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Assessment of 3-nitrobenzanthrone reductase activity in mammalian tissues by normal-phase HPLC with fluorescence detection

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Abstract

3-Nitrobenzanthrone (3-NBA) is a potent mutagen and possible human carcinogen present in diesel exhaust and airborne particulate matter. Nitroreduction is believed to play a crucial role in nitroarene activation and mutagenicity; however, quantification of nitroreduction rate in mammalian samples has proved difficult. In this study, we present a sensitive method to quantify 3-nitrobenzanthrone reductase activity in murine tissues via normal-phase HPLC with fluorescence detection of the reduced product 3-aminobenzanthrone (3-ABA). Calibration linearity was obtained for pure 3-ABA concentrations of 1–500 ng/ml ($r^2 > 0.99$), with a detection limit of 0.25 ng/ml (S/N = 3). Incubation time, substrate concentration, and protein concentration in the reaction mixture were optimized, and the detection limit of the enzyme assay is 0.97 pmol/min/mg protein. The apparent K_m and V_{max} for post-mitochondrial supernatant from Muta™Mouse liver (i.e., liver S9) were 23.9 μM and 70.2 pmol/min/mg protein, respectively. Analysis of replicate samples of Muta™Mouse liver and lung S9 yielded mean activity values of 39.0 ± 3.0 and 61.1 ± 4.3 pmol/min/mg, respectively. ANOVA revealed significant effects of tissue type and incubation condition (i.e., with or without N_2). The results show significantly higher activity in lung, and, in contrast to that observed for 1-nitropyrene, incubation in open air (i.e., without N_2 bubbling) causes only a marginal decrease in activity. Quantification of 3-NBA nitroreductase activity in murine tissues will provide insight into the published tissue-specific mutagenic activity of 3-NBA.

Keywords: Nitroreductase; Nitroarenes; HPLC; 3-Aminobenzanthrone

1. Introduction

3-Nitrobenzanthrone (3-NBA, Fig. 1) is a potent bacterial mutagen and suspected human carcinogen known to be present in diesel exhaust, airborne particulate matter, and more recently detected in soil and water [1–3]. In bacterial assays such as the Salmonella mutagenicity test, 3-NBA yields a mutagenic potency of 208,000 revertants/nmol (TA98 without S9), a value that is comparable to the potency of 1,8-dinitropyrene, the most potent direct-acting bacterial mutagen reported in the literature (257,000 revertants/nmol in TA98 without S9) [1]. In mammalian cells, 3-NBA induces micronuclei, as well as gene mutations at the *tk* and *hprt* loci in several cell lines [4–6]. In vivo experiments (per os) revealed an increased mutation frequency at the *cII* locus in several organs of the transgenic Muta™Mouse (e.g., liver, lung, colon) [7]. Finally, 3-NBA has been shown to induce DNA adducts in cultured animal cells [8–11], and a variety of tissues following in vivo exposure [12,13].

Nitro polycyclic aromatic hydrocarbons (nitroPAHs) such as 3-NBA require metabolic reduction in order to generate reactive electrophiles (e.g., nitrenium ion) that react with DNA. Mechanistic information about the biochemical pathways involved in the conversion from parent nitroPAH to DNA damaging electrophile comes from numerous sources. For example, the direct-acting mutagenic activity of 3-NBA

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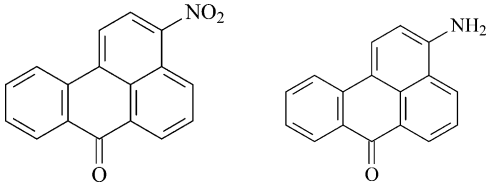

3-nitrobenzanthrone 3-aminobenzanthrone

Fig. 1. Chemical structure of 3-nitrobenzanthrone (CAS 1711-34-9) and its corresponding amino metabolite 3-aminobenzanthrone.

is decreased in nitroreductase-deficient *Salmonella* TA98NR in comparison with the parent TA98 [1]. Levels of 3-NBA–DNA adducts formed in vitro are positively correlated with NADPH:P450 reductase activities in human hepatic microsome samples, and inhibition of NADPH:P450 reductase by α-lipoic acid results in decreased adduct formation [11]. Furthermore, the frequency of DNA adducts are 45- to 78-fold higher in Chinese hamster lung V79 cells over-expressing human NADPH:P450 reductase compared with the parental V79MZ cells [11].

A mechanism for 3-NBA activation, recently proposed by Arlt et al. [14], requires an initial nitroreduction by cytosolic reductases such as xanthine oxidase, DT-diaphorase, aldehyde oxidase, or microsomal NADPH:P450 reductase to produce an unstable hydroxylarylamine. This unstable intermediate can then follow one of several alternative pathways to yield a reactive electrophile that can damage DNA. Spontaneous dehydrogenation of the protonated hydroxylamine can yield reactive nitrenium or carbocations. Acetylation of the hydroxylarylamine by *N,O*-acetyltransferase yields an acetylated product, which can readily provide the reactive cations via subsequent deacetylation. Alternatively, further reduction of the hydroxylarylamine will result in the formation of a stable amine. In the presence of cytochrome P4501A2 in the liver, the amine product can be converted back to the hydroxylarylamine intermediate [15]. Interestingly, there is currently no evidence to support ring oxidation of 3-NBA by cytochrome P450 enzymes, a reaction that has been observed for other nitro compounds such as 1-nitropyrene [16]. 3-Aminobenzanthrone (3-ABA) (Fig. 1) has been identified as the major product of 3-NBA reduction in rat lung type II alveolar cells [17], and the presence of 3-ABA in the urine of mine workers has been used as a marker of diesel exhaust exposure [18].

