Journal of Chromatography B, 879 (2011) 1476–1484

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

In-source formation of *N*-acetyl-*p*-benzoquinone imine (NAPQI), the putatively toxic acetaminophen (paracetamol) metabolite, after derivatization with pentafluorobenzyl bromide and GC–ECNICI-MS analysis☆

Dimitrios Tsikas*, Arne Trettin, Alexander A. Zörner, Frank-Mathias Gutzki

Institute of Clinical Pharmacology, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany

ARTICLE INFO

Article history:
Received 15 July 2010
Accepted 20 January 2011
Available online 4 February 2011

Keywords:
Cytochrome P450
Derivatization
Electron-capture negative-ion chemical ionization
Electrospray ionization
Pentafluorobenzyl bromide

ABSTRACT

Pentafluorobenzyl (PFB) bromide (PFB-Br) is a versatile derivatization reagent for numerous classes of compounds. Under electron-capture negative-ion chemical ionization (ECNICI) conditions PFB derivatives of acidic compounds readily and abundantly ionize to produce intense anions due to $[M-PFB]^-$. In the present article we investigated the PFB-Br derivatization of unlabelled acetaminophen (*N*-acetyl-*p*-aminophenol, NAPAP-d_0; paracetamol; MW 151) and tetradeuterated acetaminophen (NAPAP-d_4; MW 155) in anhydrous acetonitrile and their GC–ECNICI-MS behavior using methane as the buffer gas. In addition to the expected anions $[M-PFB]^-$ at *m/z* 150 from NAPAP-d_0 and *m/z* 154 from NAPAP-d_4, we observed highly reproducibly almost equally intense anions at *m/z* 149 and *m/z* 153, respectively. Selected ion monitoring of these ions is suitable for specific and sensitive quantification of acetaminophen in human plasma and urine. Detailed investigations suggest in-source formation of *N*-acetyl-*p*-benzoquinone imine (NAPQI; MW 149), the putatively toxic acetaminophen metabolite, from the PFB ether derivative of NAPAP. GC–ECNICI-MS of non-derivatized NAPAP did not produce NAPQI. The peak area ratio of *m/z* 149 to *m/z* 150 and of *m/z* 153 to *m/z* 154 decreased with increasing ion-source temperature in the range 100–250 °C. Most likely, NAPQI formed in the ion-source captures secondary electrons to become negatively charged (i.e., $[NAPQI]^-$) and thus detectable. Formation of NAPQI was not observed under electron ionization (EI) conditions, i.e., by GC–EI-MS, from derivatized and non-derivatized NAPAP. NAPQI was not detectable in flow injection analysis LC–MS of native NAPAP in positive electrospray ionization (ESI) mode, whereas in negative ESI mode low extent NAPQI formation was observed (<5%). Our results suggest that oxidation of drug derivatives in the ion-sources of mass spectrometers may form intermediates that are produced from activated drugs in enzyme-catalyzed reactions.

1. Introduction

Acetaminophen (paracetamol, *N*-(4-hydroxyphenyl)acetamide, *N*-acetyl-*p*-aminophenol, NAPAP; $HO–C_6H_4–NH–COCH_3$, MW 151) belongs to the most frequently used drugs worldwide. Acetaminophen is rapidly and extensively metabolized to its glucuronic and sulfuric acids which are excreted in the urine [1,2] (Fig. 1). Oxidation of acetaminophen by various isoforms of the phase I enzyme system cytochrome P450 (CYP) leads to formation of the highly reactive *N*-(4-oxo-2,5-cyclohexadien-1-ylidene)acetamide which is better known as *N*-acetyl-*p*-benzoquinone imine, NAPQI ($O=C_6H_4=N–COCH_3$, MW 149) [3] (Fig. 1). Enzymatic conjugation of NAPQI with the tripeptide glutathione (GSH) is the first step in the mercapturic acid pathway which finally leads to formation of the *N*-acetylcysteine derivative, the mercapturate of NAPQI, which is subsequently excreted in the urine (Fig. 1). At high doses acetaminophen is hepatotoxic and may lead to coma and death. NAPQI is regarded the ultimate toxic metabolite of acetaminophen. Interestingly, chemical and electrochemical oxidation of acetaminophen in the laboratory may also lead to NAPQI formation [4–8], and, in the presence of GSH, *in situ* generated NAPQI may be converted non-enzymatically to its GSH conjugate [8]. Formally, NAPQI may be produced from acetaminophen by subtracting each of one H atom from the hydroxylic and the amide groups and release of two electrons (R1).

$$\mathbf{H}O–C_6H_4–N\mathbf{H}–COCH_3 \rightarrow O=C_6H_4=N–COCH_3 + 2\mathbf{H}^+ + 2e^- \quad (R1)$$

☆ This paper is part of the special issue "Enhancement of Analysis by Analytical Derivatization", Jack Rosenfeld (Guest Editor).

* Corresponding author. Tel.: +49 511 532 3984; fax: +49 511 532 2750.
E-mail address: tsikas.dimitros@mh-hannover.de (D. Tsikas).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.01.025

