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Detection of benzo[*a*]pyrene sulfate and glucuronide conjugates in cell culture medium by directly coupled microbore high-performance liquid chromatography–fast atom bombardment mass spectrometry

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ABSTRACT

An improved method is described for detecting glucuronide and sulfate conjugates of benzo[*a*]pyrene in medium from cell cultures treated with benzo[*a*]pyrene. This method is based on a microbore high-performance liquid chromatograph directly coupled to a high-resolution continuous-flow fast atom bombardment mass spectrometer. Sulfate and glucuronide conjugates, as well as some structural isomers of glucuronide conjugates, were fully separated by the reversed-phase microbore high-performance liquid chromatography conditions used in this study. Since the method does not rely on the use of radiolabeled materials, it may be used to detect conjugates of a wide variety of hydrocarbons. The high sensitivity and selectivity of the method were demonstrated by detecting conjugates in the media of cell cultures derived from mice, hamsters and humans.

INTRODUCTION

Polycyclic aromatic hydrocarbons are one of the most common pollutants of the environment. Some of these compounds, including benzo[*a*]pyrene (BP), are carcinogenic in animals [1]. BP is metabolized by mixed function oxidases and hydrolyases to phenols, diols, and epoxides [2]. One class of metabolites, the "bay-region" diol epoxides, react with DNA and are believed to be one of the principal intermediates through which BP induces cancer [3,4]. Although some BP is converted to bay-region diol epoxides, most of the phase I metabolites are conjugated with sulfate, glucuronic acid, or glutathione to form water-soluble conjugates that are rapidly excreted [2]. Conjugation of BP metabolites is an important step in detoxification of BP because it competes with reactions that lead to formation of the bay-region diol epoxides [5,6]. As we attempt to understand and manipulate this competition, it is important to have analytical methods suitable for detecting and quantifying conjugates produced in cell cultures and *in vivo*.

Analytical methods normally used for detecting and quantifying hydrocarbon metabolites in cell culture medium require the use of radiolabeled hydrocarbons. Primary unconjugated metabolites are removed from media by extraction with non-polar solvents, leaving the polar conjugates in the aqueous phase. Glucuronide and sulfate conjugates can be analyzed directly [7–11]. Although these conjugates have been separated by silica gel thin-layer chromatography (TLC) [8], alumina columns [9], and ion-pairing high-performance liquid chromatography (HPLC) [10], separation of structural isomers has generally not been possible. Alternatively, conjugates

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can be hydrolyzed with β-glucuronidase or arylsulfatase to their corresponding non-polar metabolites, which are separated by HPLC and detected by their radioactivity [12–15]. Structural isomers of conjugates have been identified through indirect analysis of conjugates via their hydrolysis products. Although this indirect approach has given important structural information, it suffers from the fact that some conjugates are poor substrates for the hydrolases [7]. In addition, this approach is limited to those conjugates for which hydrolases are available.

The utility of radiotracers for metabolic studies in cell cultures is related to the fact that the metabolites must only be separated from each other, but not necessarily from other substances which are present in the medium. Detecting metabolites by their UV absorbance has generally not been practical for cell culture studies because of difficulties in separating small quantities of metabolites from other substances that also absorb in the UV range. Although radiotracer studies are particularly easy to implement and are suited to quantitative measurements, they are restricted to hydrocarbons for which labeled materials are available. In addition, synthesis and disposal of radiolabeled hydrocarbon metabolites are expensive.

As an alternative to radiolabeled materials, we have developed new methods based on microbore HPLC and continuous-flow fast atom bombardment mass spectrometry (FAB-MS) for the detection and quantitation of BP sulfates and glucuronides [16,17]. In addition to facilitating investigations of a wide variety of hydrocarbons for which radiolabeled forms are not available, this approach is attractive because it gives structural information via the mass-to-charge ratios of the molecular and fragment ions, and because it might be useful for determining hydrocarbon exposure levels. Previous reports from this laboratory describe how cell culture medium can be fractionated by microbore HPLC, collected, and analyzed by continuous-flow FAB-MS [16,17]. These investigations demonstrated that this two-step appraoch is suitable for detecting and quantifying hydrocarbon metabolites present in cell culture media. This report describes how a microbore HPLC system can be directly coupled to a high-resolution mass spectrometer and used to make similar measurements in a single step. In addition, it is demonstrated that some of the structural isomers of BP glucuronides can be separated by microbore reversed-phase HPLC.

