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PHASE-SYSTEM SWITCHING AS AN ON-LINE SAMPLE PRETREATMENT IN THE BIOANALYSIS OF MITOMYCIN C USING SUPERCRITICAL FLUID CHROMATOGRAPHY

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SUMMARY

One of the problems of the application of supercritical fluid chromatography (SFC) in bioanalysis is the fact that many sample pretreatment procedures deliver the solutes of interest in a polar solvent, which upon injection will dramatically disturb the phase system characteristics of the SFC system. The phase-system switching approach, recently introduced for liquid chromatography—mass spectrometry, can be used to avoid this problem. Plasma samples containing the thermolabile and pH-sensitive cytostatic drug Mitomycin C (MMC) were injected onto a short precolumn. After washing and drying of the precolumn the compound of interest was desorbed using a supercritical fluid and analyzed by SFC. Up to 1 ml of plasma containing 20 ng of MMC has been analyzed in this way.

INTRODUCTION

Supercritical fluid chromatography (SFC) is a powerful tool for solving analytical problems in various areas. The perspectives of SFC have been discussed in several excellent reviews[1–5]. Our interest in SFC lies in its potential to solve some of the challenging problems in bioanalysis, for SFC has several attractive features. Being complementary to gas chromatography (GC) and liquid chromatography (LC), SFC broadens the field of chromatography. Several applications indicating the bioanalytical potential of SFC have been described, *e.g.*, refs. 6–9. The possibilities of open-capillary columns in SFC, *i.e.*, very high efficiencies, which are useful in difficult separations for instance of enantiomeric compounds[10], the use of sensitive and selective GC detectors such as the nitrogen–phosphorus detector[11] and the ease of coupling with a mass spectrometer[4], have all been demonstrated with compounds of biological and pharmaceutical origins. Interesting features of packed columns in SFC are the possibility to perform rapid separations with moderate efficiencies[2], and the use of powerful LC detectors, such as UV absorbance and fluorescence. An important advantage of packed columns over open-capillary columns is the higher sample loadability of the former.

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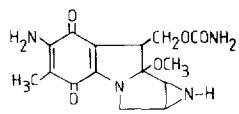
Before SFC can be successfully applied in bioanalysis some additional analytical technology has to be developed. An important aspect of bioanalysis is the selection of an appropriate sample pretreatment. Many procedures are based either on liquid–liquid extractions or on liquid–solid isolations. Especially in bioanalysis, the solutes will be reconstituted in relatively polar solvents after these pretreatment procedures. In SFC the direct injection of such a solution may disturb the chromatographic system dramatically, considering the strong influence of small percentages of organic modifier on the retention characteristics in SFC[6]. Therefore, a new approach has been developed in our laboratory. In order to avoid the injection of polar solvents in the SFC system the phase-system switching (PSS) approach, which was originally developed for liquid chromatography–mass spectrometry[12,13], has been adopted and applied in SFC. The aqueous sample, *e.g.*, plasma is pumped through a small precolumn containing an hydrophobic adsorbent. Then the precolumn is washed with water in order to remove hydrophilic compounds and subsequently dried with a stream of nitrogen in order to remove the polar solvent. Desorption of the analytes and transfer to the separation column is performed using a supercritical fluid, which is also used for the chromatographic separation. In this way the introduction of polar solvents into the SFC system can be avoided. An additional perspective of this approach lies in the possibility to combine the phase-system switching step with an on-line sample pretreatment based on liquid–solid extraction. The potential of this approach is demonstrated by the bioanalysis of the anti-cancer drug Mitomycin C (MMC)[14,15] in human plasma. For the analysis of MMC in biological fluids and tissue homogenates a fully automated system based on the use of a dialysis membrane, a short concentration column and an HPLC separation has been described[15]. In the present investigations MMC is merely used as a test compound, particularly because it is thermally labile and is degraded in solutions of both low and high pH[14].