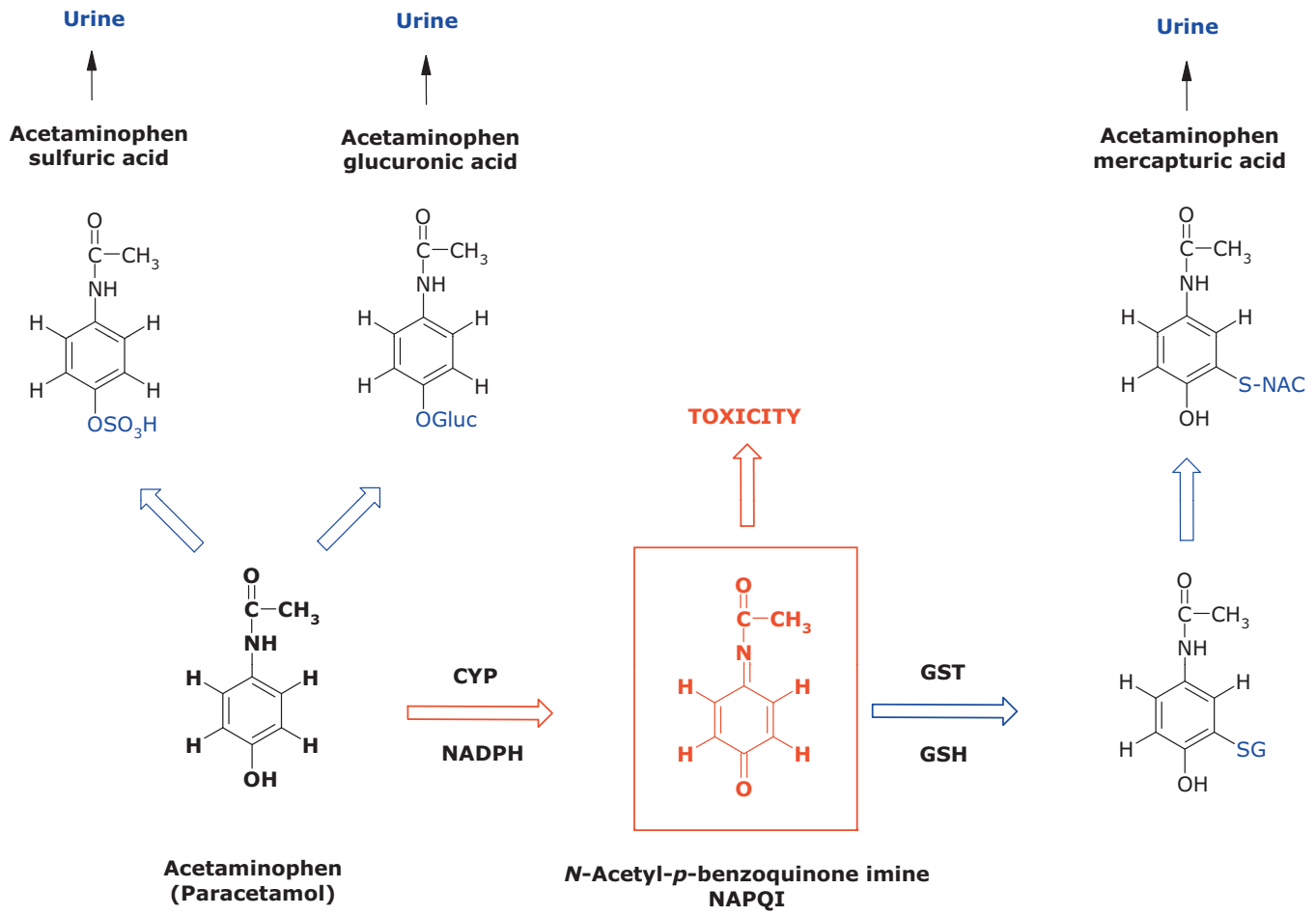

Fig. 1. Schematic of acetaminophen (paracetamol) major metabolic pathways. Enzymatic conjugation of the phenolic hydroxyl group of acetaminophen with sulfate and glucuronic acid leads to formation of the sulfuric and glucuronic acids, respectively, which are excreted in the urine. These conjugation reactions are mainly responsible for the elimination of acetaminophen. CYP-catalyzed oxidation of acetaminophen yields *N*-acetyl-*p*-benzoquinone imine (NAPQI), a highly reactive hepatotoxic and nephrotoxic intermediate. NAPQI is inactivated by chemical and enzyme-catalyzed conjugation with GSH. Gluc, glucuronic acid; CYP, cytochrome P450; GSH, glutathione; GST, GSH *S*-transferase; NAC, *N*-acetyl-cysteine.

In previous work [9–11], we found that base-catalyzed derivatization of acidic compounds with pentafluorobenzyl bromide (PFB-Br) to their pentafluorobenzyl (PFB) derivatives allows for highly sensitive analysis by GC–MS or GC–MS/MS in the electron-capture negative-ion chemical ionization (ECNICI) mode due to formation of highly intense anions $[M-PFB]^-$. In the present study we investigated the base-catalyzed derivatization of unlabelled acetaminophen (NAPAP-d_0) and the commercially available deuterium-labelled acetaminophen (NAPAP-d_4) in anhydrous acetonitrile using PFB-Br and the base *N*,*N*-diisopropylethylamine as the catalyst (Fig. 2). We found that this derivatization procedure generates exclusively the PFB ether of acetaminophen, i.e., PFB–O–C_6H_4–NH–$COCH_3$ (MW 331) and PFB–O–C_6D_4–NH–$COCH_3$ (MW 335), respectively. Expectedly, ECNICI produced abundant anions at *m*/*z* 153 and *m*/*z* 154 due to $[M-PFB]^-$ which are characteristic for PFB derivatives of acidic compounds [9]. In addition, we observed almost equally intense ions at *m*/*z* 149 and *m*/*z* 153, respectively, suggesting an additional rather unusual ionization process. This prompted us to identify the structure of these ions and the underlying mechanisms and perform a series of experiments for this purpose. These investigations included GC–MS analyses in the ECNICI mode (GC–ECNICI-MS) and in the EI mode (GC–EI-MS), GC–ECNICI-MS/MS analyses, as well as flow injection analyses (FIA) LC–MS of native, i.e., non-derivatized NAPAP-d_0 and NAPAP-d_4, in the negative and positive electrospray ionization (ESI) mode. Our study suggests that in the ion-sources of the quadrupole mass spectrometers used in the present study, ECNICI of the PFB ethers of NAPAP-d_0 and NAPAP-d_4 produces electrically uncharged NAPQI, i.e., NAPQI-d_0 and NAPQI-d_4. To the best of our knowledge this is the first report on the formation of NAPQI in GC–MS instruments.

2. Experimental

2.1. Chemicals and materials

Tetradeuterated acetaminophen {*N*-(4-hydroxyphenyl-[2,3,5,6-$^2H_4$])acetamide, NAPAP-d_4, CAS No 64315-36-2}, chemical purity declared as 95%, isotopic purity declared as 99.4% at ^2H, was from CDN Isotopes (Quebec, Canada). Unlabelled acetaminophen (NAPAP-d_0, 99% chemical purity, CAS No 103-90-2), 2,3,4,5,6-pentafluorobenzyl bromide and *N*,*N*-diisopropylethylamine were obtained from Sigma–Aldrich (Steinheim, Germany). 3-Nitro-acetaminophen was prepared from acetaminophen and nitrate by using concentrated sulfuric acid as described elsewhere for 3-nitro-tyrosine [12]. Toluene was purchased from Baker (Deventer, The Netherlands). All organic solvents were purchased from Mallinckrodt Baker (Griesheim, Germany).

2.2. Derivatization procedure for acetaminophen with PFB-Br

For the derivatization of acetaminophen with PFB-Br we chose a procedure that is commonly used for the preparation of PFB

