Phenylguanine Found in Urine after Benzene Exposure

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Comparative investigations with synthetic N^7-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 immunoassays (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 N^7-phenylguanine that may possibly be hydroxylated. These adducts differ also from O^6-, N^2- and C8-phenylguanine, respectively. — Environ Health Perspect 104(Suppl 6):1159–1163 (1996)

Key words: DNA adducts, benzene, N^7-phenylguanine, urine, ^{14}C, HPLC, GC–MS, monoclonal antibodies

Introduction

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

Following the hypothesis that N^7-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 N^7-guanine adducts of aflatoxin B_1 (*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 N^7-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 N^7-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 N^7-phenylguanine. One of the peaks (32 min) exhibits the same retention time and, after separation, an identical fluorometric behavior as that of the synthetic N^7-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 N^7-phenylguanine.

To compare N^7-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 C_{18} 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 N^7-phenylguanine. The data obtained suggested that N^7-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

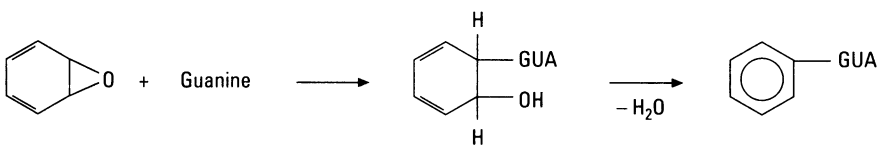

Figure 1. Proposed scheme for the reaction of benzene epoxide with guanine.

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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.

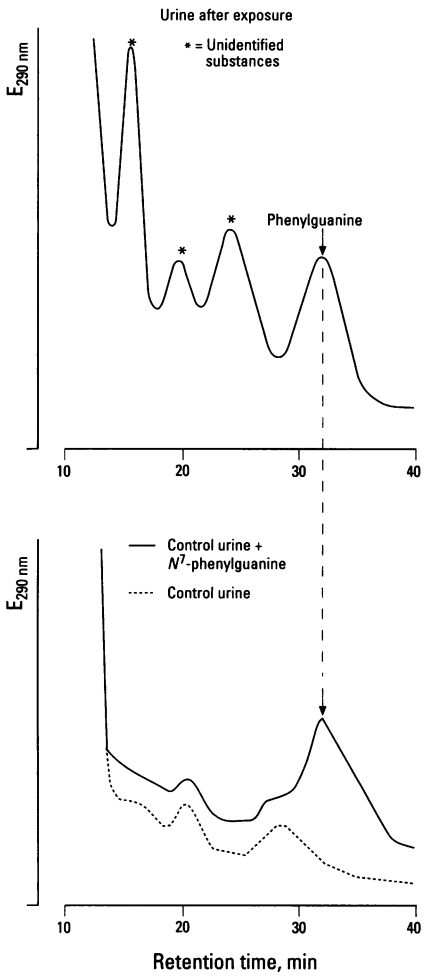

Figure 2. Chromatographic separation of phenylguanine in urine samples (cation exchange chromatography/UV detection).

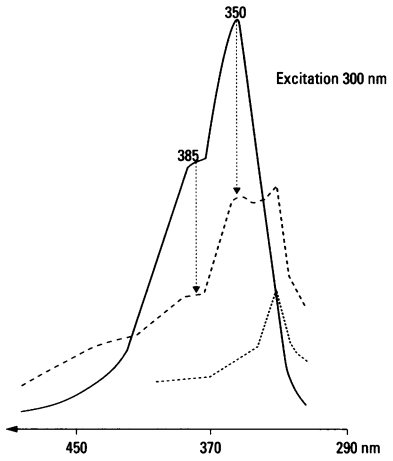

Figure 3. Fluorescence spectra of the reference (——) and the fractionated sample (-------) with the same fluorescence maxima at 385 and 350 nm. The third maximum detected (not marked) is attributed to the aqueous buffer solution (·····).

