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Gas chromatographic–mass spectrometric analysis of diols and tetrols from reactions of polycyclic aromatic hydrocarbon epoxides with hemoglobin

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ABSTRACT

We have evaluated both electron ionization (EI) and negative-ion chemical ionization (NICI) methods for the analysis of trimethylsilyl derivatives of a series of polycyclic aromatic hydrocarbon (PAH) alcohols including styrene diol, benzo[*e*]pyrene diol and tetrols, cyclopenta[*c,d*]pyrene diols, benzo[*a*]pyrene-4,5-diols, chrysene tetrols, benz[*a*]anthracene tetrols I and II, and *syn*- and *anti*-benzo[*a*]pyrene tetrols. NICI is the more sensitive method for all compounds except styrene diol. Detection limits are compound-dependent and range from 1 fmol for cyclopenta[*c,d*]pyrene diol to 1 pmol for benzo[*e*]pyrene diol. The EI detection limit for styrene diol is 60 fmol. PAH alcohols related to the compounds listed above were observed following hydrolysis of hemoglobin which had been reacted with PAH epoxides *in vitro*. Benzo[*a*]pyrene tetrols and a chrysene tetrol were observed following hydrolysis of hemoglobin isolated from human smokers' blood. Hydrolysis of styrene oxide treated hemoglobin in ^{18}O-labeled water revealed at least two mechanisms of ester hydrolysis, including the $B_{AL}1$ pathway.

INTRODUCTION

Most chemical carcinogens require conversion to electrophiles, either by metabolism or as a result of non-enzymatic chemical pathways, in order to effect their carcinogenitiey [1–4]. Reactions of these active intermediates with DNA are presumably the initiating carcinogenic events, but reactions with other biological macromolecules also occur. Some of these reactions, *e.g.* those with proteins, lead

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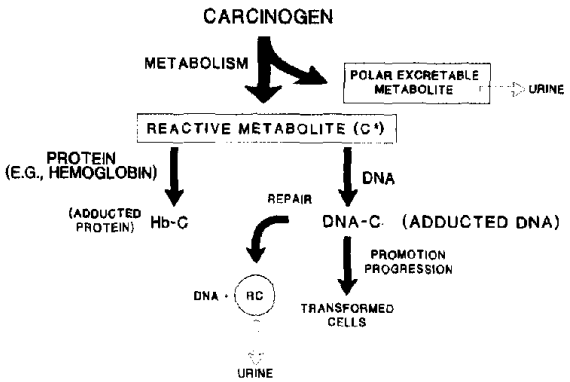


Fig. 1. General scheme for molecular dosimetry based on carcinogen metabolism and formation of nucleic acid and protein adducts.

to stable adducts that are potentially useful as dosimeters for exposure to the active forms of the carcinogen. Measurement of these adducts is presumably a better index of individual risk than is the overall environmental level of the preabsorbed and unmetabolized compound [5–7]. These interactions are summarized in Fig. 1. The metabolism of polycyclic aromatic hydrocarbons (PAHs) and subsequent reactions of the active metabolites with macromolecules follow this