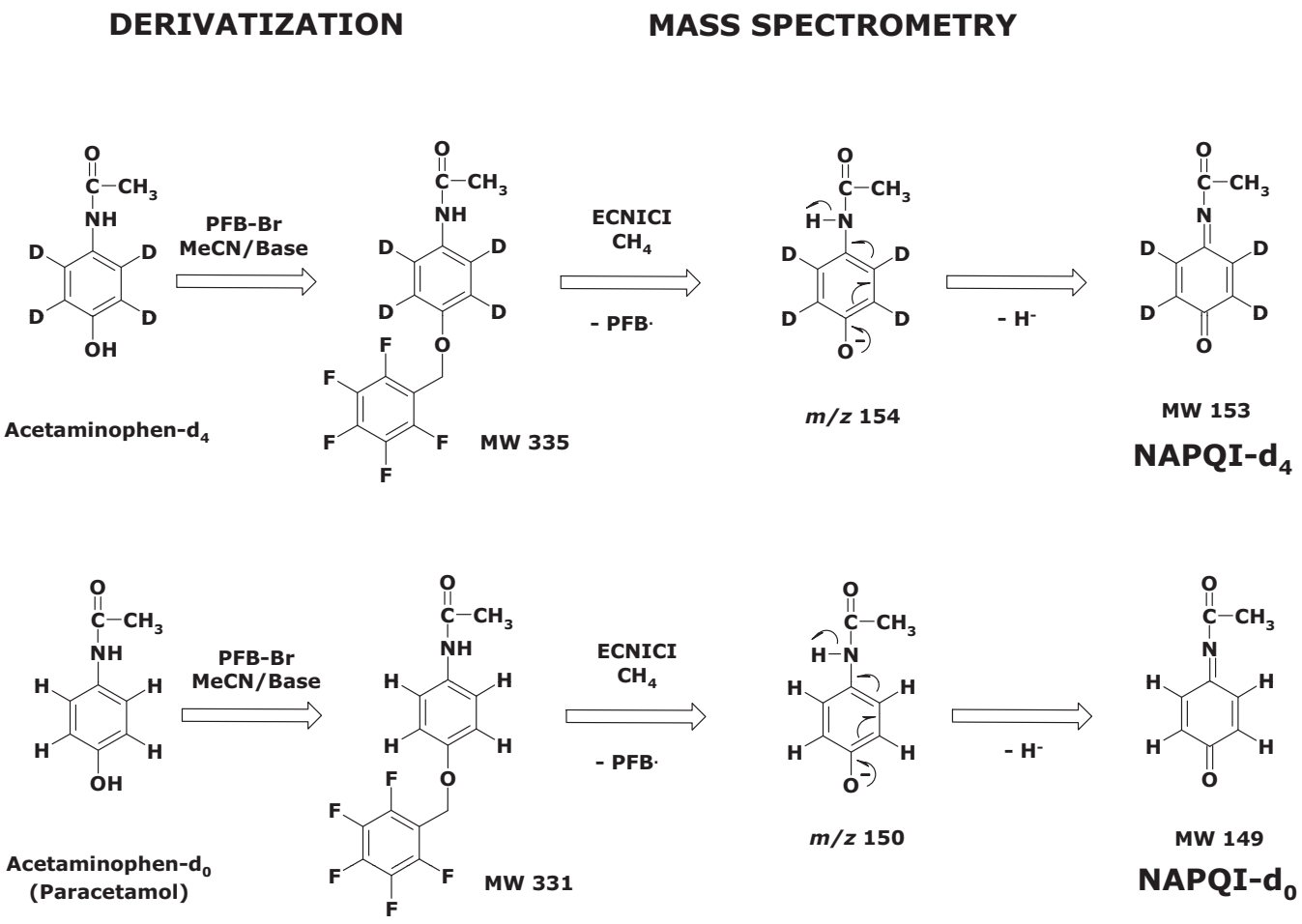

Fig. 2. Base-catalyzed derivatization of unlabelled (lower panel) and tetradeuterated (upper panel) acetaminophen with PFB-Br in MeCN to form the PFB ether derivative, and its ECNICI (CH_4) to produce unlabelled (lower panel) and tetradeuterated (upper panel) NAPQI. PFB-Br, pentafluorobenzyl bromide; MeCN, acetonitrile; PFB, pentafluorobenzyl; ECNICI, electron-capture negative-ion chemical ionization.

esters of fatty acids [9] (Fig. 2, left panel). Stock solutions of NAPAP-d_0 and NAPAP-d_4 were prepared in anhydrous acetonitrile and stored at 4 °C. For derivatization, aliquots of these and other acetaminophen-containing solutions were transferred to glass vials and the solvent was evaporated to dryness under a gentle stream of nitrogen gas. Subsequently, 100 μL anhydrous acetonitrile, 10 μL *N*,*N*-diisopropylethylamine serving as the catalyst and 10 μL of a 30 vol.% solution of PFB-Br in anhydrous acetonitrile were added. After incubation of the reaction mixtures at 30 °C for 1 h solvents and reagents were evaporated to dryness under a stream of nitrogen gas, and the residues were taken up in 1000 μL of toluene.

2.3. GC–MS and GC–MS/MS conditions

GC–MS analyses were performed on a ThermoElectron DSQ quadrupole mass spectrometer connected directly to a ThermoElectron Focus gas chromatograph and to an autosampler AS 3000 (ThermoElectron, Dreieich, Germany). A fused-silica capillary column Optima delta-6 (30 m × 0.25 mm i.d., 0.25-μm film thickness) from Macherey-Nagel (Düren, Germany) was used. Aliquots (1 μL) of the toluene solutions were injected in the splitless mode. The following oven temperature program was used with helium as the carrier gas at a constant flow rate of 1.2 mL/min: 1 min at 90 °C, then increased to 330 °C at a rate of 15 °C/min; the oven temperature of 330 °C was held for 1 min. Interface and injector were kept at 300 °C and 280 °C, respectively. The ion-source temperature was 250 °C in most analyses or varied between 250 °C and 100 °C. If not otherwise specified, electron energy and emission current were set to 70 eV and 100 μA, respectively, for EI and ECNICI with methane as the reagent gas at a flow rate of 2.4 mL/min. The electron multiplier voltage was set to 1600 V.

GC–MS/MS analyses were performed in ECNICI mode on a triple-stage quadrupole (TSQ) mass spectrometer ThermoElectron TSQ 7000 (Finnigan MAT, San Jose, CA) directly interfaced with a Trace 2000 series gas chromatograph equipped with an autosampler AS 2000 (CE Instruments Austin, TX). Chromatographic separation was carried out on an Optima-5-HT fused silica column (15 m × 0.25 mm i.d., 0.1 μm film thickness) from Macherey-Nagel (Düren, Germany). The following oven temperature program was used: 1 min at 90 °C, then increased to 330 °C at a rate of 15 °C/min, and held for 1 min at 330 °C. Interface and ion-source were kept at 320 °C and 180 °C, respectively. Electron energy and emission current were set to 70 eV and 300 μA, respectively. Methane (530 Pa) was used as the reagent gas and argon was the collision gas (0.2 Pa pressure in the collision chamber). Collision energy was set to 15 eV and electron multiplier voltage was 2000 V. Aliquots (1 μL) were injected in the splitless mode by using a BEST PTV injector, at an injector temperature of 280 °C. Mass spectra were generated using a rate of 1 s per scan.

2.4. LC–MS and LC–MS/MS conditions

FIA-LC–MS and FIA-LC–MS/MS of non-derivatized NAPAP-d_0 and NAPAP-d_4 were performed on a XEVO TQ MS instrument from

Waters (Eschborn, Germany) under positive ESI and negative ESI conditions. Mass spectra were generated using dilutions of NAPAP-d_0 and NAPAP-d_4 (each 1 μM) in water–methanol (30:70, v/v) which were infused with a flow rate of 10 μL/min into a constantly flowing (0.2 mL/min) mobile phase consisting of water–methanol (30:70, v/v) and containing 1 mM ammonium acetate. The source temperature and the capillary voltage were set to 150 °C and 3.5 kV, respectively; the desolvation gas (nitrogen) flow rate was 800 L/h at 600 °C. MS and MS/MS spectra were generated by scanning the mass range from *m/z* 20 to *m/z* 250 within 0.5 s during a total recording time of 3 s. In FIA-LC–MS/MS, argon was used as the collision gas at a flow rate of 0.15 mL/min, and the total recording time was increased to 30 s.

3. Results

3.1. Derivatization of acetaminophen with PFB-Br and GC–MS and GC–MS/MS analyses

Under ECNICI conditions, PFB esters of carboxylic acids readily ionize to form carboxylate anions $[M-PFB]^-$ whereby losing their PFB moiety (181 Da) as a radical [9]. Because of the acidity of the phenolic hydroxyl group, we assumed that derivatization of acetaminophen with PFB-Br would produce a PFB derivative which, under ECNICI conditions, would generate the phenolate anion $[M-PFB]^-$ but not the molecular anion $[M]^{\bullet-}$. Indeed, the mass spectra shown in Fig. 3 suggest formation of a simply *O*-PFB alkylated acetaminophen, i.e., the PFB ether acetaminophen derivative (NAPAP-PFB).

ECNICI of the PFB ether derivatives of NAPAP-d_0 (MW 331) and NAPAP-d_4 (MW 335) generated the expected anions $[M-PFB]^-$ at *m/z* 150 and *m/z* 154, respectively (Fig. 3A and B). However, in addition to these ions, we unexpectedly observed almost equally intense anions at *m/z* 149 $[M-PFB-H]^{\bullet-}$ and *m/z* 153 $[M-PFB-H]^{\bullet-}$, respectively. This observation suggests an alternative ionization mechanism or a consecutive in ion-source loss of a non-ring H atom (1 Da) from the anions $[M-PFB]^-$ *m/z* 150 and *m/z* 154, i.e., the H of the acetamide group, to produce an electrically neutral molecule. We are assuming that loss of a hydride, i.e., H^-, from $[M-PFB]^-$ allows for the formation of an electron-capturing 5-double bond conjugated system. A likely structure for these species could be *N*-acetyl-*p*-benzoquinone imine, i.e., unlabelled NAPQI (NAPQI-d_0; Fig. 3A) and deuterium-labelled NAPQI (NAPQI-d_4; Fig. 3B). In order to finally produce the negatively charged NAPQI radical anions $[NAPQI\text{-}d_0]^{\bullet-}$ and $[NAPQI\text{-}d_4]^{\bullet-}$, uncharged NAPQI-$d_0$ and NAPQI-d_4 must then capture secondary electrons which are abundantly present in the ion-source under ECNICI conditions (see Section 4).

