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Ion-pair chromatography of methotrexate in a column-switching system using an alkyl-diol silica precolumn for direct injection of plasma

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Abstract

The retention behaviour of methotrexate as an ion-pair with tetrabutylammonium in a column-switching system, based on an alkyl-diol silica C_8 precolumn, combined with an analytical column, LiChrospher RP 18, was studied. Methotrexate is mainly present as a divalent anion at pH 7.4, however, the retention data was consistent with the formation of a 1+1 ion pair with the counter ion. The concentration of the tetrabutylammonium and the acetonitrile in the mobile phase could be used to regulate the retention in the system. Relevant chromatographic parameters to estimate the enrichment effect in the column-switching system are also identified and discussed. The column-switching system was applied to direct injection of plasma (100 μl) giving a limit of detection of 10 ng/ml for methotrexate using UV detection at 307 nm.

Keywords: Column switching; Retention models; Ion-pair adsorption; Sample enrichment; Methotrexate

1. Introduction

Methotrexate (4-amino-10-methylfolic acid, MTX) is an acidic compound (Fig. 1) with dissociation constants of 3.36 (α-carboxyl), 4.70 (γ-carboxyl) and 5.71 (N-1) [1], respectively. On a reversed-phase column MTX can typically be retained either in acidic form [2,3] or as an ion-pair using anionic [4] or cationic counter ions [5], depending on the pH of the mobile phase. However, in the direct injection of plasma it is preferable to remove proteinaceous compounds in their native state [6,7], by using a pH 7.4 buffer. Therefore, an ion-pair chromatographic system with tetrabutylammonium ion as the counter ion and acetonitrile as the organic modifier in the buffer was chosen. In this paper, studies on the retention behaviour of MTX on the precolumn and the analytical column are presented. Retention models on the retention of MTX on the reversed-phase silica gel are proposed, providing an understanding of the influence of the concentration of the counter ion and the organic modifier. Although the mechanism of ion-pair adsorption has been studied

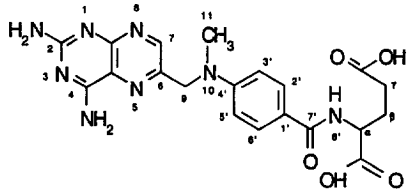

Fig. 1. Structure of methotrexate.

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intensively in different systems [8,9], reports on the behaviour of divalent anions in ion-pair systems are less common.

The enrichment effect is an important factor in the column-switching system, making it possible to transfer a large fraction from one column to another without substantial loss of resolution. The theoretical estimation as well as the application of this effect on handling of biological samples in a simple or a coupled HPLC system have been discussed [9–16]. In this paper, efforts are made on further describing and utilizing this phenomenon to design relevant parameters and to estimate the enrichment effect in the system.

Based on the studies on retention behaviour and enrichment effects, a column-switching system for the direct injection of plasma samples for the automated determination of MTX and its metabolite in the low-dose therapy (5–10 mg/ml) was developed. A precolumn of alkyl-diol silica (ADS) C_8, a type of restricted-access media [17–19], was coupled to an analytical column, LiChrospher RP 18, via an automatic six-port switching valve.

2. Experimental

2.1. Reagents and materials

Methotrexate (MTX) was purchased from Sigma (St. Louis, MO, USA). Acetonitrile of gradient grade, water of HPLC-grade, 1 *M* sodium hydroxide of analytical grade, ortho-phosphoric acid (85 wt.% solution in water) and tetrabutylammonium hydrogen sulphate (TBA, LiChropur) were obtained from Merck (Darmstadt, Germany). Micro test-tubes of 1.5-ml capacity were provided by Eppendorf (Hamburg, Germany).

2.2. Preparation of standards and plasma samples

All stock and working standard solutions of MTX were kept in plastic micro test-tubes to avoid the adsorption of MTX to glassware [20] and were covered with aluminium foil to protect them from light. No degradation of MTX was observed by UV detection for the stock solution stored at −20°C for more than three months.

Blank human plasma was obtained from the Blood Bank (University Hospital, Munich, Germany). The spiked plasma was made by vortex-mixing the working standard with the blank plasma with less than 10% dilution factors. Plasma samples were prepared daily from freshly thawed plasma and were centrifuged for 5 min at 4000 *g* prior to injection. The injection volume was 100 μl.

