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Determination of albumin adducts of (+)-*anti*-benzo[*a*]pyrene-diol-epoxide using an high-performance liquid chromatographic column switching technique for sample preparation and gas chromatography–mass spectrometry for the final detection

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Abstract

A novel method has been developed for the determination of (+)-*anti*-benzo[*a*]pyrene-diol-epoxide [(+)-*anti*-BPDE] albumin adducts in the low-picogram range. Blood from rats and humans was investigated for the validation of the method. Instead of the usual acid hydrolysis we used alkaline conditions for the cleavage of the esters formed with asparagic or glutamic acid residues of albumin. Alkaline hydrolysis gave rise to benzo[*a*]pyrene-r-7,*t*-8,*t*-9,*c*-10-tetrahydrotetrol (BT I-1) which was separated from the matrix by HPLC with a column switching technique. The analytes were collected by an automated fraction collector and after silylation determined with GC–MS using negative chemical ionization. Adduct concentrations were calculated by the internal standard method. Benzo[*a*]pyrene-r-7,*t*-8,*c*-9,*c*-10-tetrahydrotetrol (BT-II-2) was used as an internal standard because of its similar physicochemical properties and its absence from human samples. To determine the recovery of the analytical procedure benzo[*a*]pyrene-r-7,*t*-8,*t*-9,*t*-10-tetrahydrotetrol (BT I-2) was added at the end of the sample clean-up. Single ion recording mode was applied for the detection of the analyte and the standards using the abundant fragment ion *m*/*z* 284 for quantitation of the three tetrols. The mean recovery of the internal standard BT II-2 was about 50%. The limit of detection was 0.15 pg per injection corresponding to 0.01 fmol/mg albumin. Regression coefficients of the calibration curves were r^2=0.99 and r^2=0.98 for BT I-1 concentration ranges of 4–400 ng/l and 4–40 ng/l, respectively. The mean coefficient of variation for duplicate analyses of human albumin samples was found to be 22%.
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1. Introduction

Benzo[*a*]pyrene (BaP) is a widely distributed environmental carcinogen primarily generated by incomplete combustion of organic materials. (+)-*anti*-BPDE is the ultimate carcinogen formed during the mammalian metabolism of benzo[*a*]pyrene [1]. Polycyclic aromatic hydrocarbons (PAHs) are chemically inert and not able to cause tumors. For excretion and detoxification, PAHs are metabolized by mixed-functional oxidases of the cytochrome P450 family [2]. The electrophilic intermediates, primarily epoxides, can bind covalently to cellular macromolecules such as DNA and proteins [3].

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Formation of DNA adducts is supposed to be a critical step in tumor initiation [4]. Therefore, the level of DNA adducts is a valuable parameter for the estimation of cancer risk. Since availability of DNA from target cells is limited, surrogate markers are used instead. Protein adducts have been found to correlate fairly well with DNA adducts [5] and thus can serve as markers for the biologically effective dose of carcinogens. Another advantage of measuring adducts of blood proteins instead of DNA adducts is that they are not subjected to repair. As a result, the adducts can accumulate over the lifetime of the protein. Human serum albumin is a plasma protein with a half-life of 21–25 days [6]. Albumin is synthesized in the liver and it serves as a transport vehicle of lipophilic compounds. (+)-*anti*-BPDE and (−)-*syn*-BPDE are reported to form esters with asparagic or glutamic acid residues of albumin which yield four different isomeric BaP-tetrahydrotetrols (Fig. 1) [7].

Alkaline hydrolysis of the adducts gives rise to the tetrahydrotetrol BT I-1 which is finally determined. Different methods have been published for the quantitation of BPDE–albumin adducts. The results reported are dependent on the method used and are highly variable. GC–MS methods with negative chemical ionization (NCI) turned out to be the most sensitive and selective detection method for the quantitation of the tetrahydrotetrols of benzo[*a*]pyrene [8,9].

We have developed a method using HPLC for sample clean-up and GC–NCI-MS for the final determination of the BaP-tetrols. Instead of the usual acid hydrolysis for the ester cleavage we used alkaline conditions, which significantly reduce the matrix-derived problems in the analytical procedure.

