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Evaluation and routine application of the novel restricted-access precolumn packing material Alkyl-Diol Silica: coupled-column high-performance liquid chromatographic analysis of the photoreactive drug 8-methoxypsoralen in plasma

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Abstract

A fully automated coupled-column HPLC method for on-line sample processing and determination of the photoreactive drug 8-methoxypsoralen (8-MOP) in plasma has been developed. The method is based on the novel internal-surface reversed-phase precolumn packing materials Alkyl-Diol Silica (ADS). This new family of restricted-access materials has a hydrophilic, electroneutral outer particle surface and a hydrophobic internal pore surface. The supports tolerate the direct and repetitive injection of proteinaceous fluids such as plasma and allow a classical C_{18}-, C_8- or C_4-reversed-phase partitioning at the internal (pore) surface. The total protein load, i.e. the lifetime of the precolumn used in this study (C_8-Alkyl-Diol Silica, 25 μm, 25 × 4 mm I.D.), exceeds more than 100 ml of plasma. 8-MOP was detected by its native fluorescence (excitation 312 nm, emission 540 nm). Validation of the method revealed a quantitative and matrix-independent recovery (99.5–101.3% measured at five concentrations between 21.3 and 625.2 ng of 8-MOP per milliliter of plasma), linearity over a wide range of 8-MOP concentrations (1.2–3070 ng of 8-MOP/ml, $r = 0.999$), low limits of detection (0.39 ng of 8-MOP/ml) and quantitation (0.79 ng of 8-MOP/ml) and a high between-run (C.V. 1.47%, $n = 10$) and within-run (C.V. 1.33%, $n = 10$) reproducibility. This paper introduces coupled-column HPLC as a suitable method for on-site analysis of drug plasma profiles (bedside-monitoring).

1. Introduction

8-Methoxypsoralen, (9-methoxy-7H-furo[3,2-g][1]benzopyran-7-one, 8-MOP, methoxsalen; Fig. 1), a photosensitizing furocoumarin, is used for the treatment of skin diseases such as vitiligo [1,2] and psoriasis [3]. The therapy consists of oral ingestion of the drug followed by irradiation of the patient with ultraviolet A light (UVA, 320–400 nm) and depends on having a therapeutic level (50–100 ng of 8-MOP/ml) of 8-methoxypsoralen in plasma at the time of exposure to UVA, two hours after application. In 1987, Edelson and coworkers [4] presented a new therapy for cutaneous T-cell lymphoma involving an extracorporeal activation of 8-MOP with ultraviolet A light (photopheresis) after oral administration of the drug. Recently, photo-

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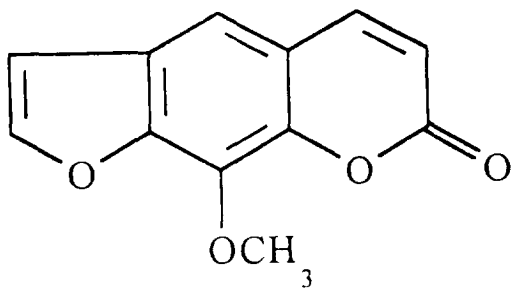

Fig. 1. Structure of 8-methoxypsoralen.

pheresis has been used for the treatment of heart transplant rejection [5]. As large inter- and intra-individual variations in the pharmacokinetics of 8-MOP have been reported [6], monitoring of the drug levels is necessary for successful treatment. Up to now HPLC [7–14] and GC [15–17] have been applied to determine 8-methoxypsoralen in biological fluids. The published methods involve time-consuming sample pretreatment steps such as liquid–liquid or solid-phase extraction, use radiolabeled compounds or are insufficiently sensitive or precise to perform 8-methoxypsoralen analysis during photochemotherapy.