2.3. Chromatographic instrumentation and conditions

The retention studies were performed on a simple chromatographic system containing a pump, Model L-6200 (Merck), a Rheodyne injector 7125 (Berkeley, CA, USA), an analytical column, LiChrospher RP-18, 5 μm, 125×4 mm I.D., an ultraviolet detector set at 307 nm and a D-2500 integrator, all from Merck. The values of pH and ionic strength of the mobile phases mentioned below refer to the phosphate buffer system. Thiourea was used to measure the void volume of columns under different mobile phase conditions.

A schematic column-switching system is given in Fig. 2. The system consisted of two pumps (P1 and P2), both of which were Model L-6200, an AS-4000 autosampler (AS) connected to a cooling system (Lauda/Tauber, Germany), a precolumn of C_8-alkyl-

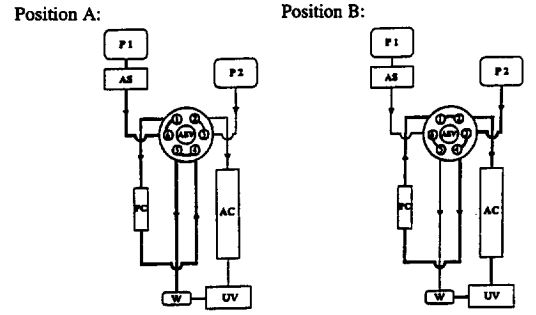

Fig. 2. Schematic diagram of column-switching HPLC system. Position A: injection and back-flush. Position B: loading. P1 = pump 1, 2 m*M* TBA in 2% acetonitrile + phosphate buffer, pH 7.4 (I = 0.05); P2 = pump 2, 5 m*M* TBA in 18% acetonitrile + phosphate buffer, pH 7.4 (I = 0.05); AS = autosampler; ASV = automatic switching valve; PC = precolumn, ADS C_8, 25×4 mm I.D., 25 μm; AC = analytical column, LiChrospher RP 18, 125×4 mm I.D., 5 μm; UV = ultraviolet detector, 307 nm; W = waste. Flow direction is indicated by arrows.

diol silica (ADS), 25 μm, 25×4 mm I.D. (PC) (Prof. Boos, Munich, Germany), an analytical column of LiChrospher RP-18 (AC), an ultraviolet detector set at 307 nm (UV), a D-2500 integrator and an automatic six-port switching valve, Model ELV 7000 (ASV) (Göttingen, Germany). All instrumentation was from Merck, except for those already mentioned above. The chromatography was performed at ambient temperature, except for the plasma samples that were thermostated at 4°C by the autosampler rack. The column-switching events were programmed by pump 2 to control the pump itself, the automated switching valve and the integrator. At position A, 100 μl of sample was loaded onto the ADS C_8 precolumn for 4 min with mobile phase 1. Then the valve was switched to position B, mobile phase 2 transferred the analyte from the precolumn to the top of the analytical column in a back-flush mode for 3 min. A new analysis cycle was started after 16 min.

Mobile phase 1, used for loading plasma sample onto the precolumn, consisted of 2 m*M* TBA in 2% acetonitrile with phosphate buffer, pH 7.4 (I=0.05). Mobile phase 2, used for the transfer and separation procedure, was 5 m*M* TBA in 18% acetonitrile with phosphate buffer pH 7.4 (I=0.05). Different percentages of organic modifier were mixed with the buffer in volume ratios. These solutions were used to prepare different concentrations of TBA. After being degassed in an ultrasonic bath for 5 min, the mobile phases were used directly at a flow-rate of 1.0 ml/min.

3. Results and discussion

3.1. Retention behaviour on the analytical column

The retention of MTX was studied with phosphate buffer, pH 7.4 (I=0.05) and acetonitrile as the organic modifier. It was possible to properly retain MTX by using an eluent containing 5 to 10% acetonitrile (see Fig. 3) with an efficient and symmetrical peak on the analytical column. The retention time of MTX was, however, rather unstable, since MTX was eluted in the steep region of the curve. Therefore, a hydrophobic counter ion, i.e. 5 m*M* TBA (Q^+), was added to the eluent. The dependence of the retention factor (k') on the

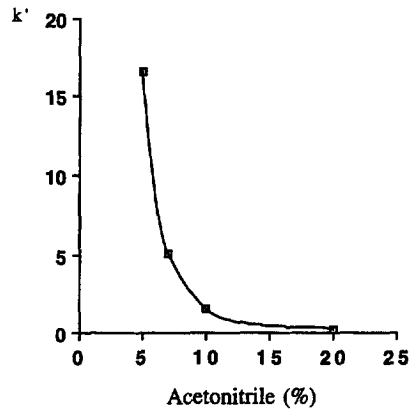

Fig. 3. Effect of acetonitrile content on the retention factor on the analytical column. Single-column LC system: mobile phase, acetonitrile in phosphate buffer, pH 7.4 (I=0.05).