The sample pretreatment system described here shows some similarities with supercritical fluid extraction (SFE). Various examples have been reported of the use of supercritical fluid extraction of solids in combination with chromatographic techniques, either high-performance liquid chromatography (HPLC)[16], GC[17] or SFC[18,19]. However, in these cases the solid samples to be analyzed are extracted directly with the supercritical fluid, and the compounds of interest are transferred to the appropriate chromatographic technique, while in the PSS system described here the compounds of interest are chromatographically retained on a solid phase material and desorbed with a supercritical fluid.

This paper describes the analysis of Mitomycin C in plasma samples and the development of the phase-system switching technique; a comparison has been made between off-line and on-line sample pretreatment.

EXPERIMENTAL

Equipment

The supercritical fluid chromatograph used was constructed from various, in most cases commercially available, components, some of which were slightly modified. A schematic diagram of the system is given in Fig. 1. The system consisted of a MWG Lauda K2R Cooling Unit (Beun de Ronde, Amsterdam, The Netherlands), a Model 2150 reciprocating HPLC pump (LKB, Bromma, Sweden), a syringe pump (BrownLee, Santa Clara, CA, U.S.A.), a MUST valve-switching unit (Spark Holland, Emmen, The Netherlands), a Constametric III reciprocating pump (Milton Roy, Riviera Beach, FL, U.S.A.), a Model 7125 injection valve (Rheodyne, Berkeley, CA, U.S.A.), a Fractovap 2101 system, which was used as a column oven (Carlo Erba, Milan, Italy), a Spectroflow 757 UV detector (ABI Kratos, Ramsey, NJ, U.S.A.) equipped with an ABI Kratos high-pressure flow cell (Brownlee Scientific) and a Type BD40 recorder (Kipp & Zn., Delft, The Netherlands).

The Model 2150 reciprocating pump was used for the delivery of the supercritical mobile phase. It was modified by replacing the PTFE inlet capillaries of the

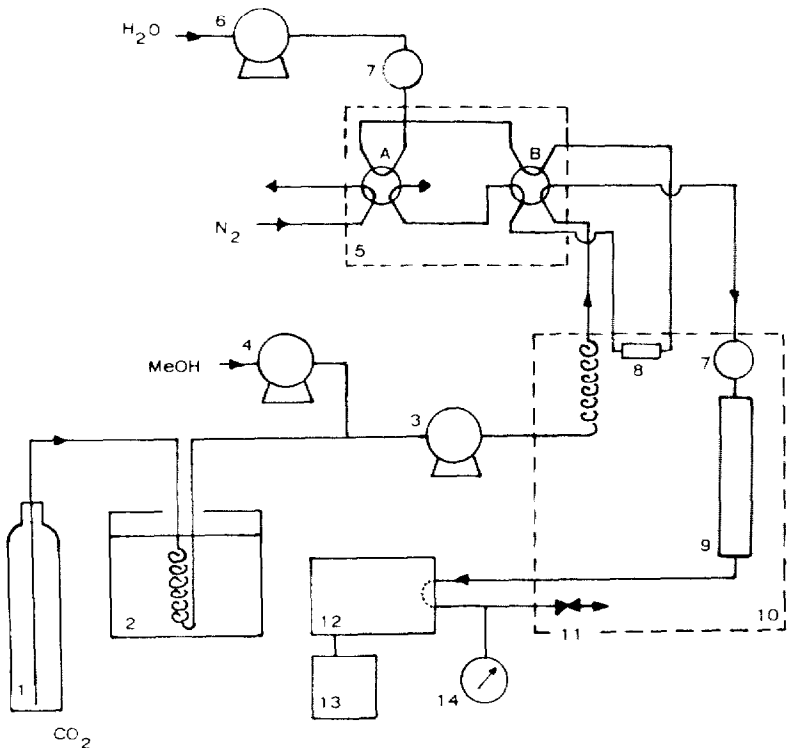


Fig. 1. Schematic diagram of the equipment used in the PSS-SFC experiments. For details see text. 1 = Carbon dioxide cylinder; 2 = cooling unit; 3 = modified reciprocating pump; 4 = syringe pump; 5 = valve switching unit; 6 = reciprocating pump; 7 = rheodyne injection valve; 8 = precolumn; 9 = analytical column; 10 = GC oven; 11 = back-pressure regulator; 12 = UV detector; 13 = recorder; 14 = manometer. MeOH = Methanol.

pump with stainless-steel capillaries, and by adding a brass water-jacket around the pump heads, through which was pumped 20% ethyleneglycol in water at −5°C. The syringe pump was used for the addition of methanol to the carbon dioxide. The third pump was used for sampling and washing the precolumn in the PSS system.

