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Journal of Chromatography B, 667 (1995) 166–172

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Determination of hemoglobin adduct levels of the carcinogen 2,4-diaminotoluene using gas chromatography–electron impact positive-ion mass spectrometry

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First received 9 June 1994; revised manuscript received 27 December 1994; accepted 27 December 1994

Abstract

A procedure to determine hemoglobin adduct yields resulting from exposure to the carcinogen 2,4-diaminotoluene (2,4-TDA) was developed using gas chromatography–electron impact positive-ion mass spectrometry. Liberated 2,4-TDA was quantified following alkaline hydrolysis of hemoglobin. Optimized derivatization of free 2,4-TDA with heptafluorobutyric anhydride allowed detection of hemoglobin adduct levels as low as 5 ng/g Hb. Pure HFBA–2,4-TDA showed a linear dynamic range of 50 to 5000 pg. The quantitative extraction and recovery of liberated 2,4-TDA (ca. 100%) following hemoglobin hydrolysis allows accurate and precise determinations of adduct yields.

1. Introduction

Xenobiotic conjugation with hemoglobin has been examined for a broad range of chemical compounds [1]. Hemoglobin conjugates serve as one of several complementary approaches to molecular markers of carcinogen dose. The hemoglobin adduct allows a determination of total cumulative exposure integrated over time throughout the lifespan of the red blood cell [2]. Hemoglobin adducts resulting from covalent modifications usually exhibit linear dose dependency, chemical stability under biological conditions, and correlate to DNA adducts under similar exposure conditions [3,4].

Aromatic amines enjoy widespread industrial and agricultural applications. Many aromatic amines have been identified as human carcinogens [5] and for their ability to induce hemoglobin adducts [6]. The aromatic amine 2,4-diaminotoluene (2,4-TDA) is a potent animal carcinogen and genotoxicant and is widely used as an industrial intermediate in the production of toluene diisocyanate and polyurethane foam, elastomers and coatings [7].

As illustrated in Fig. 1, upon oxidative metabolism by the hepatic cytochrome P-450 mixed-

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SSDI 0378-4347(94)00598-2

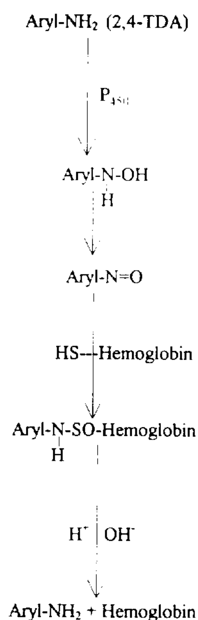

Fig. 1. Aromatic amine biotransformation. Metabolic activation of aromatic amines to intermediates capable of forming covalent conjugates with hemoglobin.

function oxidase isozymes, aromatic amines are oxidized to arylhydroxylamines (AHA). AHAs penetrate erythrocyte membranes and are further oxidized to the corresponding arylnitrosoarene (ANA) derivatives. The latter then form covalent conjugates with thiol residues of hemoglobin, resulting in the formation of the hemoglobin–aromatic amine adduct, i.e. a sulphinic acid amide [8–10]. Acidic or alkaline hydrolysis of a hemoglobin–arylnitrosoarene conjugate liberates the parent amine which can be recovered and quantified to determine the yield of hemoglobin adduct [11].

Radioisotopic studies designed to monitor covalent modification of cellular nucleophiles by aromatic amines have been useful in assessing tissue distribution and in delineating biochemical pathways. Non-radioactive, inexpensive methods for monitoring levels of hemoglobin conjugation resulting from aromatic amine exposure would be most useful in assessing tissue doses in occupational and other human cohorts. Unfortunately, the hydrolysis and recovery procedures currently employed in quantifying aromatic amine induced hemoglobin adduct levels, has contributed to variable and imprecise analytical results [12,13].

Using gas chromatography–electron impact positive-ion mass spectrometry, we have developed and optimized a sensitive, precise and accurate non-radioactive analytical method for the determination of hemoglobin adduct yields from exposure to 2,4-TDA.