An attractive method to quantify drugs in complex biological matrices such as plasma and to eliminate the necessity for any sample pretreatment is LC with column switching. In coupled-column LC the target molecules are first dynamically extracted by a sample processing precolumn and subsequently separated on an analytical column. Conventional silica-based precolumn packing materials, however, usually still require a sample clean-up step such as precipitation or dialysis prior to injection in order to remove the protein matrix. Otherwise, the protein accumulates on the packing of the precolumn, resulting in a decrease in column performance, an increased back-pressure and finally a limited number of analysis cycles. Special chromatographic supports, used in single- and coupled-column mode, allowing the direct and repetitive injection of untreated proteinaceous fluids have been developed during the last few years (for review see Refs. [18–20]). These so-called restricted-access materials exclude macromolecules such as plasma proteins without destructive accumulation but retain small target molecules by partitioning or affinity chromatography [21]. Recently, we prepared a new family of restricted-access materials, which we call Alkyl-Diol Silica (ADS) [22]. These internal-surface reversed-phase supports have been specially developed for use as precolumn packings and for HPLC-integrated extraction and enrichment of hydrophobic compounds in proteinaceous fluids by classical C_{18}-, C_8- or C_4-reversed-phase chromatography. This paper describes the first routine application of the appropriate C_8 support in a fully automated coupled-column HPLC system for direct and rapid analysis of 8-MOP in plasma.

2. Experimental

2.1. Chemicals

8-MOP was provided by Aldrich (Steinheim, Germany). HPLC-grade methanol and water were obtained from E. Merck (Darmstadt, Germany).

2.2. Sampling

Blood was collected into EDTA-containing tubes from the antecubital vein of patients and centrifuged immediately for 5 min at 4000 *g*. An aliquot of the plasma fraction was transferred into an autosampler glass vial and the temperature of the sample rack was held at 4°C. Samples not immediately analysed can be stored at −20°C in the dark for a period of more than 1 month [8].

2.3. Instrumentation

The HPLC system consisted of the following modular units: an E. Merck (Darmstadt, Germany) Model L-6000 pump (P1), a L-6200 gradient pump (P2), an AS-4000A autosampler (AS), a F-1050 fluorescence detector (FD), an D-2500 integrator, a Krannich (Göttingen, Ger-

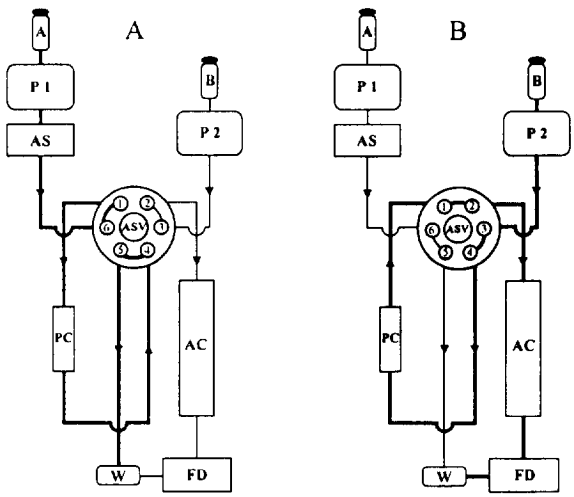

Fig. 2. Flow diagram for coupled-column analysis. (A) Valve position for precolumn loading step (LOAD). (B) Valve position for transfer step (INJECT). (W) waste, (P1/P2) pump 1/2, (AS) autosampler, (ASV) automated switching valve, (PC) precolumn, (AC) analytical column, (FD) fluorescence detector.

many) Model ELV 7000 automated switching valve (ASV), an analytical column LiChrospher RP-18, 5 μm, 125 × 4 mm I.D. (E. Merck, Darmstadt, Germany, (AC)) and a precolumn C_8-Alkyl-Diol Silica, 25 μm, 25 × 4 mm I.D. (PC). The precolumn was dry-packed and sealed using 1-μm sieves.

The instrumental set-up of the apparatus is shown in Fig. 2.

