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On-line preconcentration and high-performance liquid chromatographic determination of polycyclic aromatic hydrocarbon–DNA adducts using copper phthalocyanine trisulfonic acid as a group-specific adsorbent

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Abstract

An on-line preconcentration high-performance liquid chromatography system for the determination of polycyclic aromatic hydrocarbon–DNA adducts such as trihydroxytetrahydrobenzo[*a*]pyrene–deoxyguanotide in a DNA hydrolysate is described. Restricted-access copper phthalocyanine (CP) diol modified silica was used as a group-specific sorbent. For the determination of the benzo[*a*]pyrene adduct, two preconcentration columns (precolumns) in a dual-precolumn setup were used. Elution of the benzo[*a*]pyrene adduct from the CP precolumn was performed with 100% methanol. The analyte was re-concentrated on a C_{18} precolumn applying on-line dilution with water. The C_{18} precolumn can be desorbed with the LC mobile phase used for the analytical column. Trihydroxytetrahydrobenzo[*a*]pyrene–deoxyguanotide was determined in a DNA hydrolysate using the dual-precolumn procedure. A recovery of 95% was achieved. With fluorescence detection, a mass detection limit of 0.1 pmol injection was obtained.

Keywords: Sample preparation; Polynuclear aromatic hydrocarbons; DNA; Copper phthalocyanine trisulfonate; Benzo[*a*]-pyrene; Trihydroxybenzo[*a*]pyrene–deoxyguanosylphosphate

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) arise from the incomplete combustion of organic material. The main sources of PAHs are motorised traffic, industry, for example melting-furnaces and oil refineries, and tobacco smoke [1,2]. The incomplete combustion of organic material leads to many different PAHs. Each compound can be converted into different metabolites. Some of those metabolites, particularly the dihydrodiol epoxides, react with free amino groups of DNA nucleobases [3] to form PAH–DNA adducts.

Benzo[*a*]pyrene (B[*a*]P) is the most widely investigated compound [4–6]. B[*a*]P–DNA adducts [2,3] and B[*a*]P metabolites [7] were found in experimental animals exposed to B[*a*]P and are known carcinogens to these animals. The same B[*a*]P adducts were found in humans exposed to relatively high levels of PAHs, for example mine and

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foundry workers [8]. For this reason B[*a*]P is suspected to be carcinogenic to humans. Besides B[*a*]P, other PAHs are considered to be carcinogenic [8,9]. The analysis of PAH metabolites and, especially, PAH–DNA adducts as biomarkers for monitoring exposure of humans is therefore necessary.

The main problem for the analysis of PAH–DNA adducts is the very low level in human body fluids or tissue. Typical levels were found in tissue DNA of approximately 10 to 100 PAH–DNA adducts at 10^9 nucleotides [4,9], which means that a detection limit of 0.2 fmol PAH–DNA adduct in 10 μg DNA is necessary. A second problem is the biological matrix in which these compounds occur. For these reasons a group-specific enrichment procedure is required to preconcentrate different, relatively hydrophilic, PAH–DNA adducts and to remove biological matrix components simultaneously which may interfere with the analysis.

Hayatsu [10] introduced copper phthalocyanine (CP) trisulfonate linked to cellulose or cotton as a group-specific adsorbent for PAHs. CP trisulfonate forms hydrophobic face-to-face complexes in aqueous solutions with compounds which contain a planar structure with three or more fused aromatic rings [10]. The complexes formed are dissociated by organic solvents.

Boos et al. [11] and Lintelmann et al. [1] demonstrated the possibility of on-line preconcentration of 1-hydroxypyrene and several hydroxyphenanthrenes from urine using CP. The precolumn is packed with diol-modified porous-glass [11] or silica [1] chemically modified with CP trisulfonic acid. After the preconcentration of the PAH compounds, the column was switched on-line to the HPLC column and desorption was performed with the mobile phase used for the HPLC system. This system was also used for the determination of PAHs in surface water by Brouwer et al. [12].