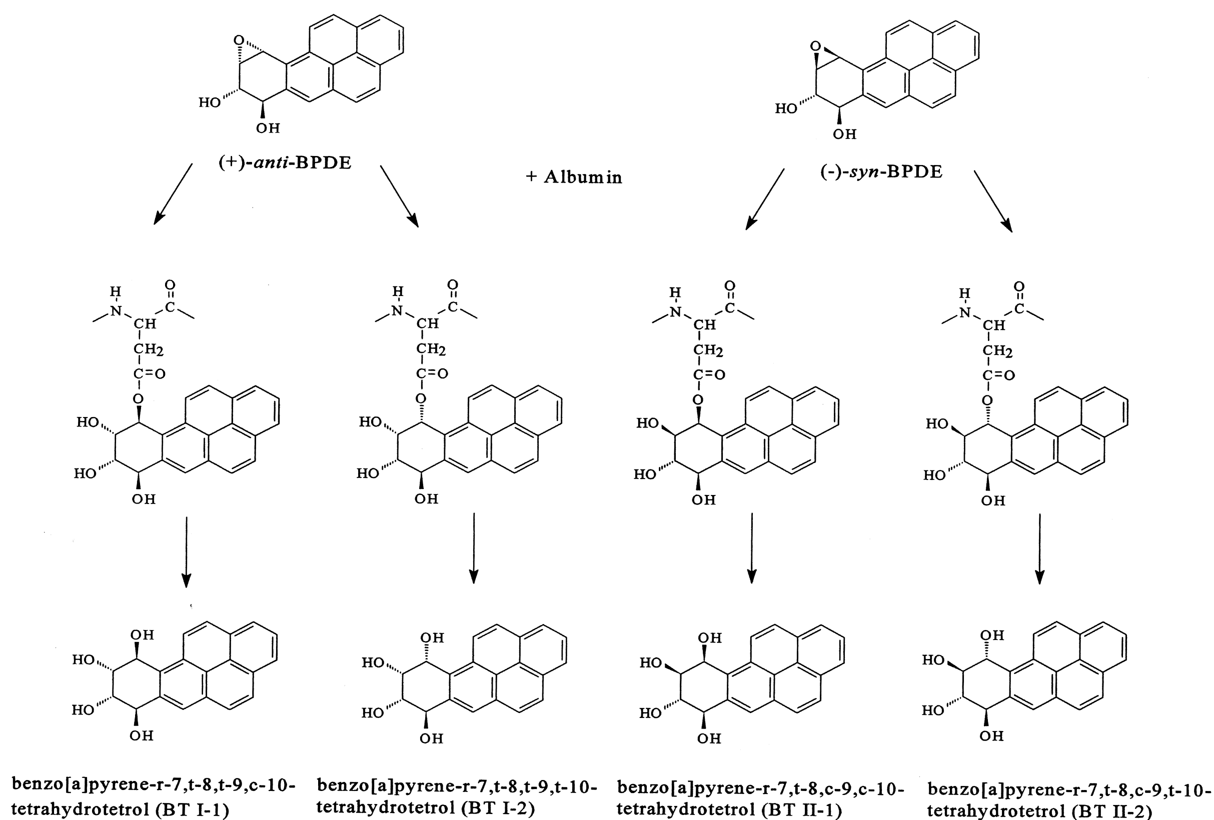

Fig. 1. Albumin ester adducts of (+)-*anti*- and (−)-*syn*-benzo[*a*]pyrene-diol-epoxide. Ester cleavage results in the formation of four isomeric tetrols.

Additionally, the detection limit was lowered considerably by the use of a programable temperature vaporizer injector (PTV).

