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RESEARCH ARTICLE

DNA adducts of *ortho*-toluidine in human bladder

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Abstract

Background: 4-Aminobiphenyl (4-ABP) and *o*-toluidine are known human bladder carcinogens, but only 4-ABP-releasing DNA adducts are known.

Methods: Determination of 4-ABP and *o*-toluidine-releasing DNA adducts in epithelial and submucosal bladder tissues of sudden death victims (SDV: $n=46$), and bladder tumours ($n=12$) by gas chromatography/mass spectrometry.

Results: Above background, 4 and 11 of 12 tumour samples contained adducts of 4-ABP (0.057 ± 0.125 fmol/µg DNA) and *o*-toluidine (8.72 ± 4.49 fmol/µg DNA), respectively. Lower adduct levels were present in both epithelial and submucosal bladder tissues of SDV (4-ABP: 0.011 ± 0.022 and 0.019 ± 0.047 fmol/µg DNA; *o*-toluidine: 0.24 ± 0.63 and 0.27 ± 0.70 fmol/µg DNA).

Conclusion: Detection of *o*-toluidine-releasing DNA adducts support the carcinogenicity of *o*-toluidine in the human bladder.

Keywords: *4-aminobiphenyl; carcinogenicity; gas chromatography; mass spectrometry; smoking*

Introduction

In 2006 the Deutsche Forschungsgemeinschaft (DFG) Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area classified *o*-toluidine as a proven human bladder carcinogen (DFG 2006). Because of its genotoxic potential no dose-effect relationship can be derived and an occupational threshold limit value cannot be set. Two years later, the classification of *o*-toluidine was upgraded by the International Agency for Research on Cancer and is now listed as 'carcinogenic to humans' (Baan et al. 2008, IARC 2009). The corresponding monograph is still in preparation, but in a short summary it is stated that there is only moderate evidence of genotoxicity as the main mechanism of *o*-toluidine (Baan et al. 2009). This is in contrast to other aromatic amines such as 4-aminobiphenyl for which the evidence has been rated as strong. One of the reasons for this discrepancy could be the fact that the presence of DNA adducts of *o*-toluidine has not been reported so far in human tissues whereas DNA adducts of 4-aminobiphenyl have been found in bladder tissue and other possible target tissues (Talaska et al. 1991, Lin et al. 1994, Culp et al. 1997, Thompson et al. 2002, Airoldi et al. 2002, Martone et al. 1998, Ricicki et al. 2005, Zayas et al. 2007, Bessette et al. 2010). In rat experiments DNA adducts of *o*-toluidine have been either not detected using HPLC/MS/MS (Jones & Sabbioni 2003) or detected only in liver, but not bladder, after administration of *o*-toluidine or the local anaesthetic prilocaine using ^{32}P-postlabeling (Duan et al. 2008). After a single oral dose of 500 mg/kg ^{14}C-labeled *o*-toluidine, covalent binding of radioactivity has been detected in rat liver DNA (Brock et al. 1990). The failure to detect adducts in rat bladder in the study of Duan et al. (2008) could be due to the fact that ^{32}P-postlabeling is very inefficient for detection of DNA adducts of *o*-toluidine as well as the closely related *o*-anisidine (Mourato et al. 1999, Stiborova et al. 2002).

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The lack of evidence for DNA adducts of *o*-toluidine is somewhat unexpected considering its similarities to 4-aminobiphenyl. Both these aromatic amines undergo *N*-hydroxylation and yield hydrolysable haemoglobin adducts (Sabbioni & Richter 1999) (Figure 1). In humans, haemoglobin adducts of *o*-toluidine have been found at consistently higher concentrations compared to 4-aminobiphenyl and are less dependent on smoking (Richter & Branner 2002). Concentrations of *o*-toluidine haemoglobin adducts are reported to be exceptionally high in rubber industry workers (Ward et al. 1996) and in patients treated with prilocaine (Gaber et al. 2007). The ubiquitous occurrence of *o*-toluidine in the environment is further confirmed by its presence in urine of non-smokers without known occupational exposure (El-Bayoumy et al. 1986, Grimmer et al. 2000, Riedel et al. 2006, Kütting et al. 2009).

The purpose of the present study was to determine DNA adducts of *o*-toluidine in human bladder tissue using a combination of analytical methods for the determination of haemoglobin adducts from aromatic amines (Kutzer et al. 1997, Lewalter & Gries 2000) and DNA adducts from tobacco-specific nitrosamines releasing 4-hydroxypyridylbutanone (Hölzle et al. 2007, Heppel et al. 2009).