In this article we describe a modified on-line preconcentration method, based on CP-silica as sorbent, which can be used for the HPLC determination of PAH–DNA adducts. Besides the group-specific preconcentration, the removal of biological matrix components such as nucleosides and enzymes used for the hydrolysis of DNA has been investigated. Trihydroxybenzo[*a*]pyrene–deoxyguanosylphosphate was used as the model compound. Monitoring of the B[*a*]P adduct was performed by fluorescence detection.

2. Experimental

2.1. Reagents

7R,8S,9S - Trihydroxy - *10S* - (N2 - deoxyguanosyl-3′ - phosphate) - 7,8,9,10 - tetrahydrobenzo[*a*]pyrene (B[*a*]P adduct) was purchased from the National Cancer Institute Chemical Carcinogen Reference Standard Repository (Kansas City, MO, USA). HPLC-grade acetonitrile and HPLC-grade methanol were obtained from Rathburn (Walkerburn, UK). A solution of rat liver DNA with a concentration of 0.29 μg/μl, containing 0.33 μg/μl micrococcal endonuclease and 0.33 μg/μl phosphodiesterase was provided by the Dutch Institute of Public Health and Environment (RIVM). The DNA was dissolved in 1.9 m*M* Tris, 20 m*M* sodium succinate and 10 m*M* calcium chloride and incubated for 4 h at 37°C to hydrolyze the DNA to nucleosides. All aqueous solutions were prepared with water purified with a Milli-Q system from Millipore (Bedford, MA, USA).

2.2. Equipment and analytical procedure

A coupled-column HPLC system with two precolumns, Fig. 1, was used for the on-line preconcentration and analysis of the B[*a*]P adduct. The HPLC

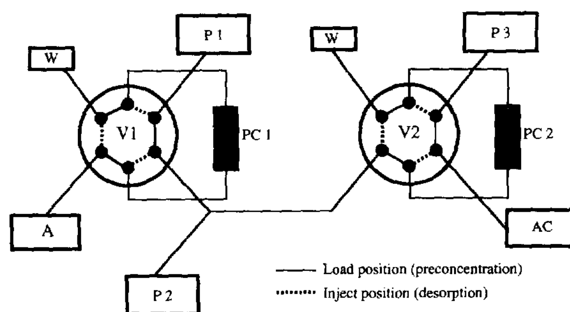

Fig. 1. Schematic representation of the dual-precolumn HPLC system. A=autosampler and dilutor, V1=first injection valve, V2=second injection valve, PC 1=CP precolumn, PC 2=C_{18} precolumn, P 1=Pump 1 (100% methanol, 0.1 ml/min), P 2=Pump 2 (water, 0.9 ml/min), P 3=Pump 3 (LC mobile phase), AC=analytical column, W=waste.

system consisted of three Spectroflow 400 pumps (Kratos, Rotterdam, Netherlands). Pump 1 was connected to a Gilson 231 autosampler with a 401 dilutor (Villiers-Le-Bel, France) and a 7010 six-port injection valve (Rheodyne, Cotati, CA, USA). The CP precolumn was connected to the autosampler injection valve. The CP precolumn (10×4 mm I.D.) was packed with CP trisulfonic acid modified silica (30 μm) which was a gift of Professor Boos (University of Munich, Germany). The silica particles were made of restricted-access material with a molecular mass cut-off of 15 000. Before sample introduction, the precolumn was equilibrated with 3 ml methanol–water (10:90, v/v) at 1 ml/min. The dilutor was used for sample loading (volume 20 μl) and for rinsing the CP precolumn after sample loading with 5 ml methanol–water (10:90, v/v) at 1 ml/min. After washing the CP precolumn to remove biological matrix components, the injection valve was switched and the analytes were desorbed with 1 ml 100% methanol at 0.1 ml/min delivered by pump 1. Directly after desorption, the sample was diluted on-line with water delivered by pump 2 (0.9 ml/min) and re-concentrated for 10 min on the second precolumn (15×4 mm I.D.) which was packed with LiChrosorb ADS C_{18} bonded silica (25 μm, M_r cut-off 15 000; Merck Darmstadt, Germany). A multiport streamswitch (Spark Holland, Emmen, Netherlands) was applied for switching the C_{18} precolumn on-line with the analytical column. Methanol–water (55:45, v/v) delivered by pump 3 at 1 ml/min was used as LC mobile phase. Desorption of the analytes with the LC mobile phase from the C_{18} precolumn to analytical column was performed in a back-flush direction to avoid peak-broadening. Table 1 gives the time schedule of the procedure. The system simultaneously is capable to preconcentrate a sample on the CP precolumn during desorption from the C_{18} precolumn and LC analysis of a previous one.

