Postlabelling-HPLC analysis of lipophilic DNA adducts from human lung

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To the memory of Alexei Likhachev

A new modification of the ^{32}P-postlabelling method was described for the analysis of lipophilic DNA in human tissues. To isolate these DNA adducts the method applied nuclease P1 enrichment before labelling and butanol extraction after labelling, followed by high performance liquid chromatography (HPLC) separation and flow-through radioactivity detection. These enrichment methods are known to work for many adducts of polycyclic aromatic hydrocarbons (PAHs). In human peripheral lung tissue from smokers the apparent level of the DNA adducts observed was 25–244 adducts per 10^8 nucelotides; in two alleged non-smokers the level of adducts was 17 and 109 adducts per 10^8 nucleotides. When the same samples were analysed by thin-layer chromatography (TLC), as in the conventional postlabelling assay, the recovery was 5% of that of the HPLC method. Nevertheless, the results from the two methods correlated. In TLC the adducts were lost because they did not remain in the origin in D1 of the TLC development. There was no large difference in recovery between the two techniques for the PAH–DNA adduct standards used. The present results are underestimates of the true adduct levels because there is no way to correct for labelling efficiency and recovery of unknown adducts. Yet they give a lower estimate of the level of lipophilic DNA adducts in human lung tissue.

Keywords: *^{32}P-postlabelling, DNA adducts, DNA damage, PAH, smoking, lung cancer.*

Abbreviations: *BP, benzo(a)pyrene; FD, fluorescence detection; HPLC, high performance liquid chromatography; PAH, polycyclic aromatic hydrocarbon; PEI, polyethyleneimine; TLC, thin-layer chromatography.*

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Introduction

The spectrum of DNA adducts in human DNA covers a wide range of chemical classes, formed by endogenous and exogenous DNA-binding agents (IARC 1994, Hemminki *et al.* 1995, Bartsch 1996). The adduct-forming chemicals include simple alkylating agents, oxidizing intermediates, difunctional aldehydes and large aromatic carcinogens (Alexandrov *et al.* 1992, Chaudhary *et al.* 1994, Kumar and Hemminki 1996, Rothman *et al.* 1996). Some individual species in each class have been quantified in human DNA but the multitude of DNA adducts observed by, for example, the ^{32}P-postlabelling technique remains unidentified. Although many adducts have been shown to be caused by environmental chemicals present in tobacco smoke or in polluted environment (Phillips *et al.* 1988, Hemminki *et al.* 1990, Rojas *et al.* 1994, 1995, Möller *et al.* 1996), numerous other adducts appear to be originating from endogenous sources (Bartsch 1996). It is a major challenge to identify and quantify the main human DNA adducts. This work is necessary for the establishment of the pathological significance of DNA adducts.

Due to the chemical diversity of DNA adducts they cannot be analysed in one step or by a single method. The ^{32}P-postlabelling technique is a versatile method that detects many adducts at the same time, but depending on the variation of the method it is selective towards certain classes of adducts (Randerath *et al.* 1981, Gupta 1985, Reddy and Randerath 1986, Beach and Gupta 1992, IARC 1993). The assay usually includes an enrichment step for adducts, because normal nucleotides, when present in molar excess over adducts, severely suppress the labelling of adducts (Hemminki *et al.* 1993). Selection of a certain enrichment method involves assumptions regarding the behaviour of the adducts. For example, application of nuclease P1 or butanol extraction in the enrichment of adducts, both commonly used for the polycyclic aromatic hydrocarbon (PAH) adducts, assumes that the adducts are recovered in the treatment. This has been shown to be the case for a number of specific PAH adducts whereas most other types of known DNA adducts are lost in either one of these enrichment methods (Gupta and Earley 1988, Beach and Gupta 1992, Segerbäck and Vodicka 1993).

In order to embark on the characterization of human DNA adducts we wanted to select a method that could detect a spectrum of adducts in a single analysis and that would be reproducible over time. According to previous experience the ^{32}P-postlabelling technique coupled to high-performance liquid chromatography (HPLC) separation and flow-through detection fulfilled the criteria of versatility and reproducibility (Möller and Zeisig 1993, Möller *et al.* 1993, 1996, Försti *et al.* 1994, Bykov *et al.* 1995, Hemminki *et al.* 1996, 1997, Kumar and Hemminki 1996). The samples were treated with nuclease P1 before postlabelling and with butanol after postlabelling to select for a defined class of DNA adducts. Based on prior knowledge many PAH adducts will be recovered in such treatment. We were however surprised to find other main adducts, probably unrelated to PAHs, in all the lung samples analysed. The HPLC method was compared with the conventional thin-layer chromatography (TLC) method.