Although there is a great deal of evidence confirming the importance of nitroreduction in 3-NBA mutagenicity [5,10], quantitative determination of the rate of 3-NBA reduction in bacteria or mammalian tissues has not been attempted. In this work, we present a sensitive method to quantify 3-nitrobenzanthrone reductase activity in mammalian cells and/or tissues. The rate of 3-NBA reduction, assessed via 3-ABA production, was determined using normal-phase HPLC with fluorescence detection.

2. Materials and methods

2.1. Chemicals and animals

Caution: 3-NBA is a potent mutagen and suspected human carcinogen. It should be handled with extreme care.

3-NBA (3-nitro-*7H*-benz[*de*]anthracen-7-one) was obtained from the Sigma Library of Rare Chemicals (Sigma–Aldrich, Oakville, ON). Custom synthesized 3-ABA was a generous gift of Volker Arlt (Institute of Cancer Research, Surrey, UK). The solvents acetonitrile, methanol (MeOH), ethyl acetate, tetrahydrofuran, dichloromethane (DCM), toluene, and *n*-hexane were all HPLC-grade (OmniSolv®, EMD Chemicals Inc., Gibbstown, NJ). All other chemicals were analytical grade. Dimethylsulfoxide (DMSO) was obtained from Sigma–Aldrich (Oakville, ON). Oxoid nutrient broth No. 2 was obtained from Oxoid Inc. (Ottawa, ON). Glucose-6-phosphate (G-6-P), reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate ($NADP^+$), and glucose-6-phosphate dehydrogenase were obtained from Sigma–Aldrich (Oakville, ON). Transgenic mouse strain 40.6, also known as the Muta™Mouse (CD/2 F_1 generated from BALB/c × DBA/2 [19]), was bred and maintained under conditions approved by the Health Canada Animal Care Committee. *Salmonella typhimurium* strains TA98 and TA100 were obtained from Molecular Toxicology Inc. (Boone, NC). *S. typhimurium* strains YG1041 and YG1042 were generous gifts of Takehiko Nohmi (National Institute of Health Sciences, Tokyo, Japan) [20].

2.2. Preparation of Muta™Mouse tissue homogenates and Salmonella lysates

Healthy male Muta™Mouse specimens were euthanized by cervical dislocation, and selected tissues (e.g., liver, lung) excised for further processing. The collected tissues were rinsed with ice-cold 50 mM phosphate buffer (pH 7.4), minced with scissors, and homogenized (PCR Tissue Homogenizing Kit, Omni International Inc., Marietta, GA) in cold buffer at a 1:5 (w/v) ratio. The homogenate was then centrifuged for 20 min at 9000 × *g* (4 °C) (Eppendorf 5415R, Brinkmann Instruments, Mississauga, ON), and the supernatant (i.e., post-mitochondrial supernatant or S9 fraction) kept at −30 °C until enzymatic analysis. To obtain bone marrow, femurs were flushed with cold phosphate buffered saline and the solution centrifuged at 10,000 × *g* (4 °C) for 1 min. Pellets were kept at −30 °C until enzymatic analysis.

Bacterial cells were grown overnight in 100 ml Oxoid nutrient broth No. 2 at 37 °C with agitation, harvested by centrifugation, and washed with 50 mM phosphate buffer (pH 7.4). Cells were disrupted by sonication (Braunsonic 1510, Branson Ultrasonics Corp., Danbury, CT) at 250 W for 6 × 30 s with 15 s intervening intervals on ice. The homogenate was centrifuged at 9000 × *g* for 20 min (4 °C) and the supernatant kept at −30 °C until enzymatic analysis.

The protein content of the homogenates was determined via the Bradford method (Bio-Rad Laboratories (Canada), Ltd., Mississauga, ON) with bovine serum albumin as a standard [21].

2.3. Enzyme reaction systems

Nitrofurazone reductase activity was assayed by measuring the temporal decrease in absorbance of nitrofurazone at 400 nm (molar extinction coefficient 12,960 M^{-1} cm^{-1} [22,23]) at room temperature (approximately 23 °C). Reaction mixtures contained (1 ml) 50 mM Tris–HCl (pH 7.0), 0.1 mM nitrofurazone, and 0.1 mM NADPH (reduced form). The reaction was initiated by adding 0.5–1.0 mg of cell or tissue preparation (i.e., S9 fraction).