The analytical column, Nucleosil 120-5 C_{18}, 5 μm, 250×4.6 mm I.D., was purchased from Machery–Nagel (Düren, Germany). Detection was performed with a Jasco (Tokyo, Japan) FP920 fluorescence detector at 339 nm excitation and 377 nm emmision. For detection of the nucleosides and the DNA hydrolysate at 254 nm a Gilson 119 UV detector was used. Data were recorded with a Axxi-Chrom 727 Chromatography Data system (Axxiom Chromatography, Calabasas, CA, USA).

3. Results and discussion

3.1. Retention of the B[a]P adduct on CP-silica

Two different coupled-column HPLC systems were used for the on-line preconcentration and analysis of the B[*a*]P adduct. Our first approach was a single-precolumn HPLC system by preconcentra-

Table 1
Timetable for the dual-precolumn analysis

Time (min)	CP precolumn position	C_{18} precolumn position	Comment
0		Inject	Desorb sample from C_{18} precolumn with LC mobile phase and analyse
1	Load		Equilibrate CP precolumn with 3 ml methanol–water (10:90, v/v).
4			Inject new sample on CP precolumn
5			Rinse CP precolumn with 5 ml methanol–water (10:90, v/v)
13		Load	Equilibrate C_{18} precolumn with 3 ml methanol–water (10:90, v/v)
16	Inject		Desorb CP precolumn with 100% methanol and re-concentrate analyte on C_{18} precolumn
26			End run

ting the analyte on the restricted-access CP precolumn. CP-silica, 30 μm, are particles with a pore diameter of approximately 6 nm [1,11]. CP is attached to the internal surface of the silica particles. The outer surface of the silica particles is modified with diol groups [13]. The advantage of the use of restricted-access material is that besides the specific adsorption of PAH compounds by CP-silica within the particles, the particles also have a size exclusion function which can be used to eliminate high-molecular-mass compounds such as enzymes. After preconcentration, the CP precolumn was switched online to the analytical column. The analyte was instantaneously desorbed from the CP precolumn, with the LC mobile phase, to the analytical column and analysed.

While the single-precolumn set-up works satisfactorily with PAHs and non-ionic metabolites (data not shown), problems occurred when the negatively charged PAH–DNA adducts were to be analyzed. The recovery of the B[*a*]P adduct when desorbed with methanol–water (55:45, v/v) was approximately 55%. Another problem was that the variation of the recovery was rather high. The recovery ranged from 40 to 68%. The low and irreproducible recovery of the B[*a*]P adduct was caused by slow desorption kinetics of the B[*a*]P adduct from the CP precolumn.

To improve desorption, the percentage methanol in the LC mobile phase was increased to methanol–water (65:35, v/v). This resulted in a recovery of 65% (n=2). However, the analysis of the B[*a*]P adduct when using more than 65% (v/v) methanol in water as LC mobile phase was not possible on the analytical column because the B[*a*]P adduct elutes unretained. The affinity of the B[*a*]P adduct to the CP precolumn was higher than the affinity to the analytical column.

For these reasons, a dual-precolumn system as shown earlier for the combination of an immunoaffinity and a C_{18} precolumn by Farjam et al. [14] was used. Desorption of analytes from the CP precolumn to the C_{18} analytical column was performed in two steps. First, elution of all compounds retained on the CP precolumn is achieved by flushing with 1 ml 100% methanol at 0.1 ml/min. Then water is added via a mixing union at a ratio 9:1. A 10-ml volume of the resulting solution containing methanol–water (10:90, v/v) is re-concentrated on the C_{18} precolumn, which serves as an interface between the CP precolumn and the C_{18} analytical column. Analytes retained on the C_{18} precolumn are eluted to the analytical column using the LC mobile phase.