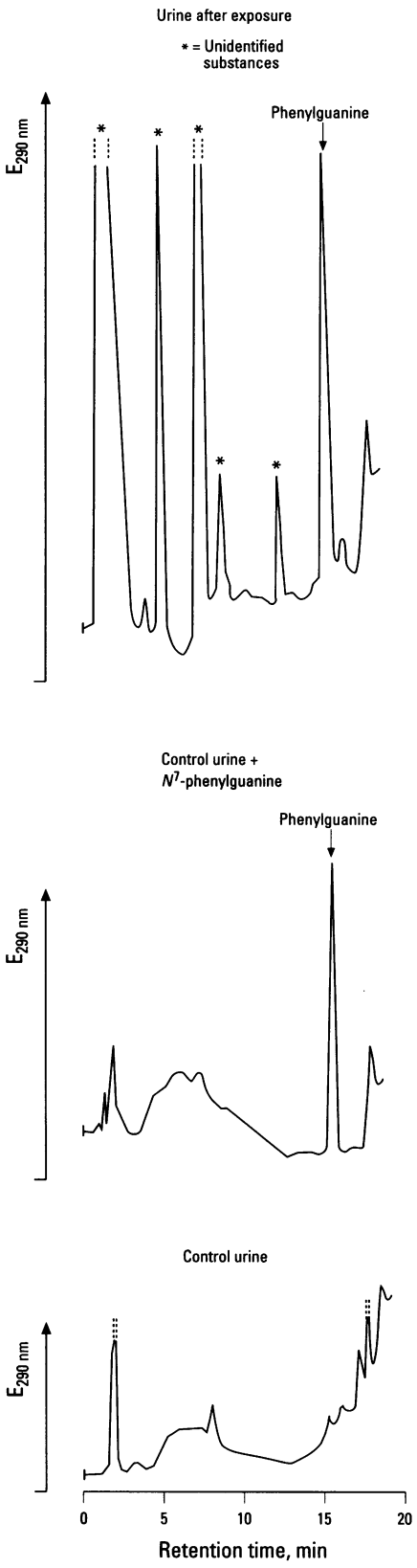

Figure 4. HPLC separation (UV detection) of phenylguanine in urine samples.

preparation of the urine samples. Such behavior could also be observed in the detection of glutathione adducts of some polycyclic aromatic hydrocarbons (*19,20*).

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 (3.3 mCi respiration 13.3 ppm/hr each) in a closed system (exsiccator, 10.3×10^{-3} m^3). The reduction of [^{14}C]benzene in the air

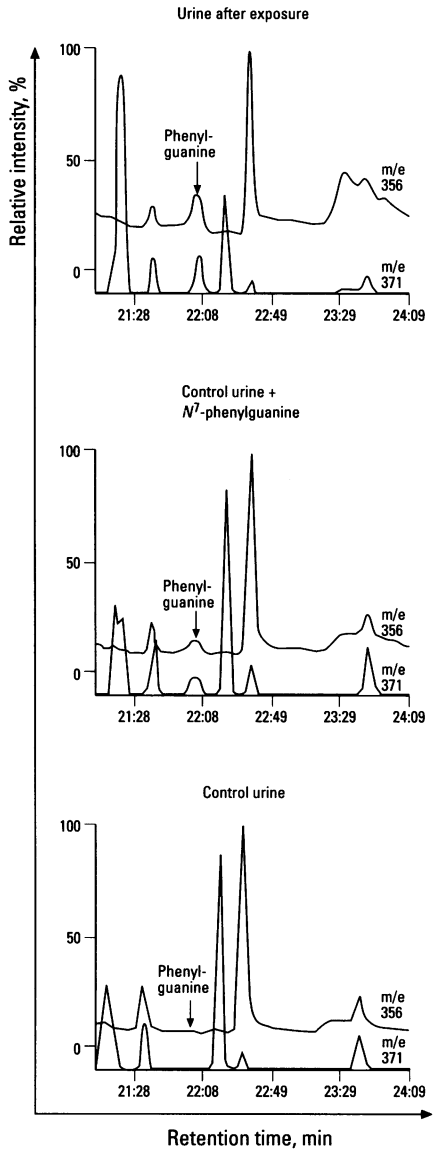

Figure 5. GC–MS measurements at m/e 371 (molecular mass of N^7-phenylguanine) and m/e 356 (mass fragment) with different urine samples.

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 femura), 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: N^7-phenylguanine, multistage synthesis according to Verkoyen et al. (*17*), O^6-phenylguanine, synthesis according to Balsinger and Montgomery (*23*), C8-phenylguanine, multistage synthesis according to Chin et al. (*24,25*), N^2-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 [^{14}C]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 ^{14}C 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%.