A flow-rate of 2 ml/min was used in all experiments. UV detection was done at the wavelength of maximum absorbance of MMC (in methanol) at 360 nm. Other components of the system were a carbon dioxide cylinder with a siphon tube (Hoekloos, Amsterdam, The Netherlands), a line filter (0.5 μm; Nupro, Willoughby, OH, U.S.A.) at the outlet of the carbon dioxide cylinder, a Rosil C_{18} analytical column (150 mm × 4.6 mm I.D.) (Alltech, Deerfield, IL, U.S.A.), a Nupro Model SS-SS1-A fine-metering valve used as a back-pressure regulator and a Bourdon-type pressure gauge. The precolumn (10 mm × 3.2 mm I.D.) was laboratory-made and hand-packed with the stationary phase material, which was enclosed between two frits.

Materials

The column used in the SFC experiments was 150 mm × 4.6 mm I.D. packed with 5-μm Rosil C_{18} material (Alltech). Similar columns packed with other materials (silica, NH_2, C_8) were also tested.

Carbon dioxide was of technical grade (grade 2.5). Analytical grade methanol from J. T. Baker Chemicals (Deventer, The Netherlands) was filtered over a 0.45-μm membrane filter (Sartorius, Göttingen, F.R.G.) before use. The water was purified using a Milli Q Water Purification System (Millipore, Bedford, MA, U.S.A.). XAD-2 (150–200 μm) was obtained from Serva (Heidelberg, F.R.G.) and Polygosil C_{18} (40–63 μm) from Macherey-Nagel (Düren, F.R.G.). Mitomycin C was from Kyowa Hakko Kogyo (Tokyo, Japan). Pooled human plasma was used, centrifuged at 1000 *g* for 10 min before injection.

RESULTS AND DISCUSSION

SFC of Mitomycin C

The behaviour of MMC in SFC has not been studied previously. In order to evaluate the chromatography of MMC under SFC conditions, various experimental parameters were varied.

Several column materials have been tested. The best results were obtained with a C_{18} stationary phase. The other materials tested were silica, aminopropyl- and octyl-silica. With the C_8 material poor peak shapes of MMC were observed, while with silica and aminopropyl material no peaks of MMC were detected, indicating a capacity factor above 25.

No elution of MMC was observed from a packed C_{18} column when pure carbon dioxide was used as the mobile phase. At least 5% methanol must be added to the carbon dioxide as a modifier in order to obtain capacity factors below 10 (at 30-MPa and 60°C). The influence of the percentage of methanol in the mobile phase on the capacity factor has been investigated at a constant back-pressure of 30 MPa. The results are given in Fig. 2. The influence of the modifier content of the mobile phase is dramatic. The capacity factor decreases fromn a value of 27 at 2.5% methanol to a value of 1 at 10% methanol. At a constant percentage of methanol added to the mobile phase the back-pressure also has some influence on the capacity factor of

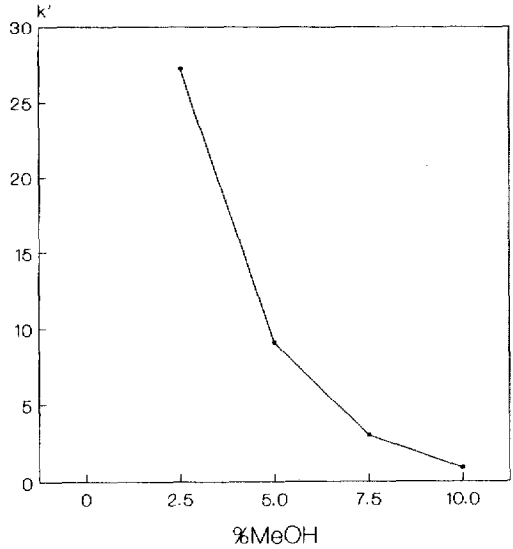


Fig. 2. Capacity factor (k') of MMC as a function of the percentage of methanol in the mobile phase. Conditions: C_{18} column (150 mm × 4.6 mm I.D., 5 μm); back-pressure 30 MPa; flow-rate 2.0 ml/min; oven temperature 50°C; UV detection at 360 nm.