The precursor anions *m/z* 149 and *m/z* 150 for NAPAP-d_0 and *m/z* 153 and *m/z* 154 for NAPAP-d_4 produced in ECNICI mode were subjected to collision-induced dissociation (CID). The product ion mass spectra generated are shown in Fig. 4. Interestingly, CID of $[M-PFB]^-$ at *m/z* 150 and *m/z* 154 did not generate product ions at *m/z* 149 and *m/z* 153, respectively, due to loss of one H radical, i.e., $[M-PFB-H]^{\bullet-}$. The product ion mass spectra contained paired ions; however, the intensities of the mass fragments observed from *m/z* 149 and *m/z* 153 were clearly different from those obtained by CID of *m/z* 150 and *m/z* 154. The major product ions at *m/z* 138 from *m/z* 153 and at *m/z* 134 from *m/z* 149 are most likely formed by loss of a methyl group as a radical $[M-PFB-H-CH_3]^{\bullet-}$. Subsequent loss of CO (MW 28 Da) would then lead to the product ions *m/z* 106 and *m/z* 110 $[M-PFB-H-CH_3-CO]^-$, respectively. By contrast, the major product ions at *m/z* 107 from *m/z* 150 and at *m/z* 111 from *m/z* 154 are likely to be formed by loss of the acetyl group as a radical (MW 43 Da) $[M-PFB-CH_3CO]^{\bullet-}$.

The 70 eV EI mass spectrum of non-derivatized acetaminophen contained the molecular cation *m/z* 151 $[M]^{\bullet+}$ (intensity, 45%), and the ions *m/z* 108 ($[M-CH_3CO]^+$, 100%), *m/z* 81 (20%; not assigned) and *m/z* 43 ($[CH_3CO]^{\bullet+}$, 35%). Interestingly, no cations $[M-2H]^+$ were observed due to loss of two H, which would correspond to positively charged NAPQI-d_0.

In order to investigate whether substituents on the aromatic ring of acetaminophen may influence the ionization of PFB ethers under ECNICI or EI conditions, we prepared 3-nitro-acetaminophen, derivatized it with PFB-Br as described for acetaminophen and analyzed its PFB ether mass spectrometrically. Under ECNICI conditions *m/z* 195 ($[M-PFB]^-$) was the sole anion formed. Thus, unlike the PFB ether of NAPAP, no ion due to ($[M-PFB-H]^-$) was observed from the PFB derivative of 3-nitro-acetaminophen (data not shown). EI of the PFB ether derivative of acetaminophen did not produce cations at *m/z* 149. Also, the EI mass spectrum of the PFB ether derivative of 3-nitro-acetaminophen did not contain a cation at *m/z* 149. It has been reported that under EI conditions the *N*,*O*-trimethylsilyl derivative of 3-nitro-acetaminophen did not produce a cation at *m/z* 149 [13].

3.2. FIA-MS and FIA-MS/MS analyses of native acetaminophen

The most intense ions of non-derivatized NAPAP-d_0 and NAPAP-d_4 under negative ESI conditions were due to $[M-H]^-$ at *m/z* 150 and *m/z* 154 (100% each) and due to $[M-CH_3CO]^{\bullet-}$ at *m/z* 107 and *m/z* 111 (80% each), respectively. A very weak (<5%) anion at *m/z* 149 due to $[M-2H]^{\bullet-}$ was observed. The most intense ions of non-derivatized NAPAP-d_0 and NAPAP-d_4 under positive ESI conditions were *m/z* 152 and *m/z* 156 (100% each) due to $[M+H]^+$, *m/z* 174 and *m/z* 178 (70% each) due to $[M+Na]^+$, and *m/z* 110 and *m/z* 114 (30% each) due to $[M-CH_2{=}CO]^+$, respectively. No cations $[M-2H]^+$ were observed due to loss of 2H, i.e., due to positively charged NAPQI-d_0 and NAPQI-d_4, respectively. CID of $[M-H]^-$ (Fig. 5) and $[M+H]^+$ (data not shown) did not generate product ions that would correspond to NAPQI-d_0 and NAPQI-d_4, respectively. Interestingly, the negative ESI product ion mass spectra of non-derivatized acetaminophen (Fig. 5) were virtually identical with the ECNICI product ion mass spectra generated from $[M-H]^-$ of the acetaminophen PFB ether derivative (Fig. 4B and D).

3.3. Investigation of parameters potentially influencing NAPQI formation

By using NAPAP-d_4 (at 65 μM) as internal standard we performed quantitative GC–ECNICI-MS analyses of NAPAP-d_0 added to human plasma (13–130 μM) in the SIM mode. For this, analytes were extracted from 100-μL aliquots of plasma with 300-μL aliquots of ethyl acetate. The ethyl acetate phase was decanted and the solvent was removed under nitrogen. Subsequently, derivatization with PFB-Br was performed as described in Section 2.2. The peak area ratio (PAR) of *m/z* 149 to *m/z* 150 for APAP-d_0 and the PAR of *m/z* 153 to *m/z* 154 for APAP-d_4 were highly reproducible. Thus, the PAR of *m/z* 149 to *m/z* 150 was determined to be 0.737 ± 0.011 (RSD, 1.4%), whereas the PAR of *m/z* 153 to *m/z* 154 was 0.791 ± 0.006 (RSD, 0.7%). Similar results were also obtained from analyzing urine samples spiked with NAPAP-d_4 (at 650 μM) and varying concentrations of NAPAP-d_0 (130–1300 μM) after extraction and derivatization as described above for plasma samples (data not shown).

In consideration of the constancy of the PAR of *m/z* 149 to *m/z* 150 and of *m/z* 153 to *m/z* 154 we investigated the effect of electron energy (EE, range 25–130 eV), emission current (EC, range 50–300 μA), methane pressure (flow rate, range 0.5–2.5 mL/min),

