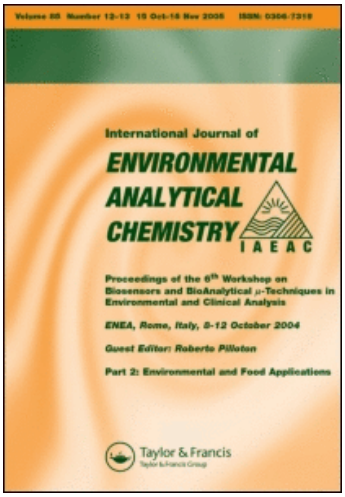

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THERMOSPRAY MASS SPECTROMETRY AS A TECHNIQUE FOR ANALYSIS OF HYDROXYLATED AND CONJUGATED BENZO(a)PYRENE DERIVATIVES*

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Thermospray (TSP)-mass spectrometry has been assessed as a technique for analyzing benzo(a)pyrene (BaP) metabolites. Negative ion spectra provided the most useful information. Spectra of hydroxylated metabolites (one to four OH groups) included $[M-H]^-$ ions and fragments attributable to progressive dehydration. Conjugated metabolites gave small or non-detectable $[M-H]^-$ or $[M-Na]^-$ ions and a major fragment at m/z 267 corresponding to $[BaPO]^-$. All the hydroxylated compounds produced $[M+OAc]^-$ adduct ions. The conjugated metabolites produced $[BaPOH+OAc]^-$ ions at m/z 327. Thermal degradation was frequently encountered, presumably occurring in the heated capillary of the TSP probe.

KEY WORDS: Mass spectrometry, thermospray, Benzo(a)pyrene, metabolites, fragmentation, HPLC.

INTRODUCTION

Thermospray (TSP) mass spectrometry is a well established technique for the introduction of compounds that are labile and of low volatility into a mass spectrometer via a high performance liquid chromatograph (HPLC). It has been extensively used for the analysis of many such compounds, including both primary and secondary metabolites. A number of reviews have been published recently, documenting various aspects of the use of TSP[1–3].

Polynuclear aromatic hydrocarbons (PAH) are a family of compounds that are generated during the incomplete combustion of fossil fuels and other carbonaceous material. They have become ubiquitous pollutants of the biosphere and concentrate in the marine environment as the result of aerial deposition, urban and industrial runoff, accidents and waste effluents such as those from sewage treatment plants. PAH are hydrophobic compounds and as such are accumulated in sediments. Due to their solubility in lipids they readily enter the food chain, where they can produce a number of toxic effects in organisms, including neoplasia. Because of the possible association between PAH contamination and

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neoplasia in fish,[4] and because PAH are readily metabolized by these biota,[5] further investigations into the metabolism of PAH by these organisms are warranted. Particularly as variations in the production of PAH metabolites may be reflected as differences in disease susceptibility.

Benzo(a)pyrene (BaP) is well recognized as a procarcinogenic PAH. This carcinogenicity is mediated by highly reactive intermediary metabolites which are capable of binding to cell macromolecules.[6] *In vitro* BaP is known to be metabolized by fish microsomes to produce many of the same products that are encountered in mammals.[7] *In vivo* both hydroxylated compounds and their conjugated (glucuronide, sulfate, glutathione) products are formed.[8] BaP metabolites are labile and of low volatility and are therefore not immediately amenable to gas chromatographic analysis, though the primary metabolites can be analyzed by this technique after derivatization. HPLC can be used for their analysis but does not provide positive information on compound identities. HPLC mass spectrometry (HPLC-MS) is a technique that may be appropriate for these compounds because both separation and the structural information required for identification may be achievable. To this end, direct liquid introduction (DLI) HPLC-MS has been used to elucidate the mass spectrometric behavior of these compounds.[9] Also, because of the lability of these metabolites, fast atom bombardment (FAB) mass spectrometry has been investigated.[10] Both of these techniques provide useful information, but also suffer from drawbacks. Because of flow rate limitations, DLI requires either the use of microbore (1 mm i.d.) HPLC columns or the diverting of >90% of the flow from a regular (e.g. 4.6 mm i.d.) analytical column. FAB-MS is a static technique and even though dynamic (flowing) FAB has been developed, the flow rate requirements are very low, of the order of 5 μl/min. TSP is a technique that offers the opportunity of analyzing and identifying BaP metabolites with commonly employed HPLC flows (approx. 1 ml/min), provided that sufficiently informative spectra can be obtained. This paper reports the first step of this process, using flow-injection methodology to determine the mass spectrometric behavior of both hydroxylated and conjugated BaP metabolites under TSP conditions.