2. Experimental

2.1. Chemicals and reagents

All reagents and solvents were of analytical grade or better. All glassware was washed with detergent, rinsed with distilled water, soaked at least 24 h in a 10% nitric acid bath, rinsed in distilled water, and oven dried at 100°C. A Millipore Super-Q water filter system produced ASTM Type 1 water for use. Toluene (Optima grade) extraction solvent, NaOH, NaCl, indicating Drierite, ethanol, ethyl ether, dimethyl sulfoxide (DMSO) and monobasic potassium phosphate were obtained from Fisher Scientific (Pittsburgh, PA, USA). 2,4-TDA (97–99% pure), sodium sulfate, heptafluorobutyric anhydride (HFBA), 2,6-dimethylaniline (2,6-DMA), and sodium dodecyl sulfate (SDS) were purchased from Aldrich (Milwaukee, WI, USA). Sodium pentobarbital was obtained from UCLA Pharmaceutical Services (Los Angeles, CA, USA). Anhydrous free ethylenediaminetetraacetic acid (EDTA) and rat hemoglobin were from Sigma (St. Louis, MO, USA).

2.2. Animal treatment

Male Fischer-344 rats (180–200 g) were obtained from Simonsen Laboratories (Gilroy, CA, USA), and housed in hanging wire cages. Animals were allowed to acclimate for two to three weeks prior to administration of 2,4-TDA; during this time they were maintained on standard laboratory diet and water. 2,4-TDA was administered by intraperitoneal (i.p.) injection in 100 μl dimethyl sulfoxide (DMSO). A minimum of

three animals were used per dose level or time point. Control animals received vehicle alone. Animals were anesthetized with 20 mg sodium pentobarbital i.p. Cardiac puncture allowed collection of whole blood in heparinized vacutainer tubes (Fisher Scientific).

2.3. Instrumentation

Mass spectrometric data were obtained on a Hewlett-Packard 5988A mass spectrometer operating in the selected-ion mode (SIM) for m/z 345 [$M - C_3F_7$] and the molecular ion base peak m/z 514 [$M^{+\cdot}$]. Positive ions were produced in a 70 eV source at 250°C. Gas chromatography was conducted on a Hewlett-Packard 5890A gas chromatograph equipped with a DB-1701 chemically bonded fused-silica capillary column (30 m × 0.32 mm I.D., 0.25 μm film thickness; J and W Scientific). Standards and samples were injected by sandwich technique in the splitless mode, with a purge delay of 0.75 min and an injection port temperature of 180°C. Helium was used as the carrier gas at 2.4 ml/min. The temperature program was: 80°C for 1 min, 80°C to 240°C at 15°C/min, and holding at 240°C for 2 min. Transfer line temperature was 250°C.

2.4. Hemoglobin extraction from whole blood

Erythrocytes were isolated by centrifuging whole blood at 1000 g for 5 min. Erythrocytes were washed 3 times in equal volumes of 0.9% NaCl solution. Four equal volumes of a 10^{-4} M EDTA solution (pH 7.5) were used to lyse membranes. The lysate was centrifuged at 6000 g for 10 min, the supernatant retained, and the protein precipitated by adding four volumes of ice-cold ethanol drop-wise while stirring. The protein was re-suspended and spun using the following wash: ethanol–water (8:2), ethanol (96%), ethanol–diethyl ether (1:3), and diethyl ether. Dialysis overnight against distilled water produced hemoglobin which was placed in a vacuum desiccator and dried in vacuo over indicating Drierite until constant weight. Hemoglobin was stored under vacuum at −80°C until analysis.

2.5. Hemoglobin hydrolysis/aromatic amine extraction

A 100-mg quantity of hemoglobin was added to 4.8 ml of saturated sodium hydroxide and 0.2 ml of 0.05% SDS. The suspension was placed in a PTFE sealed vessel and sonicated for 3 h at 37°C. Two grams of NaCl were then added, followed by 1 ml of toluene, and the solution then agitated for 10 min. After centrifugation for 5 min at 1000 g, the toluene layer was recovered and the extraction repeated four additional times. The toluene volumes were combined, 96% of the initial toluene volume being recovered.

2.6. Aromatic amine derivatization

Recovered toluene was carefully evaporated to 1 ml under a gentle nitrogen stream at 60°C. At room temperature, 20 μl of HFBA was added, the mixture was vortex-mixed for 1 min and allowed to stand for 15 min. Excess HFBA and heptafluorobutyric acid were removed by washing with 1 ml of dihydrogen potassium phosphate (pH 7.0) [14–17]. The toluene layer was recovered, its mass determined and internal standard (2,6-DMA) added according to the volume of toluene recovered (100 pg/ml). Addition of 2,6-DMA before protein hydrolysis was found to competitively retard recovery of 2,4-TDA.