2. Experimental

2.1. Chemicals

(±)-BaP-r-7,*t*-8,*t*-9,*c*-10-tetrahydrotetrol (BT I-1), (±)-BaP-r-7,*t*-8,*t*-9,*t*-10-tetrahydrotetrol (BT I- 2), (±)-BaP-r-7,*t*-8,*c*-9,*c*-10-tetrahydrotetrol (BT II-1) and (±)-BaP-r-7,*t*-8,*c*-9,*t*-10-tetrahydrotetrol (BT II-2) were purchased from the NCI Carcinogen Standard Repository (Midwest Research Institute, Kansas City, MO, USA). The chemical purity of the standards was >98% (HPLC). Pronase E (*Streptomyces griseus*, 7000 units/g) purchased from Boehringer (Mannheim, Germany) was used for enzymatic digestion. For solid-phase extraction (SPE) Bond Elut®-C_{18} cartridges (3 cm^3/500 mg, Varian, Harbour City, USA) were used. HPLC-grade methanol and water were obtained from Promochem (Wesel, Germany). Organic solvents for sample preparation were distilled before use. N,O-Bis(trimethylsilyl)-acetamide (BSA) with 5% trimethylchlorosilane (TMCS) was obtained from Fluka (Buchs, Switzerland). All reagents were of analytical grade.

2.2. Instrumentation

The HPLC system consisted of the model 1050 from Hewlett-Packard (gradient pump and autosampler, Waldbronn, Germany) an automated six-port switching valve from Valco (Switzerland), a second isocratic HPLC-pump (L-6000A) from Merck (Darmstadt, Germany), and a fluorescence detector (1046A) from Hewlett-Packard. The analytical column (Symmetry® C_{18}, 250×4.6 mm, 5 μm) from Waters (Eschborn, Germany) was placed in a heatable column oven. The enrichment column (LiChrospher® RP18-ADS, 20×4 mm) was obtained from Merck. The HPLC eluate was collected with an automated fraction collector (SuperFrac, Pharmacia Biotech, Uppsala, Sweden).

The gas chromatographic instrumentation consisted of the GC 8000 Top and MD 800 from CE Instruments (Egelsbach, Germany) equipped with a PTV injector. A DB 5MS (30 m×0.25 mm I.D., 0.25 μm film thickness) capillary GC column (J&W Scientific, Folsom, CA, USA) was interfaced directly with the MS source. Ultrahigh-purity methane (99.995%, Linde, Munich, Germany) was used as the reagent gas for negative chemical ionization.

2.3. Animal treatment

Two female Sprague–Dawley rats were treated with benzo[*a*]pyrene by intraperitoneal injection of 6.25 μmol/kg and 31.24 μmol/kg in corn oil (daily, five times per week over 4 weeks). An untreated rat was used as control sample. Blood was obtained 3 days after the last administration. Plasma and red blood cells were separated immediately after blood sampling and stored at −20°C.

2.4. Human blood samples

Blood from not occupationally exposed, healthy volunteers was drawn into EDTA-containing monovettes. Plasma was separated from red blood cells by centrifugation (3220 *g*, 15 min, 4°C) and kept frozen until analysis (−20°C).

2.5. Sample preparation

Albumin was precipitated from 10 ml plasma by adding 10 ml saturated ammonium sulfate solution and adjusting the pH to 4.5. After isolation by centrifugation (3220 *g*, 60 min, 4°C) the protein was solubilized in 10 ml of a buffer containing 10 m*M* Tris and 1 m*M* EDTA (pH 8.3). The albumin concentration was measured photometrically by the method of Pinnell and Northam (BCP kit, Sigma, Deisenhofen, Germany). An equivalent of 100–200 mg albumin together with 330 pg BT II-2 was digested overnight with 20 mg pronase (equivalent to 140 units) from *Streptomyces griseus* (final volume 20 ml). The BaP-related esters were cleaved under vacuum conditions (~20 mbar) at pH 11 and 80°C for 2 h. The resulting solution was applied to C_{18} SPE cartridges. The cartridges were washed with 10 ml distilled water to remove unbound material and dried by centrifugation (3220 *g*, 15 min, 10°C). The adsorbed analytes were eluted with 5 ml methanol. After evaporation by a rotary evaporator, the residue

was resuspended in 4 ml water and the solution was extracted twice with 2×4 ml ethyl acetate. The organic solvent was removed by a rotary evaporator and the dry residue was dissolved in 1 ml water–methanol (80:20; v/v) using a sonicator to ensure complete solution. Fig. 2 shows the scheme of the sample preparation steps.