percentage of acetonitrile in the mobile phase is given in Fig. 4. Comparing retention with and without TBA at 20% acetonitrile in the eluent indicates that k' is enhanced about fifteen times with the addition of 5 m*M* TBA in the eluent. This is obviously a strong ion-pair effect and it is further seen that the dependence of k' on the acetonitrile is much less steep than without TBA being present (Fig. 3), so acetonitrile can be used to properly regulate the retention. Naturally, the concentration of the counter ion (Q^+) also affects the retention on the column. Fig. 5 shows results from a variation of the TBA concentration (0–10 m*M*) with 20% acetoni-

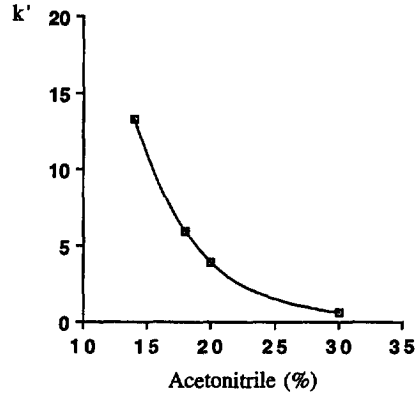

Fig. 4. Regulation of retention on the analytical column by varying the acetonitrile content in the presence of TBA in the mobile phase. Single-column LC system: mobile phase, acetonitrile and 5 m*M* TBA in phosphate buffer, pH 7.4 (I=0.05).

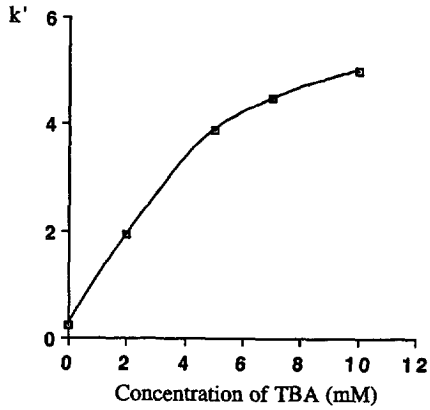

Fig. 5. The retention factor on the analytical column as a funtion of the concentration of TBA in the mobile phase. Single-column LC system: mobile phase, TBA in 20% acetonitrile + phosphate buffer, pH 7.4 (I=0.05).

trile in phosphate buffer, pH 7.4 (I=0.05). The retention factor of TBA initially increases linearly with increasing concentration of TBA, but at TBA≥ 5 mM, the curve reaches a plateau. Optimized conditions were chosen with respect to retention of MTX and potential interfering endogenous compounds and were, for the analytical column, 5 mM TBA in 18% acetonitrile with phosphate buffer, pH 7.4 (I=0.05).

3.1.1. Retention model: concentration of counter ion

At pH 7.4, MTX is mainly present as the divalent anion in an aqueous solution, based on its pK_a values (pK_{a_1}=3.36, pK_{a_2}=4.7). Applying the stoichiometric ion-pair adsorption model [8] for retention with TBA, the experimental data fitted better with the formation of the 1+1 complex (QHA). It can be shown that such a complex will dominate the retention if the following inequality holds:

$$K_{QHA}a_{H^+} > 10\,K_{Q_2A}K_{a_2}[Q^+] \quad (1)$$

where K_{QHA} and K_{Q2A} are the respective ion-pair adsorption equilibrium constants and $[Q^+]$ is the counter ion concentration.

The retention factor of MTX in the chromatographic system, can, according to the ion-pair adsorption model, be expressed as [8,9]:

$$k'_{HA} = \frac{qK^0K_{QHA}a_{H^+}[Q^+]}{K_{a_2}(1+K_{QX}[Q^+][X^-])} \quad (2)$$

where it is assumed in a similar fashion that $H_2PO_4^-$ (X^-) is the main co-ion competing with MTX for the adsorption site [and not HPO_4^{2-} which also is present in high concentrations at pH 7.4, (pK_{a_2}~7.2)]. K^0 is the total adsorption capacity of the stationary phase and q is the phase ratio. Eq. (2) indicates that k'_{HA} can be varied by the concentration of TBA and the competing ion in the mobile phase. Inverting Eq. (2) to

$$\frac{1}{k'_{HA}} = \frac{K_{a_2}}{qK^0K_{QHA}a_{H^+}[Q^+]} + \frac{K_{QX}[X^-]}{qK^0K_{QHA}a_{H^+}} \quad (3)$$

shows that a linear relationship should be obtained by computing $1/k'_{HA}$ against $1/[Q^+]$, keeping the pH constant. The plot of the experimental data is shown in Fig. 6, illustrating a reasonable fit as support for the assumption that the formation of the 1+1 complex dominates over the 2+1 complex.