Fig. 2 shows the chromatograms obtained by direct injection of the B[*a*]P adduct, after preconcentration of the B[*a*]P adduct with the single-precolumn setup and with the dual-precolumn setup. The improvement of the recovery when the dual-precolumn setup was applied is significant, the recovery of the B[*a*]P adduct compared to direct injection being 95% (relative standard deviation is 1%, n=11).

3.2. Analytical data

Validation of the dual-precolumn setup provides a calibration curve for the B[*a*]P adduct with a concentration range from $4.60 \cdot 10^{-9}$ to $1.15 \cdot 10^{-7}$ *M*. The calibration plot was characterised by $y=4.12 \cdot 10^{11}x+14.25$, $r^2=0.999$ (n=4). When storing the B[*a*]P adduct at −20°C, the day-to-day variation was within 1.5% (n=3). The absolute detection limit for

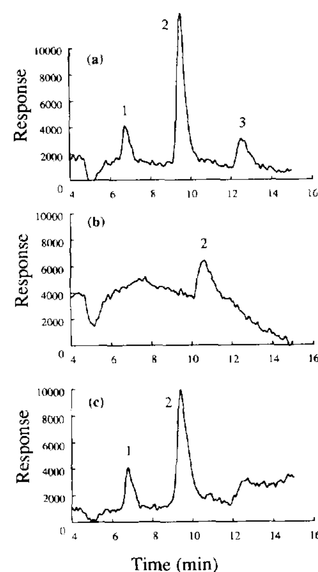

Fig. 2. Chromatograms of the B[*a*]P adduct ($2 \cdot 10^{-8}$ *M*) obtained by direct injection (a) or preconcentration with the single (b) or dual-precolumn setup (c), performed by fluorescence detection. 1: unknown, 2: B[*a*]P adduct, 3: unknown. For chromatographic conditions, see text.

the B[a]P adduct was 0.1 pmol ($S/N=3$). No breakthrough of the B[a]P adduct through the CP nor the C_{18} precolumn was observed for washing volumes up to 30 ml methanol–water (10:90, v/v).

3.3. Elimination of nucleosides

Besides the preconcentration of PAH–DNA adducts by the CP precolumn, the removal of the biological matrix components is an important element of the present procedure. Both enzymes used for hydrolysis and unmodified nucleosides are present in a high excess and may interfere with the determination of PAH–DNA adducts. For characteristic samples approximately 10 to 100 PAH–DNA adducts at 10^9 unmodified nucleosides are found [9,15]. To examine the selectivity of the CP precolumn, the elimination of adenosine, guanosine and 2-deoxyadenosine was investigated with the single-precolumn system. Water was used as a preconcentration solvent and methanol–water (20:80, v/v) as LC mobile phase and as desorption solvent. The nucleosides were monitored by UV detection.

The three nucleosides investigated, adenosine, guanosine and 2-deoxyadenosine, were not retained on the CP precolumn even when high concentrations up to 10^{-4} M were injected. However when high concentrations are used, it is necessary to apply a washing volume of at least 5 ml water to flush the nucleosides completely through the CP precolumn.

Adenosine was also analysed with the dual-precolumn system. Methanol–water (30:70, v/v) was applied to desorb the C_{18} precolumn and as LC mobile phase. A total amount of 0.5 mg was injected. When rinsing the CP precolumn with 30 ml methanol–water (10:90, v/v) and preconcentrating the adenosine on the C_{18} precolumn for 10 min, only 0.5 ng was detected. The remaining adenosine can be easily separated from the PAH–DNA adducts on the analytical column when methanol–water (55:45, v/v) was used as LC mobile phase.