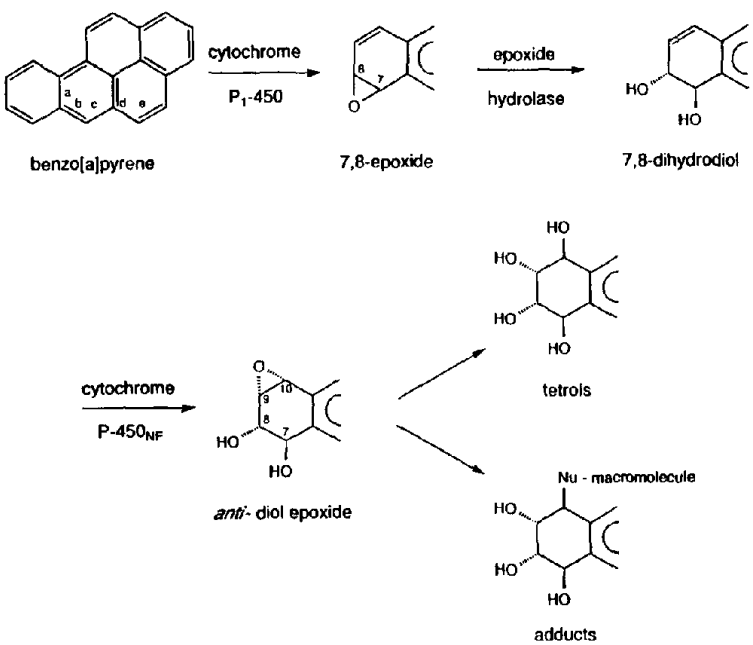


Fig. 2. Metabolism of benzo[*a*]pyrene, a typical polycyclic aromatic hydrocarbon, and reaction of resulting epoxide intermediates with macromolecules to form adducts.

scheme closely (Fig. 2). The ultimate carcinogens that arise from these compounds are typically the corresponding epoxides and diol epoxides [2]. Reactions with both DNA and protein usually occur between a benzylic site on the epoxide and a nucleophilic site on the macromolecule. Human and mouse hemoglobin, for example, both form carboxylate esters from *anti*-benzo[*a*]pyrene diol epoxide (a-BaPDE) which release benzo[*a*]pyrene (BaP) tetrols on hydrolysis [8,9]. Quantitation of these tetrols forms the basis for BaP dosimetry [9].

Dosimetry has often been done with specific target compounds to which humans are known to be exposed. One of the objectives of our research, however, is to develop methods which can be applicable in principle to classes of compounds, or which can detect compounds to which exposure might not have been expected. As part of this work, we have evaluated the mass spectra of oxidized derivatives of a number of PAHs in order to determine which derivatives and ionization methods are appropriate for the quantitation of these compounds when present at low levels in biological samples.

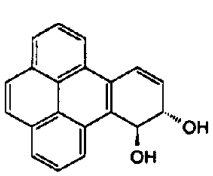
EXPERIMENTAL

Materials

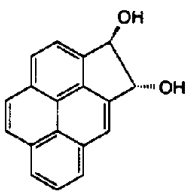
The structures for the following compounds are shown in Fig. 3. Benzo[*e*]pyrene-*trans*-9,10-dihydrodiol (BePD) was bought from the NCI Chemical Carcinogen Repository (Midwest Research Institute, Kansas City, MO, U.S.A.). Tritiated styrene oxide was purchased from Amersham (Arlington Heights, IL, U.S.A.). *cis*- and *trans*-benzo[*a*]pyrene-4,5-diol (c-BaP45D, t-BaP45D), *cis*- and *trans*-cyclopenta[*c,d*]pyrene diol (c-CPPD, t-CPPD), styrene diol (SD), *cis*- and *trans-anti*-chrysene tetrol (ca-CT, ta-CT), *cis*- and *trans-anti*-benz[*a*]anthracene tetrol I (ca-BaAT-I, ta-BaAT-I), *cis*- and *trans-anti*-benz[*a*]anthracene tetrol II (ca-BaAT-II, ta-BaAt-II), *cis*- and *trans-anti*-benzo[*a*]pyrene tetrol (ca-BaPT, ts-BaPT), *cis*- and *trans-syn*-benzo[*a*]pyrene tetrol (cs-BaPT, ts-BaPT), and the four isomers of benzo[*e*]pyrene tetrol ($BePT_{a-d}$) were prepared by hydrolysis of the corresponding epoxides. Trisyl Z was purchased from Pierce (Rockford, IL, U.S.A.). Pronase E (protease Type XXV) was obtained from Sigma (St. Louis, MO, U.S.A.).

Instrumentation

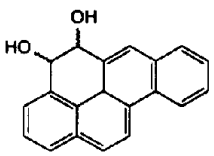
Gas chromatographic–mass spectral (GC–MS) analyses were done on a Hewlett Packard Model 5987A which was manually tuned each day with perfluorotributylamine for maximum overall sensitivity. Ultra-high purity methane (Med-Tech, Medford, MA, U.S.A) was used for both positive (PCI) and negative-ion chemical ionization (NICI) experiments with source pressures of 105–135 and 40–65 N/m^2, respectively. The source temperatures were 150°C for NICI, 200°C for PCI and 240°C for electron ionization (EI). Electron voltages were 170 eV for both NICI and PCI and 70 eV for EI. A direct capillary interface maintained at



