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Preliminary experiments for the analysis of tetrahydrotetrols of benz[*a*]anthracene and benzo[*a*]pyrene derived from their hemoglobin adducts using a coupled-column high-performance liquid chromatographic system

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Abstract

Improved technologies for the detection of polycyclic aromatic hydrocarbon adducts are required for human biomonitoring. Therefore, a coupled-column high-performance liquid chromatographic method, with system-integrated sample processing, has been developed and its applicability for determination of tetrahydrotetrols of polycyclic aromatic hydrocarbons in acid hydrolysates of human hemoglobin has been investigated. A novel column-switching technique applying 'thermotransfer' is used to separate tetrahydrotetrols of benzo[*a*]pyrene and benz[*a*]anthracene more efficiently. Derivatives of polycyclic aromatic hydrocarbons from blood hydrolysates are concentrated on a pre-column and then transferred to the analytical column by applying an electrical current to heat the solvent eluting the pre-column. This method allows for rapid and quantitative transfer of the analytes from the pre-column to the analytical column, after HPLC-integrated sample processing.

Keywords: Column switching; Benz[*a*]anthracene; Benzo[*a*]pyrene; Polynuclear aromatic hydrocarbons; Tetrahydrotetrols; Haemoglobin

1. Introduction

Benzo[*a*]pyrene (BP) and benz[*a*]anthracene (BA) are two important carcinogenic polycyclic aromatic hydrocarbons (PAHs). After absorption by the human body through inhalation, ingestion or dermal permeation, PAHs undergo metabolic activation via the action of cytochromes P-450 isozymes and epoxide hydrolase. This metabolic activation results in the formation of diol-epoxides. These activated electrophiles subsequently form adducts with biological macromolecules (DNA bases and amino acids) [1,2]. Though the extent of formation of carcinogen–macromolecular adducts is ultimately dependent on external exposure to PAHs, the level of adduct formation varies greatly among the human population even when external exposure is similar. This interindividual variation in adduct formation presumably results from a complex combination of genetic polymorphisms, and induced and inhibited states of the enzymes responsible for metabolism of the parent hydrocarbons and procarcinogenic inter-

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mediates [3]. Detection of these adducts, biomonitoring or biological effect monitoring, therefore allows determination of activated carcinogens at the molecular level (biological effective dose). Thus, both the external dose and the individual metabolic activation is integrated in this measure [4].

DNA adducts usually constitute mutagenic events, whereas protein adducts can serve as biological markers, integrating the biological effective dose over the lifetime of the protein (e.g. hemoglobin 120 days), since there is no repair mechanism for protein adducts [5].

For the analysis of benzo[*a*]pyrene–hemoglobin adducts several methods have been reported. These methods use either high-performance liquid chromatography (HPLC) with on-line fluorescence detection [6], off-line synchronous fluorescence spectroscopy [7,8] or gas chromatography–mass spectrometry (GC–MS) [7,9] to determine tetrahydrotetrols derived by either enzymatic or acid hydrolysis. Extraction and enrichment of the tetrahydrotetrols from the biological matrix has usually been achieved by liquid–liquid extraction [7,9]; however, this procedure is time-consuming and often causes problems through generation of emulsions. A very effective way for sample concentration and purification is immunoaffinity chromatography, but this approach is laborious and requires development of antibodies [10,11]. For GC or GC–MS the analytes have to be further altered; trimethylsilyl derivatives have been used. In contrast, for HPLC there is no need for further derivatization, but the reported methods have not achieved the resolution and sensitivity of GC–MS.

In general, the major disadvantage of the previously reported methods is the need for multiple manual, time-consuming and inefficient procedures. To overcome these problems a coupled-column HPLC method with integrated sample processing on a selective pre-column for the determination of BA- and BP-tetrahydrotetrols in blood has been developed.