3-NBA reductase reaction mixtures (total volume 1.0 ml) consisted of 50 mM sodium phosphate buffer (pH 7.4), 3.0 mM $MgCl_2$, 1.0 mM NADH, 1.0 mM $NADP^+$, 4.0 mM glucose-6-phosphate, and 1 unit of glucose-6-phosphate dehydrogenase. This NADPH generating system was then purged with ultra-pure N_2 gas (BOC Canada Ltd., Mississauga, ON) for 30 min on ice before adding substrate (i.e., 3-NBA) to a final concentration of 20 μM. The reaction was initiated by adding 0.5–1.0 mg protein of the prepared tissue/cell fractions (i.e., S9 fraction) and incubated anaerobically at 37 °C for 30 min. The reaction was stopped by adding 100 μl methanol, and the product was extracted with 1.0 ml toluene/ethyl acetate (1:1, v/v). The extraction was performed three times; the combined solvent evaporated under ultra-pure N_2, and the residue dissolved in 0.5 ml ethyl acetate. The amount of 3-ABA produced was determined using normal-phase HPLC (see below). All calibration standards and analytical samples were dissolved in ethyl acetate.

2.4. Normal-phase HPLC detection of 3-aminobenzanthrone

The HPLC system consisted of a Waters 600E multi-solvent delivery system, a Waters 2767 sample manager with 20 μl injection loop (Waters Corporation, Milford, Massachusetts), a Tosoh TSK gel CN80Ts cyanopropyl column (5 μm particle, 80 Å pore, 4.6 mm × 250 mm i.d., running at ambient temperature) (Tosoh Bioscience, Montgomeryville, Pennsylvania), and a Waters 2475 Multi-λ fluorescence detector. *n*-Hexane/ethyl acetate (60:40, v/v) was used as mobile phase with a flow rate of 1.0 ml/min. Excitation and emission wavelengths for 3-ABA detection were 470 and 570 nm, respectively. Fluorescence detector gain was set to 1 and energy units full scale (EUFS) was set at 1000. Linear calibration curves, which were run daily, were obtained for concentrations between 1.0 and 500 ng/ml ($r^2 > 0.99$). Calibration curves were analyzed by ordinary least-squares linear regression. There was no evidence of variance heteroscedasticity, and no weighting was applied.

3-ABA calibration standards in ethyl acetate were prepared immediately before each calibration run. Additional analyses (not shown) confirmed that the 10 ng/ml 3-ABA standard was stable for at least 1 week at 4 °C. Since 3-ABA is sensitive to metals, the HPLC system employed only PEEK (polyetheretherketone) tubing (Tsuyoshi Murahashi, Kyoto Pharmaceutical University, personal communication).

2.5. Data analyses

Fluorescence and photodiode array detector signals were collected and integrated using MassLynx 4.0 (Waters Corporation, Milford, Massachusetts). The concentration of the catalytic product, 3-ABA, was determined using a linear calibration curve with integrated peak area data from standards between 1.0 and 500 ng/ml. Specific enzyme activity was calculated as the 3-ABA production rate (pmol/min/mg protein). The apparent K_m and V_{max} values were calculated from plots of enzyme activity versus substrate via least-squares, non-linear regression analysis (SAS version 8.02 for Windows [24]). Treatment effects (e.g., tissue type, incubation time, nitrogen bubbling) were examined using least-squares ANOVA or paired-sample *t*-tests (SigmaStat 2.03 for Windows, SPSS Inc., Chicago, IL). For the latter analysis, the homogeneity of variance assumption was verified using the Bartlett test [25].

3. Results and discussion

3.1. Chromatography conditions for 3-aminobenzanthrone quantification

Murahashi et al. developed a sensitive HPLC method to quantify trace amounts of 3-nitrobenzanthrone in surface soil and water following catalytic reduction (i.e., reflux with Raney Ni) and HPLC detection of 3-aminobenzanthrone [3]. We adapted this sensitive analytical method to quantify 3-nitrobenzanthrone reductase activity. However, 3-ABA fluorescence is highly solvent dependant, displaying minimal fluorescence in polar solvents such as methanol and water, and elevated fluorescence in low polarity solvents such as ethyl acetate and *n*-hexane [3]. Maximal fluorescence and optimized excitation/emission wavelengths for 3-ABA in a variety of solvents were determined using a multi-wavelength fluorescence detector scanning from 200 to 800 nm. The results obtained, summarized in Table 1, showed slight variations in the maximum excitation and emission wavelengths and maximum fluorescence in *n*-hexane. In addition, the results showed a significant negative relationship ($r^2 = 0.89$, $p < 0.002$) between solvent polarity (expressed as PI or polarity index) and relative fluorescence intensity (Fig. 2). *n*-Hexane or other non-polar solvents proved to be ideal choices as LC mobile phases.

Normal-phase HPLC employed a cyanopropyl bonded-phase silica column as the stationary phase, and although *n*-hexane permits maximal fluorescence of 3-ABA, it proved too weak to elute 3-ABA from the column. Addition of

Table 1
Maximal fluorescence intensities (RFU) of 3-aminobenzanthrone (310 ng/ml) in a variety of solvents

Solvent	Polarity index	Fluorescence intensity	Excitation (nm)	Emission (nm)
Hexane	0.00	7869.48	461	536
Toluene	2.40	6453.02	468	550
DCM	3.10	4490.31	468	530
Tetrahydrofuran	4.00	2722.02	473	574
Ethyl acetate	4.40	3477.12	470	570
MeOH	5.10	28.60	513	604
Acetonitrile	5.80	852.59	472	596

increasing proportions of ethyl acetate increased eluotropic strength for acceptable 3-ABA elution. An acceptable retention time of 6.9 min was obtained with *n*-hexane/ethyl acetate (60:40, v/v) at a flow rate of 1.0 ml/min (Fig. 3A).