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MATERIALS AND METHODS

Chemicals

RNase A, RNase T1, micrococcal nuclease, spleen phosphodiesterase, snake venom phosphodiesterase and prostatic acid phosphatase were obtained from Sigma (St Louis, MO, USA). Proteinase K and P1

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Boehringer Mannheim Biochemica (Mannheim, Germany). T4 polynucleotide kinase was from United States Biochemicals (Cleveland, OH, USA) and [γ-^{32}P]ATP from Amersham International (Little Chalfont, UK). Methanol was HPLC grade (J. T. Baker B. V., Deventer, The Netherlands). All other chemicals were of analytical grade and were either from Sigma or from E. Merck (Darmstadt, Germany).

Sampling, DNA isolation and adduct standards

All the patients underwent lung surgery, seven because of lung cancer and the two others for cyst and sclerosis. All the patients were male, with no prior chemo- or radiotherapy nor occupational exposure. The mean age was 50 years (age not recorded for one patient). All but two were recorded as smokers of 20–25 cigarettes per day. Peripheral lung tissue, distant from the tumour, was removed in the operation and frozen for DNA isolation.

Tissue was first homogenized in *ca* 10 volumes of saline and the mixture was extracted with 0.5 volumes of phenol. The aqueous phase was mixed with two volumes of ethanol : cresol (9 : 1) and nucleic acids were spooled on a glass rod after 30 min. The rod was washed with 70% ethanol, nucleic acids were dissolved and reprecipitated with ethanol. Nucleic acids were dissolved again, treated with RNase A and DNA was precipitated with ethanol. The DNA preparation contained varying amounts of RNA at this stage, as was evident in HPLC analysis of an aliquot treated with nuclease P1 and alkaline phosphatase. To degrade the remaining RNA, 5 µl RNase A (10 µg µl^{-1}) and 2 µl RNase T1 (20 U µl^{-1}) were added in 0.5 ml 50 mM Tris (pH 8.0), followed by incubation at 37 °C for 1 h. The solution was extracted sequentially with an equal volume of phenol (pre-equilibrated with 0.1 M Tris, pH 8.0), phenol : sevag (chloroform/isoamyl alcohol, 24 : 1), 1 : 1, and sevag. DNA was precipitated from the aqueous phase by adding 0.1 volume of 5 M NaCl and one volume of chilled ethanol, followed by centrifuging at 13000 rpm for 5 min. The DNA pellet was then washed once with 70% ethanol and dissolved in Millipore water. DNA concentration was determined by absorbance at λ 260 nm and DNA purity by the $A_{260\,nm}/A_{280\,nm}$ ratio, which ranged between 1.6 and 1.8 in water (corresponding to 1.8–2.0 in buffer).

PAH–DNA adduct standards were prepared by incubating parent hydrocarbons in the presence of an Arochlor 1254-induced rat liver S9 fraction, and were provided by Dr D. Segerbäck (Segerbäck and Vodicka 1993). The 'benzo(*a*)pyrene (BP)–DNA' was obtained by including BP in the incubation mixture, 'PAH–DNA' was obtained by including 12 different PAHs: pyrene, 2-methyanthracene, benzo(*g,h,i*)perylene, fluoranthene, benzo(*e*)pyrene, BP, dibenz(*a,h*)anthracene, benzo(*b*)fluorathene, chrysene, benz(*a*)anthracene, cyclopenta(*c,d*)pyrene and indeno(*c,d*)pyrene. A commercial BP diol epoxide-derived adduct at the N^2 position of 2′-deoxyguanosine-3′-monophosphate (BPDE–dGMP) was also used (Midwest Research, Kansas City).

DNA digestion and postlabelling using the 'monophosphate method'

The monophosphate modification of the postlabelling procedure was described by Randerath *et al.* (1989). A scheme of the monophosphate method, as compared with the conventional bisphosphate method, is shown in Figure 1. DNA (10 µg) was hydrolysed with a mixture of nuclease P1 (20 mU per µg DNA) and prostatic acid phosphatase (0.2 µg per µg DNA) at pH 5. After incubation at 37 °C for 45 min, the reaction was terminated by adding 100 µl chilled ethanol. Proteins were allowed to precipitate for 20 min at – 20 °C and after centrifugation water–ethanol solution was transferred to the fresh tube and evaporated to dryness. The modified nucleotides were converted to ^{32}P-postlabelled diphosphates in the labelling mixture (2 µl) containing 2.4 U T4 polynucleotide kinase and 2.3 pmol ATP (7 µCi [γ^{32}P]ATP, 3000 Ci mmol^{-1}). The reaction was carried out at pH 9.6. After labelling, the mixture was treated with snake venom phosphodiesterase