2. Experimental

2.1. Chemicals

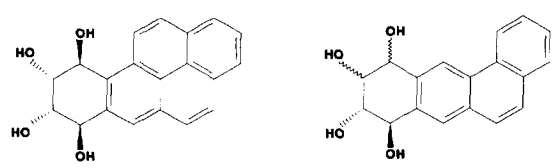
(±)-r-7,t-8,t-9,c-10-Tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene and benz[*a*]anthracene-*trans*-8,9-dihydrodiol-10,11-epoxide (*anti*) were obtained from the NCI Carcinogen Standard Repository (Midwest Research Institute, Kansas City, MO, USA). 8,9,10,11-Tetrahydroxy-8,9,10,11-tetrahydrobenz[*a*]-anthracene (BA-tetrahydrotetrol) was obtained by hydrolysis of benz[*a*]anthracene-*trans*-8,9-dihydrodiol-10,11-epoxide (*anti*) with acidified water (pH 3). Chemical structures of the tetrahydrotetrols of BA and BP are shown in Fig. 1. HPLC-grade methanol was obtained from Merck (Darmstadt, Germany). HPLC-grade water was generated using a Milli-Q deionization system (Millipore, Eschborn, Germany). All other chemicals were of the highest purity available.

2.2. Instrumentation

The HPLC system consisted of a gradient pump (L-6200) with interface (D-6000) and an UV detector (655A) from Merck, an automated six-port switching valve from Valco (Switzerland), a second pump (Model 590) from Waters (Eschborn, Germany) and a fluorescence detector LS-4 (Perkin-Elmer, Rockville, MD, USA). The analytical column (LiChrospher 100 RP-18 endcapped, 5 μm, 250×4 mm) from Merck was placed in a Peltier cooled column thermostat (Walfort and Partner, Reinhardshagen, Germany). The pre-column (copper phthalocyanine-modified polymer, 20×4 mm I.D.) was a BioAdduc available from Walfort and Partner. The electric power supply Voltcraft TNG 235 was obtained from Conrad (Hirschau, Germany), 2×2.5 A max.

2.3. Blood samples and tetrahydrotetrol isolation

Blood samples (4 ml), obtained from human volunteer donors, were centrifuged (3000 *g* for 10 min) and the pellet was washed three times with



A B

Fig. 1. Structures of (±)-r-7,t-8,t-9,C-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (A) and 8,9,10,11-tetrahydroxy-8,9,10,11-tetrahydrobenz[*a*]anthracene (B).

physiological saline (2 ml, 0.9%). Cell lysis was achieved by addition of water (7 ml) to the pellet. The solution was centrifuged (12 000 *g*, 25 min) to remove cell debris. The resulting supernatant containing hemoglobin was retained. For method development authentic standard tetrahydrotetrols were added to the hemoglobin. The samples were hydrolyzed with HCl (0.1 *M*, 1 h, 80°C) [7]. After centrifugation (3000 *g*, 10 min) an aliquot (5 ml) of the supernatant was injected onto the HPLC system.

2.4. Chromatography

The HPLC system (Fig. 2) consisted of a conventional gradient pump system, with the following components added to facilitate integrated sample processing of the biological materials: an additional pump (P1) with an additional injection valve (IV1) for sample-injection on a pre-column, filled with a special packing material showing high selectivity for the analytes. These additional components were connected to the basic HPLC system through an automated six-port switching valve, allowing for programmed connection and disconnection of pre-column and analytical column. The injection valve (IV2) next to the gradient pump (P2) allows direct injection of standards onto the analytical column with the switching valve in the 'load' position for determination of recoveries on the pre-column.

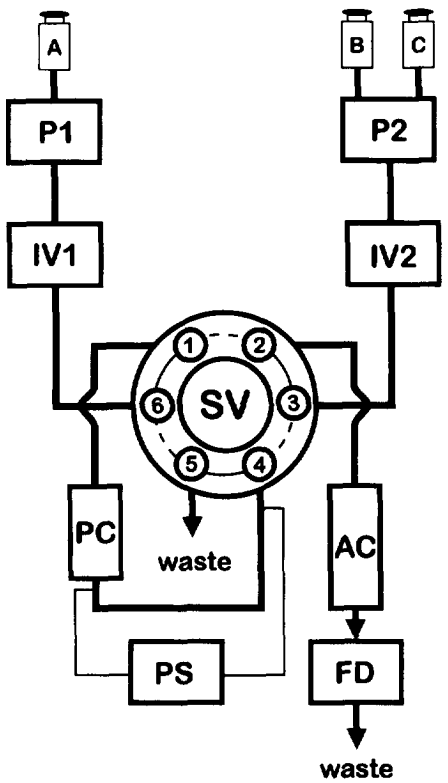


Fig. 2. Module configuration and switching-valve positions: (A, B) water; (C) methanol; P1=pump; P2=gradient pump; IV=injection valves; SV=switching valve; PC=pre-column; AC=analytical column; FD=fluorescence detector; PS=power supply for heating the capillary; solid line=flow in switching valve position 'load'; dashed line=flow in switching valve position 'inject'.