An E. Merck HPLC system equipped with a series-connected L-6200 gradient pump, an AS-4000A autosampler, the given ADS precolumn, a L-4250 UV detector and a D-2500 integrator was used to record the protein elution profiles and to determine the capacity factors of the analyte.

2.4. Chromatographic parameters

For coupled-column analysis, the Alkyl-Diol Silica precolumn and the reversed-phase analytical column were connected via an electrically-driven automated six-port switching valve. The positions of the switching valve are listed in Table 1. During the analysis cycle, pump 1, which delivers the sample (injection volume: 25 μl), operates at a flow-rate of 1.0 ml/min with eluent A (water).

The programmable pump 2 delivers eluent B (methanol–water, 60:40, v/v) at a flow-rate of 0.8 ml/min. The microprocessor unit of the programmable gradient pump controls the pump itself, the automated switching valve and the integrator.

Detection was performed using the natural fluorescence of 8-MOP (excitation 312 nm, emission 540 nm). The best signal-to-noise ratio in our system was achieved at an emission wavelength of 540 nm.

2.5. Calibration

A stock solution of 8-MOP in HPLC-grade methanol (approximately 100 μg of 8-MOP/ml) was prepared and the concentration photometrically confirmed at 300 nm (log $\epsilon = 4.16$, [23]). The calibration standards ranging from 21.3 to 625.2 ng of 8-MOP/ml were prepared by diluting the stock solution with HPLC-grade water. Protected from light, the stock solution and the calibration standards are stable at 4°C for more than one month. The calibration standards were

Table 1
Timetable for switching-valve positions (cf. Fig. 2)

Time (min)	Position	Precolumn	Analytical column
0.0–8.0	A	Sample loading	(re)Conditioning
8.0–13.0	B	Analyte transfer	Analyte transfer, separation
13.0–15.0	A	Reconditioning	Separation

analysed in the coupled-column mode to obtain the calibration graph. After checking of the linearity an external three-point calibration was applied in routine analysis.

3. Results and discussion

3.1. Alkyl-Diol Silica (ADS)

This new family of chemically and enzymatically modified precolumn packing materials, which was recently introduced by us [22,24], belongs to the group of restricted-access media having a physical diffusion barrier [18–20]. These materials are specifically designed for the direct and repetitive injection of untreated biological fluids, the quantitative elimination of macromolecules such as proteins in the interstitial void volume, as well as for the dynamic extraction and on-column enrichment of small solutes by classical reversed-phase partitioning. The Alkyl-Diol packings (particle diameter: 25 μm) are synthesized by bonding a hydrophilic phase, i.e. glycerylpropyl- (diol-) groups, to 60 Å LiChrospher silica. In a second step the diol-groups are reacted with fatty acid chlorides having different *n*-alkyl chain length (C_4, C_8, C_{18}). Finally, the packings are treated with pancreatic lipase (EC 3.1.1.3) and/or esterase (EC 3.1.1.1) to remove the hydrophobic partitioning moieties (fatty acids) exclusively from the outer surface by enzymatic hydrolysis. The molecular mass cut-off of the modified packings is approximately 15 kDa. This exclusion limit prevents macromolecules from interacting with the hydrophobic partitioning phase (*n*-alkyl moieties) which is covalently bound to the internal surface of the particles. The outer surface (glycerylpropyl, i.e. diol moieties) of the support is hydrophilic, electroneutral and non-adsorptive towards proteins (Fig. 3).