EXPERIMENTAL

BaP metabolites were obtained from the National Cancer Institute, Chemical Carcinogens Repositories (National Institutes of Health, Bethesda, MD). The compounds that were used for this study are given in Table 1. Samples were dissolved in deionized water or in deionized water containing 0.1 M ammonium acetate and introduced into the mass spectrometer via loop injection onto the TSP interface using a Rheodyne (Model 7125) injection valve (6 μl injection loop). BaP metabolite concentrations were 200 or 500 μg/ml with up to 6 μl being injected into the interface. An aqueous solution of 0.1 M ammonium acetate was used as the mobile phase, and was delivered (1 ml/min) by a Perkin Elmer Series 4 HPLC System (Perkin-Elmer Corp., Norwalk, CT).

The TSP interface and source were purchased from Vestec Corp. (Houston, TX).

Table 1 Characteristic negative ions and average relative abundances for benzo(a)pyrene metabolites using thermospray ionization

Compound (isomers)	*Mol. wt.*	*Ions*
Nonconjugated BaP metabolites		*m/z (relative intensity)*
Monohydroxy BaP (1, 3, 6, 7, 9, 11)	268	267(100), 283(30), 327(1)
BaP-trans-dihydrodiol (4, 5; 7, 8; 9, 10)	286	267(100), 285(5), 301(2), 345(50)
BaP-tetrahydrotriol (r7, t8, t9)	304	267(100), 283(30), 285(20), 301(25), 303(10), 319(10), 363(90), 379(25)
BaP-tetrahydrotetrol (r7, t8, t9, t10; r7, t8, t9, c10; r7, t8, c9, t10; r7, t8, c9, c10)	320	267(30), 283(80), 301(90), 319(50), 345(25), 378(95), 379(100)
Conjugated BaP metabolites		
BaP-glucuronide (1, 9)	444	235(40), 267(100), 327(10), 443(5)
BaP-7, 8-dihydro-7-glucuronide-8-OH	462	235(25), 267(100), 283(20), 327(20), 443(8), 461(5)
BaP-7-sulfate (sodium salt)	370	235(5), 267(100), 327(15)

The original design was augmented by addition of a separate probe tip heater. However, thermal contact between the source, the probe tip and the capillary allowed thermal interaction between the three heated regions, resulting in inadequate control of these temperatures. Therefore, less improvement in performance was achieved than anticipated. The TSP source was mounted on an Extrel ELQ 400-2 mass spectrometer (Extrel Corp., Pittsburgh, PA). Typically, the source was operated at 230–240 °C while the tip of the interface was normally maintained at 199–205 °C. The choice of temperatures was determined by the need to maximize the molecular ion while preventing the tailing of the peak. Instrument tuning and mass calibration were accomplished with polyethylene glycol of approximate molecular weight 600 (Sigma Chemicals, St. Louis, MO.).

RESULTS AND DISCUSSION

Negative ion detection was found to provide both the most useful spectra and the greatest sensitivity. Emphasis is therefore placed on the negative ion spectra in the discussion below. Table 1 shows the major ions and their relative intensities for the compounds investigated, as well as their molecular weights. These spectra represent composites of the different isomers studied. The spectra of a number of scans were averaged for each isomer, typically from up to three successive injections of the compounds. Then, because there were no obvious differences between isomers the composite spectra given in Table 1 were derived. The averaging of spectra for the individual compounds was necessary because of the instability of the ion beam, which appeared to be due to a combination of the high pressure in the source and to fluctuations in that pressure. The latter were

Table 2 Fragment ions of hydroxylated BaP metabolites. (Values in parentheses = relative intensities)

Compound	$M-H$ *m/z*	$M-H_2O-H$ *m/z*	$M-2H_2O-H$ *m/z*	$M-2H_2O-OH$ *m/z*
BaPOH	267(100)	—	—	—
$BaPH_2(OH)_2$	285(5)	⟶ 267(100)	—	—
$BaPH_4(OH)_3$	303(10)	⟶ 285(20)	⟶ 267(100)	—
$BaPH_4(OH)_4$	319(50)	⟶ 301(90)	⟶ 283(80)	⟶ 267(30)

attributed to the use of a single piston HPLC system. In general the spectra were consistent from scan to scan but averaging was important for any single compound because a sudden change in pressure could cause the apparent disappearance of an ion in any one spectrum. That ion would reappear in the next scan as the source pressure stabilized.