EXPERIMENTAL

Isolation of BP conjugates from BP-treated cells

The mouse hepatoma cell line Hepa-1c1c7, early passage hamster embryo cell cultures, and the human hepatoma cell line HepG2 were grown to confluence in 150-cm^2 flasks and re-fed with fresh media. After 24 h the cells were treated with BP (Chemical Carcinogen Repository, NCI, through the Midwestern Research Institute, Kansas City, MO, USA; 0.5–1.0 μg/ml of media) for 10 h. Medium from these three flasks, as well as from three additional flasks in which the cells had not been treated with BP, was recovered at the end of the incubation period and stored at −20°C. Aliquots (400 μl) of each type of media were used to prepare samples for analysis by HPLC–MS. Non-polar materials in the media, including non-polar BP metabolites, were removed by extracting with a mixture of chloroform–methanol water [18]. The aqueous layer from each extract was dried, dissolved in 2 ml of water and loaded on a C_{18} Sep-Pak cartridge (Waters Assoc., Milford, MA, USA). The cartridge was washed with 2 ml of water, followed by 2 ml of methanol. The methanol fraction was filtered (4 mm, 0.45 μm Nylon 66, Alltech Assoc., Deerfield, IL, USA), dried, and redissolved in 40 μl of an aqueous solution, 40% of which was a mixture of methanol–acetonitrile (1:3). Portions of this solution were used for HPLC–MS analysis.

Microbore HPLC–MS

The apparatus used for directly coupled HPLC–MS system (Fig. 1) consisted of a gradient microbore HPLC system (Applied Biosystems, Santa Clara, CA, USA), two sample injectors, a reversed-phase column (C_{18}, 250 mm × 1

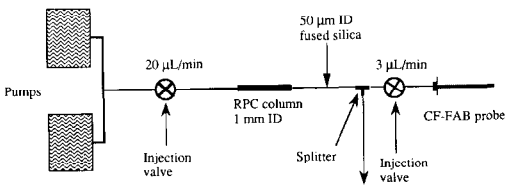

Fig. 1. Microbore HPLC interface used for HPLC–MS analysis of BP conjugates.

mm I.D., 5 μm particle size, Applied Biosystems), and an effluent splitter. A Rheodyne 7125 injector with a 20-μl external loop (Alltech Assoc.) was used for injecting samples before the column (HPLC–MS), and a Valco CW14 injector (Valco, Houston, TX, USA) was used for injecting samples after the column. The post-column injector was used primarily to generate signals for tuning the instrument and verifying the accuracy of the mass scale of the mass spectrometer. The column was protected with a guard column (20 mm × 2 mm packed bed C_{18} pellicular, Alltech Assoc.). The HPLC–MS mobile phase was a mixture of water with 0.1% trifluoroacetic acid (solvent A) and 75:25 acetonitrile–methanol with 0.1% trifluoroacetic acid (solvent B). BP conjugates were eluted with a gradient of 40–70% B in 30 min, followed by a gradient of 70–100% B in 10 min.