Material and methods

Samples of human urinary bladder

Samples of urinary bladder without visible signs of autolysis were taken from 46 sudden death victims (SDV) at routine autopsy (mean time after death 32 h, range 5 to 72 h) in the Institute of Forensic Medicine of the Ludwig-Maximilians-University, Munich, Germany. Tissue samples were separated into epithelial and submucosal layers, and stored at -20°C. Bladder tumour samples, freshly mounted on glass slides, were obtained from patients undergoing surgery at the Department of Urology, Caritas-St. Josef Medical Center, University of Regensburg, Regensburg, Germany and stored at -20°C until shipment to Munich. Samples of urine were taken from 35 of 46 SDV and 11 of 12 cancer patients for determination of acute smoking status by analyzing for the presence of cotinine. Additional information on smoking status of SDV was obtained from records of the criminal police office (n = 7) and analysis of toenails for nicotine (n = 33). The study was performed with approval from the local Ethic Committees of the Universities of Munich and Regensburg, Germany.

Reagents and standards

4-Aminobiphenyl and *o*-toluidine were purchased from Sigma-Aldrich (Deisenhofen, Germany), d_5-4-aminobiphenyl, d_3-cotinine and d_3-nicotine from TRC (Toronto, Canada), d_9-*o*-toluidine from CDN Isotopes (Point-Claire, Quebec, Canada). Solvents were of GC quality (Suprasolv, Merck, Darmstadt, Germany). Bidistilled water was prepared freshly (Destamat, Heraeus-Kendro, Langensebold, Germany). Calf thymus DNA, heptafluorobutyric acid and all other chemicals of analytical grade were purchased from Sigma-Aldrich.

DNA extraction from bladder tissue

DNA was isolated from about 100 mg tissue using the Invisorb Spin Tissue Midi kit (Invitek, Berlin, Germany) which includes lysis of proteins and RNA prior to binding of genomic DNA to spin filter columns allowing efficient removal of all impurities during washing steps (Heppel et al. 2009). Purity and concentration of DNA were determined spectrophotometrically at 230, 260 and 280 nm in a LabelGuard microliter cell coupled to a NanoPhotometer (Implen, Munich, Germany).

Hydrolysis of DNA and derivatization of aromatic amines

The DNA solution was acidified with 100 µl 4 M HCl and 1 pg each of d_5-4-ABP and d_9-*o*-toluidine added as internal standards. DNA was hydrolyzed at 80°C for 3 h, extracted twice with 500 µl CH_2Cl_2 and the organic phase discarded. The aqueous layer was adjusted to pH 7.5 with 400 µl 1 M NaOH and 100 µl 0.5 M Na_2HPO_4 buffer, extracted three times with 300 µl $CHCl_3$ and extracted aromatic amines immediately derivatized by adding 10 µl of heptafluorobutyric acid (HFBA). After 15 min at room temperature, the reaction was stopped with 50 µl of methanol and after addition of 250 µl toluene carefully reduced to dryness in a SpeedVac vacuum concentrator (Bachofer, Reutlingen, Germany). The residue was dissolved in 80 µl toluene and transferred to a 100 µl conical glass insert and again reduced to dryness under a stream of nitrogen. Samples were stored at -20°C until further analysis.

Gas chromatography-mass spectrometry

Analysis was performed by GC/MS/NCI on a Carlo Erba QMD 1000 (Fisons Instruments, Mainz-Kastel, Germany) with methane as ionization gas (Gaber et al. 2007). The samples were reconstituted with 10 µl toluene and 1 µl was injected in the splitless mode on a Factor Four capillary column VF-17 ms (30 m × 0.25 mm I.D., film thickness 0.25 µm; Varian, Darmstadt, Germany) operated with helium as carrier gas at a pressure of 80 kPa. The temperatures of the injection port and the source were set to 250°C, and the transfer