Nitroreductase activity in enteric bacteria such as *S. typhimurium* is usually measured spectrophotometrically using nitrofurazone as a convenient substrate [22,23]. Initial attempts to adapt this method to mammalian samples proved unsuccessful (results not shown). Although the method could readily be employed to assess nitroreductase activity in *S. typhimurium* strains TA98, TA100 (i.e., 17 and 53 nmol/min/mg protein, respectively), and the nitroreductase (i.e., *cnr*) enhanced strains YG1041 and YG1042 (i.e., 660 and 568 nmol/min/mg protein, respectively), the method was not sufficiently sensitive, or perhaps inappropriate, to detect nitroreductase activity in Muta^{TM}Mouse tissue samples. Consequently, subsequent efforts to assess nitroreductase activity in Muta^{TM}Mouse samples employed the aforementioned HPLC method.

3.2. Calibration curve, 3-ABA detection limit, and matrix effects

Fig. 3A shows a typical chromatogram of a 10 ng/ml 3-ABA standard run under the aforementioned conditions. Replicate runs showed good reproducibility and a relative standard deviation of 3.6% for this concentration ($n = 6$). The relative standard deviation for a 100 ng/ml solution was 1.0% ($n = 5$). Good calibration linearity was observed for 3-ABA concentrations from 1 to 500 ng/ml ($r^2 = 0.99$). Fig. 3B shows a chromatographic run of a blank reaction mixture (i.e., buffer system, S9) extract that should not contain 3-ABA. A small peak was identified around the retention time of 6.9 min with an average integrated peak area of 20 units ($n = 3$). This extremely small peak may be the result of injection carry over from the previous run. Using a signal-to-noise ratio of 3.0, the integrated peak area of 20 units yields a calculated 3-ABA detection limit 0.25 ng/ml.

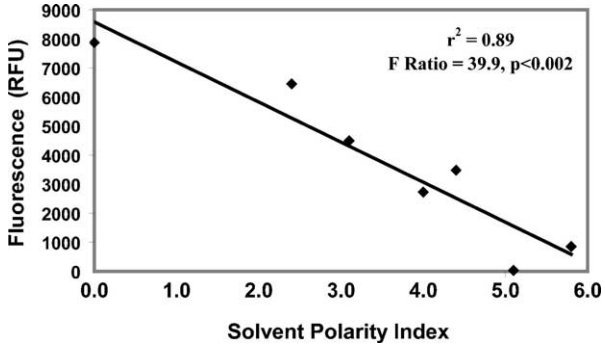

Fig. 2. Effect of solvent polarity on 3-aminobenzanthrone fluorescence intensity. The excitation and emission wavelengths are shown in Table 1.

A major concern regarding the application of a sensitive 3-ABA detection method to a biological enzyme assay is the effect of the biological matrix on the fluorescence intensity of 3-ABA, and the efficiency of 3-ABA extraction from the reaction mixture. To address this issue, 75 ng of 3-ABA was spiked into an extract of a blank biological reaction mixture (i.e., no substrate), dried, and re-dissolved in 0.5 ml ethyl acetate for chromatographic analysis. Fig. 3C shows the resulting chromatogram with strong 3-ABA peak. Replicate runs yielded a mean 3-ABA signal that is 91.1 ± 0.5% of that expected from the 3-ABA calibration curve ($n = 3$). Moreover, when 75 ng 3-ABA was added to a heat-inactivated enzyme reaction system, 3-ABA recovery and detection was 79.7 ± 8.2% of that expected from the 3-ABA calibration curve ($n = 3$). Collectively, these results confirm good efficiency for extraction of 3-ABA from the enzyme reaction mixture, and only minor interference from the extract of the biological matrix.

Fig. 4A shows the spontaneous reduction of 3-NBA to 3-ABA in a reaction mixture without enzyme. The peak area (i.e., 1477 units) indicates a concentration of approximately 5 ng/ml. Fig. 4B and C show typical chromatograms of extracts from the enzymatic reduction reaction with either Muta^{TM}Mouse liver S9 or lung S9, respectively. Under the optimal reaction conditions (see below), the specific net reductase activities were 39.0 ± 3.0 and 61.1 ± 4.3 pmol/min/mg protein for liver S9 and lung S9, respectively ($n = 4$). Considering the observed rate of spontaneous reduction, two times the activity in the biological blank was used to calculate the detection limit for the determination of specific enzyme activity. The calculation yielded a 3-NBA reductase activity detection limit of slightly less than 1 pmol/min/mg protein (i.e., 0.97 pmol/min/mg protein).