2.6. HPLC separation and fraction collection

The total solution (1 ml) was injected into the HPLC system. For sample clean-up the analytes were loaded on an RP18-alkyl-diol-silica (RP18-ADS) enrichment column and flushed for 15 min with water–methanol (90:10, v/v) delivered by pump 1. Simultaneously the analytical column was equilibrated for the subsequent separation with pump 2 (Fig. 3A). By switching of the six-port valve the enrichment column was connected with the analytical column and the analytes were eluted in the back-flush mode (Fig. 3B).

The analytical separation was performed with a gradient consisting of a KH_2PO_4 buffer and methanol by increasing the methanol content from 45 to 72% (Table 1). The column temperature was 50°C and the flow-rates of pumps 1 and 2 were set at 1 ml/min. Fluorescence detection of the analytes was performed at an excitation wavelength of 246 nm and an emission wavelength of 390 nm. The fraction of the eluate containing the analyte and the internal standard was collected by an automated fraction collector.

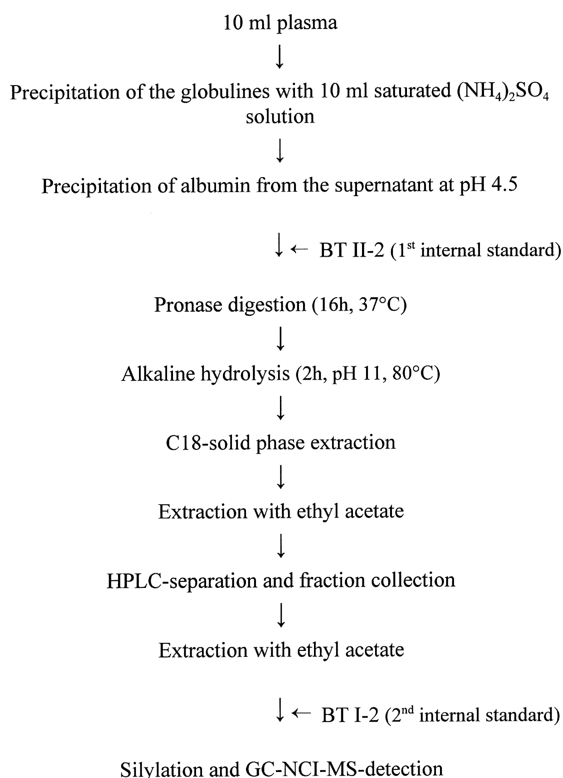

Fig. 2. Scheme of the sample preparation an analysis of the BPDE–albumin adducts

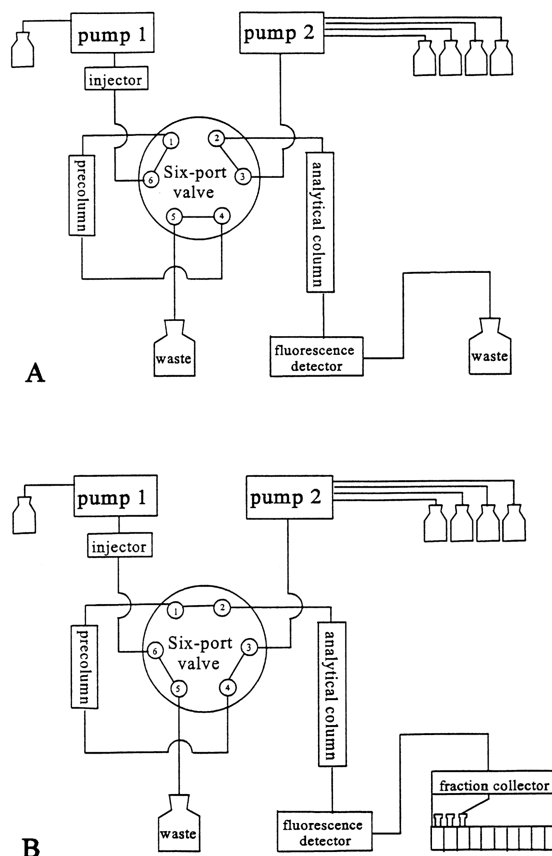

Fig. 3. Scheme of the HPLC system with coupled column technique; (A) loading of the enrichment column and equilibration of the analytical column (B) transfer of the analytes and fraction collection.