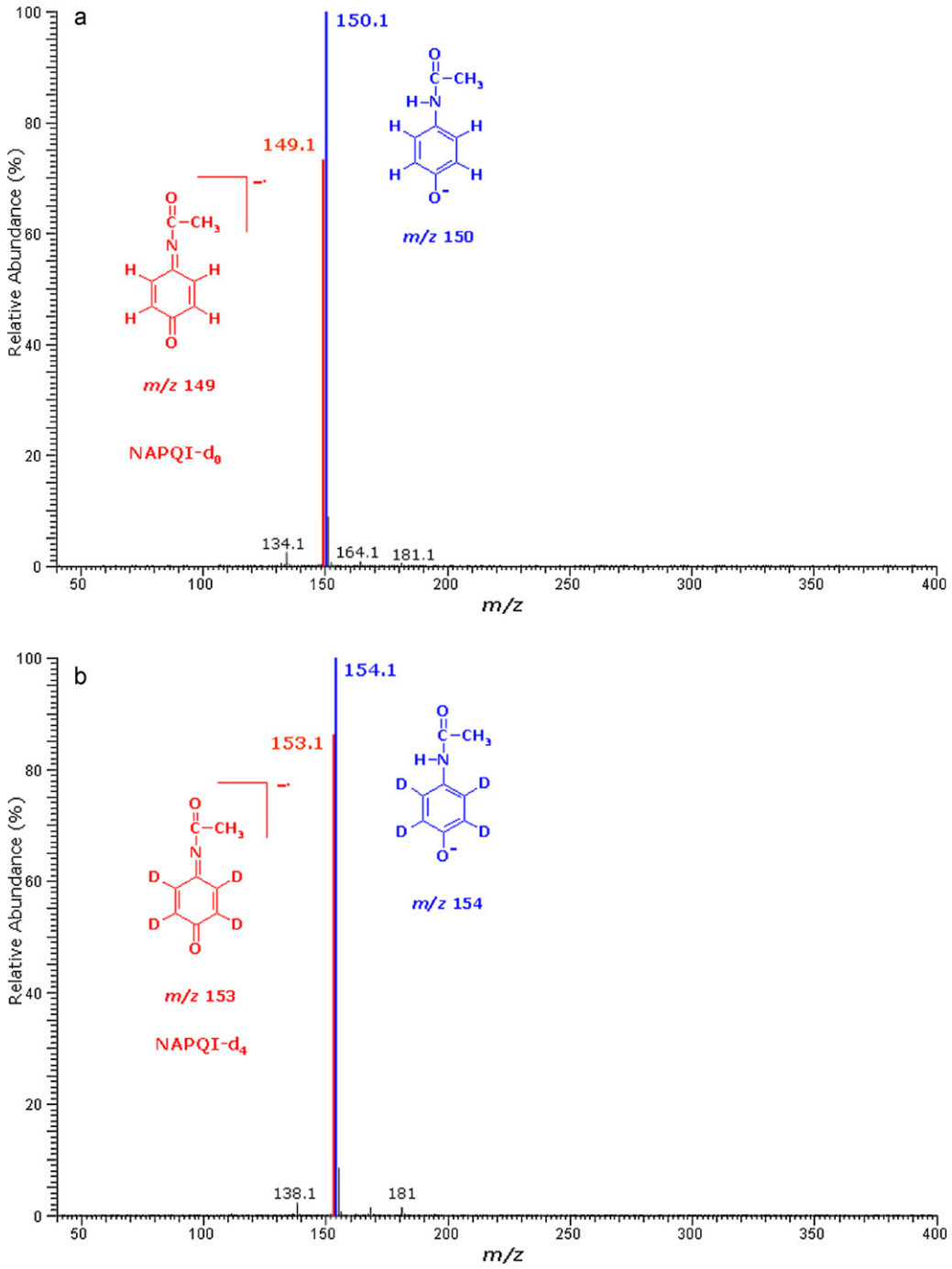

Fig. 3. GC–ECNICI-MS spectra of the PFB ether derivatives of unlabelled (A) and tetradeuterated (B) acetaminophen. The DSQ instrument was used.

and ion-source temperature (range 100–250 °C) on the peak area and the above mentioned PAR by using a mixture containing NAPAP-d_0 and NAPAP-d_4 from a spiked urine sample (500 and 650 μM, respectively). EE, EC and methane pressure had no appreciable effect on the PAR but a considerable effect on the peak area (data not shown). The greatest effect both on the PAR of *m*/*z* 149 to *m*/*z* 150 and of *m*/*z* 153 to *m*/*z* 154 (Fig. 6), and on the peak area (data not shown) exerted the ion-source temperature. The lower the ion-source temperature was, the higher was the PAR of *m*/*z* 149 to *m*/*z* 150 and of *m*/*z* 153 to *m*/*z* 154 (Fig. 6). Thus, decreasing the ion-source temperature from 250 °C to 100 °C increased both PAR values by a factor of about 3 in each case. It is noteworthy that the decrease in the ion-source temperature was associated with a small but continuous increase of the ratio of PAR of *m*/*z* 153 to *m*/*z* 154 to the PAR of *m*/*z* 149 to *m*/*z* 150 (Fig. 6). These findings suggest that NAPQI formation from NAPAP-PFB under ECNICI conditions is mechanistically based and that lower ion-source temperatures favor NAPQI formation.

A similar effect of the ion-source temperature on the PAR of $[M-H]^-$ to $[M]^-$ under ECNICI conditions was also observed for PFB derivatives of unlabelled and ^{15}N-labelled nitrite, i.e., PFB-$^{14}NO_2$ (i.e., *m*/*z* 226 to *m*/*z* 227) and PFB-$^{15}NO_2$ (i.e., *m*/*z* 227 to *m*/*z* 228) [14], respectively (data not shown). However, the PAR was much higher and decreased from about 12.5:1 at 100 °C ion-source temperature to only 11.3:1 at 250 °C ion-source temperature.

4. Discussion

Pentafluorobenzyl bromide (PFB-Br) is a versatile derivatization reagent for many inorganic anions and various classes of organic compounds. Derivatization with PFB-Br can be performed both in aqueous phase, for instance for inorganic anions such as nitrate within a wide pH-range [11], and in anhydrous organic solvents such as acetonitrile, commonly in the presence of a base serving as the catalyst. PFB esters of carboxylic groups-containing compounds such as prostaglandins and thromboxane allow for highly sensitive quantification by GC–ECINICI-MS/MS [9]. Due to the acidity of the hydroxyl group of acetaminophen, we assumed that derivatization of acetaminophen with PFB-Br would also produce strongly electron-capturing PFB ether derivatives, i.e., NAPAP-PFB. Analogous to the PFB esters of fatty acids, we also assumed that NAPAP-PFB would allow for highly sensitive quantitative determination of acetaminophen in biological samples by GC–ECNICI-MS and GC–ECNICI-MS/MS (Fig. 2).

As expected, reaction of acetaminophen with PFB-Br in acetonitrile produces the acetaminophen-PFB ether derivative (see reaction (R2)). Under ECNICI conditions, the reagent or buffer gas, such as methane in the present study, is ionized by primary electrons (e_P^-) emitted from the cathode to produce positively charged reagent gas molecules and secondary electrons (e_S^-) (see (R3)). Our