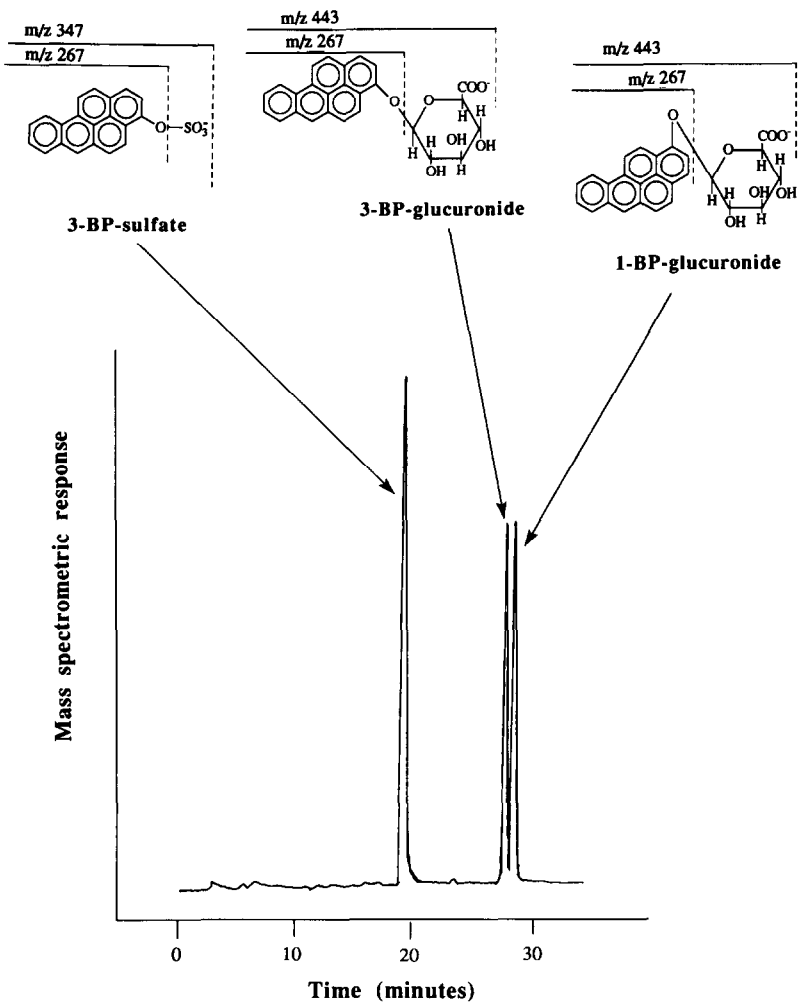

Fig. 2. Single-ion recording of *m/z* 267.0810 (resolution 10 000) for injection of a mixture of 10 ng each of BP 3-sulfate, BP 1-glucuronide, and BP 3-glucuronide.

The eluent was monitored at 254 nm with an Applied Biosystems 757 UV detector equipped with a 1-μl flow cell. The flow of 20 μl/min through the column was split approximately 1:6 to give a flow-rate of 3 μl/min into the mass spectrometer via a continuous-flow probe described previously [19]. The microbore HPLC system was attached to a Kratos MS50RF mass spectrometer which was operated in the negative-ion mode with an 8-kV ion accelerating potential. The signal from a mass window 100 ppm wide (resolution 10 000) and centered at *m/z* 267.0810, 347.0378, or 443.1129 was recorded in the single-ion monitoring mode with an HPLC data acquisition system (Dynamax, Rainin Instruments, Woburn, MA, USA).

RESULTS AND DISCUSSION

The HPLC–MS apparatus illustrated in Fig. 1 has been optimized for detection, identification, and quantitation of low levels of polar metabolites present in complex mixtures, such as cell culture medium. Reversed-phase microbore HPLC was selected for isolation of BP conjugates because it generally gives high levels of efficiency and recovery for polar substances, and because the dilution factor is minimized. This latter feature is particularly attractive when the HPLC system is directly coupled to a continuous-flow fast atom bombardment mass spectrometer since the flow into the mass spectrometer must be restricted. With a flow-rate of 20 μl/min through the 1 mm I.D. column and a flow of 3 μl/min into the mass spectrometer, only 15% of the sample was used for mass spectral analysis. Hence, a six-fold improvement in the system sensitivity would be possible if a smaller column and hence lower flow-rate were used [20]. The 1 mm I.D. column was used in the present investigation because we wanted to load large volumes (up to 20 μl) of sample and because, in our hands, 1 mm I.D. columns have been significantly more durable than 0.3 mm I.D. columns. Furthermore, the sensitivity of the system with the larger column was adequate for cell culture studies.

The chromatographic performance of this system is demonstrated for a mixture of BP 3-sulfate, BP 3-glucuronide, and BP 1-glucuronide in Fig. 2. The chromatogram, obtained by single-ion monitoring at *m/z* 267.0810, which is the exact mass of a fragment common to all three conjugates, shows that these three standards can be completely separated by microbore reversed-phase HPLC. Elution of the sulfate conjugate before the glucuronide suggests that it is an anion at pH 2.0, which is consistent with its pK_a of 1. At pH 2, the glucuronides are not ionized (pK_a 5) and are therefore more highly retained by the column. It follows that the 0.1% trifluoroacetic acid in the mobile phase is used to establish the pH and is not functioning as an ion-pairing agent.

The excellent separation of BP sulfate and BP glucuronides (resolution 14) is notable since Merrick and Selkirk [11] were unable to completely resolve these conjugates after optimizing conditions for reversed-phase HPLC. It is likewise notable that BP 1-glucuronide and BP 3-glucuronide were fully resolved by the current HPLC method. Although the improved separation may be attributed in part to the use of a microbore column, the selectivity of the silica used to pack the column as well as the use of a ternary mobile phase are probably more important factors. Plakunov *et al.* [10] have also separated BP sulfates from BP glucuronides (resolution 1) by HPLC. Their procedure, performed at pH 6 where both conjugates are ionized, used tetrabutylammonium bromide as an ion-pairing agent.