Table 1
Gradient program for the HPLC separation

Time (min)	Solvent A (%)	Position of switching valve	Procedure
0.0	45	A	Equilibration of the analytical column
14.9	45	A	
15.0	45	B	Transfer of the analytes
21.9	56	B	
22.0	56	A	Analytical separation
32.0	72	A	
32.1	100	A	
3.0	100	A	Flushing of the analytical column

Flow-rate pumps 1 and 2: 1 ml/min.
Solvent A: methanol; solvent B: 20 m*M* KH_2PO_4, pH 4.5
Injection volume: 1 ml.
Column temperature: 50°C.
Fluorescence detection: λ_{Ex}: 246 nm; λ_{Em}: 390 nm.

2.7. GC–MS analysis

The HPLC fraction (6 ml) containing the analyte and the internal standard BT II-2 was evaporated by a rotary evaporator to remove the methanol completely. The resulting aqueous solution (2 ml) was diluted with 4 ml water and extracted twice with 2×4 ml ethyl acetate. The combined organic phases were reduced to 0.5 ml by a rotary evaporator. 220 pg BT I-2 was added as the second internal standard to determine the recovery of the first internal standard BT II-2. The sample was dried under a stream of nitrogen and the analytes were derivatized with silylating agent by adding 10 μl BSA with 5% TMCS to the dry sample (room temperature, 15 min). Forty μl of hexane were added and 10 μl of the resulting solution were injected into the GC–MS. The derivatives were analysed using NCI with methane as a reagent gas. Selected ion recording mode (SIR) was used for quantitation.

The GC-oven temperature was held at 100°C for 3 min, followed by an increase to 320°C at a rate of 30°C/min. The temperature was kept at 320°C for 5 min. The PTV temperature was initially set at 50°C for 1 min and then raised to 300°C within 50 s. The carrier gas (He) had a constant flow-rate of 0.8 ml/min. MS conditions were as follows: ion source 200°C, interface 280°C, emission current 350 μA, electron energy 70 eV, dwell time 0.08 s, interchannel delay 0.02 s. The ions *m/z* 446.1 [M−$(CH_3)_3$Si-O-Si$(CH_3)_3$] and *m/z* 284 [M−2 $(CH_3)_3$Si-O-Si$(CH_3)_3$] were the most significant fragments of the analyte as well as of the first and second standard. For quantitation of the results the ion *m/z* 284 (relative abundance 46%) was used while the fragment ions at *m/z* 446.1 (relative abundance 30%) and *m/z* 447.1 (relative abundance 24%) served for confirmation. Adduct concentrations were calculated by a five-point calibration curve constructed by measuring freshly derivatized standards with a BT I-1 concentration range of 4–400 ng/l and a constant concentration of 6.6 μg/l BT II-2. Each standard was prepared singly and run in duplicate. Ten wash cycles of the autosampler syringe before each injection, and injection of 10 μl hexane after each standard sequence were run to exclude carry over from the system. Pure water was prepared parallel to the protein samples as control blanks.

3. Results and discussion

We found that the alkaline hydrolysis of the BPDE-ester adducts yields less HPLC background and therefore more reproducible results than acid hydrolysis. It may be that under acidic conditions peptide bonds are cleaved resulting in low-molecular-mass compounds which may interfere with the chromatographic procedure and simulate the presence of tetrols. This may probably also explain the higher adduct concentrations obtained with HPLC

and fluorescence detection after acid hydrolysis [10]. Nevertheless, HPLC sample clean-up was found to be absolutely necessary to obtain interpretable GC–MS results. Coupled column HPLC turned out to be a suitable method for this purpose with good reproducibility. This method allows the injection of relatively large sample volumes without loss of resolution in the analytical separation. In addition, the usage of RP18-ADS material as an enrichment column considerably prolonged the lifetime of the analytical column. The external surface of these spherical particles is covered with hydrophilic electroneutral diol groups protecting the sorbent from contamination by proteins. The internal hydrophobic surface (C_{18} alkyl chains) of the porous material is accessible for small sample constituents only. Thus, low-molecular-mass compounds can be effectively enriched and separated from the matrix [11].