3.3. Optimization of enzyme reaction conditions

Several factors, including incubation time and the amount of total protein added to the reaction mixture, were found to affect the measured 3-NBA reductase activity of Muta^{TM}Mouse liver S9. The results shown in Fig. 5 indicate that the formation of 3-ABA increases with increasing incubation time, reaching a maximum approximately 30 min after the start of the reaction. The slight decrease in measured 3-ABA formation at 60 min, which is not statistically significant at $p < 0.05$, may be caused by transformation of 3-ABA by enzymes such as cytochrome P4501A2, *N*-acetyltransferase

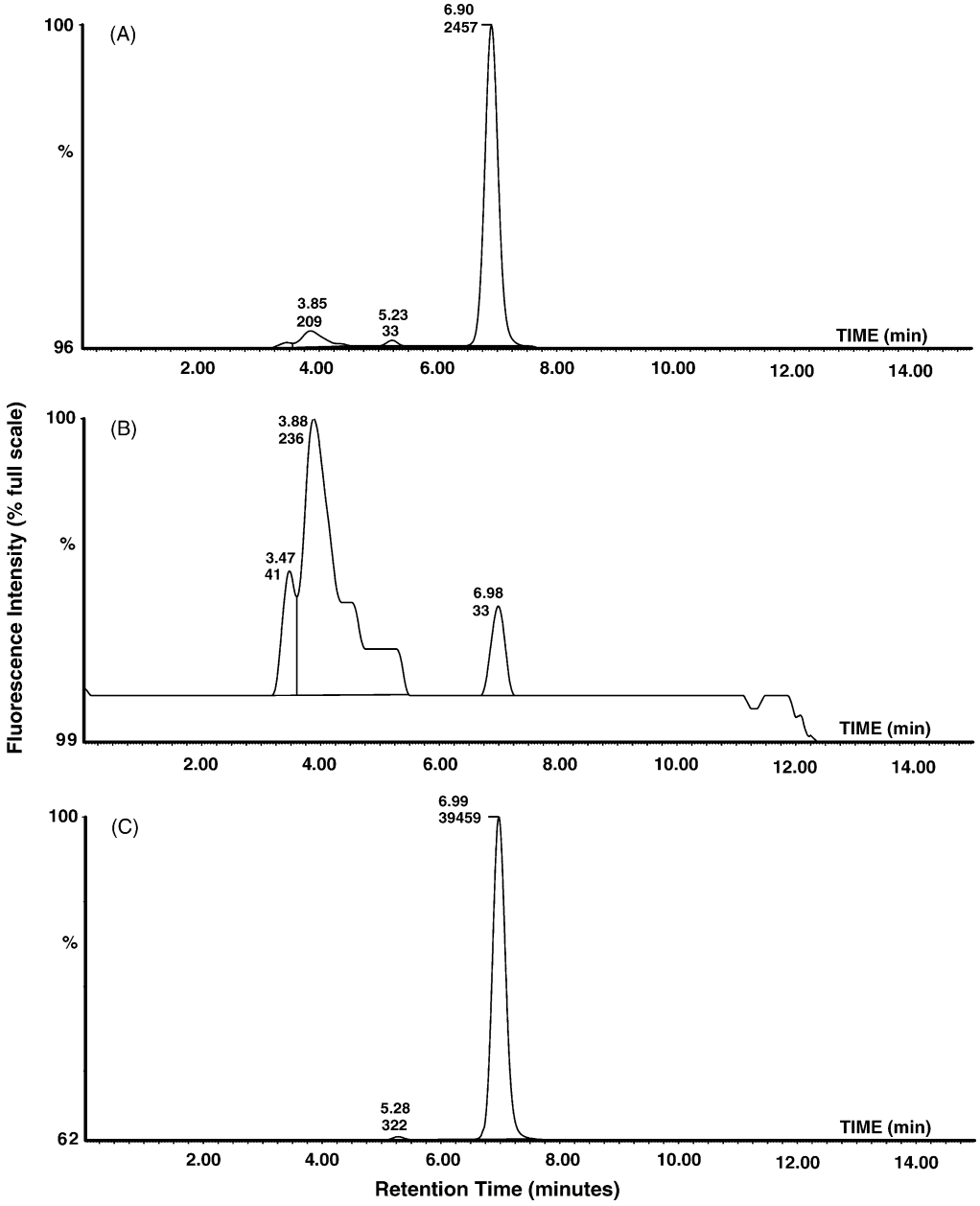

Fig. 3. Chromatograms of (A) a 10 ng/ml 3-ABA standard solution; (B) an extract of a blank enzymatic reaction mixture without substrate; and (C) 75 ng 3-ABA spiked into an extract of a blank enzymatic reaction mixture (final concentration 150 ng/ml). Upper values are chromatographic retention times; lower values are integrated peak areas. *n*-Hexane/ethyl acetate (60:40, v/v) mobile phase on a Tosoh TSK gel CN80Ts cyanopropyl column (4.6 mm × 250 mm, i.d.). Flow rate, 1.0 ml/min. Excitation wavelength 470 nm, emission wavelength 570 nm.

(NAT) or sulfotransferase (SULT), which have been shown to be involved in further metabolism of 3-ABA [10,15]. When reaction time was held constant (30 min), increases in the protein content of the reaction mixture from 0.13 to 1.5 mg total protein yielded a rapid increase in 3-ABA formation up to a protein content of 0.5 mg (Fig. 6). Additional increases in protein content did not yield further increases in 3-ABA production and the relationship begins to display zero order kinetics above 0.5 mg. When both reaction time and protein concentration were held constant (i.e., 30 min, 1 mg protein), increases in substrate concentration (i.e., 3-NBA) yielded a steady increase in the rate of 3-ABA formation. The results, illustrated in Fig. 7, show a linear increase in the rate of 3-ABA formation up to approximately 20 μM 3-NBA, beyond which the reaction begins to approach zero order kinetics.