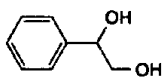
BePD



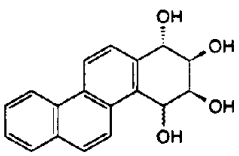
c- & *t*-CPPD



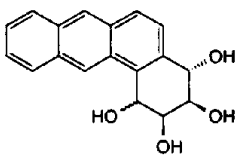
c- & *t*-BaP45D



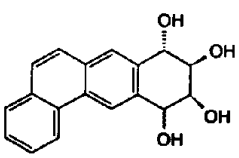
SD



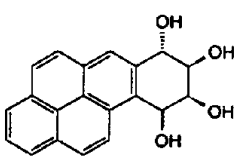
ca- & *ta*-CT



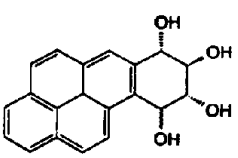
ca- & *ta*-BaAT-I



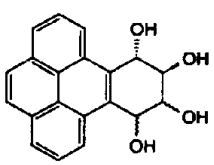
ca- & *ta*-BaAT-II



ca- & *ta*-BaPT



cs- & *ts*-BaPT



$BePT_{a-d}$

Fig. 3. Structures of the polycyclic aromatic hydrocarbon dihydrodiols and tetrahydrotetrols studied

240°C connected the GC to the MS system. The injection port and transfer lines were maintained at 250°C. The GC column was a 30 m × 0.25 mm I.D. fused-silica capillary coated with DB-1 by the manufacturer (J & W Scientific, Rancho Cordova, CA, U.S.A.). Samples were injected in the splitless mode (splitless valve open 0.5–0.9 min).

Derivatization and chromatography

The PAH diols or tetrols in reactivials or Eppendorff tubes were treated for 5 min or less at room temperature with 5–10 μl of Trisyl Z to form the trimethylsilyl (TMS) derivatives. The resulting solutions were then injected directly (1 μl) into the GC–MS system. A typical GC oven program used was as follows: 150°C isothermal for 1 min; ramp to 330°C at 20°C/min; 330°C isothermal for 4 min.

Reaction with hemoglobin in vitro

Epoxides or diol epoxides in 35 μl of tetrahydrofuran were incubated with human erythrocytes from 1 ml of whole blood (a 60 molar excess of hemoglobin to epoxide) in 1 ml of phosphate-buffered saline (PBS). The cells were then lysed

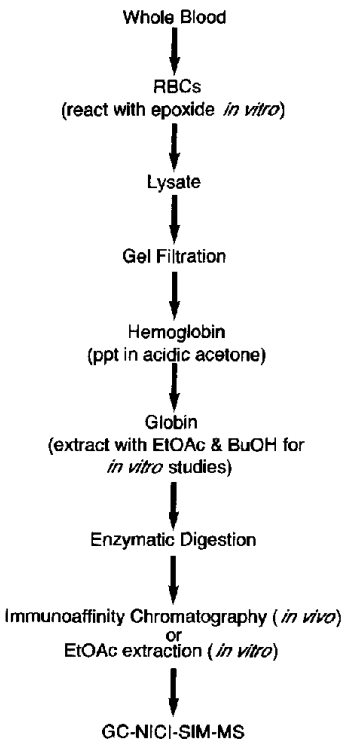


Fig. 4. Work-up of hemoglobin for analysis of esters arising from reactions *in vitro* with polycyclic aromatic hydrocarbon epoxides and diol epoxides.

in deionized water and the globin precipitated with acidic acetone at $< -10°C$. The air-dried globin was dissolved in water and extracted with ethyl acetate and 1-butanol (4 volumes each) and then digested twice with 5% (w/w) pronase at pH 8. The digest was exhaustively extracted with ethyl acetate. The organic layer was taken to dryness, derivatized with Trisyl Z and analyzed by GC–MS (see Fig. 4).

Analysis of human hemoglobin

Human hemoglobin was generally worked up as described above for the experiments *in vitro* except that the digest was concentrated on an immunoaffinity column containing an immobilized monoclonal antibody which had been raised against a-BaPDE adducts and which had affinity for various PAH alcohols. The *in vitro* and *in vivo* experimental methods are described in greater detail in another report [10].