In order to achieve complete elution of the analytes from the pre-column during transfer, electrical power was applied in the final set-up to the capillary between the switching valve (position 4) and the pre-column. Because of its ohm-resistance the stainless-steel capillary is momentarily heated by applying electrical power, thereby heating the eluent passing through it.

For detection of the analytes their natural fluorescence was used: for BA-tetrahydrotetrol λ_{ex}=333 nm, λ_{em} 371 nm; for BP-tetrahydrotetrol λ_{ex}=341 nm, λ_{em}=400 nm.

Both tetrahydrotetrols, *anti*-BA and *anti*-BP, exist in two diastereomers each, with the hydroxyl-group in position 11 (BA) or 10 (BP) in *cis* or *trans* position (with respect to the hydroxyl group in position 7). But during acid hydrolysis one of the two stereoisomers epimerizes to the more stable one. In case of BP-tetrahydrotetrol, the diastereomer with the hydroxyl-group in position 10 in *trans* position (benzo[*a*]pyrene-r-7,t-8,t-9,t-10-tetrahydrotetrol) epimerizes almost quantitatively into the stable isomer with the hydroxyl-group in position 10 in *cis*-position (benzo[*a*]pyrene-r-7,t-8,t-9,c-10-tetrahydrotetrol) [12]. Thus only one of the diastereomers is detected after acid hydrolysis.

Eluent composition, switching valve position and status of the electrical current are given in Table 1.

3. Results and discussion

Coupled-column chromatography for determination of PAH-metabolites in human body fluids has

Table 1
Time table for coupled-column HPLC analysis of tetrahydrotetrols of BA and BP

Time (min)	B (%)	C (%)	Position switching valve	Electrical power	Procedure
0	60	40	load	off	Sample processing and equilibration of the analytical column
18	60	40	inject	on	Transfer of the analytes
23	60	40	load	off	Analytical separation
43	20	80	load	off	
43.1	0	100	inject	off	Clean-up
48	0	100	load	off	
48.1	60	40	load	off	Recondition

Flow-rate pump 1 is 0.5 ml/min with eluent A, pump 2 0.7 ml/min with gradient eluent B and C.
A, B=water; C=methanol.
Injection volume: 5 ml biological sample.
Column temperature: 15°C.
Fluorescence detection: BA-tetrahydrotetrol, λ_{ex}=333 nm, λ_{em}=371 nm; BP-tetrahydrotetrol, λ_{ex}=341 nm, λ_{em}=400 nm.

previously been developed [13,14]. Therefore it is not described in detail here, only the significant modifications are given. These modifications address the large difference in chromatographic behavior of the analytes of interest (BP-tetrahydrotetrol and BA-tetrahydrotetrol).

One difference from the original approach is the use of a polymer-based instead of the silica-based pre-column packing material. This was necessary to achieve adequate retention of BA-tetrahydrotetrol on the pre-column. Though it is stated [13,15–19] that sorbents of the copper phthalocyanine trisulfonate type selectively retain compounds with a structure of at least three fused planar rings from aqueous solutions, poor results for BA-tetrahydrotetrol were obtained. This was probably due to the presence of four hydroxyl-groups at the non-bay/non-K region of BA-tetrahydrotetrol, which somehow affect the effective hydrophobic and steric interactions between the pre-column packing material and BA-tetrahydrotetrol, leading to decreased interaction. However, good retention of the tetrahydrotetrols of both BP and BA using a polymer-based pre-column packing material were obtained. This effect may be due to greater hydrophobicity of the polymeric solid phase, leading to increased non-specific interaction between the analyte and pre-column packing material. Alternatively, the increased amount of copper phthalocyanine trisulfonate ligand on the polymer surface compared to the silica support may cause this effect [20].