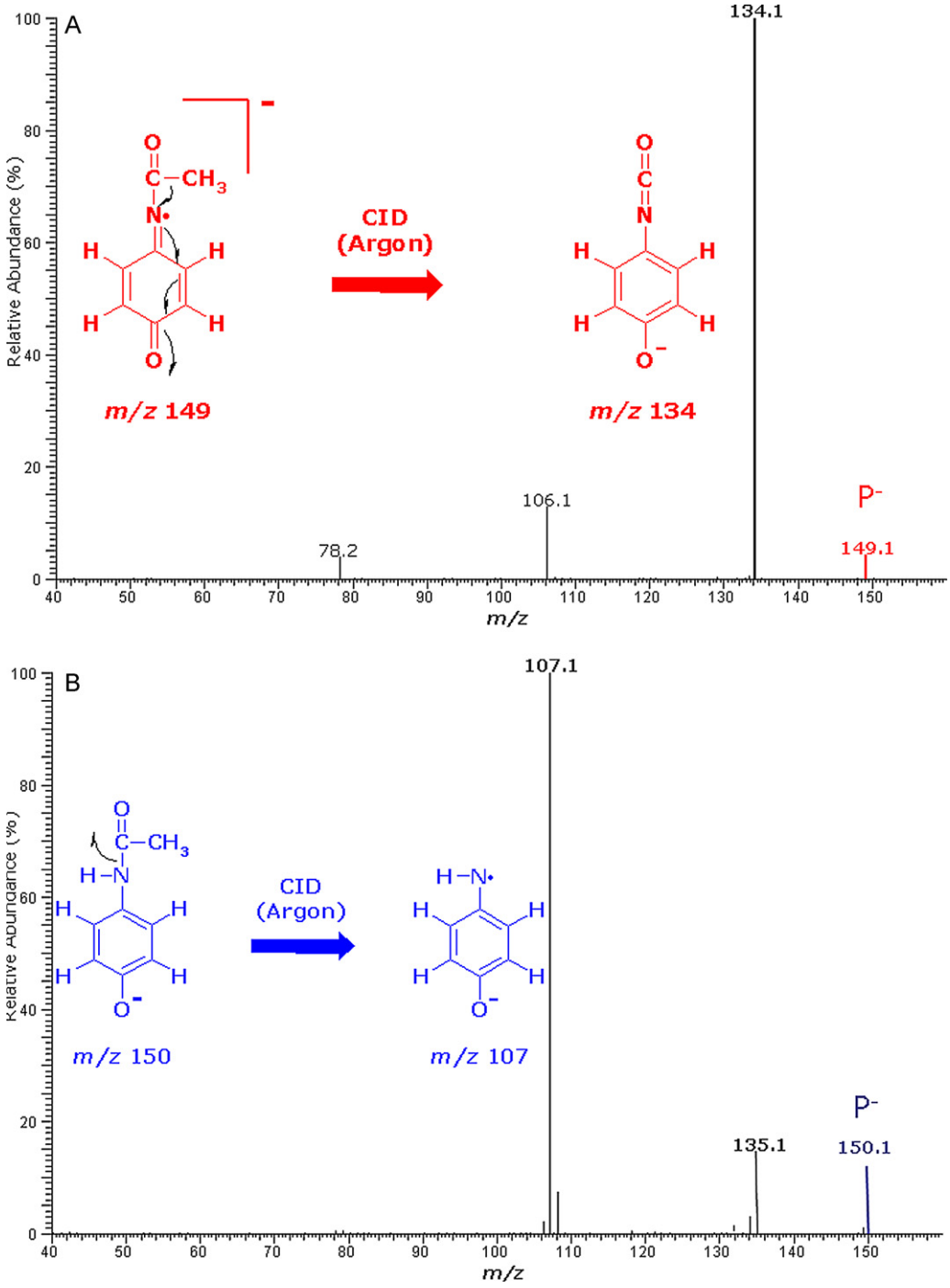

Fig. 4. Product ion mass spectra generated by CID of *m*/*z* 149 (A) and *m*/*z* 150 (B) for unlabelled acetaminophen, and of *m*/*z* 153 (C) and *m*/*z* 154 (D) for deuterium-labelled acetaminophen. The TSQ instrument was used. P^-, precursor ion subjected to CID; CID, collision-induced dissociation.

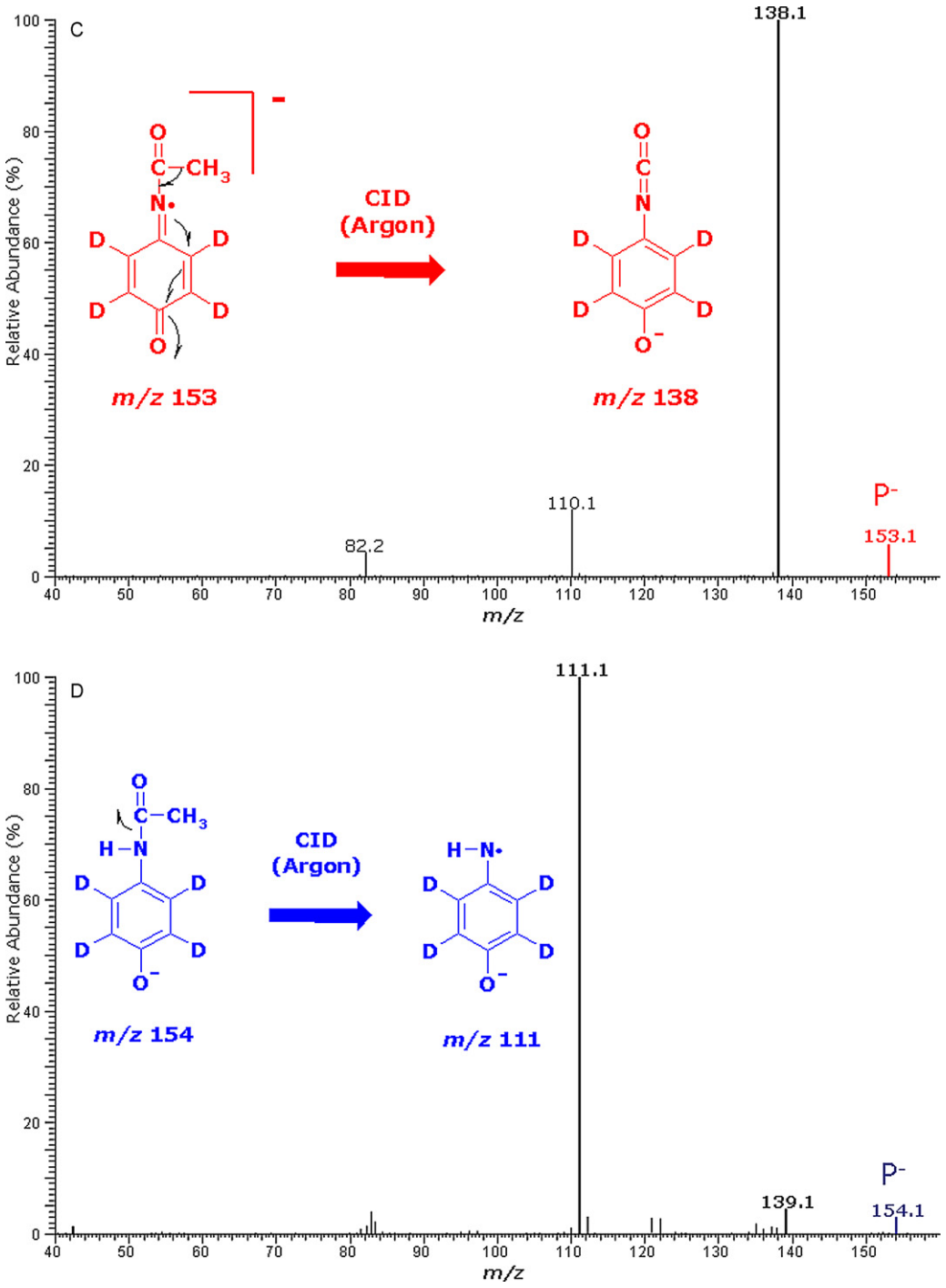

Fig. 4. (*Continued*).

study shows that the PFB ether of acetaminophen ionizes under ECNICI conditions to form an intense negatively charged ion due to $[M-PFB]^-$ (R5) after capture of one secondary electron (R4), in complete analogy to the PFB esters of carboxylic acids which produce intense carboxylate anions [9]. Thus, high-abundance formation of $[M-PFB]^-$ should allow for highly sensitive quantification of acetaminophen in biological samples analogous to PFB esters of fatty acids [9].

$$CH_3CO\text{–}NH\text{–}C_6H_4\text{–}OH + \text{PFB-Br} \rightarrow CH_3CO\text{–}NH\text{–}C_6H_4\text{–}O\text{–}PFB + HBr \quad (R2)$$

$$CH_4 + e_P^- \rightarrow CH_4^{\bullet +} + 2e_S^- \quad (R3)$$

$$CH_3CO\text{–}NH\text{–}C_6H_4\text{–}O\text{–}PFB + e_S^- \rightarrow [CH_3CO\text{–}NH\text{–}C_6H_4\text{–}O\text{–}PFB]^{\bullet -} \quad (R4)$$

$$[CH_3CO\text{–}NH\text{–}C_6H_4\text{–}O\text{–}PFB]^{\bullet -} \rightarrow [CH_3CO\text{–}NH\text{–}C_6H_4\text{–}O]^- + PFB^{\bullet} \quad (R5)$$

In addition to this expected rather regular ionization, our study strongly suggests that under ECNICI conditions a large part of the originally formed phenolate anion of acetaminophen (i.e.,