In all cases, except for the sulfate conjugate, an $[M-H]^-$ ion was observed. There was no $[M]^-$ molecular ion. The intensity of the $[M-H]^-$ ion was, however, highly variable. For monohydroxy BaP, the $[M-H]^-$ ion at m/z 267 was the base peak. For other compounds, the $[M-H]^-$ ion intensity varied from 5–50%. There was no obvious relationship between molecular structure and the intensity of the $[M-H]^-$ ion. Aside from the $[M-H]^-$ ion, the spectra of these compounds exhibited the presence of a number of other fragment and adduct ions.

The fragmentation processes for the hydroxylated BaP metabolites are illustrated in Table 2. The energy transferred to the molecule by the thermospray process was sufficient to cause the loss of one water molecule from dihydrodiols, two water molecules from tetrahydrotriols, and two water molecules along with a hydroxyl group from tetrahydrotetrols. In each case, this resulted in the formation of the m/z 267 ion, which is $[BaPO]^-$ ($[C_{20}H_{11}O]^-$), the oxygenated ion of the basic polynuclear aromatic ring structure. It can be seen that the fragmentation process leading to this structure is relatively facile; particularly for the dihydrodiol and tetrahydrotriol, where the m/z 267 ions are the base peaks of the spectra. For the tetrahydrotetrols the same process applies, though the intensity of the m/z 267 ion is lower (30%). This is presumed to be due to the dissipation of energy via the formation of the m/z 301 and m/z 283 ions. The analysis of a number of isomers, three dihydrodiols (positional isomers) and four tetrahydrotetrols (stereo-isomers), provided no evidence of variations in fragmentation pattern related to the isomeric structures of the compounds. Such isomer specific fragmentation has been observed with direct liquid introduction (DLI) HPLC-MS.[9] This uniformity of spectra from different isomers may, in part, be due to the necessity of averaging spectra because of the fluctuations in ion source pressure.

The spectra of all the conjugated (secondary) metabolites were dominated by an aglycon ion at m/z 267. This ion, which resulted from the breaking of the glycosidic bond, was the base peak for these compounds. The negative ion spectra of two BaP-glucuronides and a BaP-sulfate are shown in Figure 1. For the BaP-9-glucuronide (Figure 1A) a small, 5%, $[M-H]^-$ ion was observed at m/z 443. A similar intensity $[M-H]^-$ ion at m/z 461 occurred for BaP-7,8-dihydro-7-