3.1.2. Organic modifier

As demonstrated in Fig. 5, there is a linear relationship between k' and $[Q^+]$ at low concentrations of TBA. It is therefore reasonable to neglect the effect of the competing ions on the retention with ≤5 mM TBA in the mobile phase. The distribution processes, by varying the acetonitrile concentration (S), can then be illustrated by the processes:

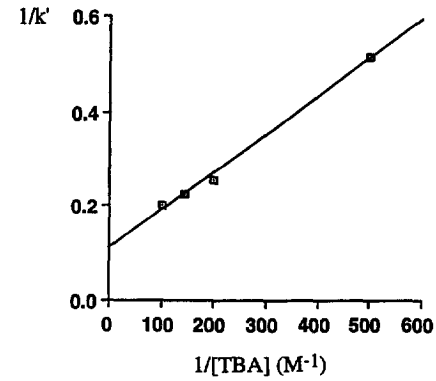

Fig. 6. Relationship between $1/k'$ and $1/[Q^+]$. For conditions see Fig. 5.

$$HA_m^- + Q_m^+ + F_s = QHAF_s \quad (4)$$

and

$$nS_m + F_s = S_nF_s \quad (5)$$

where F_s is the free adsorption site. Thus, k'_{HA} in the chromatographic system can be expressed as:

$$k'_{HA} = \frac{qK^0K_{QHA}[Q^+]}{1 + K_S[S]^n} \quad (6)$$

where K_S is the equilibrium constant for acetonitrile adsorption on the solid phase and n is the number of acetonitrile molecules competing for the adsorption site. Eq. (6) shows that the retention of MTX decreases with increasing acetonitrile concentration in the mobile phase, when the concentration of the counter ion is fixed. If $K_S[S] \gg 1$, Eq. (6) can be transformed to a simple logarithm form:

$$\log k'_{HA} = \log qK^0 + \log K_{QHA}[Q^+] - \log K_S - n \log[S] \quad (7)$$

Eq. (7) indicates a linear relationship between log k'_{HA} and log [S] on condition that q, K^0, K_{QHA}, K_S and $[Q^+]$ are constant. The graphic computation (Fig. 7a) shows a fairly good linearity within the concentration range studied. The number of acetonitrile molecules competing with the ion-pair complex is about four, according to the slope of the curve.

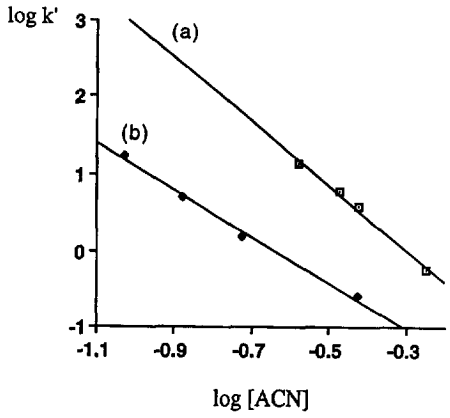

Fig. 7. Relationship between log k' and the logarithm of the acetonitrile concentration. (a) With 5 mM TBA in the mobile phase; (b) without TBA.

Even when the counter ion TBA is absent in the mobile phase, MTX is adsorbed on the stationary phase as shown in Figs. 3 and 5. This is evidence for ion-pair formation with sodium ion (Na^+) in the mobile phase. An expression of k'_{HA} analogous to that given in Eq. (7) can be derived:

$$k'_{HA} = \frac{qK^0K_{NaHA}[Na^+]}{1 + K_S[S]^n} \quad (8)$$

This means that k'_{HA} can be regulated by the concentration of sodium ion and acetonitrile in the mobile phase. Making the same assumptions as for Eq. (6), Eq. (9) is derived:

$$\log k'_{HA} = \log qK^0 + \log K_{NaHA}[Na^+] - \log K_S - n \log[S] \quad (9)$$

A plot of log k'_{HA} versus log [S], calculated from Fig. 3, results in a linear curve (Fig. 7b), supporting the validity of Eq. (9) within the studied acetonitrile range. The slope of the curve is three. The difference in the slopes of Fig. 7a and 7b is reasonable, since it indicates that more acetonitrile molecules are needed to compete with the larger TBA ion-pair compared to the Na^+ ion-pair. However, additional data on the effect of a variation of sodium ion in the mobile phase is needed to confirm the retention model of this kind.