MMC. However, this influence is less dramatic than the influence of the modifier content. This is in agreement with published data for other compounds, *e.g.*, see ref. 6. With 10% methanol in the mobile phase and a temperature of 50°C the capacity factor decreases from 1.6 at 18 MPa to 0.8 at 33 MPa. Variation of the temperature does not result in significant changes as far as the retention characteristics are concerned. However, due to thermal degradation of the MMC, the peak heights and peak areas decrease rapidly at temperatures above 60°C.

As seen from the typical MMC chromatogram in Fig. 3a, the peak of MMC suffers from tailing. At first sight, this may be due to the interaction of MMC with the free silanol groups at the surface of the stationary phase. Similar effects have been reported by others[20] with highly polar compounds. However, with 12% methanol present in the mobile phase such an interaction is not expected.

In most of the experiments described in this paper, 12% methanol was used at a column temperature of 50–60°C at a back-pressure of 28–30 MPa. Under these optimized conditions a calibration plot for MMC in SFC analysis was obtained, which was linear over at least two decades from 10 to 500 ng per injection. The detection limit based on a signal-to-noise ratio of 3 was calculated to be 0.4 ng, which compares well with the detection limit of 0.25 ng found in HPLC[15].

Off-line sample pretreatment

Two off-line sample pretreatment procedures have been tested. The first procedure consists of the denaturation of proteins by methanol and centrifugation of the samples (10 min at 1000 *g*). The 5-μl injections of the supernatant in the SFC system resulted in pressure fluctuations in the system and an unstable UV signal. Therefore, a more elaborate procedure based on liquid–solid isolation was tested[21]. The procedure

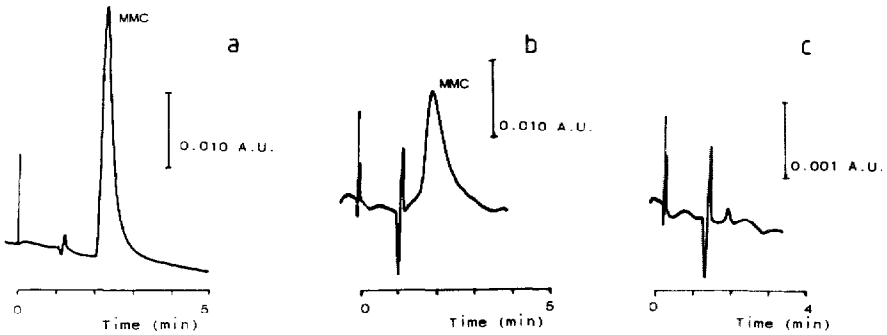


Fig. 3. Chromatograms of MMC dissolved in methanol: with injection of (a) 5 μl of 40 μg/ml MMC in methanol, (b) 5 μl of extract after the off-line simple pretreatment of 1 ml plasma containing 100 μg MMC and (c) 5 μl of extract after the off-line sample pretreatment of 1 ml blank plasma. Conditions: as in Fig. 2, except that the percentage of methanol is 12%.

consists of the following steps. A Pasteur pipette is filled with 100 mg XAD-2 (particle size 150–200 μm). The solid phase material is first activated with 6 ml of methanol and washed with 6 ml of water. Then the plasma sample (1 ml) is extracted. After washing the material with 6 ml of water, the analytes are eluted with 6 ml of methanol. The eluate is evaporated to dryness by blowing nitrogen gas over it at ambient temperature. The residue is dissolved in 100 μl of methanol. A 5-μl volume of this solution is injected directly onto the analytical column. This procedure is straightforward; recoveries are 93%. A typical chromatogram is given in Fig. 3b. The determination limit is estimated to be 30 ng/ml.