Besides the applications shown in this paper the ADS materials have been used for the bioanalysis of the anthracycline cytostatic epirubicin and its metabolites [25], the analysis of digoxin with on-line immunochemical detection

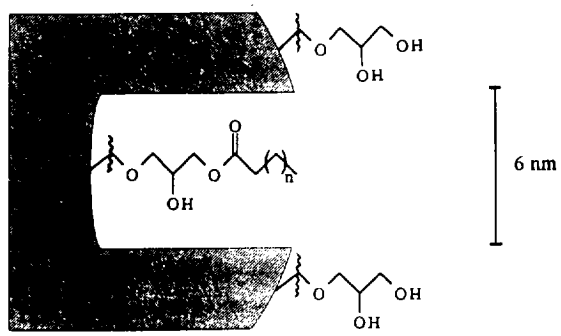

Fig. 3. Schematic representation of bonded-phase topography of Alkyl-Diol Silica particulate; $n = 1$: butyryl, $n = 5$: capryloyl, $n = 15$: stearoyl.

[26,27] as well as for the coupled-column analysis of local anaesthetics [28].

3.2. Selection of the appropriate ADS precolumn packing material

When using a restricted-access precolumn packing in a coupled-column system, the selection of the packing material and the appropriate mobile-phase composition is important. To avoid protein precipitation, the concentration of the organic modifier as well as the pH and ionic strength of the eluent, with which the sample is loaded onto the precolumn, must be non-denaturing. Pinkerton showed that the concentration of the organic modifier should be kept below 25% for acetonitrile, 20% for isopropanol and 10% for tetrahydrofuran [29]. Fig. 4 shows

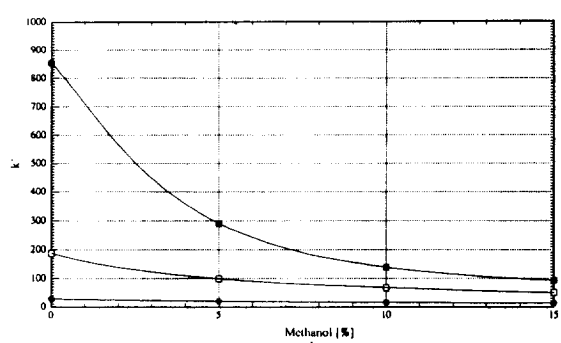

Fig. 4. Capacity factors (k') of 8-MOP on C_4- (●), C_8- (□) and C_{18}-ADS (■) precolumn packing materials (25 × 4 mm I.D., 25 μm, eluent: water–methanol mixtures, flow-rate: 1.0 ml/min, detection: UV 220 nm, hold-up time marker: thiourea) as a function of the methanol content of the eluent.

the effect of methanol content on the capacity factors of 8-MOP comparing C_4-, C_8- and C_{18}-ADS precolumn packing materials.

As shown in Fig. 4, only the C_8- and C_{18}-ADS sorbents prevent any loss of the analyte by breakthrough during the precolumn loading step. In order to minimize the adsorption and enrichment of interfering components we chose the less hydrophobic C_8-ADS packing and a pure aqueous mobile phase. Under these conditions, the analyte is concentrated on top of the precolumn and peak-broadening is reduced (peak compression).

3.3. Lifetime of the precolumn

In order to determine the lifetime of the packing a series of ca. 200 analysis cycles using a C_8-ADS precolumn (25 × 4 mm I.D., 25 μm) was run and the injection volume was increased to 500 μl of human plasma. The sample loading eluent was water and the loading time was 18 min at a flow-rate 1.0 ml/min. No increase in back-pressure or loss of extraction efficiency with respect to the analyte could be observed. Under these conditions, the total injected protein load exceeds more than 100 ml of human plasma sample.