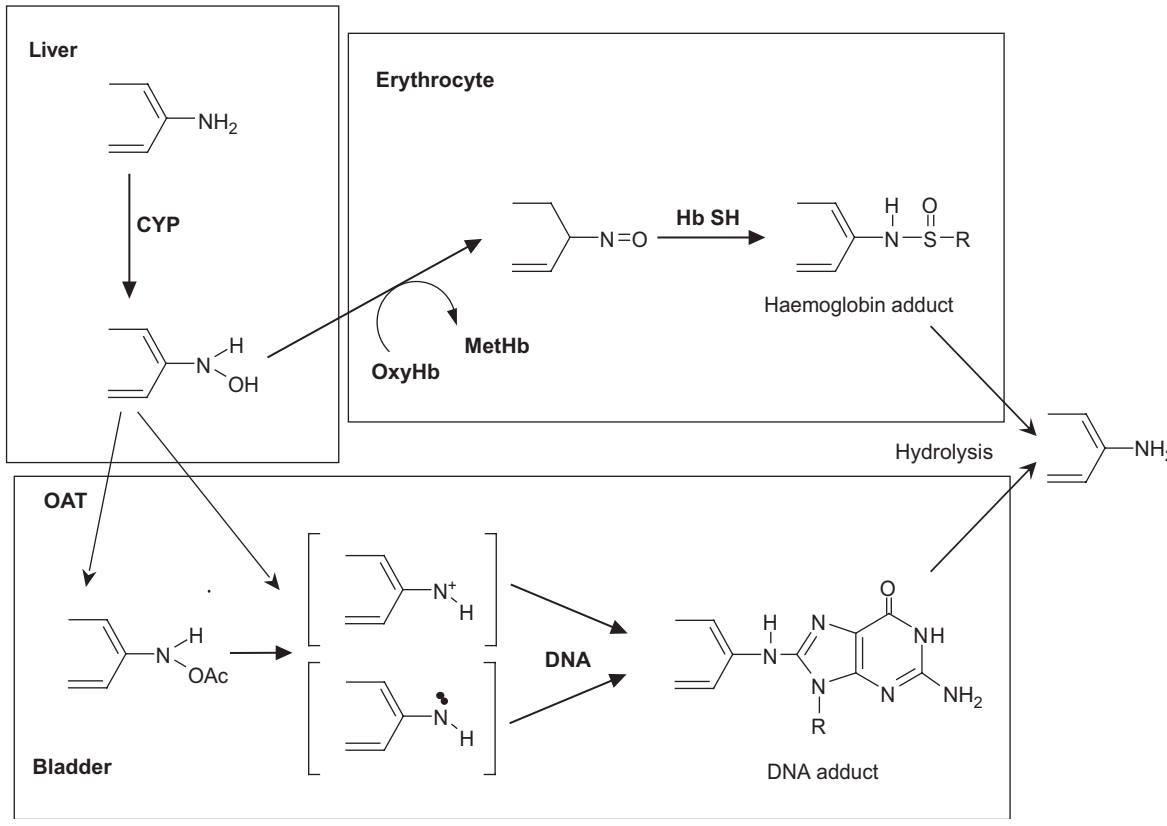

Figure 1. Postulated metabolic pathways of arylamines leading to haemoglobin and DNA adducts; modified from Sabbioni & Richter (1999).

line to 270°C. The column oven was initially maintained at 80°C for 1 min, and then programmed to 210°C at 12°C min^{-1}, to 300°C at 15°C min^{-1}, and held at 300°C for 10 min. Quantification was performed by selective-ion monitoring (SIM) at m/z 283, 290, 345 and 350 for the HFBA derivatives of *o*-toluidine, d_9-*o*-toluidine, 4–ABP and d_5-4-ABP, respectively. All samples were analyzed in duplicate. A blank sample of DNA elution buffer spiked with the internal standards was analyzed with each daily batch of 4-6 samples to control for background contamination. Internal standards did not lose deuterium during hydrolysis. As reported by Lewalter & Gries (2000) the two deuteriums of the amino group of d_9-*o*-toluidine are lost.

Determination of smoking status

Smoking status in tumour patients was determined by self-report. Smokers having stopped smoking more than 4 weeks prior to surgery were excluded from the group of active smokers. Additionally, free cotinine in urine of tumour patients was determined by GC/MS (Stepanov & Hecht 2005). Urine from SDV was routinely analyzed for cotinine with ELISA by a commercial lab (Labor Becker, Olgemöller & Kollegen, Munich, Germany). A cut-off value of 100 ng/ml urine (Tricker 2006) was used to distinguish between active smokers and non-smokers. Toenails from SDV were analyzed for nicotine by GC/MS (Schütte-Borkovec et al. 2009).

Statistical Analyses

Values less than 2-fold higher than background values were designated as not detectable and included as zero values in calculation of mean ± standard deviation. All values were non-normally distributed as determined by the Shapiro-Wilk test for normality. Therefore, statistical analyses of differences between groups were performed by the two-tailed Mann-Whitney test. All data analyses were performed using Prism 4 for Windows (GraphPad Software Inc., San Diego, CA, USA).