3.4. Preconcentration of the B[a]P adduct from DNA hydrolysate

To study the applicability of the present method for biological matrixes, DNA hydrolysate was spiked with the B[a]P adduct in a ratio of approximately one B[a]P adduct at 40 000 nucleosides. The sample consists of $1.1 \cdot 10^{-8}$ M PAH–DNA adduct and approximately $4.5 \cdot 10^{-4}$ M nucleosides. Besides nucleosides, the hydrolysate contained the enzymes needed for the hydrolysis. A 20-μl aliquot of this sample was injected without any further sample preparation. Chromatograms representing the injections of the B[a]P adduct in water and the B[a]P adduct in hydrolysate are shown in Fig. 3. The recovery of the B[a]P adduct from the DNA hydrolysate was 97% (relative standard deviation is 3%, $n=4$). Although the ratio of B[a]P adduct to nucleosides is rather high, the total amount of DNA hydrolysate injected is normal for real samples. However, for a more realistic ratio of B[a]P adduct to nucleosides, the detection limit has to be improved.

Besides fluorescence detection to monitor the B[a]P adduct, UV detection was performed also (Fig. 4) to monitor the hydrolysate. In addition to the chromatograms of the preconcentration of the B[a]P adduct in water and in hydrolysate, also a UV chromatogram of a direct injection of the B[a]P adduct in hydrolysate is shown. The direct injection of the B[a]P adduct in hydrolysate resulted in a large disturbance of the baseline, caused by the biological matrix. The UV chromatograms demonstrate that the present preconcentration procedure can be used to eliminate the UV-absorbing compounds of the bio-

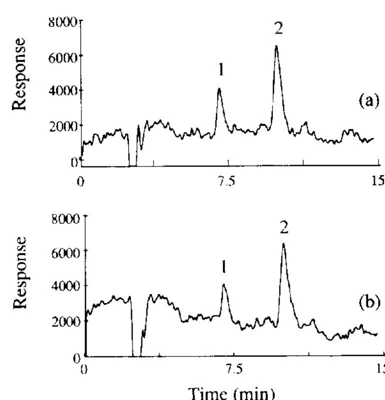

Fig. 3. Chromatograms of the B[a]P adduct ($1.1 \cdot 10^{-8}$ M) dissolved in water (a) or DNA hydrolysate ($4.5 \cdot 10^{-4}$ M) (b) obtained by preconcentration with the dual-precolumn setup, performed by fluorescence detection. 1: unknown, 2: B[a]P adduct. For chromatographic conditions, see text.

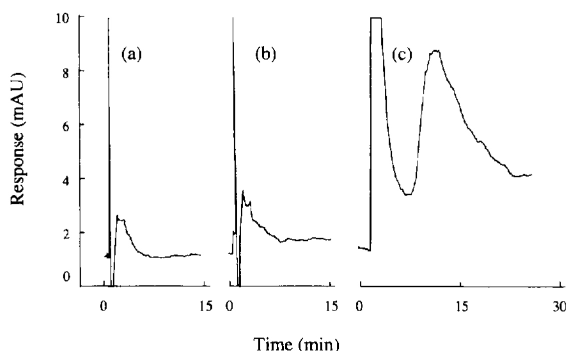

Fig. 4. Chromatograms of the B[*a*]P adduct ($1.1\cdot10^{-8}$ *M*) dissolved in water (a) or DNA hydrolysate ($4.5\cdot10^{-4}$ *M*) (b) obtained by preconcentration with the dual-precolumns and direct injection of the B[*a*]P adduct ($1.1\cdot10^{-8}$ *M*) dissolved in DNA hydrolysate ($4.5\cdot10^{-4}$ *M*) (c), performed by UV detection. For chromatographic conditions, see text.

logical matrix in which PAH–DNA adducts have to be measured.

4. Conclusions

CP trisulfonic acid is a highly selective sorbent for PAH compounds. A dual-precolumn setup was applied for the determination of the B[*a*]P adduct. The fully automated on-line preconcentration procedure described above can preconcentrate B[*a*]P adducts from a biological matrix without any additional sample preparation. Besides the B[*a*]P adduct, other PAH derivatives are expected to adsorb on the CP precolumn if they contain at least 3 aromatic fused rings. However, the detection limits achieved with a normal fluorescence detector need an improvement of a factor of 500 to measure B[*a*]P adducts at the level normally found in tissue. Mass spectrometry or laser induced fluorescence detection may be helpful for this.

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