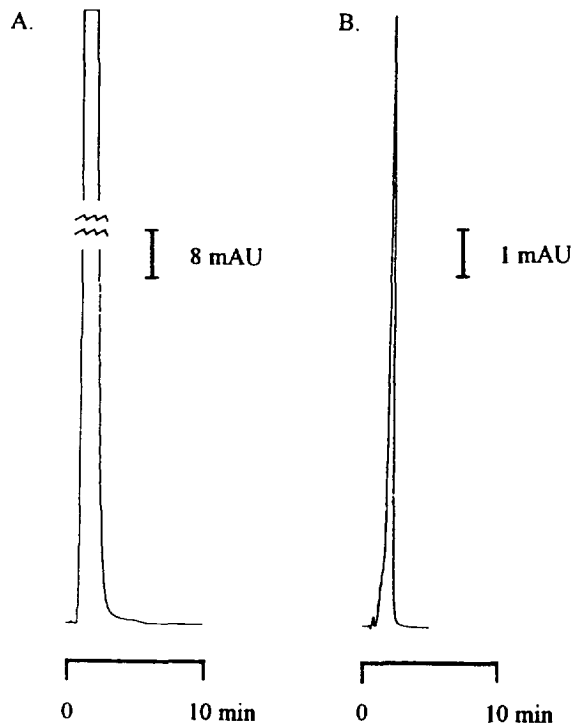

Fig. 5. Precolumn elution profiles. (A) Sample processing step: matrix elution profile after injection of 25 μl of plasma. Detection: UV 280 nm; eluent: water; flow-rate: 1 ml/min. (B) Transfer step: elution profile of 8-MOP (120.5 ng). Detection: UV 300 nm; eluent: methanol–water (60:40, v/v); flow-rate: 0.8 ml/min.

3.4. Coupled-column analysis cycle

In the coupled-column mode, HPLC-integrated sample processing and 8-MOP separation are characterized by four steps:

(1) Sample application. The sample (25 μl plasma or standard solution) is loaded with eluent A (HPLC-grade water) via the autosampler through valve positions 6-1 onto the ADS-precolumn. During 8 min the protein matrix and other interfering components are quantitatively eliminated (c.f. Fig 5A). The analyte, 8-MOP, is simultaneously enriched by reversed-phase partitioning, while the analytical column is equilibrated with methanol–water (60:40, v/v) by the gradient pump (P2).

(2) Transfer. After automated valve switching to the position INJECT, the analyte is eluted from the precolumn (PC) in the backflush mode by eluent B (methanol–water, 60:40, v/v) and transferred in a single, narrow elution band (c.f. Fig. 5B) through valve positions 1-2 to the top of the series-connected analytical column.

(3) Separation. Separation is carried out under isocratic conditions (eluent B, methanol–water, 60:40, v/v).

(4) Reconditioning. After automated valve switching to the position LOAD, the precolumn is reequilibrated during the last 2 min of the analysis cycle. A new cycle can be started every 15 min.

Fig. 6 shows representative chromatograms obtained by coupled-column analysis of a standard solution of 8-MOP and plasma samples before and after oral application of the drug.

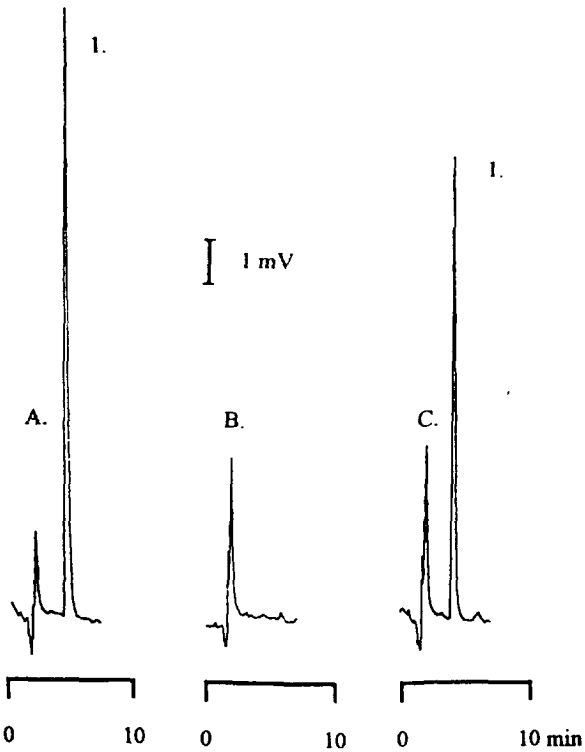

Fig. 6. Coupled-column analysis of 8-MOP. (A) 25 μl of 8-MOP standard solution (307 ng/ml), peak 1: 8-MOP. (B) 25 μl of plasma prior to oral application of 8-MOP. (C) 25 μl of plasma 2 h after oral application of 20 mg of 8-MOP. 8-MOP plasma level: 233 ng/ml, peak 1: 8-MOP.