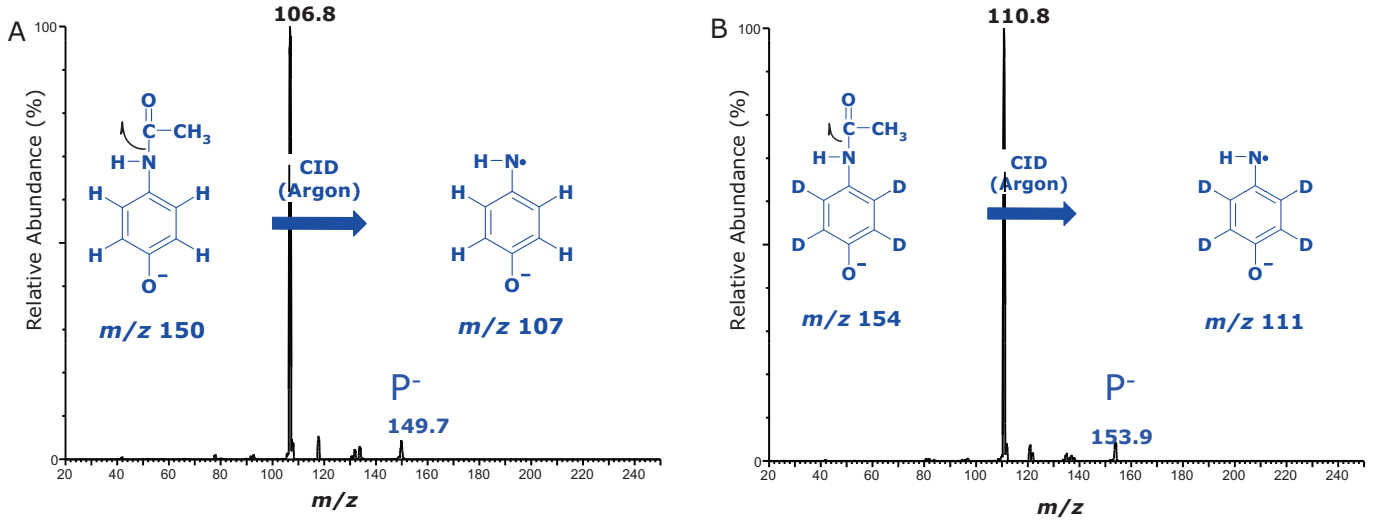

Fig. 5. Negative ion electrospray ionization (ESI) product ion mass spectra generated by CID of *m/z* 150 (A) for unlabelled acetaminophen and of *m/z* 154 (B) for deuterium-labelled acetaminophen (both non-derivatized). The XEVO TQ instrument was used. P^-, precursor ion subjected to CID; CID, collision-induced dissociation.

$[CH_3CO–NH–C_6H_4–O]^-$) undergoes further reactions in the ion-source. It is likely that this process involves loss of the sole H atom of the acetamide group as a hydride to form uncharged NAPQI (i.e., $CH_3CO–N{=}C_6H_4{=}O$) (see (R6)), the intermediate toxic metabolite of acetaminophen formed by CYP-catalyzed oxidation of acetaminophen [1–7]. The lower the ion-source temperature was, the more NAPQI was formed. Thus, at 250 °C the NAPQI:NAPAP molar ratio was 0.74:1, whereas this ratio was 2.1:1 at the ion-source temperature of 100 °C. Interesting was the finding that this ratio was constantly higher for NAPQI-d_4:NAPAP-d_4 compared to NAPQI-d_0:NAPAP-d_0, suggesting an isotope effect. We assume that under ECNICI conditions, NAPQI formed in the ion-source captures a secondary low-energy electron to produce negatively charged radical NAPQI, i.e., $[CH_3CO–N{=}C_6H_4{=}O]^{\bullet -}$ (see (R7)), which is then separated by the mass analyzer and finally detected. The driving force for the in-source oxidation of acetaminophen phenolate to NAPQI could be the formation of a highly conjugated system comprising 5 double bonds, which is moreover strongly electron-capturing (Fig. 2, right panel). This mechanism is supported by literature data showing that many compounds rich in double bonds, including many aromatic compounds, readily ionize to form molecular anions $[M]^{\bullet -}$ under ECNICI conditions [15–19].

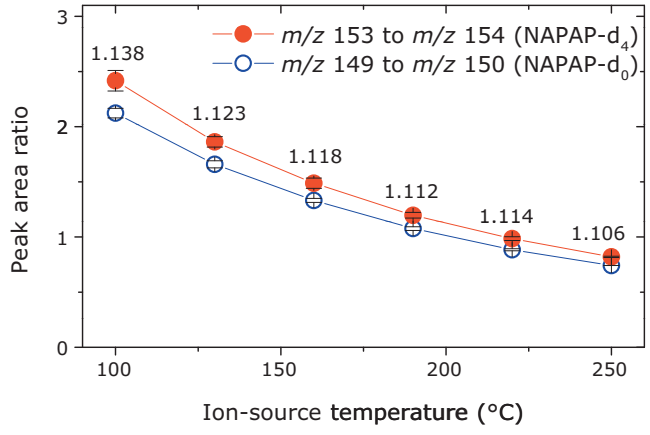

Fig. 6. Relationship between PAR of *m/z* 149 and *m/z* 150 or of *m/z* 153 and *m/z* 154 and ion-source temperature. EE, EC and methane were kept constant at 70 eV, 100 μA and 2.5 mL/min, respectively. SIM of *m/z* 149 and *m/z* 150 for unlabelled acetaminophen (NAPAP-d_0) and of *m/z* 153 and *m/z* 154 for the internal standard (NAPAP-d_4) which were added to a urine sample at final added concentrations of 500 μM and 650 μM, respectively. The DSQ instrument was used.

$$[CH_3CO–NH–C_6H_4–O]^- \rightarrow CH_3CO–N{=}C_6H_4{=}O + H^- \quad \text{(R6)}$$

$$CH_3CO–N{=}C_6H_4{=}O + e_S^- \rightarrow [CH_3CO–N{=}C_6H_4{=}O]^{\bullet -} \quad \text{(R7)}$$

It is worth mentioning that NAPQI formation was not observed under EI conditions in GC–MS from non-derivatized acetaminophen or from NAPAP-PFB. Also, in FIA-LC–MS positive ESI NAPQI formation from non-derivatized NAPAP was not observed. In negative ESI mode, FIA-LC–MS of non-derivatized acetaminophen produced a very weak anion (<5%) at *m/z* 149 which could be due to $[NAPQI]^-$. That the PFB ether derivate of 3-nitro-acetaminophen did not produce $[M–PFB–H]^-$ in addition to $[M–PFB]^-$ under ECNICI conditions, suggests that the nitro group at the *meta*-position of 3-nitro-acetaminophen prevents oxidation of the phenolate of 3-nitro-acetaminophen to 3-nitro-NAPQI in the ion-source.

It seems that the conditions prevailing in ECNICI in GC–MS are similar to those occurring in aqueous alkaline solutions of phenolic compounds in the presence of transition metal ions or in electrochemistry, which finally promote their oxidation. The fragmentations observed in the present study such as loss of PFB, CH_3CO and CO radicals are usual. However, the formal abstraction of H^- from the acetaminophenolate seen in this study is rather unusual. Obviously, the anion chemistry under ECNICI conditions, notably of fluorine-containing compounds, is rather complex and associated with formation of unusual species [14,20–23].

Due to considerable advance in analytical chemistry, recently several new unusual acetaminophen metabolites, for instance NAPAP-S-S-NAPAP and NAPAP-sulfate-NAPAP, have been proposed to occur in urine of rats given high doses of acetaminophen (e.g., 1600 mg per kg bodyweight) [2]. This study has also revealed that acetaminophen metabolism is associated with considerable changes in many different endogenous compounds including antioxidants and energy-related metabolites [2]. These findings suggest that despite intensive investigation over the past decades the mechanisms by which toxic intermediates such as NAPQI are formed from CYP-catalyzed acetaminophen metabolism are still incompletely understood. Moreover, despite the particular importance of oxygenation reactions that involve heme groups, mechanisms of heme reactions in enzymes such as CYP450, nitric oxide synthase (NOS) and heme oxygenase (HO) are still not well-understood [24].

