S-Phenylcysteine in Albumin as a Benzene Biomarker

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Biological markers of internal dose are useful for improving the extrapolation of health effects from exposures to high levels of toxic air pollutants in animals to low, ambient exposures in humans. Previous results from our laboratory have shown that benzene is metabolized by humans to form the adduct S-phenylcysteine (SPC). Levels of SPC measured in humans occupationally exposed to benzene were increased linearly relative to exposure concentrations ranging from 0 to 23.1 ppm for 8 hr/day, 5 days/week. However, the method of measurement used was laborious, prone to imprecision and interferences, and insufficiently sensitive for the low-dose exposures anticipated in the United States (100 ppb). An improved chemical method was necessary before SPC adducts in albumin could be used as a benzene biomarker. A simple, sensitive method to measure SPC adducts is being developed and is based on the cleavage of the cysteine sulfhydryl from blood proteins treated with Raney nickel (RN) in deuterium oxide. The product of the reaction with RN is monodeuterobenzene. SPC treated with RN released monodeuterobenzene in a concentration-dependent fashion. SPC was measured by RN treatment of globin from rats repeatedly exposed by inhalation to 600 ppm benzene. SPC levels measured using the RN approach were 690 ± 390 pmol SPC/mg Hb (mean ± % difference, n = 2), as opposed to 290 ± 45 pmol SPC/mg Hb (mean ± SEM, n = 3) as measured by our previous method. This method may facilitate the cost-effective, routine analysis of SPC in large populations of people exposed to ambient levels of benzene. — Environ Health Perspect 104(Suppl 6):1147–1149 (1996)

Key words: adduct, albumin, benzene, biomarker

Introduction

Risk assessment for toxic air pollutants is often based on the extrapolation of health effects from high levels of exposure in animals to ambient exposures in human. The underlying biological and chemical processes that constitute exposure and consequent effects may not be understood, which weakens the validity of these extrapolations. One means of more accurately defining these processes is by the use of biological markers of internal dose. Our laboratory has been developing biological markers to the human carcinogen benzene; results have shown that benzene is, in part, metabolized by humans to an intermediate that binds to cysteine groups in albumin to form the adduct S-phenylcysteine (SPC). We have measured the formation of SPC in albumin in workers after benzene exposure (1) and levels of SPC in humans occupationally exposed to average concentrations of 0.4, 4.4, 8.4, and 23.1 ppm benzene 8 hr/day, 5 days/week. SPC increased linearly in the exposed group, giving a statistically significant slope (p < 0.001) of 0.044 ± 0.008 pmol/mg albumin × ppm), with an intercept of 0.135 ± 0.095 pmol/mg albumin. Although SPC in albumin may prove useful as a biomarker of benzene exposure, considerable sensitivity would be required to measure SPC in the albumin of people routinely exposed to benzene in environmental settings. Measuring SPC routinely by our current method would be difficult because it is laborious and prone to imprecision and interferences. An alternate approach to measure SPC was therefore sought.

This article describes a new method based on the cleavage of the cysteine sulfhydryl from the albumin by treatment with Raney nickel (RN) in D₂O. For SPC the product of the reaction was the release of monodeuterobenzene (MDB) (Figure 1). The released MDB was purged from the RN solution with helium and trapped by passing the vapor through a cartridge containing the adsorbent substance Carbopack (Alltech, Deerfield, IL). The trapped benzene was then desorbed from the cartridge using a Tekmar thermal desorption apparatus (Tekmar, Cincinnati, OH). The benzene released from the desorption apparatus was analyzed by gas chromatography-mass spectrometer (GC–MS). Incomplete reaction of the SPC with RN and inefficiencies in the purging and desorption process were corrected by adding a known amount of [¹³C₆]SPC standard to the reaction mixture. Initial results of the assay that established its efficacy.

Materials and Methods

Reagents

The SPC and [¹³C₆]SPC have been synthesized (2). The deuterium oxide (99% <) was acquired from Aldrich (Milwaukee, WI) and an aqueous suspension of RN. To purge as much benzene from the suspension as possible, the water was removed from the RN on a vacuum line and displaced with D₂O prior to use. The RN was kept tightly sealed until used. Carbopack was acquired from Alltech. All other reagents were acquired from Aldrich and were of >99% purity.

![Figure 1. Reaction of S-phenylcysteine with Raney nickel in D₂O to produce monodeuterobenzene.](image-url)
Globin Isolation and Hydrolysis

Globin was isolated from the red blood cells as previously described (1). The isolation process frees the heme group from the globin protein. Globin was hydrolyzed in flame-sealed vials by adding 10 ml of 6 N HCl to 500 mg of protein. After purging with dry nitrogen, the vials were sealed and incubated at 110°C for 24 hr. The vials were opened, and the hydrolysates brought to dryness with a stream of dry nitrogen. Aliquots of the [13C6]SPC internal standard solution (about 50 pmol of internal standard) were added and the samples reconstituted in D2O.