Phase-system switching and on-line sample pretreatment

A liquid–solid isolation procedure as described above can easily be implemented on-line in an HPLC system by the use of valve-switching techniques. This approach is used for instance in the automatic HPLC determination method for MMC[15]. However, in combination with SFC a phase-system switching step must be performed in order to step from aqueous solutions to supercritical mixtures of carbon dioxide and methanol. A system has been designed which performs such a phase-system switching in combination with SFC. The procedure consists of five distinct steps. In the first step the plasma sample, after centrifugation, is injected into a stream of water, which transports the sample to a short precolumn. Adsorption onto the surface of the precolumn material takes place. In the second step the precolumn is washed with 1 ml water. In the third step the precolumn is dried, removing residual water, with a stream of nitrogen flowing in the backflush direction. In the fourth step the compounds are desorbed from the precolumn by a stream of supercritical carbon dioxide with 12% methanol and transferred to the separation column. In the last step the compounds are separated on the analytical column and selectively detected at 360 nm with an UV detector. After each experiment the precolumn is washed with 10 ml of water. The various steps in this procedure have been studied in more detail.

Using an adsorption step from aqueous solutions and a desorption step with 12.5% acetonitrile in water, the retention characteristics of the precolumn in the

analysis of MMC in plasma were investigated. It was found that nearly complete recoveries (between 90 and 104%) can be obtained in such a system.

Injection of 5 μl water into the SFC system does not result in serious problems the first few times, but cannot be done frequently as it results in pressure fluctuations and disturbance of the UV signals. This is expected, as only 0.1–0.2% water can be dissolved in supercritical carbon dioxide under the present conditions. By using solutions of MMC in mixtures of water and methanol the influence on the chromatography of MMC has been investigated. It was found that the peak shapes rapidly deteriorate with increasing water content of the sample. From these experiments it can be concluded that it is necessary to dry the precolumn. Drying of a precolumn has also been described by Noorozian *et al.*[22] in LC–GC experiments. In the present experiments a stream of nitrogen at a flow-rate 300–400 ml/min was used to dry the precolumn. It has been found that drying times up to 25 min at ambient temperature do not result in complete removal of the water on the precolumn. Therefore, it was decided to place the precolumn in the SFC column oven at 50–60°C. Drying at 60°C for 10 min resulted in significant decrease of the chromatographic signals, probably due to thermal degradation of the MMC. Lowering the temperature to 50°C gave better results.

Two types of adsorbents have been investigated. The silica-based Polygosil C_{18} material gave irreproducible results. Better results were obtained with the XAD-2 resin material with particle size 150–200 μm.

When during the procedure the supercritical fluid is switched to the dried precolumn a significant pressure drop of about 6 MPa takes place in the system. However, it takes about 30 s to restore the original condition.

The liquid–solid isolation step is surprisingly selective. UV detection at 215 nm after sampling blank plasma samples onto the precolumn and analyzing with the same procedure results in a broad peak, with similar capacity factors as for MMC. However, considering the complexity of plasma, far more extensive signals are expected. In the analysis of MMC in plasma samples the compounds coeluted from the

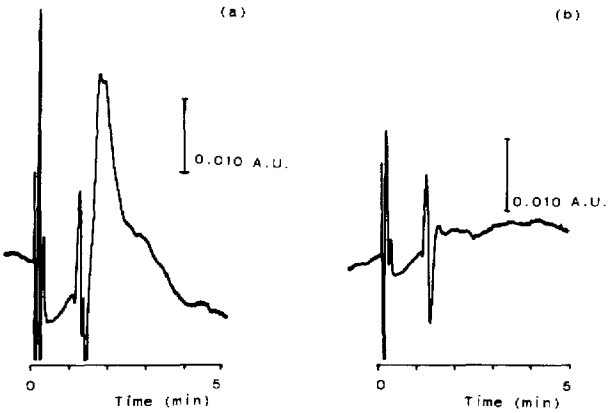


Fig. 4. Chromatograms after injection of 20 μl of blank plasma onto the precolumn with UV detection at (a) 215 nm and (b) 360 nm. Conditions: precolumn (10 mm × 3.2 mm I.D.) packed with XAD-2; sampling with 1.0 ml/min for 1 min; other conditions as in Fig. 3.