This study has been directed at detecting BP metabolites of known structure. As a result, molecular ions ($[M - H]^-$) or fragment ions characteristic of these metabolites were detected in the single-ion monitoring mode. When members of a particular class of metabolites have a common ion, they can be detected by monitoring this ion. For example, most of the phenolic metabolites, conjugated or non-conjugated, give a peak at *m/z* 267.0810 (see Fig. 2). Hence all of these metabolites could be detected by monitoring a narrow mass range centered on this mass. Results presented in Fig. 2 illustrate how this ion can be used to detect different BP conjugates. Identification of these metabolites would be made principally by their chromatographic retention time.

For the present measurements, high resolution ($m/\Delta m$ 10 000) was used to compensate for incomplete purification of the BP conjugates by HPLC. High mass resolution strongly discriminates against ions that do not have the desired elemental composition and substantially increases the selectivity of the detector. As a result, BP conjugates can be detected even though different substances, some of which may also give ions at the same nominal m/z as the BP conjugates, may co-elute with the BP conjugates. These ions, which would interfere with detection of the BP metabolites, may be due to co-eluting substances present in the media, or they may be part of the FAB-MS background.

Additional structural information could, in principle, be obtained from full-scan mass spectra. For example, the sulfate and glucuronide conjugates of BP can be distinguished by their full-scan negative-ion mass spectra [16,17]. However, full-scan mass spectra can be difficult to obtain when operating in the high-resolution mode because of the reduced sensitivity of the mass spectrometer. As a result, full-scan mass spectra of extracts of cell culture medium were of low quality. Structurally diagnostic full-scan mass spectra of BP metabolites could likely be made in the low-resolution mode if additional purification steps were used.

Detection and identification of BP sulfate in media from a cell culture (HepG2) by directly coupled microbore HPLC–MS is illustrated by results presented in Fig. 3. Single-ion chromatograms in Fig. 3a–c indicate the signal for m/z 267.0810 for (a) reference BP 3-sulfate, (b) medium from cells treated with BP, and (c) medium from cells that were not treated with BP. It is evident from these results that a substance in medium from BP-treated cells giving this ion elutes from the microbore HPLC column at the same time as authentic BP sulfate, and that this substance is not present in medium from cells that were not treated with BP. Identification of this material as BP sulfate was substantiated by monitoring the molecular ion ($[M - H]^-$, m/z 347.0378). Single-ion chromatograms given in Fig. 3d and e show that this ion is obtained from medium from cells treated with BP, and is not obtained from medium from cells that were not treated with BP. Collectively, these results show that the BP-sulfate is a principal conjugate formed by HepG2 cells, and that there is no evidence for other metabolites with similar chromatographic and mass spectral properties. This conclusion is consistent with results presented by Plakunov *et al.* [10] who used radioactively labeled BP to show that both BP 3-sulfate and BP