Results

Of the 46 SDV, 16 cases died of cardiovascular diseases, 9 cases died from accidents, 7 cases died from the misuse of addictive drugs, 5 cases committed suicide, 3 cases died from homicide, one each from influenza and lung cancer and the remaining 4 cases died from unknown causes. Twenty SDV were autopsied on the day of death and 26 cases within two to three days after death. Cadavers

were stored in coolers before autopsy and did not show visible signs of autolysis prior to autopsy. Sixteen SDV were female and 30 male, 19 were classified as current smokers or users of other nicotine-containing products (7 females and 12 males) based on either cotinine levels in urine (12 cases) or records from the criminal police office (7 cases). The remaining 27 SDV were classified as non-smokers (9 females and 18 males) by cotinine values in urine (23 cases), records from the criminal police office (2 cases), and analysis of nicotine in toenails (2 cases). The mean age of SDV was 50 ± 21 years; significantly higher in females compared to males (60 ± 19 vs. 45 ± 20 years, p <0.05) and with no significant differences according to smoking status.

Tumours in all 12 cancer patients were verified as urothelial carcinoma. Data on age, gender, smoking status and occupation are summarized in Table 1. Patients were predominantly males (11 of 12), actual smokers (5) or ex-smokers (4) and were of older age compared to SDV (64 ± 11 vs. 50 ± 21 years, p <0.05). They have not undergone chemotherapy and have not been treated with local anaesthetics including prilocaine prior to surgery.

o-Toluidine-releasing DNA adducts were detected in 11 of 12 bladder tumour samples (Figure 2, Tables 1 and 2). In contrast, 4-ABP was detected in only 4 samples at levels twofold higher than background (Figure 3, Tables 1 and 2). Adduct levels of *o*-toluidine (8.72 ± 4.49 fmol μg^{-1} DNA) were significantly higher than those of 4-ABP (0.057 ± 0.125 fmol μg^{-1} DNA, p <0.001). Actual smoking status had no significant effect on adduct levels from both arylamines. Smokers had 40% lower adduct levels from *o*-toluidine compared to non-smokers (6.12 ± 5.19 vs. 10.58 ± 3.06 fmol μg^{-1} DNA, n.s.).

o-Toluidine-releasing DNA adduct levels were similar in bladder epithelial (0.24 ± 0.63 fmol μg^{-1} DNA; detectable in 13 samples) and submucosal tissues (0.27 ± 0.70 fmol μg^{-1} DNA detectable in 10 samples) of 46 SDV, and significantly lower compared to tumour tissue (p <0.001; Table 2). 4-ABP-releasing DNA adduct levels in bladder tissue of SDV were only slightly lower than in tumour samples, and detectable in 32 of 46 samples from the epithelial layer (0.011 ± 0.022 fmol μg^{-1} DNA) and 28 samples from the submucosal layer (0.019 ± 0.047 fmol μg^{-1} DNA). The differences in 4-ABP adduct levels between both tissue layers and with tumour tissue did not reach significance. There were no significant differences in adduct levels of aromatic amines in bladder tissue of SDV according to gender, age and time elapsed between death and autopsy (Table 2).

Discussion

Our results support the notion of Skipper et al. (2010) that *o*-toluidine is indeed a human bladder carcinogen. The presence of *o*-toluidine-releasing DNA adducts in human bladder tissue gives strong evidence for genotoxicity of *o*-toluidine. According to results of a large population-based case-control study in Los Angeles County, California, monocyclic aromatic amines may account for a substantial proportion of bladder cancers among the general population (Gan et al. 2004). In this study, arylamine-haemoglobin adducts of 2,6-dimethylaniline, 3,5-dimethylaniline, and 3-ethylaniline stemming from as yet unknown environmental sources were all independently associated with bladder cancer risk in

Table 1. Characteristics of bladder tumour patients diagnosed with urothelial carcinoma and DNA adduct levels in tumour tissue.

Patient#	Age (years)	Gender	Current smoking status	Occupation	*o*-Toluidine (fmol μg^{-1} DNA)	4-ABP (fmol μg^{-1} DNA)	Cotinine ($\mu g\ l^{-1}$ urine)
1580	79	male	Smoker	Farmer	n.d.	0.080	260.9
3006	71	male	Ex-Smoker	Carpenter	12.007	n.d.	34.9
9875	60	male	Non-smoker	Metal plater	8.656	0.437	n.d.
10503	59	male	Smoker	Farmer	13.243	n.d.	2.8
10937	56	male	Smoker	Male nurse	4.008	n.d.	257.8
11946	75	female	Smoker	Housewife	9.379	0.098	265.3
14565	69	male	Non-smoker	Bricklayer	12.565	n.d.	1.6
20536	71	male	Ex-Smoker	Construction engineer	14.544	n.d.	n.d.
20977	69	male	Ex-Smoker	Metal worker	10.052	n.d.	n.d.
22813	49	male	Smoker	Metal worker	3.974	0.066	160.8
23333	44	male	Ex-Smoker	Heating installer	11.138	n.d.	n.a.
26462	71	male	Non-smoker	Filling station attendant	5.100	n.d.	n.d.
n	12				12	12	11
mean[a]	64				8.722	0.057	89.5
S.D.	11				4.491	0.125	120.0

S.D., standard deviation; n.d., not detected; n.a., not available

[a]for calculation of mean and standard deviation the values of not detected samples were set to zero.