The apparent K_m and V_{max} of the reaction, calculated via non-linear least-squares regression, were 23.9 μM and 70.2 pmol/min/mg protein, respectively. Although the calculated kinetic parameters are interesting, comparisons with published values for other organisms and/or enzyme systems is not possible, and potentially misleading. Nitro compound reduction in mammals is a multi-step process involving sev-

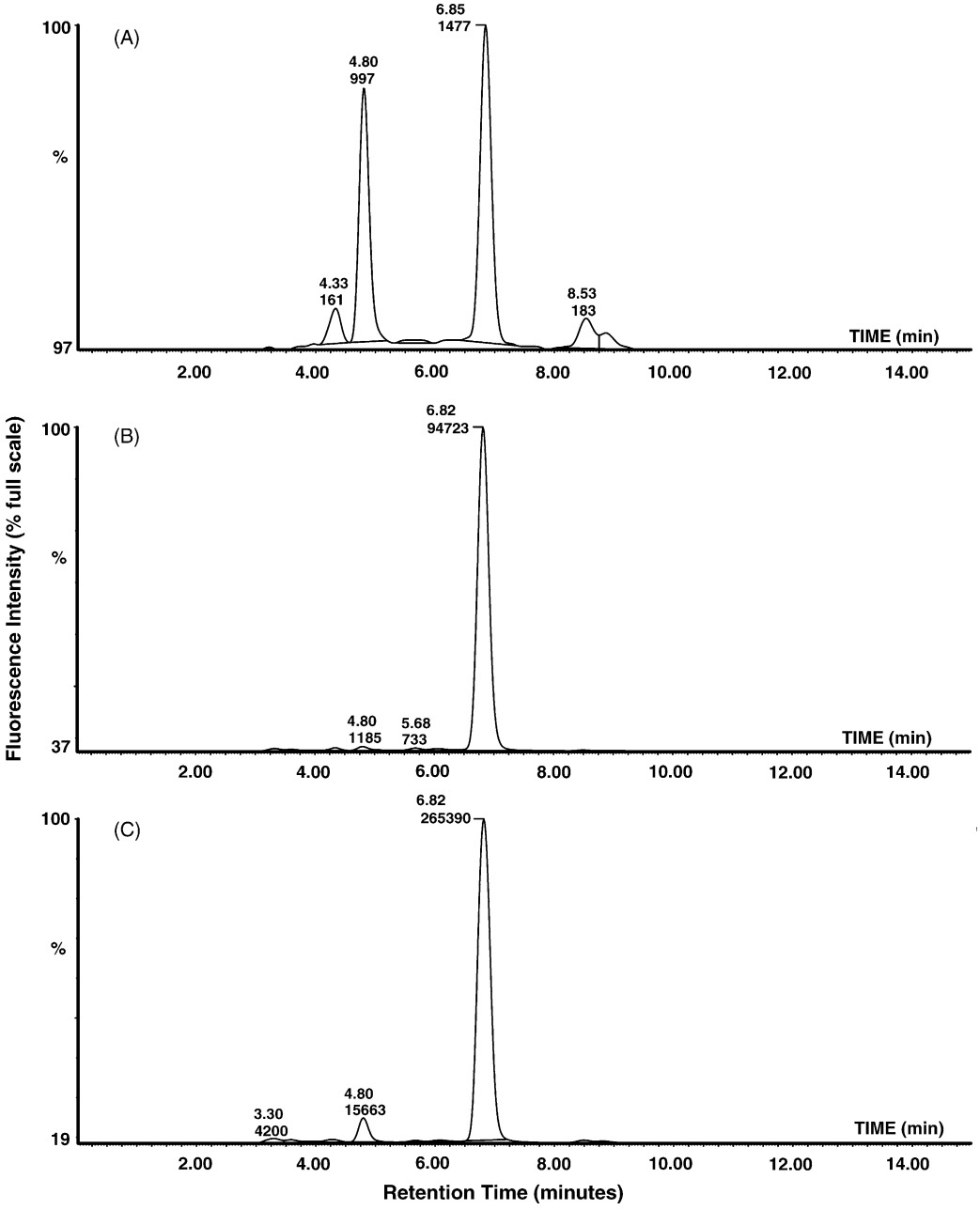

Fig. 4. Chromatograms showing spontaneous and enzymatic reduction of 3-NBA. (A) Spontaneous 3-ABA production in a reaction mixture without enzyme. (B) Chromatogram showing 3-ABA production in a reaction mixture containing liver S9. (C) Chromatogram showing 3-ABA production in a reaction mixture containing lung S9. Upper values are chromatographic retention times; lower values are integrated peak areas. Details regarding the reaction and chromatography conditions are provided in the text.

eral intermediates (e.g., nitroso, hydroxylamino), and in all likelihood, more than one enzyme [10,15]. Consequently, nitro compound reduction will not adhere to the principles of ordinary Michaelis–Menton kinetics. Bacterial nitroreduction is thought to follow a ping-pong or double displacement mechanism in which the enzyme reacts sequentially with more than one substrate and releases more than one product [26,27]. Although ping-pong kinetics can yield a family of familiar Lineweaver–Burke plots, the K_m and V_{max} values relating to formation of the final product (e.g., 3-ABA) will be dependent of the concentrations of several substrates (e.g., reduced NADP) [28]. Further characterization of the kinetic properties of the relevant murine enzymes would require purification of the active enzymes or enzyme complexes, and characterization of the precise cofactor requirements.