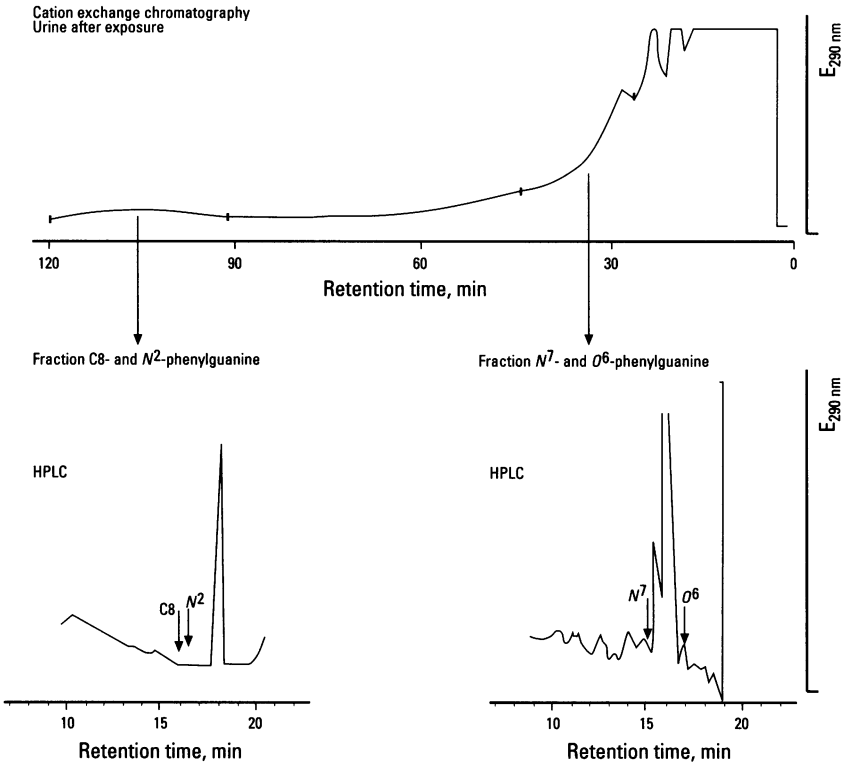

Figure 6. Chromatographic separation of a rat urine sample after exposure to benzene (500 mg/kg bw) using cation exchange chromatography and HPLC of collected phenylguanine fractions

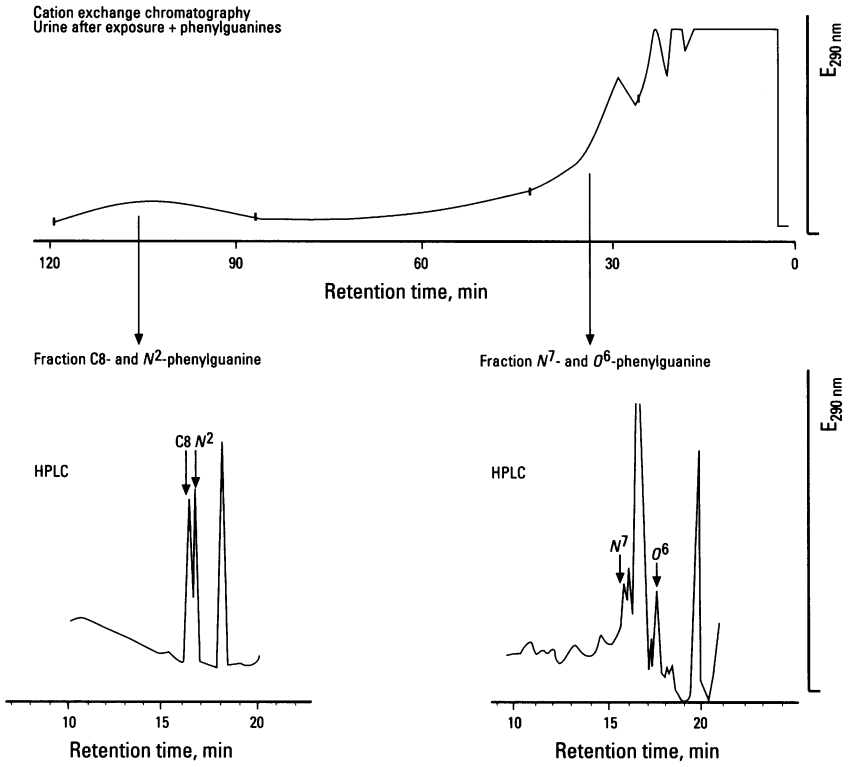

Figure 7. Chromatographic separation of a rat urine sample after exposure to benzene (500 mg/kg bw) spiked with 100 ng N^7- and O^6-phenylguanine and 200 ng C8- and N^2-phenylguanine per milliliter, injection volume 50 µl, using cation exchange chromatography and HPLC of collected phenylguanine fractions.