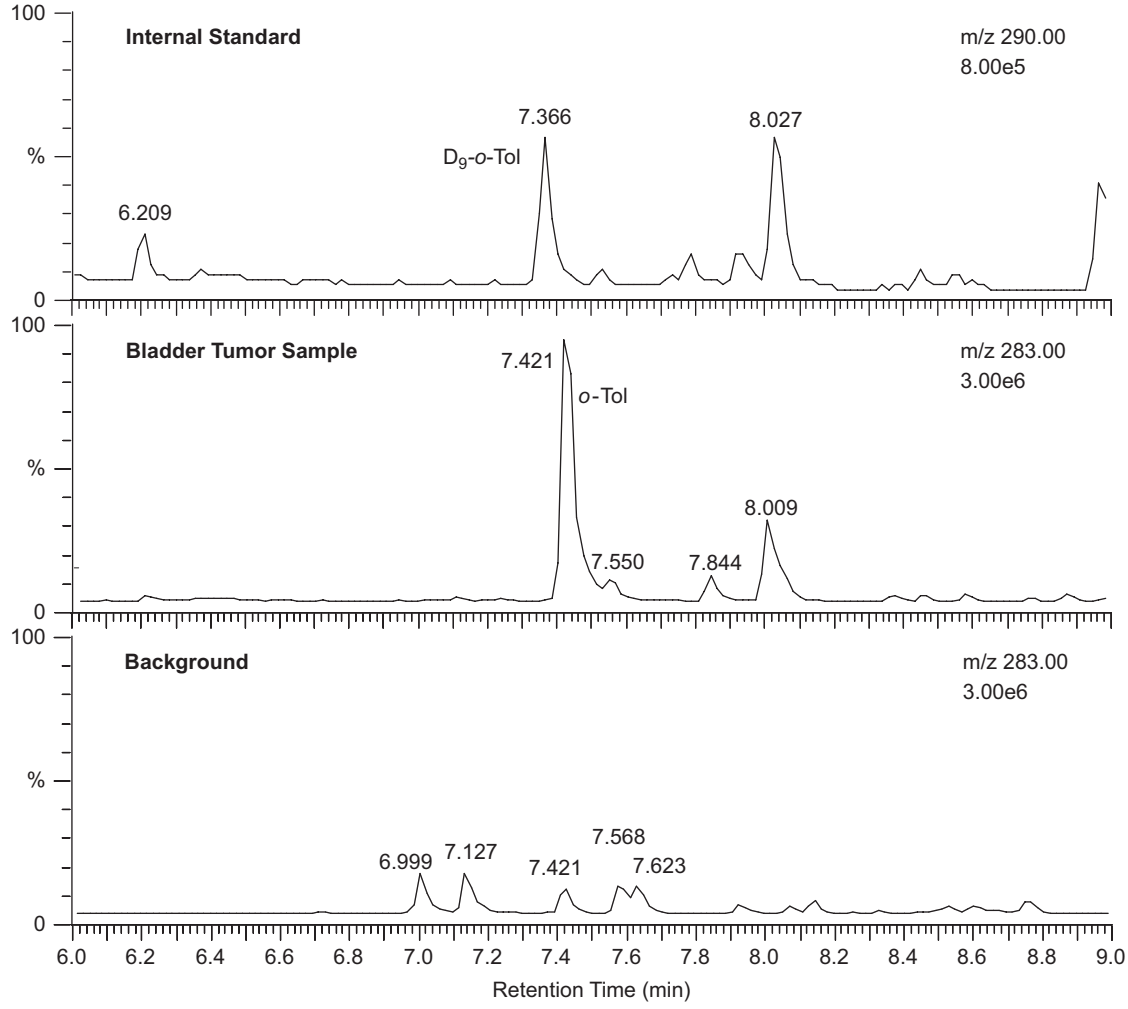

Figure 2. GC-MS chromatograms of the HFBA derivatives of d_9-*o*-toluidine (internal standard), *o*-toluidine in a bladder carcinoma (Table 1; patient #9875, male 60 y old non-smoker) and a water blank.

non-smokers. *o*-Toluidine has not been included in the analyses although in contrast to the other monocyclic aromatic amines it produces bladder tumours in animal experiments (Sabbioni & Richter 1999, Pliss 2004).