The retention times of the analyte and the internal standard in the subsequent analytical separation were found to become more stable at a lower pH of the KH_2PO_4 buffer. A pH value of 4.5 proved to be the optimum for this purpose. The recovery of the online enrichment was found to be >95% for standard solutions. Fig. 4 shows a representative HPLC chromatogram of a human albumin sample.

The corresponding GC–NCI-MS chromatogram of the same albumin sample is shown in Fig. 5A. The adduct level is about 0.05 fmol/mg albumin. In human blood samples, only the (+)-*anti*-BPDE–albumin adducts was found. Female Sprague–Dawley rats treated with BaP exhibited albumin adducts of both (+)-*anti*-BPDE and (−)-*syn*-BPDE. Fig. 5B shows an albumin sample of a rat treated with 31.24 μmol/kg BaP. The main products formed are BT II-2 (about 400 fmol/mg albumin) and BT I-1 (about 80 fmol/mg albumin). BT I-2 and BT II-1 are formed to a lesser extent.

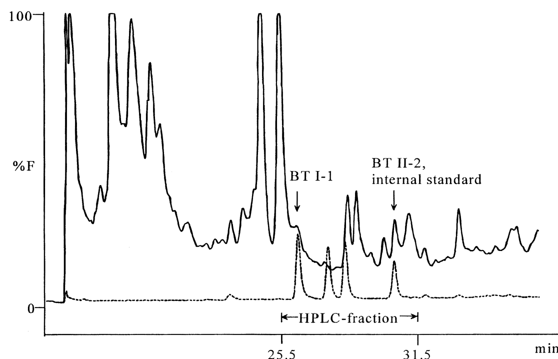

Fig. 4. HPLC chromatogram of a human albumin sample. For comparison a standard chromatogram showing the four BTs I-1, II-1, I-2 and II-2 was overlaid. The fraction eluting between 25.5 and 31.5 min was collected.

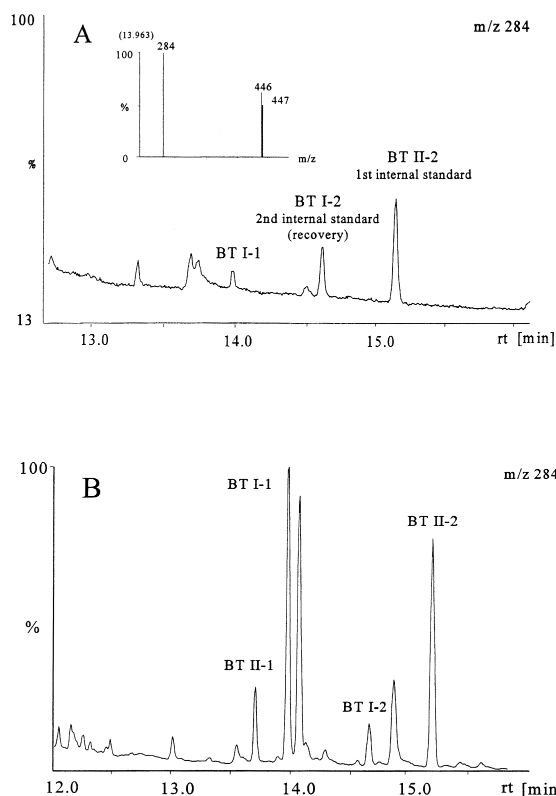

Fig. 5. (A) GC–NCI-MS-SIR chromatogram (*m/z* 284) of trimethylsilyl-derivatives of the human albumin sample shown in Fig. 4. The inset shows the fragmentation pattern with the ions *m/z* 284, 446.1 and 447.1. (B) GC–NCI-MS-SIR (*m/z* 284) chromatogram of albumin adducts isolated from a rat treated with benzo[*a*]pyrene. The rat sample was not spiked with standards. Unidentified peaks at 14.05 min and 14.90 min.

These results are in agreement with those obtained from mice treated with [^{3}H]BaP [12] .