RESULTS AND DISCUSSION

The general MS characteristics of the TMS derivatives of the PAH dihydrodiols and tetrahydrotetrols under EI and PICI conditions were consistent with previous reports [11,12]. The NICI mass spectra of these compounds were generally simple and characteristic for the diols and tetrols as groups. The TMS derivatives of the tetrahydrotetrols showed major fragment ions at $M-162$ corresponding to loss of $C_6H_{18}Si_2O$. The dihydrodiol derivatives gave base peaks at $M-90$ following loss of $(CH_3)_3SiOH$. Molecular ions were usually absent except

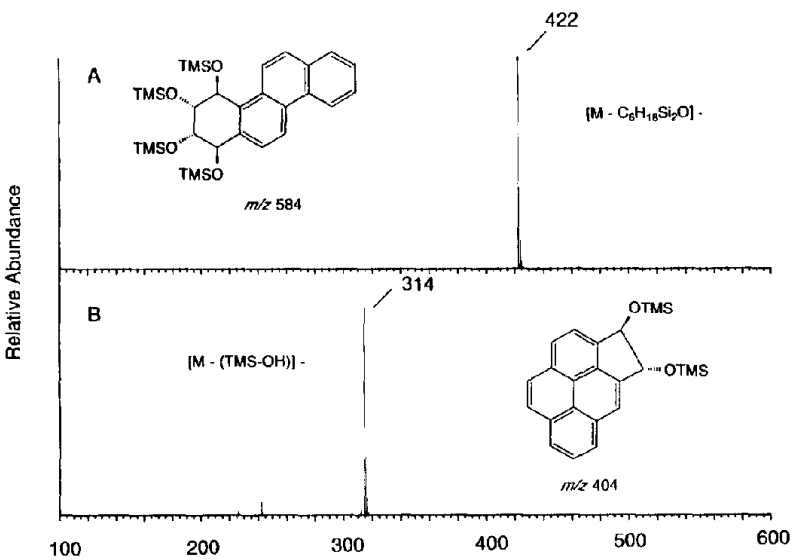


Fig. 5. Negative-ion chemical ionization mass spectra for the trimethylsilyl derivatives of chrysene-1,2,3,4-tetrahydrotetrol (A) and cyclopenta[*c,d*]pyrene-3,4-dihydrodiol (B).

at high analyte concentrations and then only in one or two cases. The chromatograms of the TMS derivatives of BePD usually contained three peaks, two of which were consistent with the phenols expected following dehydration. The peak corresponding to the intact molecule accounted for less than a third of the total material. Typical NICI mass spectra for CT and CPPD are shown in Fig. 5A and B, respectively.

In keeping with our experience in other systems [13–15], along with the high electron affinity of polycyclic aromatics, we expected that the best combination of sensitivity and selectivity for these compounds in complex matrices would be obtained with NICI; this proved generally true except for SD. The base peak for SD diTMS under EI conditions is the apparent substituted tropylium analogue at m/z 179. The detection limit for styrene oxide in selected-ion monitoring (SIM) analyses on this ion was about 60 fmol. Table I lists the ions used for quantitation and the corresponding detection limits for all the compounds listed in Fig. 3. The criterion for detection was a signal-to-noise ratio of $\geq$ 10. It should also be noted that, as with all chromatographic and especially MS techniques, the operational sensitivity depends on the complexity of the mixture and on the condition of the instrument. Mass spectrometers with freshly cleaned sources and new electron multipliers will generally perform best.

These methods were used, in conjunction with other techniques, to examine human blood from others and from umbilical cords following birth. BaP tetrols and, somewhat surprisingly, a chrysene tetrol, were identified in some of the samples [16].

The hydrolysis of the adducted globin to release the PAH alcohols has fortu-

TABLE I

PAH ALCOHOL PER TMS DERIVATIVE SIM-MS DETECTION LIMITS AND IONS MONITORED

Fragmentation by NICI (CH_4), except for SD, which was by EI.