Interestingly, in our subjects without known occupational exposure to aromatic amines adduct levels of *o*-toluidine were significantly higher than for 4-ABP confirming previous observations showing considerably higher haemoglobin adduct levels for *o*-toluidine compared to 4-ABP (Richter & Branner 2002). Both aromatic amines can be readily detected in tobacco smoke (Patrianakos & Hoffmann 1979, Grimmer et al. 1995, Stabbert et al. 2003), however, differences in haemoglobin adduct levels between smokers and non-smokers are much lower for *o*-toluidine than for 4-ABP (Richter & Branner 2002). This suggests a significant contribution of environmental sources other than tobacco smoke to *o*-toluidine exposure. In 2001, the world-wide production volume of *o*-toluidine was estimated to be 59,000 tonnes and the main industrial uses were in the synthesis of herbicides, rubber chemicals, dye and pigment intermediates, resin hardeners, fungicide intermediates, and pharmaceutical intermediates (OECD 2004). If not handled with due caution, substantial exposure to *o*-toluidine has been shown to occur in occupational settings (Ward et al. 1996, Korinth et al. 2007). The local anaesthetic prilocaine is another significant source of *o*-toluidine body burden (Gaber et al. 2007). The contribution of other sources to human exposure is not well characterized but may include food, water, ambient air and coloured textiles from developing countries (Neurath et al. 1977, Palmiotto et al. 2001, IFOP 2001, OECD 2004). More recently, increasing use of poly(*o*-toluidine) for electrochromic devices, polymeric batteries, photovoltaic cells, energy storage, opto-electronic devices and display devices has also been reported (Elmansouri et al. 2009).

Blank samples of DNA elution buffer were analyzed with each daily series of 4-6 DNA samples to control for inadvertent contamination of reagents by unknown environmental sources of *o*-toluidine. Background

Table 2. DNA adducts in bladder tissue of sudden death victims and human bladder tumours.

		Sudden death victims					Tumour patients	
		o-Toluidine		4-ABP			*o*-Toluidine	4-ABP
	n	epithelial	submucosal	epithelial	submucosal	*n*		
All	46	0.236 ± 0.625[d](13)	0.267 ± 0.697[d](10)	0.011 ± 0.022(32)	0.019 ± 0.047[a](28)	12	8.722 ± 4.491(11)	0.057 ± 0.125[b](4)
Non-smoker	27	0.136 ± 0.280[d](7)	0.292 ± 0.784[d](5)	0.012 ± 0.026[c](20)	0.015 ± 0.041(14)	7	10.580 ± 3.055(7)	0.061 ± 0.165[b](1)
Smoker	19	0.378 ± 0.910[c](6)	0.232 ± 0.571[c](5)	0.010 ± 0.017(12)	0.024 ± 0.055(14)	5	6.120 ± 5.193(4)	0.049 ± 0.046[a](3)
Male	30	0.273 ± 0.742(8)	0.218 ± 0.514(7)	0.015 ± 0.026(24)	0.018 ± 0.045(19)			
Female	16	0.165 ± 0.316(5)	0.359 ± 0.966(3)	0.005 ± 0.008(8)	0.022 ± 0.053(9)			
Age ≤50 y	22	0.328 ± 0.858(5)	0.281 ± 0.775(6)	0.013 ± 0.023(16)	0.023 ± 0.054(13)			
Age >50 y	24	0.151 ± 0.278(8)	0.255 ± 0.634(4)	0.009 ± 0.025(16)	0.016 ± 0.041(15)			
Autopsy ≤24 h	20	0.024 ± 0.107(1)	0.247 ± 0.799(4)	0.017 ± 0.028(17)	0.026 ± 0.057(14)			
Autopsy >24 h	26	0.399 ± 0.795(12)	0.302 ± 0.623(6)	0.007 ± 0.015(15)	0.014 ± 0.039(14)			

Data were analyzed by Mann-Whitney test; presented as fmol μg^{-1} DNA; mean ± standard deviation (number of positive samples), values of not detected samples were set to zero.

