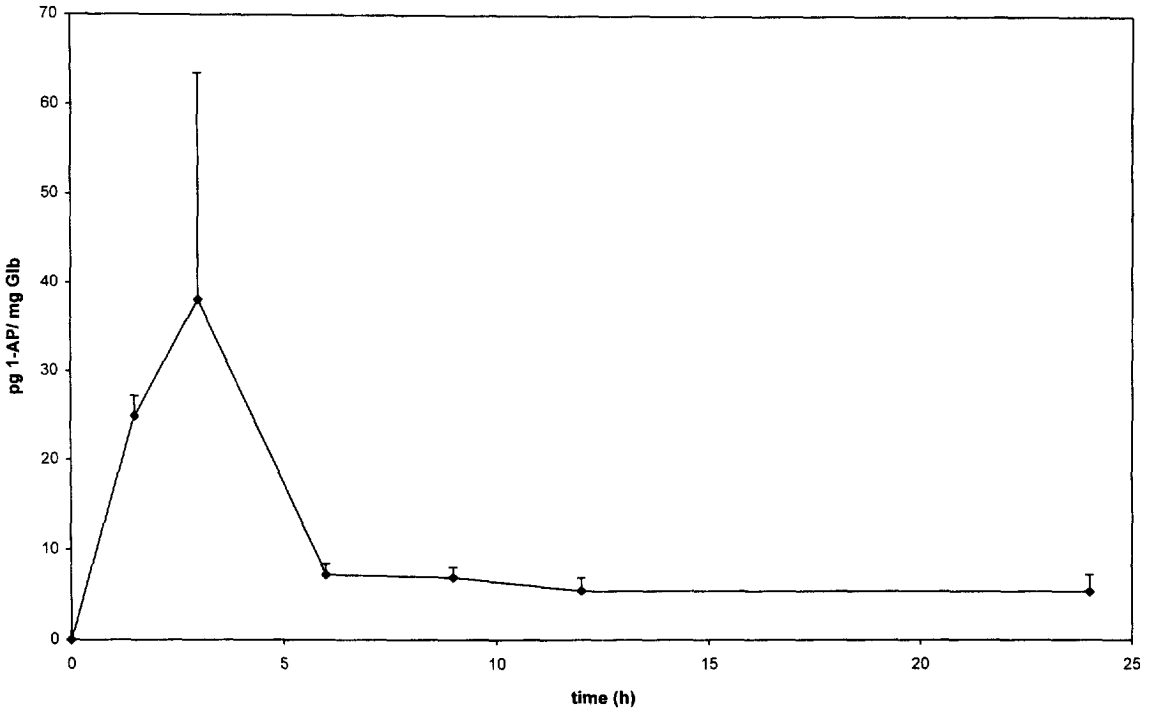

Fig. 6. Time course of the yield of 1-AP as released product upon hydrolysis of Hb adducts following oral administration of a single dose of 1-NP. Hydrolysis was conducted under nitrogen, in 1 *M* NaOH (final volume) for 60 min. The experiments were conducted twice, on two different days and in duplicate. The arithmetic mean of three rats per time point with standard deviation (error bars) are presented.

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