2.7. HFBA–2,4-diaminotoluene derivative standard synthesis

2,4-TDA was dissolved in toluene using ultrasonication. A 10-fold molar excess of HFBA was added and the mixture vortex-mixed for 1 min. After standing 15 min, the toluene, excess HFBA and heptafluorobutyric acid were evaporated under a gentle nitrogen stream at 60°C, leaving a white precipitate. The precipitate was dried and weighed. The yield of HFBA–2,4-TDA derivative was 92 ± 3%. The standard was

then dissolved in toluene and an aliquot subjected to GC–MS to determine purity.

2.8. Standard additions

The standard additions analytical method [18] was used to determine if the protein matrix affected amine recovery from hemoglobin samples isolated from animals administered 0.5 mg/kg 2,4-TDA. A 100-mg amount of hemoglobin was quartered into four equal volumes, then $0\times$, $1.5\times$, $2.0\times$, and $2.5\times$ the expected mass of 2,4-TDA to be recovered from the animal was spiked into hemoglobin before work-up. A linear regression analysis of the three spikes containing known amounts of 2,4-TDA allowed the unknown 2,4-TDA mass to be interpolated. The 7% loss of 2,4-TDA mass retarded by suppressing interferences from protein matrix elements was not statistically significant from that obtained by an internal standards analysis at $p = 0.025$.

3. Results and discussion

3.1. Linear range and detection limit

Peak-area ratios from the synthesized derivative and internal standard were used to create a standard calibration curve in triplicate. Linear regression analysis allowed determination of the linear dynamic range. The limit of detection was determined to be 50 pg HFBA–2,4-TDA with a signal-to-noise ratio of 3.2:1. The linear response range extended from 50 to 5000 pg of HFBA–2,4-TDA. The purity of the synthesized derivative was determined by mass spectrometry to be 96%. The predominant contaminant originated from toluene and was benzaldehyde.

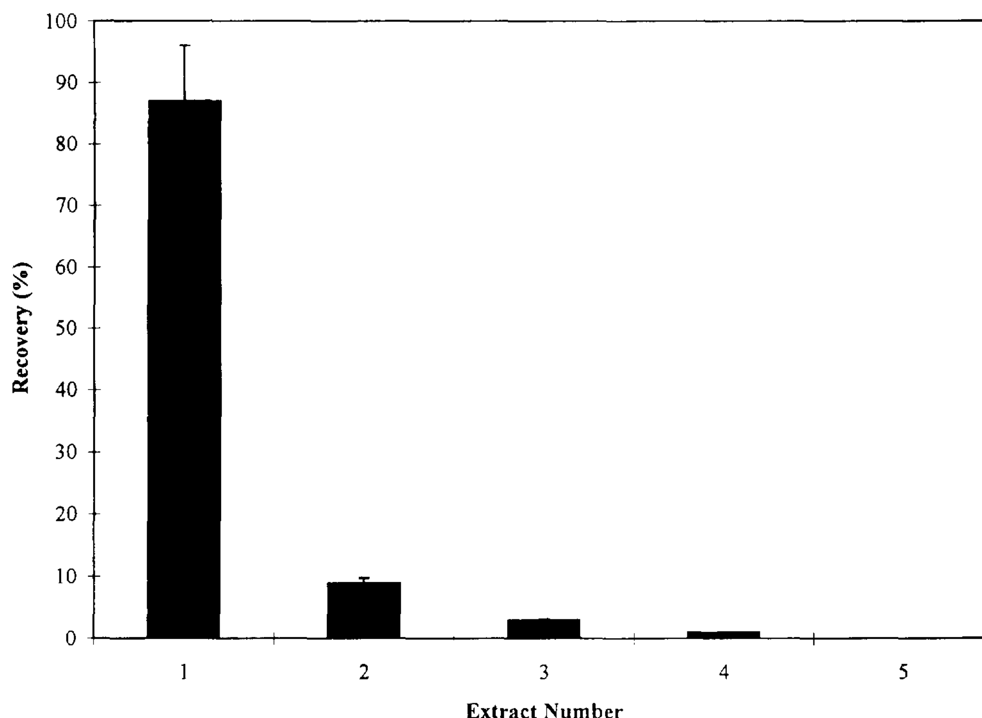

Fig. 2. Recovery of 2,4-TDA from male Fischer-344 rat hemoglobin. A 100-mg quantity of rat hemoglobin was hydrolyzed and extracted to determine the minimum number of extracts necessary to achieve quantitative recovery. Each extract represents the mean ± S.D. of three independent trials.