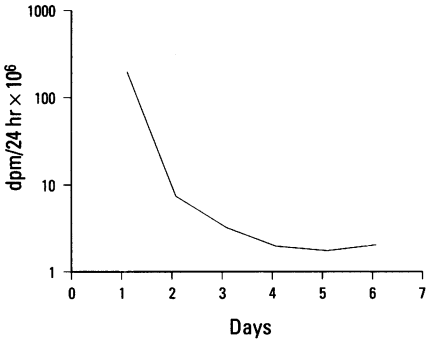

Figure 8. Excretion kinetics of labeled compounds in 24-hr urine samples of rats after exposure to [^{14}C]benzene (mean values of three rats).

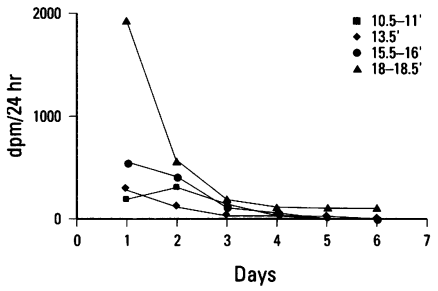

Figure 9. Excretion kinetics of ^{14}C-labeled compounds in 24-hr urine samples of rats(C8-/N^2-phenylguanine).

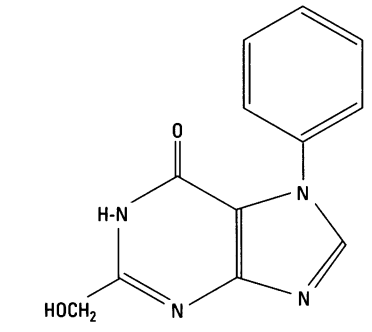

Figure 11. Structure of 2-hydroxymethyl 7-phenyl-hypoxanthine.

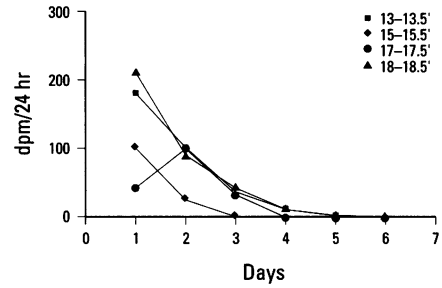

Figure 10. Excretion kinetics of ^{14}C-labeled compounds in 24-hr urine samples of rats (N^7-/O^6-phenylguanine).

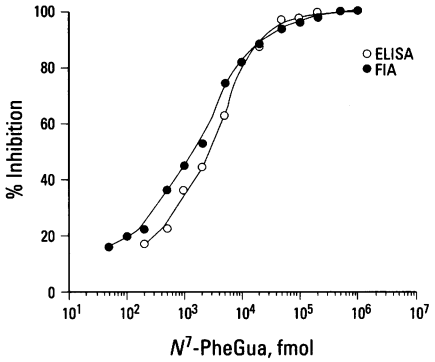

Figure 12. Competitive inhibition of mAb CE6/G11 binding to N^7-phenylguanine in enzyme-linked immunosorbent assay and fluorescent immunoassay.

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 N^7/O^6-phenylguanine fraction. Their excretion was completed on day 4, with the exception of the late-eluting compound (Figure 9). In the C8/N^2-phenylguanine fraction, four compounds were also detected, showing retention times of 13.5, 15 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 N^7-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), N^7-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 N^7-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 [^{14}C]benzene demonstrates the occurrence of ^{14}C-labeled compounds in the urine of rats exposed to [^{14}C]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 phenylguanine(s) found differ also with respect to their immunologic behavior from N^7-phenylguanine.

All our findings are in accordance with the hypothesis that compound(s) slightly different from N^7-phenylguanine, possibly containing a hydroxy function, are excreted as guanine adduct(s) of benzene.

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