Phenylguanine Found in Urine after Benzene Exposure

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Comparative investigations with synthetic N7-phenylguanine were carried out to clarify whether this compound is eliminated via the urine of rats as a benzene-derived nucleic acid adduct. As sensitive methods for detecting trace amounts of the compound, gas chromatography–mass spectroscopy, high performance liquid chromatography, and two immunosassays (enzyme-linked immunosorbent assay and fluoroimmunoassay) with appropriate monoclonal antibodies were used. The results indicate the excretion of several benzene-related guanine adducts slightly different from N7-phenylguanine that may possibly be hydroxylated. These adducts differ also from O6-, N2- and C8-phenylguanine, respectively. — Environ Health Perspect 104(Suppl 6):1159-1163 (1996)

Key words: DNA adducts, benzene, N7-phenylguanine, urine, [14C], HPLC, GC–MS, monoclonal antibodies

Introduction

There is ample evidence that benzene uptake by mammals leads to the formation of nucleic acid adducts after biotransformation of the carcinogen (1–13). Identification of (3′OH)benzethenono-(N1,N2)-deoxyguanosine as a DNA adduct by Iowa et al. (3,14) was confirmed by Snyder et al. (4) and Kaur et al. (5).

Following the hypothesis that N7-phenylguanine could be formed by the reaction of benzene epoxide with guanine (Figure 1), attempts were undertaken in our laboratory to identify it as an in vivo adduct after administering single, high doses of benzene to male Wistar rats ip. Having in mind that well known N7-guanine adducts of aflatoxin B1 (15) and benzo[a]pyrene (16) are excreted in the urine, our investigations focused on urine analyses. We were aware of the limitations of such an approach. No decision is possible as to whether guanine derivatives are generated by arylation of DNA, RNA, or free guanine. Furthermore, the amounts excreted should reflect water solubility of the adduct rather than production rates. Highly sensitive and specific methods are required for the urine analyses. On the other hand, if N7-phenylguanine is formed, it should be eliminated from DNA very rapidly because beside excision repair mechanisms, it will depurinate and may accumulate in body fluids.

Urine Analyses Using Cation Exchange Chromatography, HPLC, and GC–MS

After having synthesized N7-phenylguanine (17), we analyzed urine samples in comparison investigations, methods for which are reported by Norpoth et al. (18).

In Figure 2 the separation of urine components after exposure with cation exchange chromatography (UV detection) is presented in comparison to the spectra of the control urine and of the control urine containing the synthetic N7-phenylguanine. One of the peaks (32 min) exhibits the same retention time and, after separation, an identical fluorometric behavior as that of the synthetic N7-phenylguanine (Figure 3). The substances that represent the other peaks have not yet been identified.

Our hypothesis of the adduct formation was further confirmed by high performance liquid chromatography (HPLC) measurements with reversed phase carrier material (Figure 4). The urine fraction containing the phenylguanine was isolated by cation exchange chromatography and after a clean-up with Sep Pak C18 cartridges (Baker, Phillipsburg, NJ) this fraction was measured by HPLC. As shown in Figure 4, the urine of rats treated with 50 μl benzene, ip, in contrast to that of untreated rats, produced several peaks. Again, one of the peaks showed the same retention time as the synthetic N7-phenylguanine.

To compare N7-phenylguanine with other possible phenyl adducts present in the urine, silylation and gas chromatography–mass spectrometry (GC–MS) analyses were performed. After separation with cation exchange chromatography and clean-up with Sep Pak C18 cartridges, the sample was silylated and fractionated by capillary gas chromatography. The detection was performed with a Kratos MS 80 mass spectrometer (Kratos, Manchester, UK) (Figure 5).

Measurements revealed the retention time of a compound with a molecular mass of 371 and a mass fragment of 356, as observed with the synthetic N7-phenylguanine. The data obtained suggested that N7-phenylguanine can be detected in the urine of rats treated with high doses of benzene. Discussing this conclusion we underlined that another adduct may be formed originally; for example, a hydroxy compound (similar to the products observed in the metabolism of benzo[a]pyrene), which will be transformed into the dehydroxylated phenylguanine during the

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Abbreviations used: HPLC, high performance liquid chromatography; GC–MS, gas chromatography–mass spectrometry; ELISA, enzyme-linked immunosorbent assay; FIA, fluoroimmunoassay; mAb, monoclonal antibody.

![Figure 1. Proposed scheme for the reaction of benzene epoxide with guanine.](image-url)
Investigations with $^{14}$C-Benzene and Unlabeled Benzene Using a Refined HPLC Technique

Three male Wistar rats (average weight: 270 g) were exposed to $^{14}$C-benzene (113 mCi/mmol, purchased from Amersham Buchler, Braunschweig, Germany) in a closed system (exsiccator, 10.3 $\times$ 10$^{-3}$ m$^3$). The reduction of $^{14}$C-benzene in the air preparation of the urine samples. Such behavior could also be observed in the detection of glutathione adducts of some polycyclic aromatic hydrocarbons (19, 20).

Figure 2. Chromatographic separation of phenylguanine in urine samples (cation exchange chromatography/UV detection).