3.2. Extraction efficiency

Recoveries of parent amines in hemoglobin adduct studies have been found to typically range between 40 and 70%. The ability of aromatic amines to hydrogen bond and their propensity to form mixed micelles in detergent (SDS) under alkaline conditions contribute to their imprecise and non-quantitative recovery [19,20]. As a result, extraction of parent amines following hemoglobin hydrolysis has proven problematic [12,13]. Optimal hemoglobin hydrolysis conditions were necessary to maximize 2,4-TDA recovery. Fig. 2 shows optimized 2,4-TDA recovery from rat hemoglobin hydrolysates using saturated NaOH and 0.05% SDS. A 100-mg quantity of hemoglobin was spiked with 500 ng of 2,4-TDA and hydrolysed for a 3-h period with varying detergent and NaOH concentrations. Following NaCl saturation, hemoglobin was agitated for 10 min and 2,4-TDA was extracted to determine the minimum number of extractions necessary to achieve quantitative recovery. Optimal hydrolysis occurred with 4.8 ml of saturated NaOH and 0.2 ml of SDS (0.05%). Optimal 2,4-TDA recovery was achieved using toluene which also minimized co-extraction of polar hydrolysis material. SDS concentrations higher than 0.05% decreased the amine recovery while increasing NaOH concentration increased the recovery. Solubilization of the protein and optimization of detergent and NaOH concentrations were necessary to maximize 2,4-TDA recovery.

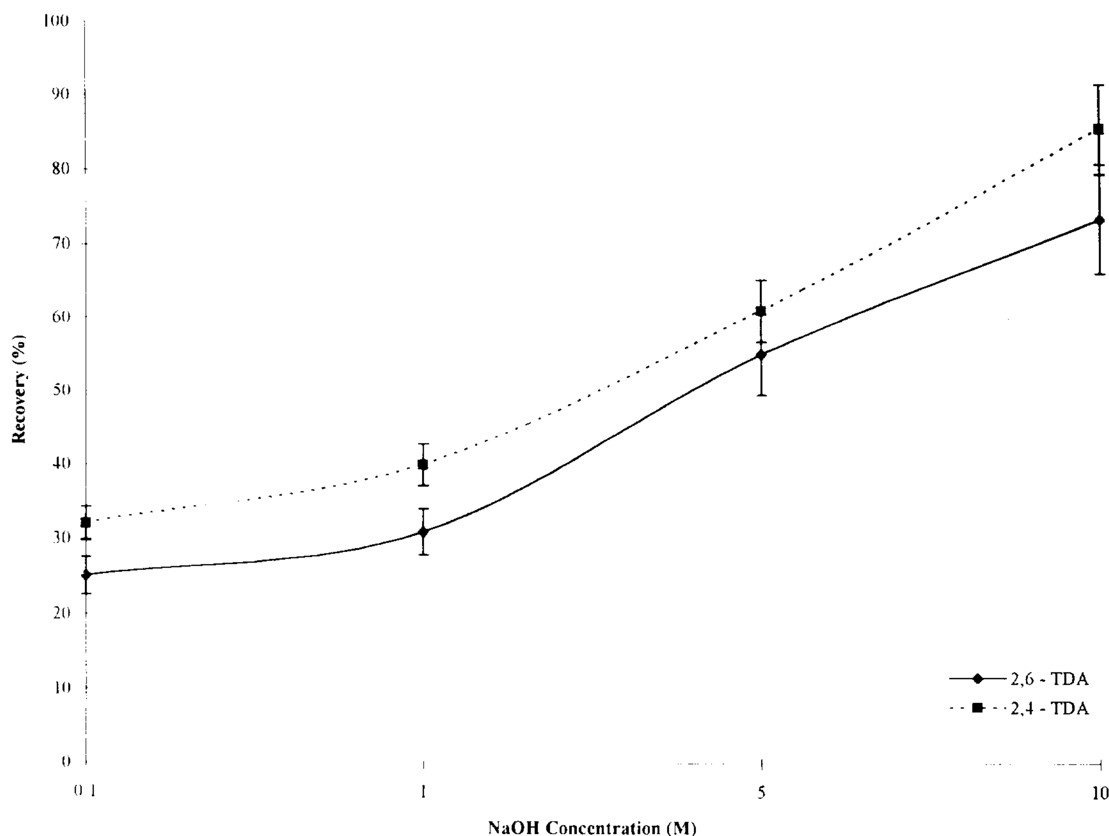

Fig. 3. Dependence of 2,4-TDA and 2,6-TDA recovery on NaOH concentrations. A 100-mg quantity of hemoglobin from male Fischer-344 rats was spiked with two isomers of TDA (1000 ng) and extracted into toluene upon hydrolysis with various NaOH concentrations. Both NaOH and SDS concentrations influenced recovery of diamines into toluene. Data points represent mean ± S.D. from five independent experimental trials.