3.1. Sample processing

After injection of the hydrolyzed hemoglobin sample into IV 1, with the switching valve in the 'load' position (flow through valve positions 6, 1, 4, 5), the analytes of interest are selectively retained on the pre-column. Unwanted biological matrix and other extraneous materials are eluted to waste (HPLC-integrated sample processing). The complete elution of the matrix is monitored using a UV detector at the effluent of the pre-column. During this step the analytical column is equilibrated (P2).

3.2. Transfer

Following sample processing the switching valve is rotated to the 'inject' position (flow through valve positions 3, 4, 1, 2) and the analytes are transferred to the now connected analytical column. Elution of the tetrahydrotetrols from the pre-column is achieved when the steric and hydrophobic interactions between the pre-column packing material and the analytes are abolished. Normally this is achieved by solely increasing the methanol content of the eluent (methanol–water) delivered to the pre-column [13,14]. But the methanol content is crucial to both automated sample processing and analytical separation. Quantitative elution of the analytes from the pre-column after sample processing is usually achieved with 60% methanol. However, subsequent

separation of the analytes on the analytical column requires a water–methanol gradient to extend from 40 to 100% to achieve a good separation of the substances of interest.

Using three different instrumental set-ups, the influence of the temperature on the extent of interactions between the pre-column stationary phase and the tetrahydrotetrols was investigated to determine conditions for quantitative elution of the analytes from the pre-column with a low methanol content of 40%.

(1) Table 2 shows the recoveries of the tetrahydrotetrols on the pre-column at different temperatures, maintaining all other variables constant. In the first set-up examined the whole pre-column was heated in a waterbath. With increasing temperatures the recovery of BP-tetrahydrotetrol improves, whereas it decreases for BA-tetrahydrotetrol (Table 2, 25–60°C). This can be explained by a reduction of the interactions between the pre-column packing material and the analytes through increased temperature. This reduction has two different effects for the tetrahydrotetrols. On the one hand it allows a quantitative elution of the more hydrophobic BP-tetrahydrotetrol from the pre-column during the transfer step even with a low methanol content. Alternatively it reduces the already poor retention of the more polar BA-tetrahydrotetrol on the pre-column, leading to a loss of this compound during sample processing before analyte transfer. Therefore, the preferable system would include a cold pre-column during sample processing (quantitative retention of BA-tetrahydrotetrol) and a hot pre-column for the transfer step (quantitative elution for BP-tetrahydrotetrol).

Table 2
Recoveries of tetrahydrotetrols of BA and BP on the pre-column using different set-ups and different temperatures

	BA-tetrahydrotetrol (%)	BP-tetrahydrotetrol (%)
With entire pre-column heated in a waterbath with temperature:		
25°C	68.2	67.0
40°C	66.1	91.8
50°C	53.2	95.8
60°C	38.1	96.2
With only the capillary heated; temperature waterbath:		
90°C	77.9	86.0
99°C	76.2	89.9
Thermotransfer using electric power		
	96.2	100.0

BA=benz[*a*]anthracene; BP=benzo[*a*]pyrene. The recoveries were calculated comparing the peak areas of the peaks obtained after direct analysis of a standard on the analytical column (valve position 'load') with the peak areas resulting of a coupled-column analysis with HPLC-integrated sample processing on the pre-column.

(2) Only the capillary leading from the switching valve (position 4) to the pre-column is heated. Thus the pre-column is at ambient temperature during the sample processing (solvent flow from switching valve position 1 to the pre-column), preventing loss of BA-tetrahydrotetrol. During transfer, however, with the switching valve in the 'inject' position, the direction of the flow is inverted (from switching-valve position 4 to the pre-column), resulting in heated solvent entering the pre-column and eluting the tetrahydrotetrols to a higher extent (Table 2, 90°C and 99°C). Thus by heating of just this particular capillary the elution power of the low-methanol eluent (even with the low methanol content of 40%) can be selectively increased during transfer without affecting the retention of the analytes during sample processing.

(3) An electric current was used for heating the capillary, to obviate the inconvenience of placing a boiling waterbath proximal to the HPLC equipment and to obtain even higher recoveries (Table 2, thermotransfer). Electric power was applied to the capillary (between switching-valve position 4 and the pre-column) during transfer step (switching valve in 'inject' position), while the methanol mixture (40%) is flowing through this capillary into the pre-column. Because of its ohm-resistance the capillary and therefore the solvent is momentarily heated, avoiding slow convection of the heat from the waterbath through the capillary to the solvent as in the previous set-up. This results in a very sharp elution peak of the analytes, which are then transferred to the analytical column for separation and detection. The principal idea and the precise set-up of the electrical power supply is described in Ref. [21].