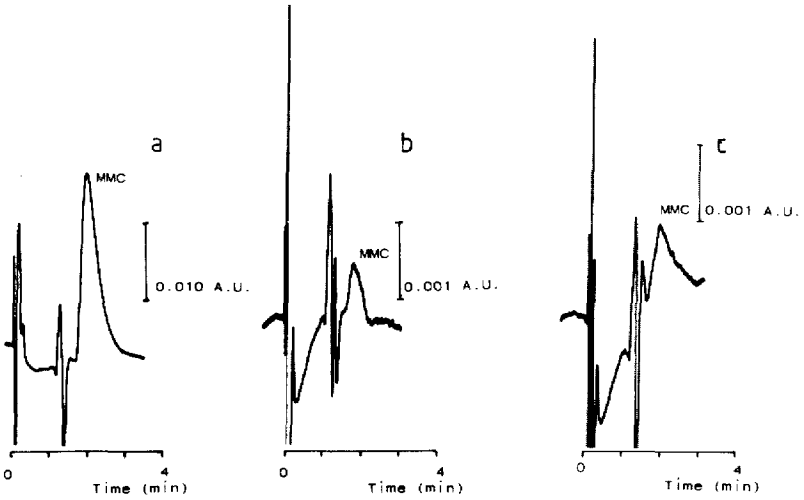


Fig. 5. Chromatograms after on-line liquid–solid extraction of MMC from plasma samples with sampling of (a) 20 μl plasma containing 200 ng MMC, (b) 20 μl plasma containing 20 ng MMC and (c) 1 ml plasma containing 20 ng MMC. For conditions see Fig. 4.

precolumn do not interfere. At the wavelength of maximum absorbance of MMC, *i.e.*, 360 nm, the blank plasma samples give clean UV signals (see Fig. 4). These results indicate that the phase-system switching approach in this case is quite a selective sample pretreatment procedure.

One of the drawbacks of the off-line sample pretreatment procedure lies in the fact that it is hardly possible to dissolve the residue reproducibly in volumes smaller than 100 μl. As only 5 μl of the methanolic solution can be injected on the SFC column, it is not possible to analyze directly the equivalent of 1 ml plasma in such a system. In the on-line sample pretreatment system the volume of the plasma samples injected has been varied between 20 and 1000 μl, keeping the absolute amount of MMC in the samples constant (either 20 or 200 ng). Typical chromatograms are given in Fig. 5, showing that it is possible to analyze with the system described 1 ml of plasma directly. Typical recoveries in the determination are 70% (with an oven temperature of 50°C). Thus, some (thermal) degradation of MMC appears to take place during the procedure, as there is no evidence for significant break-through of MMC on the precolumn. However, lower temperatures cannot be used, as SFC will not be stable under those conditions. The calculated detection limit of the complete procedure is 1.48 ng MMC. The determination limit is about 5 ng/ml.

With the precolumn used in these experiments it is possible to inject, at least 15 times, 1 ml of plasma before the precolumn has to be replaced.

CONCLUSIONS

The analysis of Mitomycin C in plasma samples with SFC is described. MMC is used merely as a test compound. It has been shown that MMC can be analyzed successfully by using an off-line liquid–solid isolation procedure. However, the off-

line procedure is time-consuming and laborious. An on-line liquid–solid isolation can also be performed by using valve-switching techniques. In this system 1 ml of plasma can be sampled directly. On the precolumn a phase-system switching is performed, in which the polar liquid phase which disturbs the SFC analysis is removed, and the compound of interest is desorbed from the precolumn with a supercritical mixture of carbon dioxide and methanol, also used as the mobile phase in SFC. The determination limit of this procedure is 5 ng/ml. The present system can easily be coupled to a continuous-flow system equipped with a dialysis membrane in order to obtain a fully automated system for Mitomycin C analysis. Other applications of the phase-system switching approach in SFC are now under investigation and will be reported separately.

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