While facile hydrolysis is sufficient to liberate the parent amine, the NaOH concentration used in hydrolysis was found to be critical in the recovery of 2,4-TDA (Fig. 3). Increasing the hydrolysis time to 24 h did not liberate additional amine relative to 3-h hydrolysis, nor did increase of the hydrolysis temperature to 60°C liberate further 2,4-TDA relative to room temperature hydrolysis. Acidic hemoglobin hydrolysis did not result in additional 2,4-TDA release, but peaks from acidic hydrolysate extracts were more difficult to resolve. Alkaline hydrolysis was selected as the method of choice.

3.3. Hemoglobin adducts

The principal organic released from the adduct under these analysis conditions was the liberated parent amine, 2,4-TDA. Comparison of chromatograms of hydrolysate extracts from control animals and dosed animals indicated the presence of only one major adduct under these conditions (Fig. 4). The ability to accurately measure adduct levels throughout the entire dose range was uniform. Hemoglobin adduct yield was consistently greater than 92% in animals receiving 0.5–250 mg/kg. There was a dose response of adduct concentration with increasing doses of 2,4-TDA. In order to determine the time of maximal adduct concentration, animals were dosed at time 0, and sequentially sacrificed over a 30-day time window. The highest hemoglobin adduct concentrations were observed 18–24 h following administration of 2,4-TDA. Adduct levels at day 30 decreased 88% from adduct levels measured at day one. The dose-dependent adduct level increase is inversely correlated with an uncharacteristic decrease in the hemoglobin binding index [12] of 2,4-TDA. This relationship deserves further study. Adduct levels as a function of 2,4-TDA dose are reported in Table 1.

Table 1
Hemoglobin adduct levels from single i.p. administration of 2,4-TDA to male Fischer-344 rats

2,4-DTA dose (mg/kg) ($n = 5$)	Hemoglobin adduct levels (mean ± S.D.) (ng/g Hb.)
0	0
0.5	39.4 ± 3.3
50	96.7 ± 10.8
250	185 ± 25

Adduct levels were determined 18–24 h post administration.

4. Conclusions

A non-radioactive analytical method suitable for the determination of 2,4-TDA–hemoglobin adducts was developed. The ability to accurately and precisely monitor 2,4-TDA induced hemoglobin adduct levels should provide additional information regarding the proportion of exposure available for macromolecular modification.

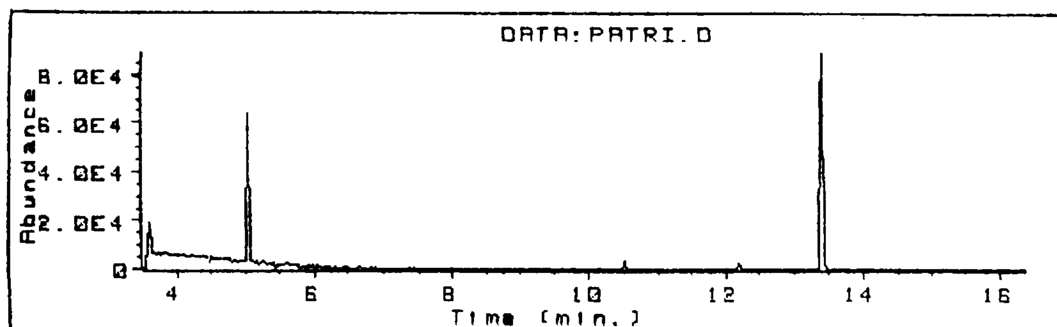

Fig. 4. Selected-ion monitoring chromatogram. Internal standard, 2,6-DMA (5.9 min) and liberated parent amine 2,4-TDA (13.4 min) from hydrolyzed hemoglobin isolated from Fischer-344 rat dosed with 250 mg/kg 2,4-TDA.

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