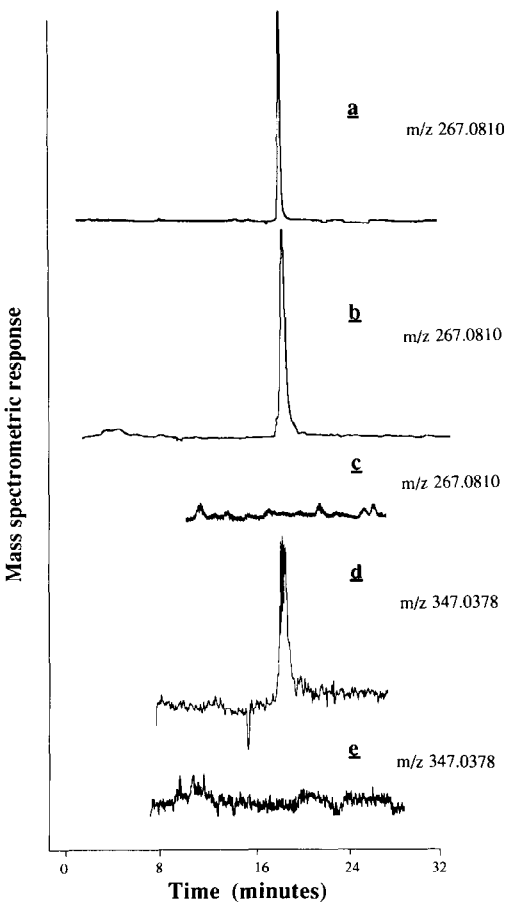

Fig. 3. HPLC–MS single-ion recordings (resolution 10 000) of an extract of medium from a HepG2 cell culture. Ions at m/z 267.0810 may be formed from many different BP conjugates, while ions at m/z 347.0378 are characteristic of BP sulfate. (a) m/z 267.0810 for 5 ng of reference BP 3-sulfate; (b) m/z 267.0810 for 400 μl of medium from cells treated with BP; (c) m/z 267.0810 for 400 μl of medium from cells that were not treated with BP; (d) m/z 347.0378 for 40 μl of medium from cells treated with BP; (e) m/z 347.0378 for 40 μl of medium from cells that were not treated with BP.

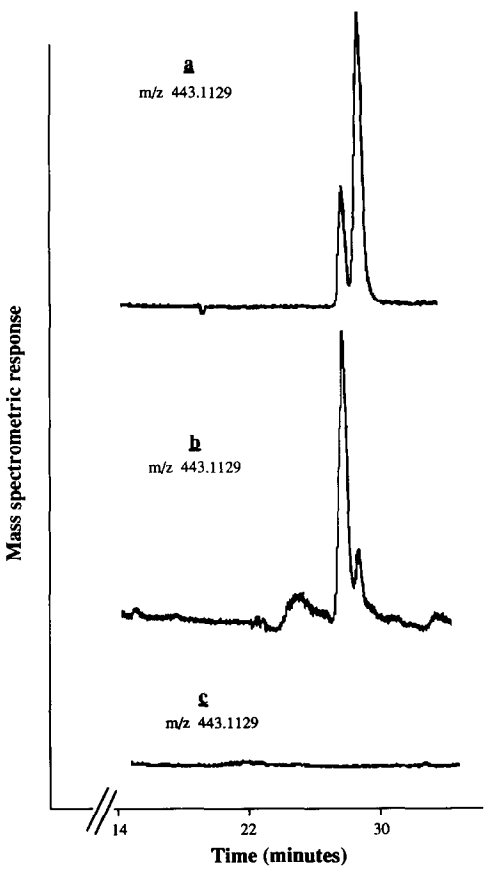

Fig. 4. HPLC–MS single-ion recordings (resolution 10 000) of m/z 443.1129, which is diagnostic for BP-glucuronides. (a) Reference BP 1-glucuronide and BP 3-glucuronide; (b) 200 μl of medium from BP-treated Hepa-1c1c7 cells; (c) 200 μl of medium from Hepa-1c1c7 cells that were not treated with BP.

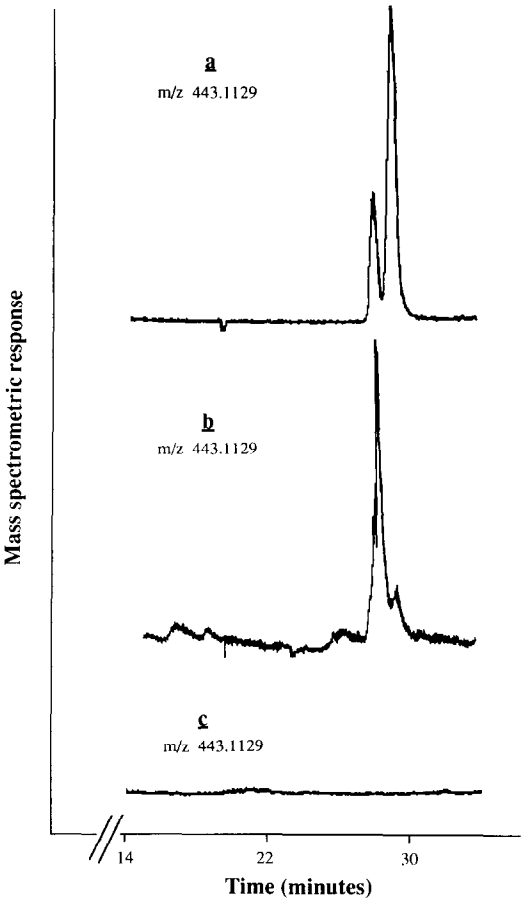

Fig. 5. HPLC–MS single-ion recordings (resolution 10 000) of m/z 443.1129, which is diagnostic for BP glucuronides. (a) Reference BP 1-glucuronide and BP-3-glucuronide; (b) 200 μl of medium from BP-treated hamster embryo cells; (c) 200 μl of medium from hamster embryo cells that were not treated with BP.