3.5. Validation

The matrix-independent recovery was measured using aqueous standard solutions of the analyte. For the determination of the matrix-dependent recovery, pooled plasma samples were spiked with a known amount of 8-MOP standard solution (Table 2).

The linearity between detector response (peak area) and 8-MOP concentrations covers the range 1.2-3070 ng of 8-MOP per milliliter of plasma ($r = 0.999$).

To obtain data on the reliability of the system, within-day and day-to-day precision were determined. The coefficients of variation were 1.33% for within-day analysis ($n = 10$) and 1.47% for day-to-day analysis ($n = 10$) of plasma samples containing 307 ng of 8-MOP/ml.

The limits of detection (LOD) and quantitation (LOQ), calculated according to Ref. [30], were 0.39 ng and 0.79 ng of 8-MOP/ml, respectively.

The HPLC system described above is able to process 96 samples per 24 hours. Using a modified set-up (tandem precolumn configuration) with alternating precolumn sample enrichment [31] we were able to increase the sample through-put to 144 samples per 24 hours.

We applied this method to the analysis of 8-MOP plasma levels in extracorporeal photochemotherapy. In this regimen, the drug, activated by UVA light, binds to pyrimidine bases of the T-lymphocyte DNA. After reinfusion of the photomodified cells a long-term adjunct immunosuppressive effect is clinically observed [32]. Consequently, neither 8-MOP administration nor UVA irradiation is effective separately. The success of the treatment depends on the proper synchronization of sufficient 8-MOP plasma levels (50-100 ng of 8-MOP/ml) with the time of exposure to UVA light. After oral ingestion of

Table 2
Matrix-independent recovery from standard solutions and matrix-dependent recovery from plasma

Concentration 8-MOP (ng/ml)	Recovery from standard solutions (%)	C.V. (%)	Recovery from plasma (%)	C.V. (%)
21.3	100.3	1.7	100.4	4.5
53.3	100.9	1.6	101.3	1.5
106.5	100.8	1.3	99.5	1.9
260.0	100.1	0.4	101.1	0.6
625.2	101.0	0.4	100.7	0.2

Injection volume: 25 μl, $n = 5$.

the drug whole blood samples were drawn every 30 min, centrifuged, the resulting plasma fraction transferred into an autosampler glass vial and the analysis cycle started. As soon as the therapeutic 8-MOP level was reached, irradiation was initiated. Using this on-site HPLC analysis (bedside monitoring) therapeutic efficiency could be improved.

4. Conclusions

This paper demonstrates the suitability of restricted-access materials as precolumn packings in coupled-column HPLC for bioanalysis. The use of the novel reversed-phase precolumn packing material Alkyl-Diol Silica enabled us to develop a fully automated coupled-column HPLC method for the routine determination of the photoreactive drug 8-MOP in plasma. The essential features of the method are:

–the chromatographic performance of the novel precolumn packing,

–no manual sample pretreatment, except for a centrifugation step,

–improved precision and accuracy as well as a considerable reduction of analysis time, compared to conventional manual methods and

–no requirement for an internal standard.

A coupled-column system using restricted-access precolumn packings for integrated sample processing should have a broad applicability in the fields of pharmacokinetics and drug monitoring (as shown in this paper), clinical chemistry (e.g. catecholamines [21]), toxicology, forensic chemistry and occupational medicine (e.g. internal PAH-exposure [33]).

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