[a,b]4-ABP values significantly different from correspondent values for *o*-toluidine, $p < 0.05$ (a) and $p < 0.001$ (b)

[c,d]Significantly different from values for tumour patients, $p < 0.05$ (c), $p < 0.0001$ (d)

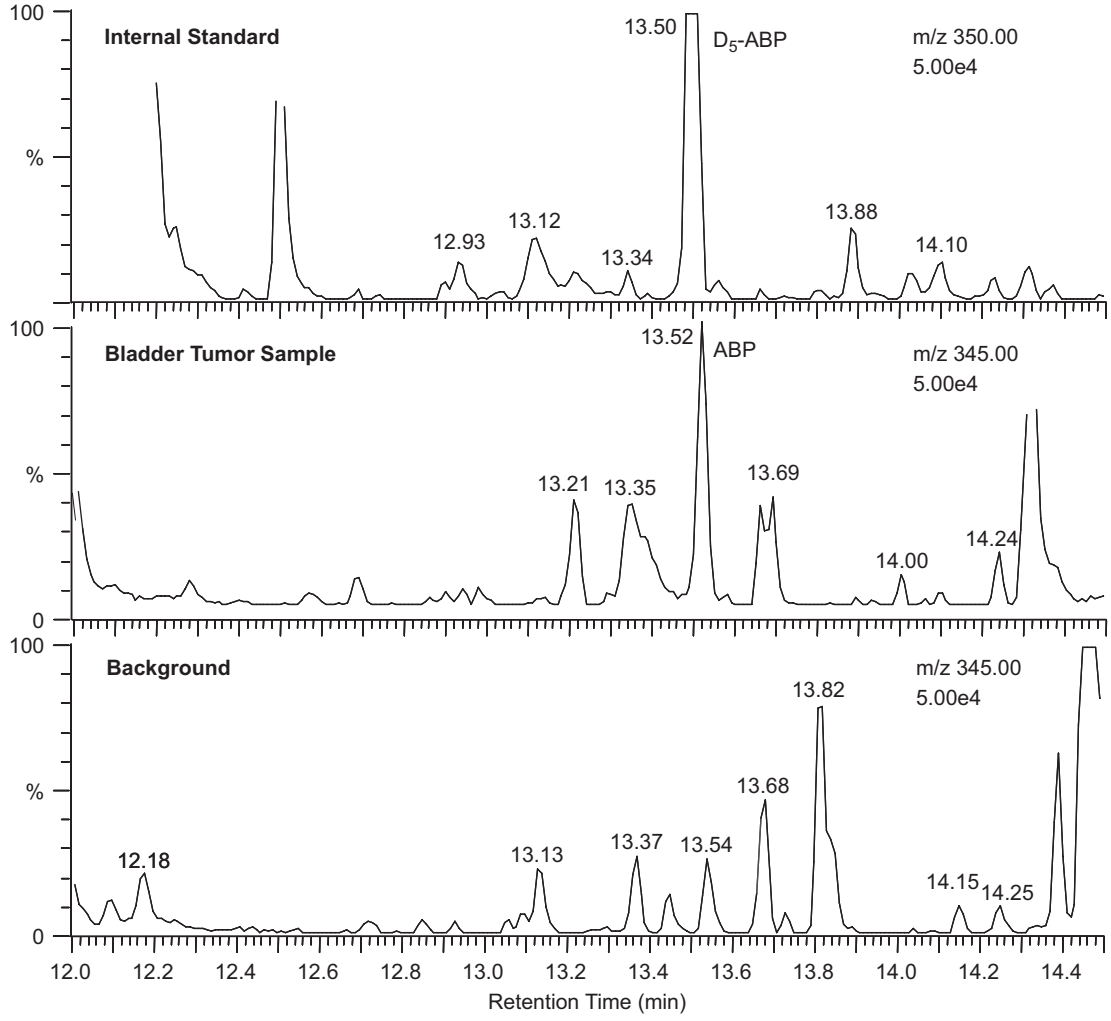

Figure 3. GC-MS chromatograms of the HFBA derivatives of d_5-4-ABP (internal standard), 4-ABP in a bladder carcinoma (Table 1; patient #3006, male 71 y old smoker) and a water blank.

contamination by *o*-toluidine was identified to occur in a previous study on the determination of haemoglobin adducts (Kutzer et al. 1997) and is accentuated in the current study due to the lower amounts of *o*-toluidine bound to DNA. The background values of both 4-ABP and *o*-toluidine were larger than the detection limit of the MS

detector which was defined by a signal-to-background ratio of 1:5 for chromatograms acquired in the SIM mode (Lewalter & Gries 2000). Consequently, the classical analytical approach in which the limits of detection (LOD) and limit of quantification (LOQ) are defined, could not be applied. Only adducts levels exceeding the daily background by more than 2-fold were considered to be above the detection limit. Using a more stringent definition of 3-fold above background would have reduced detectable *o*-toluidine adducts in epithelial and submucosal tissue of SDV from 13 to 3 and from 10 to 5 samples, respectively. In contrast, the high *o*-toluidine adduct levels in tumour tissue of 11 bladder cancer patients (mean 4.5-fold higher, minimal 3.2-fold, maximal 8.4-fold) would all remain detectable.