Through a self-constructed device the application of the electrical power can now be regulated by the gradient pump via a time program, just like the switching valve, making the system even more convenient.

3.3. Analytical separation

Different RP-18 columns were tested in order to find the one best suited: good separation of the analytes must be achieved with a reasonable peak shape. In addition the eluent composition for this particular column must be in accordance with the conditions needed for the transfer step. After testing several columns the one found to be most suited was an endcapped RP-18, 5 μm. With this column the influence of various parameters on the separation was investigated (gradient composition, column temperature and flow-rate). The best separation is achieved using a methanol–water gradient with a flow-rate of 0.7 ml/min at a column temperature of 15°C (Fig. 3).

3.4. Cleaning and reconditioning

In order to clean pre-column and analytical column from even very hydrophobic substances, both are eluted with pure methanol with the valve in the 'inject' position. Afterwards both columns are equilibrated for the next chromatographic run.

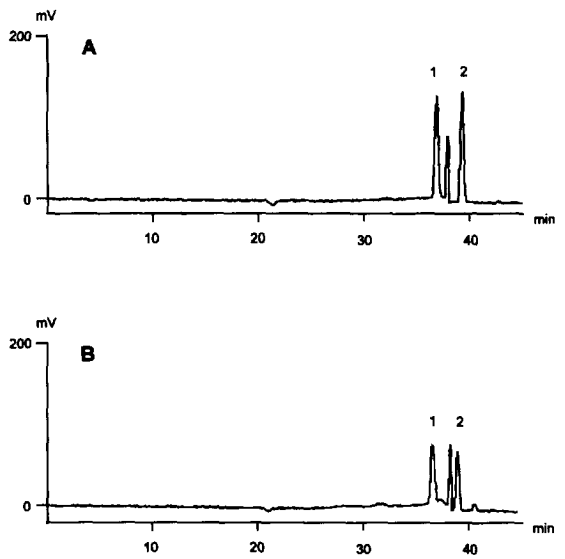


Fig. 3. Coupled-column HPLC analysis of tetrahydrotetrols of benz[a]anthracene (1) and benzo[a]pyrene (2). (A) Standard (43 ng/ml BP-tetrahydrotetrol; 6.25 μg/ml BA-tetrahydrotetrol; injection volume, 20 μl). (B) Spiked hemoglobin sample [9 ml hemoglobin solution are spiked with 50 μl standard (43 ng/ml BP-tetrahydrotetrol; 6.25 μg/ml BA-tetrahydrotetrol); injection volume, 5 ml]. The sharp signal between peak 1 and 2 is due to shifting of the wavelength of the fluorescence detector. For chromatographic conditions see Table 1.

Preliminary experiments showed for aqueous standards (5 ml) recoveries as high as 95% for BA-tetrahydrotetrol and 100% for BP-tetrahydrotetrol (Table 2). Recoveries from spiked hemoglobin samples are between 50% and 80%, depending on the amount of hemoglobin in the sample. This is probably due to absorption effects of the tetrahydrotetrols by the hemoglobin and needs further elucidation. A rough estimation of detection limits of the aqueous standards yields 10 pmol BA-tetrahydrotetrol and 60 fmol BP-tetrahydrotetrol. These high recoveries and low detection limits make this new method very promising for biological-effect monitoring of exposed humans, when compared to results obtained during other investigations [22].

4. Conclusion

A method for the determination of hemoglobin adducts with BA and BP by analysis of their HPLC tetrahydrotetrol derivatives using a coupled-column technique has been developed. A specific innovation is the use of electrical power to heat the solvent for elution and transfer of the analytes from the pre-column to the analytical column, after HPLC-integrated sample processing. The utility of this method is its superior automation and practicability. These qualities make this method attractive for routine analysis in biological-effect monitoring. Preliminary results regarding recovery are very promising, nevertheless further development of the sample pretreatment prior to HPLC-integrated processing is required.

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