3.4. *Nitroreductase activity in Muta*^{TM}*Mouse tissues*

Nitroreductases are classified into two groups, oxygen-insensitive and oxygen-sensitive [29]. Type I nitroreductases,

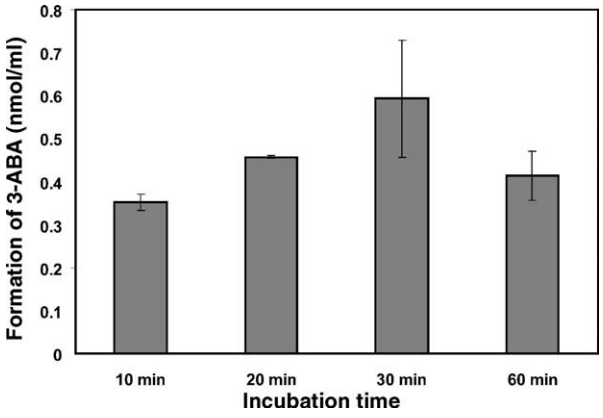

Fig. 5. Effect of incubation time on the formation of 3-ABA by Muta™Mouse liver S9. Substrate concentration was 20 μM and 1 mg protein of Muta™Mouse liver S9 was used. Values and bars represent means and standard errors ($n = 2$), respectively.

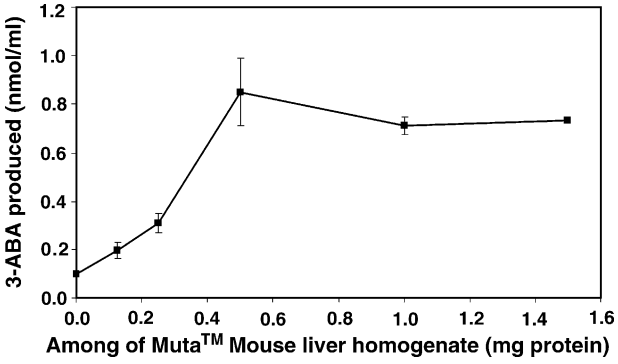

Fig. 6. Effect of total protein content on the formation of 3-ABA by Muta™Mouse liver S9. Substrate concentration was 20 μM and incubation time was 30 min. Values and bars represent means and standard errors ($n = 2$), respectively.

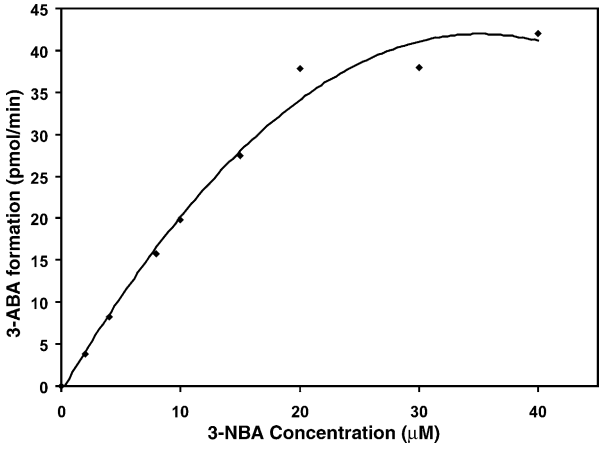

Fig. 7. Effect of 3-NBA concentration on the rate of 3-ABA formation by Muta™Mouse liver S9. One milligram protein of Muta™Mouse liver S9 was used and the incubation time was 30 min. Values represent means of two independent experiments. Non-linear least squared regression revealed apparent V_{max} and K_m values of 70.2 pmol/min/mg protein and 23.9 μM, respectively.

members of the oxygen-insensitive group, catalyze the two-electron reduction of the nitro moiety to the fully reduced amine (R-NH_2) via electrophilic reactive intermediates (i.e., nitroso and hydroxylamine) [29,30]. Type I nitroreductases include DT-diaphorase (EC 1.6.99.2) and the nitroreductases of enteric bacteria (e.g., *Escherichia coli nsfB* and *S. typhimurium cnr*) [30]. In contrast, type II nitroreductases, members of the oxygen-sensitive group, catalyze one-electron reduction, generating nitro anion radical intermediates which are readily re-oxidized back to the parent nitro compound in the presence of O_2 [30]. This latter group is thought to catalyze the formation of the fully reduced amine only under anaerobic conditions. Examples of this group include NADPH-cytochrome P-450 oxidoreductase (EC 1.6.2.4) and NADPH-b_5 oxidoreductase (EC 1.6.2.2) [30].