Measuring 4-ABP- and *o*-toluidine-releasing adducts after acid hydrolysis does not allow the determination of the contribution of individual adducts to the total adduct burden. Recently developed methods using liquid chromatography coupled to mass spectrometry have been shown to enable determination of the postulated major adduct from 4-ABP, *N*-(2′-deoxyguanosine-8-yl)-4-ABP (dG-C8-4-ABP), in human tissues (Zayas et al. 2007, Randall et al. 2010, Bessette et al. 2010). With these methods, background contamination by free aromatic amines is avoided. However, for *o*-toluidine little is known about the nature of the adducts. *o*-Toluidine-releasing DNA adducts have only been characterized after *in vitro* incubation of nucleotides or DNA with putative reactive metabolites of *o*-toluidine (Marques et al. 1996, Branco et al. 1999, Jones & Sabbioni 2003). In addition to binding to the C8 carbon of 2′-deoxyguanosine (Figure 1), other adducts binding to N and O functions of 2′-deoxyguanosine and 2′-deoxyadenosine have been reported. After oral administration of 0.5 mmol/kg *o*-toluidine to rats, no adducts have been specifically identified (Jones & Sabbioni 2003).

The efficacy of alkaline hydrolysis as used routinely for determination of 4-ABP-DNA adducts has only been tested for dG-C8-4-ABP. No data are available on hydrolysis of 4-ABP and *o*-toluidine adducts comparing alkaline with acidic hydrolysis. For DNA adducts from tobacco-specific nitrosamines acid hydrolysis has been proven to be effective for all species of adducts including phosphotriesters (Haglund et al. 2002, Lao et al. 2007a, Lao et al. 2007b). Therefore, we used our protocol for acid hydrolysis (Hölzle et al. 2007, Heppel et al. 2009) in the present study. Since stable isotope-labeled internal standards were added prior to hydrolysis, any significant loss of the aromatic amines due to decomposition occurring during hydrolysis can be excluded.

Because of the low percentage of samples with detectable DNA adduct levels from *o*-toluidine in bladder tissue of SDV (Table 2), lack of dependence on smoking status, gender and age should be interpreted with caution. No influence of smoking on adduct levels was apparent in cancer patients. The concentrations of DNA adducts from *o*-toluidine were much higher in tumour tissue compared to SDV epithelial (37-fold, $p < 0.001$) and submucosal tissue (33-fold, $p < 0.001$) of SDV. The differences were much lower for 4-ABP-releasing DNA adducts,, 5-fold and 3-fold for tumour tissue vs. SDV epithelial and submucosal tissues, respectively, and did not reach significance. One possible reason for higher adduct levels in tumour tissue could be activation of *o*-toluidine by peroxidases and cyclooxygenases. Similar mechanisms have been described for the closely related *o*-anisidine (Stiborova et al. 2001, Stiborova et al. 2002). Recently, formation of free radicals as determined by thiobarbituric acid reactive substances (TBARs) have been shown to be significantly increased in cancerous human bladder tumour tissues compared to adjacent tumour-free tissue and tissue from control patients (Bayraktar et al. 2010).

Little is known about the metabolic activation of *o*-toluidine; however, it appears that the mechanism of activation is different to that of 4-ABP. Lack of differences in haemoglobin adduct formation in smoking and non-smoking patients treated with prilocaine suggests that CYP1A2 does not seem to play a role in *o*-toluidine activation (Gaber et al. 2007). In rats, pretreatment with phenobarbital, but not ß-naphthoflavone, results in increased haemoglobin adducts from *o*-toluidine suggesting involvement of enzymes of the CYP2B family (DeBord et al. 1992). Alternative pathways of *o*-toluidine activation such as ring hydroxylation (Diaz Gómez et al. 2006) could lead to DNA adducts with different molecular weight which would not be detected with our method.

Conclusion

The presence of DNA adducts releasing *o*-toluidine in human bladder tissue clearly show that *o*-toluidine is genotoxic to humans and further support the classification of *o*-toluidine as a human carcinogen (IARC 2009). In industrial settings where *o*-toluidine is used, workers should be alerted to the hazardous nature of this compound and exposure should be avoided as far as possible. Similarly, use of prilocaine as a local anaesthetic should be discouraged especially when used at high doses in liposuction (Lindenblatt et al. 2004) or repeatedly for treatment of premature ejaculation (Dinsmore & Wyllie 2009).

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing the paper.

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