Figure 3. Fluorescence spectra of the reference (-----) and the fractionated sample (--------) with the same fluorescence maxima at 385 and 350 nm. The third maximum detected (not marked) is attributed to the aqueous buffer solution (-----).

Figure 4. HPLC separation (UV detection) of phenylguanine in urine samples.

Figure 5. GC–MS measurements at m/e 371 (molecular mass of N$^7$-phenylguanine) and m/e 356 (mass fragment) with different urine samples.
was controlled by GC measurements. After 6 hr, leaving 4 ppm benzene in the air, the animals were transferred into individual metabolic cages and 24-hr urine samples were collected over 6 days. Bone marrow (from two femur), liver, spleen, thymus, and blood were prepared. Nuclear and mitochondrial DNA were isolated separately (8) with the exception of bone marrow, where the DNA was not separated. In liver, RNA and protein were also obtained (21). Urine, urine fractions, tissue, nucleic acid, and protein labels were measured by liquid scintillation counting. For counting methods, sample preparation, and HPLC conditions, see Krewet et al. (22).

For comparison investigations four phenylguanines were synthesized according to the given methods: N7-phenylguanine, multistage synthesis according to Verkoyen et al. (17), O6-phenylguanine, synthesis according to Balsinger and Montgomery (23), C8-phenylguanine, multistage synthesis according to Chin et al. (24,25), N2-phenylguanine, multistage synthesis according to Elion et al. (26,27) and Albert et al. (28).

The analyses of urine samples from benzene-treated animals and analyses of hydrolyzed tissue DNA revealed, in comparison with the chromatograms of the same samples containing synthesized phenylguanines, that the detected substances were not identical to our references (Figures 6, 7). The peak patterns from samples of treated animals showed some deviations compared to those of control samples and the detected compounds had characteristic excretion kinetics over the examination period of 6 days. These peak patterns were also observed with only slight modifications in samples of phenobarbital-pretreated animals. The detected compounds were different from known benzene metabolites.

After the urine analyses [14C]benzene was used to decide whether the compounds detected in rat urine samples and DNA were benzene adducts different from the synthesized phenylguanines. A lower detection limit (1–100 pg) was achieved and information about the excretion kinetics was obtained. Rats exposed to radioactive benzene by inhalation showed the expected marked decrease in the urinary 14C label 48 hr after the end of exposure, but these remained unchanged from days 4 to 6 (Figure 8). Over the 6 days of urine collection, 26.3% of the dose inhaled could be detected. The label excreted daily in urine samples from day 4 to day 6 was 0.6%.
The phenylguanine fractions were further analyzed by HPLC. Twenty fractions per gradient were collected and measured by liquid-solid chromatography. Four compounds with retention times of 10.5 to 11, 13.5, 15.5 to 16, and 18 min were separated from the N7/O6-phenylguanine fraction. Their excretion was completed on day 4, with the exception of the late-eluting compound (Figure 9). In the C8/N7-phenylguanine fraction, four compounds were also detected, showing retention times of 13.5 to 15.5, 17 to 17.5 and 18 to 18.5 min. Their excretion was completed on days 3 to 5 after the end of exposure (Figure 10).

The reactivity of benzene oxide with DNA or polyguanine was examined using microsomes for the activation of benzene. Phenylguanines were not detected during the analysis of hydrolyzed nucleic acid samples (DNA or polyguanine) from microsomal incubations with benzene. After incubation of guanine and deoxyguanosine with p-benzoquinone and hydroquinone, two identical products were found that differed from all our reference substances in regard to their HPLC retention times. No such product was formed in similar incubation experiments with trans-trans-muconaldehyde.

Investigations with Monoclonal Antibodies

A definitive decision as to whether N7-phenylguanine is present in the urine of benzene-treated rats was achieved by means of enzyme-linked immunosorbent assay (ELISA) and fluoroimmunoassay (FIA) analyses (29). Using monoclonal antibodies obtained against 2-hydroxymethyl-7-phenyl hypoxanthine (Figure 11), N7-phenylguanine could be detected when added to urine samples in amounts of 100 fmol with an ELISA and 50 fmol with an FIA (Figure 12). In purified urine samples of benzene-treated rats N7-phenylguanine could not be found by applying these highly sensitive and specific techniques.

Conclusions

As demonstrated by GC–MS, benzene is metabolized in the rat to one or more guanine adducts of unknown structure, which can be detected in the urine.

Our study with [14C]benzene demonstrates the occurrence of 14C-labeled compounds in the urine of rats exposed to 14C]benzene by inhalation, which may be deoxyguanosine, guanine, or adenine adducts released from arylated DNA. The excretion of labeled compounds in urine samples remained constant from the 4th day after exposure and measurable radioactivity could be detected in biological macromolecules 6 days after the end of exposure.

As shown by highly sensitive and specific ELISA and FIA techniques, the phenylguanines(s) found differ also with respect to their immunologic behavior from N7-phenylguanine.

All our findings are in accordance with the hypothesis that compound(s) slightly different from N7-phenylguanine, possibly containing a hydroxy function, are excreted as guanine adduct(s) of benzene.

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