Raney Nickel Reduction of Globin and Analysis by Purge and Trap GC–MS

The protein hydrolysate was treated with RN catalyst for cleavage of MDB. To a 50 ml crimp-top vial we added 300 mg RN catalyst and 50 mg protein in 5 ml D2O made basic with NaOD. The contents were allowed to react at 60°C for 15 min. MDB was purged at 60°C for 15 min with a helium flow of 30 ml/min and trapped from the reaction vial. The helium was introduced using a 5-inch 18-gauge needle and exited from the vial through a deactivated silica column. The column in turn was introduced into the Carbotrap within the Tekmar. The trap was held at room temperature.

A Tekmar 5010 thermal desorption apparatus (Tekmar) with a Cryo-2 capillary interface was used to analyze the Carbotrap trap. The trap was desorbed at 400°C for 10 min into the internal trap of the Tekmar. The cryogenically focused desorbate was heated and transferred to the capillary interface, which was ballistically heated for injection on the GC–MS.

Analysis included a J&W DB1701 column held isothermally at 50°C for 1.2 min and 10°C/min to 100°C. A Hewlett Packard 5890/5970B GC–MSD (Hewlett-Packard, Wilmington, DE) was operated using selected monitoring of ions 85, 79, 78, and 50. These ions represent the internal standard, MDB, benzene background, and a confirmation ion, respectively. Limits of detection for the instrument were determined by injecting 100-μl aliquots of benzene vapor through the splitless injector.

Benzene Exposure

Globin was acquired from F344/N rats repeatedly exposed to 600 ppm benzene over 2 weeks for 6 hr per day. These studies have been described (2).

Results

To characterize the recovery of any benzene cleaved by the RN process, a 100-pg sample of benzene was spiked onto the Carbotrap. The benzene was measured by thermal desorption GC–MS, which gave a recovery of 103% relative to benzene injected directly onto the GC–MS through the splitless interface. Direct injections onto the GC–MS suggested an instrument limit of detection of about 20 pg (signal to noise of 3:1). Graded amounts of SPC standard were treated with RN in D2O, followed by purge-and-trap GC–MS (Figure 2). Response was linear over the region of interest ($r^2 > 0.99$), although a nonzero intercept was encountered for the blank and the fitted curve.

SPC was measured by RN treatment of globin from rats repeatedly exposed by inhalation to 600 ppm benzene. A chromatogram for the released MDB demonstrates the expected coelution with the internal standard (Figure 3). SPC levels measured using the RN approach were 690 ± 390 pmol SPC/mg Hb (mean ± % difference, n = 2), as opposed to 290 ± 45 pmol SPC/mg Hb as measured by our previous method (mean ± SEM, n = 3). Because of high backgrounds in blanks, lower levels of MDB released from SPC were not measured.

Discussion

The RN method has several key advantages relative to our prior approach. First, the purge-and-trap process is expected to liberate most, if not all, the cleaved benzene from the solution. Therefore, most of the analyte is delivered to the MS, as opposed to only a fraction of that delivered by the previous method. This should increase the sensitivity of the method considerably. Sensitivity is further enhanced because most MSs, including ours, are more sensitive in analyzing low versus high mass compounds when using electron impact ionization. The weights of the released MDB and the derivatized SPC are 79 and 449, respectively. In addition, only volatile materials are transferred to the GC, thus reducing the amount of nonvolatile residues and decreasing nonspecific interferences. Finally, this procedure may be automated using purge-and-trap autosamplers, which would make the method less labor intensive and more cost effective.

Ultratrace levels of benzene in aqueous samples are difficult to measure routinely because benzene is pervasive in the laboratory, and blanks for the reaction are often high and hard to reduce. To partially overcome this problem, we conducted the reaction in deuterium oxide and anticipated MDB as the product of this reaction. Background benzene still contributed to blanks because of the identical molecular weights for MDB and [13C6]benzene at unit mass resolution. The [13C6]benzene contributed signal because 6% of all benzene will contain a 13C atom, which exists in 1% natural abundance. Therefore, a factor of 17 (100/6) reduction in background resulted. Nonetheless, the apparatus (Tekmar 5000) used for these studies was not specifically designed for purge-and-trap analysis; it suffered from excessive background and memory effects and limited our ability to measure lower levels of SPC.

Of concern for any biological marker is its specificity for the toxicant of interest. SPC levels in the albumin of control individuals are not expected to be high, based on studies of the urinary excretion of the
benzene metabolite phenylmercapturic acid. Both phenylmercapturic acid and SPC should be formed by the binding of benzene oxide to cysteine groups. Phenylmercapturic acid levels have been measured in the urine of workers occupationally exposed to benzene and were found to be low in unexposed workers (3). Levels increased gradually in workers exposed to benzene levels up to 1 ppm.

While our initial results appear promising, the method can be further refined to measure these lower levels of SPC. Of greatest concern is a further reduction in the background of benzene. Use of a commercially available purge-and-trap apparatus will solve this problem. Method detection limits for benzene have been reported as low as 0.006 ppb [30 pg benzene in 5 ml water; (4)]. The purge-and-trap apparatus has recently been acquired in our laboratory; experiments using this instrument suggest these limits will be attainable.

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