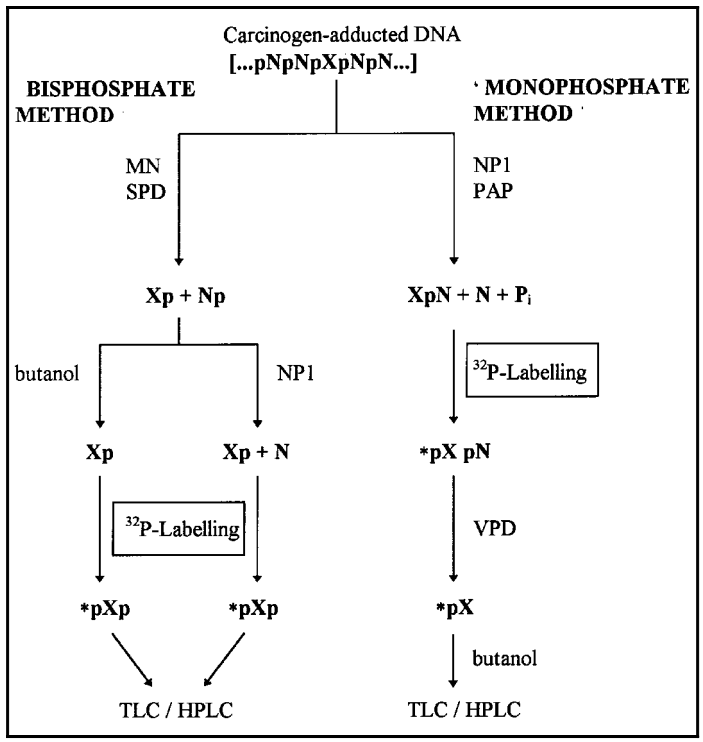

Figure 1. A scheme of the monophosphate method used in the present study as compared with the bisphosphate methods. The salient feature of the monophosphate method is digestion of DNA with nuclease P1 and prostatic acid phosphatase, leaving adducted dinucleotides for labelling and subsequent cleavage to 5′-labelled monophosphates. X = adducted nucleoside, N = unmodified nucleoside, *p = 5′-labelled phosphate group.

(0.5 mU per µg DNA) for 30 min at 37 °C to yield ^{32}P-labelled monophosphate adducts.

In order to decrease the background radioactivity and thus increase the sensitivity, the adducted nucleotides were extracted with butanol after labelling. To the labelling mixture (volume 3 µl) 2 µl of 6 mM tetrabutylammonium chloride in 0.2 M formate buffer pH 3.5, mixed with blue dextran colour, and 50 µl of 1-butanol were added. After vortexing the two phases were separated by centrifugation. The coloured water phase facilitated a complete collection of the butanol phase. The collected butanol phase was evaporated to dryness.

HPLC and TLC analysis of adducts

For HPLC analysis the mixtures were diluted to 15 µl with water or the dried sample was dissolved in 15 µl water. The whole sample was injected into the Beckman HPLC system Gold, used with a Phenomenex Kromasil C18 (2 × 250 mm, particle size 5 µm) column. A precolumn filter was installed in front of the analytical column. The volume of the sample loop was 20 µl.

Radioactivity was measured on-line with a Beckman 171 Radioisotope detector. The size of the Teflon sample loop in the flow cell was 75 µl, which was then folded into a scintillation tube containing scintillation liquid (Ready Safe, Beckman). The adducts were quantified by integration of the peak area after subtraction of background radioactivity.

Separations were carried out at ambient temperature using a binary gradient with 0.5 M ammonium formate, 20 mM phosphoric acid (pH 4.6) and methanol. Labelled adducted nucleoside bisphosphates were analysed using gradients A, which had initial conditions of 2% methanol for 5 min, after which the proportion of methanol increased linearly to 70% in 65 min and then further to 100% in 5 min. The 100% methanol was maintained for 10 min before linear decrease to 2% in 10 min.