The precision of the method and of single steps of the sample preparation have been determined. Multiple preparation of pool albumin spiked with 20 pg BT I-1 (n=8) showed a relative coefficient of variation of 17% including a relative error of 5% for the GC–NCI-MS detection. The extraction recovery

of the internal standard BT II-2 was 51±26%. It was calculated by setting the peak area of the second internal standard BT I-2 as 100% and comparing the ratio found in the sample with standard mixtures. The data obtained gave information about the efficiency of the sample work-up and were used for the validation of the method. The limit of detection was 0.15 pg per injection corresponding to 0.01 fmol/mg albumin. Regression coefficients of the calibration curves were r^2=0.99 and r^2=0.98 for BT I-1 concentration ranges of 4–400 ng/l and 4–40 ng/l, respectively.

We found detectable amounts of BT I-1 in 28 of 69 (41%) blood samples from volunteers, not occupationally exposed to PAH. The samples were run in duplicate with a mean coefficient of variation of 22%. Adduct levels ranged from below the detection limit (0.01 fmol/mg) to 0.25 fmol/mg albumin. The mean value was 0.026±0.047 fmol/mg albumin. Smokers (n=23) had significantly elevated adduct concentrations (0.045±0.060 fmol/mg) compared to nonsmokers (n=24; 0.015±0.040 fmol/mg) and passive smokers (n=22; 0.016±0.029 fmol/mg). Our findings are in fair agreement with those obtained for BPDE–hemoglobin adducts [9].

The reasons for the high adduct levels reported previously are probably the lack of selectivity of the antibody (ELISA) [13,14], and matrix-effects (HPLC) [10]. Compared to other published GC–NCI-MS methods [9,15], a higher sensitivity is achieved by the HPLC on-line enrichment with large volume injection. The time consuming fraction collection can be performed during the night hours, which significantly reduces the working time for sample preparation. Additionally, by the use of a PTV injector the limit of detection could be lowered by one order of magnitude.

In conclusion, we have developed a specific and sensitive method for the quantitation of benzo[*a*]pyrene-tetrahydrotetrols derived from BPDE–albumin adducts. The method has proven to be applicable to the determination of BPDE–albumin adduct levels of humans not occupationally exposed to PAH.

References

[1] D.M. Jerina, J.W. Daly, in: D.V. Parke, R.L. Smith (Editors), Taylor and Francis, London, 1977, p. 13.
[2] F.P. Guengerich, T. Shimada, Chem. Res. Toxicol. 4 (1991) 391.
[3] R.G. Harvey, N.E. Geacintov, Acc. Chem. Res. 21 (1988) 66.
[4] H. Autrup, R. Grafström, K. Vahakangas, C.C. Harris, Arch. Toxicol. 9 (1986) 147.
[5] H.G. Neumann, Arch. Toxicol. 56 (1984) 1.
[6] P.L. Skipper, S.R. Tannenbaum, Carcinogenesis 11 (1990) 507.
[7] B.W. Day, P.L. Zaia, K. Singh, S.R. Tannenbaum, Chem. Res. Toxicol. 7 (1994) 829.
[8] K. Taghizadeh, P.L. Skipper, Methods Enzymol. 231 (1994) 668.
[9] R. Pastorelli, J. Restano, M. Guanci, M. Maramonte, C. Magagnotti, R. Allevi, D. Lauri, R. Fanelli, L. Airoldi, Carcinogenesis 17 (1996) 2389.
[10] S. Tas, J.P. Buchet, R. Lauwerys, Int. Arch. Occup. Environ. Health 66 (1994) 343.
[11] K.-S. Boos, A. Rudolphi, S. Vielhauer, A. Walfort, D. Lubda, F. Einsenbeiß, Fresenius J. Anal. Chem. 352 (1995) 684.
[12] A.A. Melikian, P. Sun, S. Coleman, S. Amin, S.S. Hecht, Chem. Res. Toxicol. 9 (1996) 508.
[13] F.G. Crawford, J. Mayer, R.M. Santella, T.B. Cooper, R. Ottman, W.-Y. Tsai, G. Simon-Cereijido, M. Wang, D. Tang, F.P. Perera, J. Nat. Cancer Inst. 86 (1994) 1398.
[14] H. Autrup, A.B. Vestergaard, H. Okkels, Carcinogenesis 16 (1995) 1305.
[15] A.A. Melikian, S. Malpure, A. John, M. Meng, B. Schoket, S.S. Hecht, Proceedings of the 16th International Symposium of Polycyclic Aromatic Compounds, Charlotte, NC, November 1997.