9-sulfate isomers are formed by this cell line. Although the increased widths of the peaks from medium (Fig. 3b and d) suggest the presence of more than one isomer, BP 3-sulfate and BP 9-sulfate were not separated by the current chromatographic methods.

Aliquots of media extracts were injected into the microbore HPLC system with a 20-μl external loop. The rather large injection volume used for these analyses, 20 μl, did not cause any loss in chromatographic efficiency because of the gradient elution conditions used. The results presented in Fig. 3b and c were obtained by combining extracts from two 400-μl aliquots of media, dissolving the dried extracts in 40 μl, and injecting 20 μl. Hence these results indicate the response from 400 μl of media. Results presented in Fig. 3d and e indicate the HPLC–MS response for only 40 μl of media. Previous analysis of the same extract by direct-injection continuous-flow FAB–MS [16] indicated that the media contained 22.6 ng/ml BP sulfate. The BP sulfate detection limit for analysis of cell media under these conditions is illustrated in Fig. 3d. Injection of a 40-μl extract of cell media containing 0.9 ng of BP sulfate gave a peak with a signal-to-noise ratio of 6. Similar results were obtained by monitoring the fragment ion at m/z 267.0810.

The HPLC–MS system illustrated in Fig. 1 has also been used to screen media from different cell lines treated with BP for the presence of BP glucuronides. Analysis of 200 μl of medium from BP-treated Hepa-1c1c7 cell cultures by monitoring the glucuronide molecular ion ($[M - H]^-$, m/z 443.1129) gave the chromatograms in Fig. 4. The chromatogram for medium from a cell culture treated with BP, Fig. 4b, has two peaks with retention times the same as those of reference BP 1-glucuronide and BP 3-glucuronide (Fig. 4a), suggesting that these conjugates are formed by Hepa-1c1c7 cells. However, since Schaefer and Selkirk [13] found that this cell line actually produces BP 3-glucuronide and BP 9-glucuronide, it is likely that BP 9-glucuronide is not completely separated from BP 1-glucuronide and BP 3-glucuronide. Analysis of medium from a Hepa-1c1c7 cell culture that had not been treated with BP gave no response at m/z 443.1129 (Fig. 4c), indicating that the signal for the BP-treated cells is due to a BP metabolite. Based on previous analysis of the same cell media [17], the signal in Fig. 4b corresponds to injection of 50 ng of BP glucuronide.

Similar results (Fig. 5) were obtained for the Syrian hamster embryo cell cultures, which are used widely for studies of carcinogen metabolism and transformation because of their high hydrocarbon metabolizing capacity. Analysis of medium from hamster embryo cell cultures demonstrated the presence of two glucuronides (Fig. 5b). Based on previous studies, it appears that the first peak consists of BP 3-glucuronide and BP 9-glucuronide. These isomers have been reported to be the major glucuronide conjugates in these cells based on radioisotope and enzymatic cleavage of glucuronides [18]. The presence of the second peak (Fig. 5b) suggests that an additional conjugate, such as BP 1-glucuronide, is formed by these cells. Previous studies based on enzymatic hydrolysis of the glucuronides may have failed to resolve these isomeric phenols, or some of the BP glucuronide isomers may have been resistant to hydrolysis by β-glucuronidase. The chromatogram in Fig. 5b corresponds to injection of 18 ng of BP glucuronide.

The directly coupled HPLC–MS method has several advantages over the off-line methods [16,17] described previously. For example, collecting and drying HPLC fractions in off-line measurements is time-consuming and often results in sample loss, which may occur by sample sticking to the collection tube. In addition, since the exact elution time of the analyte is usually unknown, the entire analyte can be reliably retrieved only if the effluent is collected over an elution time that is somewhat greater than one peak width. This practice, which is prudent for most off-line methods, effectively decreases the chromatographic resolution. As a result, closely eluting analytes, such as BP glucuronide isomers, would be difficult to distinguish by the off-line method. It follows that the directly coupled HPLC–MS method is also useful for detecting metabolites with a particular ion in common but having different retention times. The high sensitivity and selectivity of the directly coupled HPLC–MS approach may also facilitate investigations of hydrocarbon metabolism in which cell cultures are exposed to relatively low levels of hydrocarbons.

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