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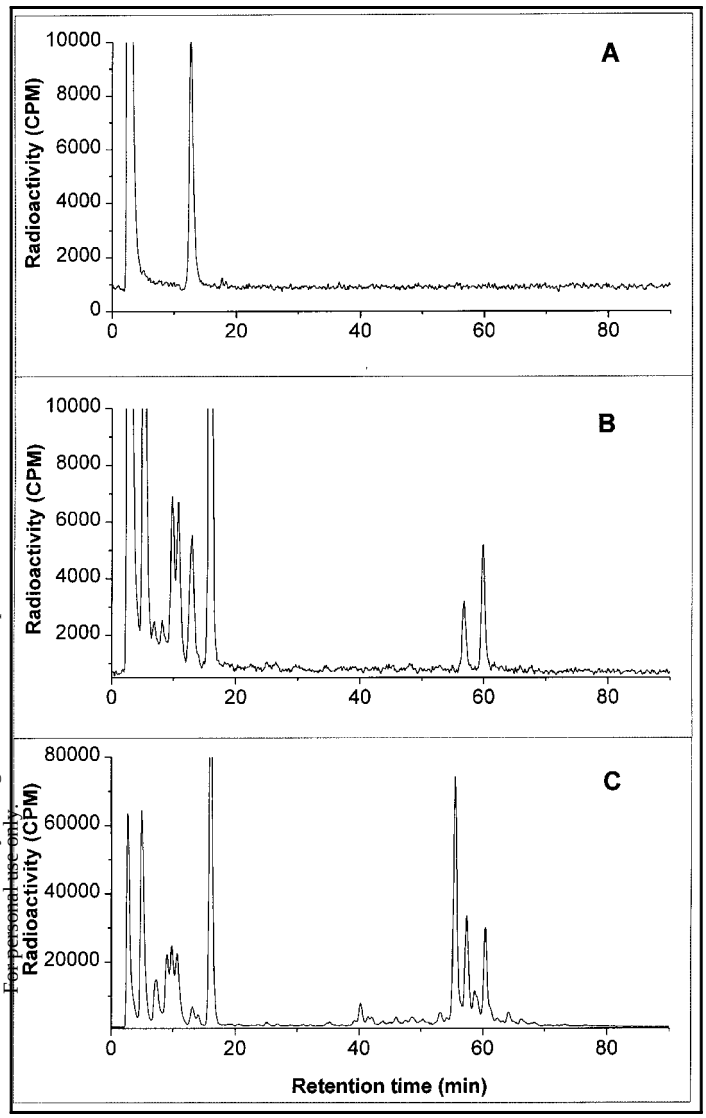

Figure 2. HPLC analysis of postlabelled samples of reagent blank containing no DNA (A), of BP–DNA standard (B) and of PAH–DNA standard (C). The peaks before 20 min include radioactive phosphate, ATP and normal nucleotides. The peak at 60 min, panel (B), is BPDE–dGMP.

For TLC the solution (3.0 μl) was transferred to a PEI–cellulose TLC plate (Macherey-Nagel). The DNA adducts were then resolved as follows: 1.0 M sodium phosphate (pH 6.0) in D1, 3.6 M lithium formate, 8.5 M urea (pH 3.5) in D3, and 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea (pH 8.0) in D4. A 2.7 cm Whatman 17 paper wick was stapled on the TLC plate in D4 development to remove the frontier background. Screen-intensified autoradiography was carried out at –86 °C for 3 days. All the adduct spots were excised together for Cerenkov counting while keeping the excised areas in different plates consistent. The radioactivity of the same area in the negative control plate was used as background value in calculating adduct levels.

The DNA adducts level was determined at least twice for each sample.

Results

The postlabelling-HPLC assay using radioactivity detection was developed based on the 'monophosphate method' where nuclease P1 was applied (Randerath *et al.* 1989). However, in order to reduce radioactivity introduced to the HPLC column, butanol extraction was carried out after postlabelling. This was very effective and reduced over 99% of the radioactivity of the postlabelling mixture. Figure 2(A) shows an HPLC-radioactivity detector analysis of a reagent blank containing no DNA. Some 9×10^6 counts min^{-1} were used in the postlabelling assay but after butanol extraction 20 000 counts min^{-1} (0.2 %) were introduced in the column as pyrophosphate and ATP (3 min and 13 min peaks, respectively). We also checked that the recovery of the BP–DNA and PAH–DNA adduct standards was good after the butanol extraction step (Figure 2(B,C)). This was done by collecting HPLC fractions between 26 and 70 min for radioactive counting. Essentially similar recoveries of radioactive adduct peaks from these standards were noted whether or not butanol extraction was carried out before the HPLC analysis. In Figure 2(B), the latter peak, eluting at 60 min, corresponded to the BPDE–dGMP standard, and was thus the diol epoxide-derived adduct. It may be surprising that the chromatogram of DNA adducts formed by 12 different PAHs (Figure 2(C)) displays a relatively simple pattern of four large peaks. However, the TLC pattern of the mixture was also composed of four spots (Segerbäck and Vodicka 1993).