We hypothesize that formation of NAPQI from acetaminophen PFB ether under ECNICI conditions in the ion-source of GC–MS instruments and CYP-catalyzed formation of NAPQI from acetaminophen could proceed via similar mechanisms. The first step in the ECNICI process is the generation of secondary electrons through the ionization of the reagent gas (here CH_4; see (R3)). In the CYP-catalyzed reaction, electrons are provided by the cofactor NADPH; abstraction of the phenolic acidic H could be catalyzed by means of carboxylates from amino acid moieties, for instance of Glu302 in human CYP [25]. In step 2, H^- could be abstracted from the acetaminophenolate by means of $CH_4^{\bullet+}$ which is abundantly present in the ion-source to produce $CH_4^{\bullet-}$ (R8), which then releases one electron and one uncharged methane molecule (see (R9)). In the CYP-catalyzed reaction, H^- abstraction most likely proceeds via the ferryl group of CYP, i.e., CYP-Fe^{4+}=O (see (R10)). Reaction (R10) is supported by recent findings indicating that heme-catalyzed oxidations require reduction of CYP–Fe^{4+}=O by H atoms [24]. It is worth mentioning that the necessity of a H source for the formation of Compound I has been often ignored in the past [24]. In case of NAPAP oxidation by CYP450 the source of the required H atom is likely to be the amide group.

$$CH_4^{\bullet+} + H^- \rightarrow CH_4^{\bullet-} + H^+ \quad \text{(R8)}$$

$$CH_4^{\bullet-} \rightarrow CH_4 + e_S^- \quad \text{(R9)}$$

$$\text{CYP–Fe}^{4+}\text{=O} + H^- \rightarrow \text{CYP–Fe}^{2+} + OH^- \quad \text{(R10)}$$

Our study on acetaminophen and previous studies on other drugs such as nefazodone [26] suggest that primarily formed drug metabolites may further degrade/hydrolyze to highly reactive and toxic compounds such as 1,4-benzoquinone imine, 1,4-benzoquinone and *p*-aminophenol [27] from acetaminophen and the hydrolysis product 2-chloro-1,4-benzoquinone from the non-tricyclic antidepressant nefazodone [26]. On the other hand, particular modification of aromatic rings such as nitration, for instance in the case of the PFB derivatives of 3-nitro-acetaminophen (present study) and 3-nitro-phenol [28], hinders formation of species like NAPQI under ECNICI conditions and possibly in CYP-catalyzed oxidations. It may be speculated that CYP-catalyzed metabolism of drugs such as acetaminophen may lead to several chemically highly reactive and possibly toxic species which may require different detoxification measures. Our study suggests that electronic activation of acetaminophen leads to formation of NAPQI and possibly to other species such as 1,4-benzoquinone imine, *p*-aminophenol and 1,4-benzoquinone. In addition, the mechanisms proposed in the present work may predict formation of further species such as acetaldehyde and cyanic acid (HN=C=O) from acetaminophen. It would be very interesting to investigate by MS-based techniques, the formation of such acetaminophen metabolites in vitro and in vivo and whether some of the metabolic changes observed in both acute and chronic dosing of acetaminophen [2] are caused by such species.

5. Conclusions

Base-catalyzed derivatization of acetaminophen with PFB-Br in anhydrous acetonitrile yields the PFB ether derivative of acetaminophen. In addition to the expected ion at *m*/*z* 150 due to $[M-PFB]^-$, we obtained highly reproducibly an intense ion at *m*/*z* 149 due to $[M-PFB-H]^-$ which is most likely the negatively charged NAPQI. The ion *m*/*z* 149 is formed in the ion-source but not in the collision chamber of the GC–MS/MS instrument. ECNICI-induced and CYP-catalyzed formation of NAPQI from acetaminophen may take place through the same mechanism and involve hydride subtraction from the acetamide group. It is proposed that the product ions observed from $[NAPQI]^{\bullet-}$ under ECNICI conditions are also formed within cells and add to the acetaminophen toxicity. The *O*-pentafluorobenzylation of acetaminophen with PFB-Br is a good example of how chemical derivatization may change physicochemical properties of analytes. Derivatization of acetaminophen to its PFB ether derivative can be utilized for sensitive and specific quantification of acetaminophen by GC–ECNICI-MS in minimum sample volume such as in blood and urine of children.

References

[1] L.P. James, P.R. Mayeux, J.A. Hinson, Drug Metab. Dispos. 31 (2003) 1499.
[2] J. Sun, L.K. Schnackenberg, R.D. Holland, T.C. Schmitt, G.H. Cantor, Y.P. Dragan, R.D. Beger, J. Chromatogr. B 871 (2008) 328.
[3] B. Testa, S.D. Krämer, Chem. Biodivers. 4 (2007) 257.
[4] I.C. Calder, M.J. Creek, P.J. Williams, J. Med. Chem. 16 (1973) 499.
[5] D.J. Miner, P.T. Kissinger, Biochem. Biopharmacol. 28 (1979) 3285.
[6] I.A. Blair, A.R. Boobis, D.S. Davies, Tetrahedron Lett. 21 (1980) 4947.
[7] D.C. Dahlin, S.D. Nelson, J. Med. Chem. 25 (1982) 885.
[8] W. Lohmann, U. Karst, Anal. Bioanal. Chem. 386 (2006) 1701.
[9] D. Tsikas, J. Chromatogr. B 717 (1998) 201.
[10] D. Tsikas, K.S. Tewes, F.M. Gutzki, E. Schwedhelm, J. Greipel, J.C. Frölich, J. Chromatogr. B 709 (1998) 79.
[11] D. Tsikas, Anal. Chem. 72 (2000) 4064.
[12] E. Schwedhelm, D. Tsikas, F.M. Gutzki, J.C. Frölich, Anal. Biochem. 276 (1999) 195.
[13] V.M. Lakshmi, F.F. Hsu, B.B. Davis, T.V. Zenser, Chem. Res. Toxicol. 13 (2000) 891.
[14] D. Tsikas, E. Schwedhelm, J.C. Frölich, J. Chromatogr. A 1067 (2005) 337.
[15] M. Grifoll, A.M. Solanas, J.M. Bayona, Contam. Toxicol. 19 (1990) 175.
[16] E. van der Heeft, A.P.J.M. de Jong, L.A. van Ginkel, H.J. van Rossum, G. Zomer, Biol. Mass Spectrom. 20 (1991) 763.
[17] F.M. Gutzki, D. Tsikas, U. Adelheid, J.C. Frölich, Biol. Mass Spectrom. 21 (1992) 97.
[18] J. Shen, X. Xia, H. Jiang, C. Li, J. Li, X. Li, S. Ding, J. Chromatogr. B 877 (2009) 1523.
[19] M. Bader, W. Rosenberger, F.M. Gutzki, D. Tsikas, J. Chromatogr. B 877 (2009) 1402.
[20] J.H.J. Dawson, N.M.M. Nibbering, Int. J. Mass Spectrom. Ion Phys. 33 (1980) 3–19.
[21] S. Ingemann, N.M.M. Nibbering, S.A. Sullivan, C.H. DePuy, J. Am. Chem. Soc. 104 (1982) 6520.
[22] H.H. Büker, N.M.M. Nibbering, D. Espinosa, F. Mongin, M. Schlosser, Tetrahedron Lett. 38 (1997) 8519.
[23] W.D. Langer, T. Velusamy, T.B. Kuiper, R. Peng, M.C. McCarthy, M.J. Travers, A. Kovacs, C.A. Gottlieb, P. Thaddeus, Astrophys. J. 480 (1997) L63.
[24] Y. Zhu, R.B. Silverman, Biochemistry 47 (2008) 2231.
[25] N.Y. Shin, Q. Liu, S.L. Stamer, D.C. Liebler, Chem. Res. Toxicol. 20 (2007) 859.
[26] A.S. Kalgutkar, A.D.N. Vaz, M.E. Lame, K.R. Henne, J. Soglia, S.X. Zhao, Y.A. Abramaov, F. Lombardo, C. Collin, Z.S. Hendsch, C.E.C.A. Hop, Drug Metab. Dispos. 33 (2005) 243.
[27] S.E. McConkey, D.M. Grant, A.E. Cribb, J. Vet. Pharmacol. Ther. 32 (2009) 585.
[28] S. Nakamura, M. Takino, S. Daihima, Analyst 126 (2001) 835.