Compound	SIM ion	SIM detection limit[a]
BePD	340	1 pmol
SD	179	60 fmol
c- and t-BaP45D	340	50 fmol
c- and t-CPPD	314	1 fmol
ca- and ta-CT	422	10 fmol
ca- and ta-BaAT-I	422	100 fmol
ca- and ta-BaAT-II	422	1 fmol
$BePT_{a-d}$	446	25 fmol
ca- and ts-BaPT	446	1 fmol
ca- and ta-BaPT	446	1 fmol

[a] Signal-to-noise ratio $\geq$ 10.

itously led to some interesting observations concerning the nature of the adducts. In earlier work in these systems, it was necessary to demonstrate that the compounds detected after hydrolysis of the proteins were in fact arising from covalent adducts rather than from strong associations involving non-specific interactions. One of the experiments designed to address this question involved hydrolysis adducted globin in ^{18}O-labeled water. If an alcohol were released from a covalent adduct, then ^{18}O incorporation into the alcohol would be observed at some pH values. Benzylic esters, for example, can hydrolyze via three different mechanisms, only one of which, *i.e.* the $B_{AL}1$ pathway, leads to ^{18}O into the released alcohol (Fig. 6). Our previous work showed that both the BaPD epoxide-derived hemoglobin and synthetic esters hydrolyze via the $B_{AL}1$ mechamism with 60 and 100%, respectively, incorporation of ^{18}O into the released alcohols [8]. Proteolysis at slightly basic pH of human hemoglobin which had been reacted with styrene oxide *in vitro* yielded SD containing about 4% ^{18}O at the benzylic position.

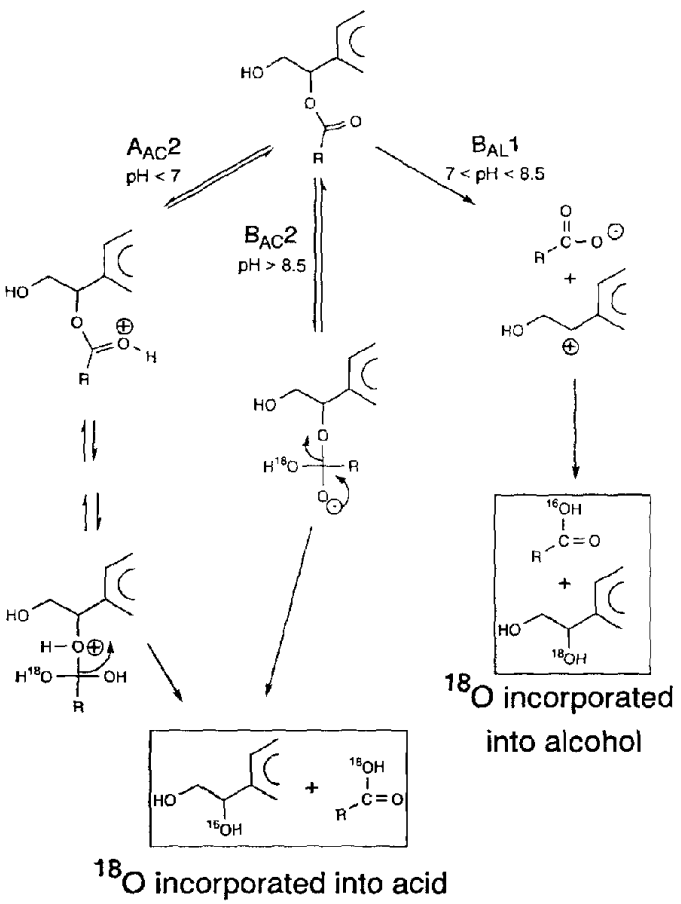


Fig. 6. Effect of pH on the mechanism of hydrolysis of benzylic esters in ^{18}O-labeled water.

Further hydrolysis under more basic conditions released SD with no detectable ^{18}O. These observations demonstrate that even the simplest PAH epoxide forms esters on hemoglobin that can hydrolyze to a small but real extent via the $B_{AL}1$ mechanism.

In summary, GC–NICI-MS is a selective and sensitive technique for the quantitation of a large number of diols and tetrols which can be obtained following hydrolysis of hemoglobin which contains carboxylate esters arising from metabolism of PAHs and reaction of the metabolites with the protein.

ACKNOWLEDGEMENTS

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