The lung samples were analysed by the postlabelling-HPLC method using radioactivity detection. Large radioactive adduct peaks were observed in many but not all samples (Figure 3). Samples in Figure 3(A–C) were from smokers and in panel D from a non-smoker. The fractions eluting between 26 and 70 min were considered to represent lipophilic material and these were collected for the determination of radioactivity. The adduct levels are shown in Table 1. The mean for smokers is 106 adducts per 10^8 nucleotides and for non-smokers 63 adducts per 10^8 nucleotides. These are very high adduct levels as far as any previous postlabelling results are concerned.

When the lung samples were analysed by TLC (Figure 4(a)) typical diagonal radioactive zones (Phillips *et al.* 1988) were detected together with some prominent spots. The total radioactive area was taken for the counting of radioactivity. The adduct levels are shown in Table 1, the mean for smokers and non-smokers being 4.5 and 3.5 adducts per 10^8 nucleotides, respectively. These levels are quite compatible with adduct data in previous studies using TLC, but only about 5% of the adduct levels recorded with HPLC.

The BP–DNA and PAH–DNA standards were also analysed by TLC (Figure 4(c, e)). The combined adduct areas were counted for radioactivity and compared with the recoveries from HPLC (Figure 2(b, c)). The recoveries in TLC were somewhat lower than those in HPLC, 80% for the BP–DNA standard and 60% for the PAH–DNA standard.

The correlation between adduct levels in HPLC and TLC was examined in the individual lung samples (Figure 5). The correlation coefficient was surprisingly high, 0.94. The relationship between the adduct levels follows the equation: Y (HPLC) = – 22 + 34X (TLC).

The reason for the difference between adduct levels in HPLC and TLC was investigated by collecting lipophilic adducts from lung tissue in HPLC (fractions eluting at 26–70 min, Figure 3) and considering the losses in various stages of the TLC analysis. A small contribution to the difference was a loss of radioactivity

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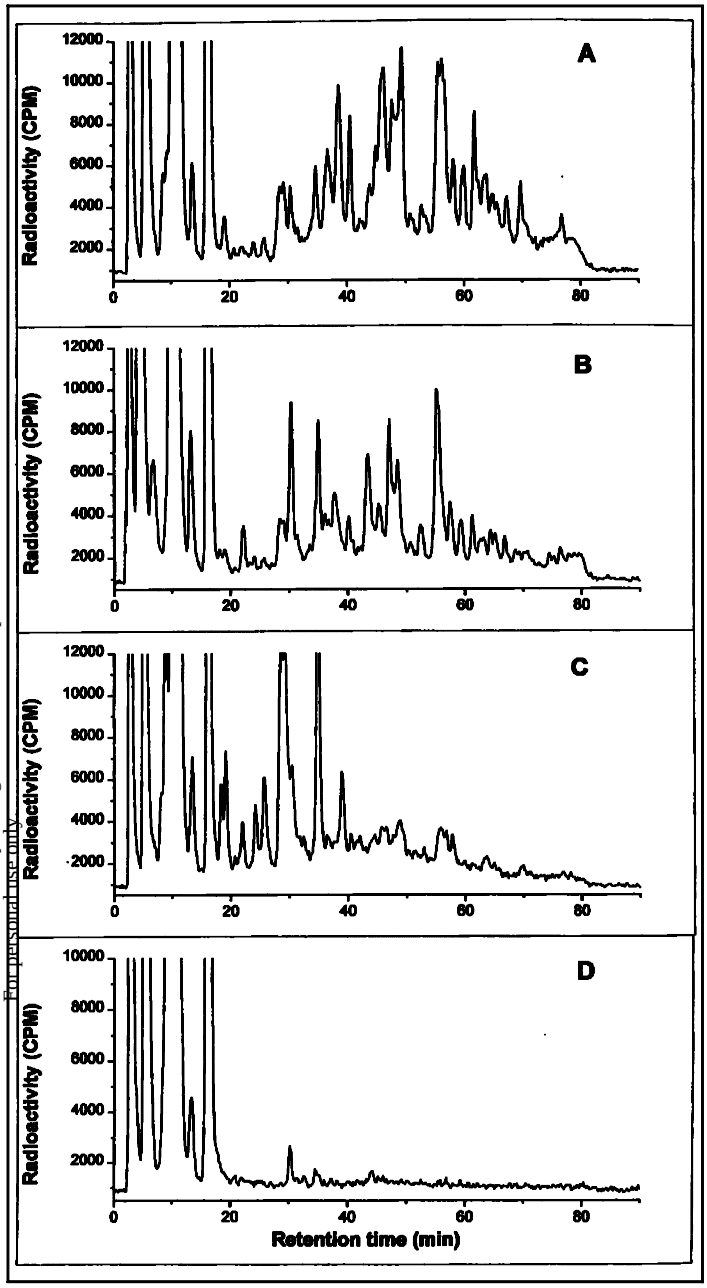