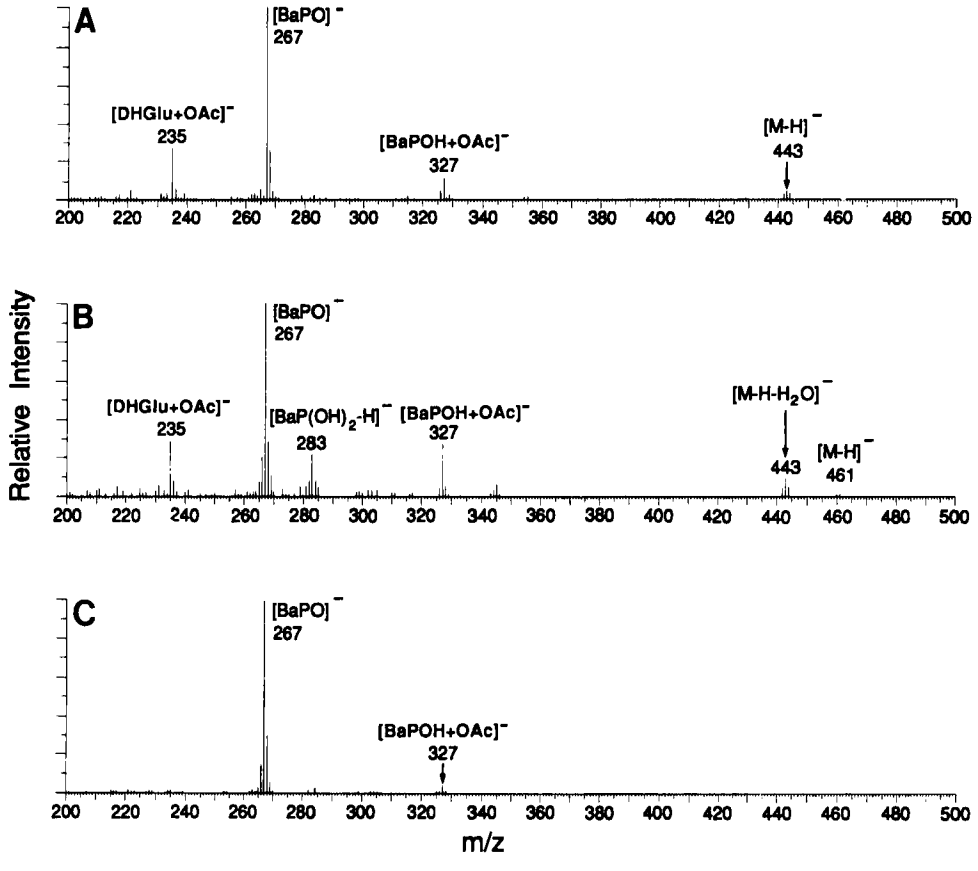

Figure 1 Negative ion TSP-mass spectra of (A) BaP-9-glucuronide, (B) BaP-7,8-dihydro-7-glucuronide-8-OH and (C) BaP-7-sulfate, sodium salt. DHGlu = dehydrated glucuronic acid. Ac = CH_3CO.

glucuronide-8-OH (Figure 1B). This compound also gave a dehydration ion at m/z 443 and an ion at m/z 283, which corresponded to $[BaP(OH)_2 - H]^-$. There was no visible $[BaPOSO_3]^-$ molecular ion for the sulfate at m/z 347 (Figure 1C), indicating that the sulfate is a less stable conjugate than the glucuronide. Other ions in these spectra are discussed later in the section on adduct ions.

Although this paper is concerned primarily with negative ions, positive ion spectra were obtained and are of relevance in a discussion of the fragmentation processes of these compounds. All positive ion spectra contained a major ion at m/z 269, which is a counterpart ion for the m/z 267 ion in the negative spectra. The relevance of the m/z 269 ion is that its only logical structure is $[BaPOH + H]^+$, and as such, it is a protonated molecular ion of monohydroxylated BaP. This indicated that, except for the monohydroxy BaP, some thermal decomposition of

Table 3 Adduct ions of hydroxylated BaP metabolites. (Values in parentheses = relative intensities, N.D. = not detected)

Compound	$[M+O-H]^-$ *m/z*	$[M+OAc]^-$ *m/z*	*Other* *m/z*
BaPOH	283(30)	327(1)	
$BaPH_2(OH)_2$	301(2)	345(50)	
$BaPH_4(OH)_3$	319(10)	363(90)	M+O+OAc, 379(25)
$BaPH_4(OH)_4$	N.D.	379(100)	M+OAc−H, 378(95), $BaP(OH)_2$+Ac, 345(25)

these compounds was occurring, presumably during passage through the TSP probe.

Aside from fragment ions, there were a number of adduct ions present in the spectra. The formation of adduct ions in TSP is well recognized.[11] For the nonconjugated (primary) metabolites, these ions are given in Table 3. The most obvious adducts were the $[M+OAc]^-$ ions ($Ac=CH_3CO$) that occurred at m/z 327, m/z 345, m/z 363 and m/z 379 for the respective levels of hydroxylation (one to four OH groups). The existence of these ions indicates that although thermal degradation in the TSP probe is important, some molecules must emerge intact from the probe, otherwise these ions would not have been observed. Adduct ions, that probably formed with residual oxygen in the mass spectrometer,[12] were commonly encountered for the nonconjugated compounds. These adducts were the $[M+O-H]^-$ ions at m/z 283, m/z 301 and m/z 319 for the monohydroxy-, dihydrodiols and tetrahydrotriol of BaP respectively. No equivalent ion was observed for the BaP-tetrahydrotetrols. This type of adduct formation is interesting because it is also a common process in negative chemical ionization (NCI), and therefore, may be indicative of the similarity between the negative ion thermospray process and NCI.[13] There are a number of other adduct ions noted in Table 3 which, though readily explainable, do not follow the more obvious sequential patterns described above.

Adduct ions were also generated from conjugated metabolites (Figure 1). However, all were adducts of fragments produced in the thermal degradation of these compounds. Thus the m/z 327 ion, which occurred for both the glucuronide and sulfate conjugates, is attributable to the formation of $[BaPOH+OAc]^-$. The m/z 235 ion present in the spectra of the glucuronides is the acetate adduct of dehydrated glucuronic acid.

The results indicate that thermospray can be successfully used for BaP metabolites, although fragmentation is greater than might have been predicted. This is particularly the case for the conjugated metabolites where compounds with relatively similar structure, such as steroid glucuronides and sulfates, have been shown to give TSP spectra in which the molecular ions are dominant.[14–15] Because of the complexity of metabolite mixtures encountered in environmentally contaminated fish[16–17] simple HPLC – MS will probably not provide the necessary separation to allow unequivocal identifications to be made. Therefore, additional

techniques will be required such as "heart-cutting" chromatography and tandem mass spectrometry.

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