The presence of oxygen in the reaction mixture has been shown to reduce the in vitro reduction of 1-nitropyrene by mammalian liver S9 [31,32]. Consequently, experimental quantification of reductase activity in samples of mammalian tissues requires continuous bubbling of the reaction system with an inert gas such as argon or nitrogen. In a pilot study examining the 1-nitropyrene nitroreductase activity of Muta™Mouse liver S9, a significant three-fold increase ($p < 0.0003$, $n = 4$) in activity was observed between the measurements made following nitrogen addition (11.5 ± 1.6 pmol/min/mg protein) and the measurements made in open air (3.5 ± 0.7 pmol/min/mg protein). This result, consistent with previous findings regarding the involvement of type II enzymes in the mammalian reduction of 1-nitropyrene [31,32], confirmed that gentle bubbling of nitrogen effectively purged oxygen from the experimental system.

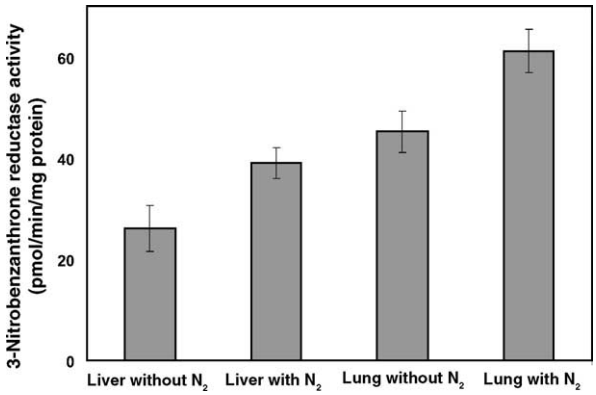

Fig. 8. Effect of incubation condition and tissue type on 3-NBA reductase activity. Total 0.5 mg protein of Muta™Mouse liver or lung S9 was used and the incubation time was 30 min. Values and bars represent means and standard errors, respectively, of four individual Muta™Mouse specimens. Paired *t*-test results show a significant difference between liver with and without N_2 ($p < 0.04$), as well as between lung with and without N_2 ($p < 0.03$). A Two-way ANOVA showed significant tissue type (F-ratio = 22.8, $p < 0.001$) and nitrogen (F-ratio = 11.1, $p < 0.008$) effects, but no tissue type–nitrogen interaction.

3-NBA reductase activity of Muta^{TM}Mouse liver and lung S9 fractions was measured both with and without a 30 min purging with ultra-pure nitrogen gas prior to addition of the substrate and tissue S9 fraction. The results obtained for the aerobic condition revealed 3-nitrobenzanthrone reductase activities in liver and lung of 26 ± 4.6 pmol/min/mg protein (mean ± standard errors) and 45.2 ± 4.1 pmol/min/mg protein, respectively (Fig. 8). Nitrogen purging increased the reductase activity by only 30% to 39.0 ± 3.0 pmol/min/mg protein and 61.1 ± 4.3 pmol/min/mg protein, for liver and lung, respectively (Fig. 8). Nevertheless, statistical analyses showed significant differences in nitroreductase activity between open air and nitrogen purged samples for both liver ($p < 0.04$, $N = 4$), and lung S9 ($p < 0.03$, $N = 4$). Furthermore, a two-way ANOVA showed significant effects of both tissue type and nitrogen bubbling, but no interaction between the tissue type and nitrogen effects. Higher nitroreductase activity in lung samples has also been reported by Ask et al. using nilutamide, a nitroaromatic drug, to examine the nitroreductase activity in Sprague–Dawley rats [33].

3.5. Conclusion

This work describes a sensitive normal-phase HPLC method to assess 3-NBA nitroreductase activity by quantifying the rate of production of the reduced product 3-aminobenzanthrone (3-ABA). Protein concentration, substrate concentration, and incubation time were optimized, and under optimal conditions, S9 fractions from Muta^{TM}Mouse liver and lung yielded 3-NBA reductase activities in the 39–61 pmol/min/mg protein range. Interestingly, the range of Muta^{TM}Mouse 3-NBA reductase values is approximately 1.8–3.5-fold greater than that measured for *S. typhimurium* strains TA98 and TA100 (i.e., 17 and 22 pmol/min/mg protein, respectively), and approximately four- to seven-fold lower than that measured in metabolically enhanced strains of *S. typhimurium* that over-express *cnr*, the *Salmonella* "classical" nitroreductase (i.e., 280 and 213 pmol/min/mg protein for *Salmonella* strains YG1041 and YG1042, respectively) [20,26]. Thus, Muta^{TM}Mouse 3-NBA reductase activity is surprisingly high relative to that of bacteria, particularly in light of the fact that nitrofurazone reductase activity for Muta^{TM}Mouse samples was below detection. It is also interesting to note that 3-NBA appears to be a substrate for both the *Salmonella* classical nitroreductase (i.e., Cnr), as well as the *Salmonella* major nitroreductase (i.e., SnrA) [26].

Although the identity of the enzymes implicated in the reduction of nitroPAHs such as 3-NBA in mammalian tissues remain largely unknown, the method developed here can be used to investigate the underlying causes of published tissue-specific differences in 3-NBA mutagenicity in vivo [7]. Ultimate isolation and identification of the enzymes involved in mammalian 3-NBA reduction is a promising area for additional research.

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