Figure 3. HPLC analysis of postlabelled human lung samples from three workers (A–C) and from a non-smoker (D). Peaks eluting between 26 and 70 min were collected for adduct determination.

washes between the directions. The primary reason was migration of adducts in direction 1 of TLC development (Figure 6). As only the origin is saved for development in other directions, most of the radioactive material recovered for HPLC analysis is lost in TLC analysis. This loss was however quite selective: no BP–DNA adducts and only a small amount of PAH–DNA adducts were lost (Figure 6).

When the HPLC-purified adduct fraction of human lung is analysed in TLC, omitting direction 1 and use of a wick in D4, most of the radioactivity migrated to the edge of the plate (Figure 4(b)). This could be expected because fast moving adducts were eliminated in D1 of TLC. By contrast, no selective losses were noted for PAH and BP adduct standards (Figure 4(d, f)).

	Sample	Adduct level by ^{32}P-TLC (adducts per 10^8 nucleotides)	Adduct level by ^{32}P-HPLC (adducts per 10^8 nucleotides)
Smokers			
	1	1.2	28
	2	7.8	244
	3	3.1	44
	4	5.4	159
	5	3.5	67
	6	4.2	177
	7	1.0	25
	Mean ± SD	3.3 ± 2.4	106 ± 87
Non-smokers			
	8	3.8	109
	9	1.0	17
	Mean	2.4	63

Table 1. Lipophilic DNA adducts in nine human lung samples detected by ^{32}P-TLC and ^{32}P-HPLC.
Each sample was analysed up to four times and means are presented.

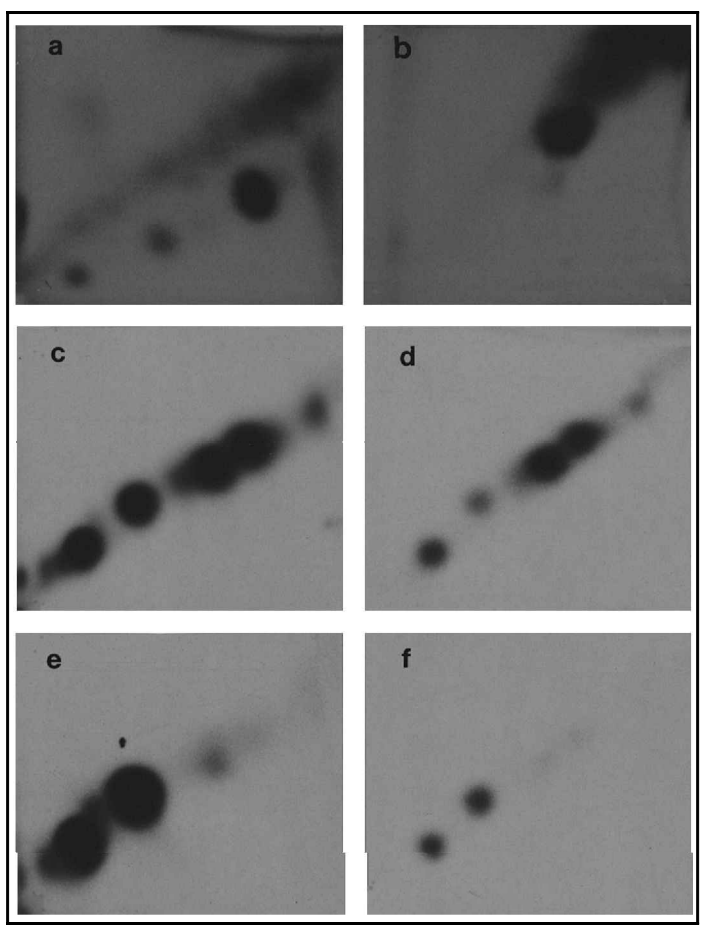

Figure 4. TLC analysis of human lung sample from a smoker (a and b), of PAH–DNA standard (c and d), and of BP–DNA standard (e and f). Samples a, c and e are analysed after postlabelling; samples b, d and f are postlabelled and purified by HPLC (fractions 26–70 min) and then analysed by TLC, omitting D1 and the wick in D4.

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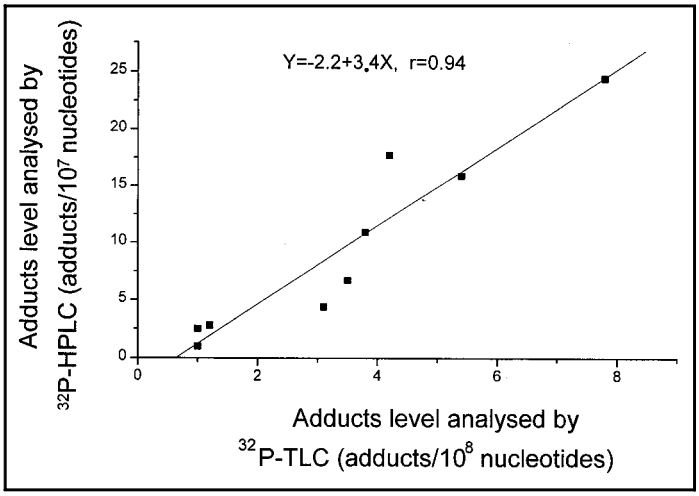

Figure 5. Correlation of the HPLC (*Y*-axis) and TLC (*X*-axis) analysis of the nine lung samples. Observe the different scales of the axes.

Discussion

We started the study in order to improve the postlabelling analysis of DNA adducts caused by PAHs, ubiquitous environmental carcinogens (IARC 1984, Boström *et al.* 1994). PAHs are also a main carcinogen class present in tobacco smoke and for this reason lung tissue was taken for analysis (IARC 1986, Phillips *et al.* 1988). DNA adducts of PAHs appear to be present in most human tissues, as shown by four independent techniques available for PAH–DNA adduct analysis: ^{32}P-postlabelling, immunoassay, high-performance liquid chromatography–fluorescence detection (HPLC–FD) and synchronous fluorescence spectroscopy (Alexandrov *et al.* 1992, Rojas *et al.* 1994, 1995). The techniques have different specificities to detect individual species of PAHs, such as BP, usually constituting 1–20% of particulate PAH. The apparent levels of PAH–DNA adducts in human tissues differ extensively depending on the method used, for example in white blood cells the postlabelling assay applying TLC often yields total PAH adduct levels lower than the observed BP–DNA adduct levels alone by the other techniques (Hemminki *et al.* 1990, 1995, van Schooten *et al.* 1992, Rojas *et al.* 1994). We assumed that the reason would be a loss of adducts in the TLC analysis (Hemminki *et al.* 1996, 1997), while in the HPLC method such losses could not take place because the whole labelled adduct mixture is injected in the machine.

It was surprising that the recovery of the total DNA adducts from the lung samples was only 5% in TLC as compared with HPLC. The main reason was shown to be the loss of adducts in D1 of TLC: only the small fraction of adducts retained in the origin is recovered in the TLC analysis while all adducts are recovered in HPLC. This loss affected different adducts selectively: the BP–DNA standard was almost completely recovered in TLC as were the adducts of 12 PAHs containing 3–6 benzene rings, formed in a microsomal system *in vitro*. It thus appeared that TLC retained and lost two entirely different sets of DNA adducts. In the study of Segerbäck and Vodicka (1993) on the recoveries of 12 different PAHs there was in fact

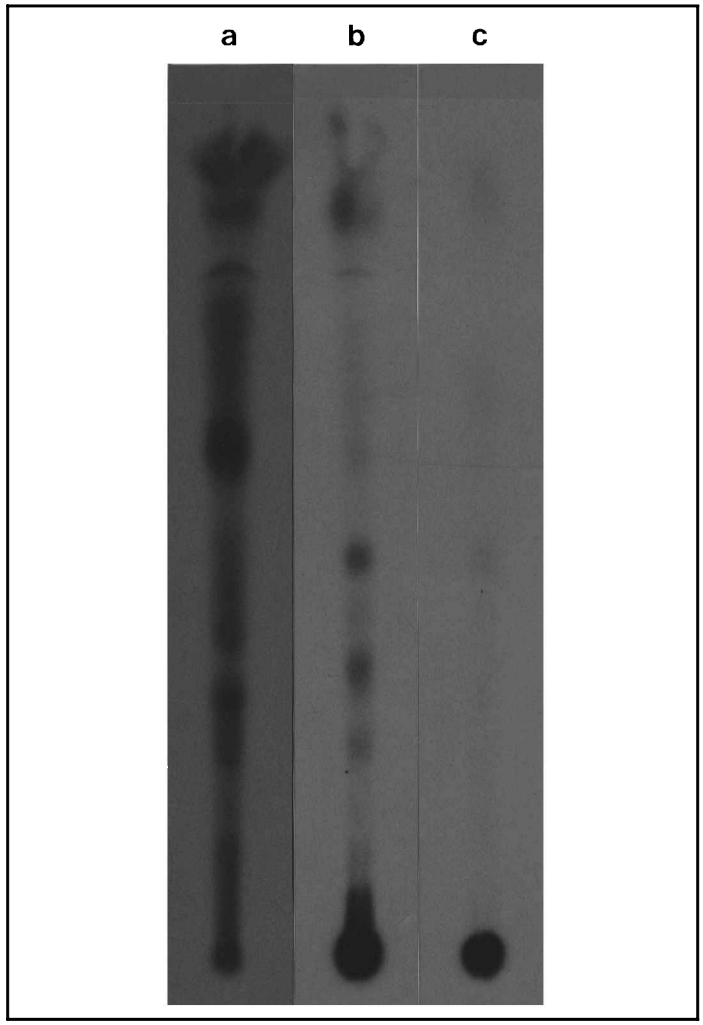

Figure 6. D1 TLC analysis of a lung sample (a), PAH–DNA (b) and BP–DNA (c). D1 was developed in 1.0 M sodium phosphate buffer, pH 6.0, as routinely used. The plates were dried and used for autoradiography.

a tendency toward poor recoveries among small PAHs, which could be expected to migrate in D1 of TLC. Interestingly, this study also found the recovery after nuclease P1 and butanol extraction to be identical for all but one PAH.

The high correlation ($r = 0.94$) of adduct levels in TLC and HPLC was unexpected. Whether tobacco smoke might be a source of these chemicals cannot be resolved in this patient series because only two alleged non-smokers were present. There was no objective data on their true non-smoking status. In all the samples studied the HPLC chromatograms showed similar main groups of adducts, although their relative abundance varied. This is further support of the same origin of the adduct forming chemicals but does not exclude endogenous sources.

A few features of the present method should be commented on. In this method the total postlabelling mixture is subjected to the HPLC analysis, a method used in few human studies so far (Hemminki *et al.* 1996, 1997, Möller *et al.* 1996). A key modification in the method was to use butanol extraction of the adducts after labelling in order to decrease the background radioactivity in the HPLC system and thus enhance the effective sensitivity. This method also enabled

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of the original sample in analysis without overloading the chromatographic system. However, in order to guarantee a good recovery of PAH adducts in butanol extraction, the adducts had to be monophosphates rather than bisphosphates, as in the conventional postlabelling method. The analysis of PAH–DNA adducts as monophosphates has been described by Randerath and co-workers (1989) and this method was applied here. The present procedure thus consisted of P1 digestion before and butanol extraction after postlabelling. Both of these enrichment methods are specific towards certain adduct classes, PAH–DNA adducts being those equally enriched by both methods (Beach and Gupta 1992). Yet we do not know at this time how nuclease P1 sensitivity compares in the monophosphate and bisphosphate methods. A disadvantage of the monophosphate method is that commercial adduct standards are not available in the form directly suitable for analysis.

Thus according to the available literature bulky lipophilic ligands bound to the N^2 position of guanine would be the adducts to be enriched by the combined P1 and butanol technique. In addition to PAHs, safrole and minor adducts of large aromatic amines would belong to this group of compounds (Gupta and Earley 1988). This indirect evidence, based on the available knowledge on environmental chemicals and DNA adducts, suggests that the adducts analysed by HPLC include PAHs. However it appears likely that other types of adducts are also present. Many of the major adduct fractions elute earlier in HPLC than the adducts of typical carcinogenic PAHs. Moreover, co-eluting adduct fractions were present in smokers and non-smokers and we have also observed similar fractions in leucocytes of non-smokers, making it unlikely that the early eluting main adduct fractions would be related to PAHs. In fact such a common adduct could be of endogenous origin. Incidentally, 1,N^2 cyclic etheno- and propano-guanine derivatives formed by 4-hydroxynonenal derivatives and crotonaldehyde are known to be resistant to nuclease P1 (Hemminki *et al.* 1991a, b).

We describe here a new modification of the postlabelling method suitable for analysis and characterization of the abundant lipophilic adducts from human tissues. The two DNA adduct enrichment methods and the HPLC separation method used have a reasonable specificity, reproducibility and a high separation power, which should enable characterization of individual adducts once standard compounds are available. We believe that these modifications will greatly help efforts to quantitatively compare DNA adducts in human tissues and assess their role in carcinogenesis.

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Acknowledgement

The study was supported by the Royal Swedish Academy of Sciences.

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Received 28 April 1997, revised form accepted 23 June 1997

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