3.2. Retention behaviour on the precolumn

MTX could be retained on the ADS precolumn with C_{18}-ligands only in the pure buffer system. Insufficient retentivity was noticed by adding 3% acetonitrile to the mobile phase, which was necessary for directing lipophilic compounds in plasma to waste and accelerating the release of drug from protein binding. Applying ion-pair conditions to the ADS C_8 surface was, therefore, chosen for the loading process. Table 1 demonstrates that the addition of 2 mM TBA in the buffer enhances the retention of MTX about ten-fold. But the broad peak implies a low efficiency of MTX on the ADS C_8 surface. This is drastically improved by adding 2% acetonitrile to the mobile phase. The small amount of organic modifier had seemingly a small effect on the retention of MTX. However, the retention value

Table 1
Ion-pair retention and peak width of MTX on ADS C_8

Mobile phase	Retention (min)	Peak width (ml)
PB, pH 7.4[a]	3.0	3
2 m*M* TBA in PB, pH 7.4[a]	~29[b]	>40
2 m*M* TBA in 2% ACN PB, pH 7.4[a]	29.6	12

Single-column LC system: column, ADS C_8, 25 μm, 25×4 mm I.D.; flow-rate, 1.0 ml/min; detection, 307 nm, 0.01 AUFS.
[a] Phosphate buffer, pH 7.4, with an ionic strength of 0.05.
[b] Approximate value caused by the peak broadening.

obtained without the addition of acetonitrile is approximate, caused by the very broad peak. Hence 2 m*M* TBA in 2% acetonitrile with phosphate buffer, pH 7.4 (I=0.05) was used as the loading mobile phase.

3.3. Enrichment effect

When the retention principle of two columns in a reversed-phase system is correctly adjusted, the extra band broadening caused by transfer of wide fractions of the eluent from the first to the second column can readily be avoided by utilizing the enrichment effect on the top of the second column, as discussed in previous papers [15,16]. Assuming the fraction is transferred in a plug form [10] and that the two columns are matching in geometry [21], the enrichment effect can be estimated by the following formula [9]:

$$V_e = \frac{V_i}{1 + k'_s} \quad (10)$$

where V_e = effective injected volume on the top of the second column; V_i = sample volume transferred from the first column to the second column; k'_s = the retention factor of the analyte on the second column using the first mobile phase. This equation may be valid in a wide sense including the sample injection zone or the transfer step with various switching modes [22]. In principle, $k'_s \gg 1$. With the back- or fore-flush mode in column-switching systems, the following equation is tenable:

$$V_i = xV_0(1 + k'_p) \quad (11)$$

where V_0 = the void volume of the primary column; x = fraction of V_0 depending on how far the analyte has reached into the precolumn; k'_p = the retention factor of the analyte on the first column using the second mobile phase. Inserting Eq. (11) into Eq. (10) gives:

$$V_e = xV_0 \frac{1 + k'_p}{k'_s} \quad (12)$$

This equation demonstrates that the enrichment effect for the transfer process involves two different retention factors derived from two different stationary phases in cross correspondence with two different mobile phases. For most commonly used column-switching system, $(1 + k'_p)$ can not be simplified as k'_p, on account of the low void volume and the fast elution on the precolumn as was done in e.g. Refs. [13,23].

According to Eq. (12), it is obvious that the lower the value of k'_p and the higher the value of k'_s, the better the enrichment effect that is achieved in the system. The former case can be reached by using a less retentive precolumn combined with a mobile phase with stronger eluting power for the second column. While a more hydrophobic analytical column together with a weak mobile phase for running the precolumn would fit the latter requirement. The desorption of MTX from the precolumn gave $V_i \leq$ 2000 μl. Since k'_s is rather high as can be seen from Fig. 7a, log $k' > 3$, V_e is < 2 μl by applying Eq. (12). Fig. 8 is a comparison of chromatograms obtained from the single and the coupled systems, illustrating the negligible extra band broadening brought about by the transfer process. However, a slight increase in retention time was observed in the coupled system, which may be attributed to the presence of a mixture of eluents on the top of the analytical column decreasing the eluting strength, and a lag time due to the desorption and the valve-switching events.

3.4. Determination MTX in plasma with UV detection

Based on the designed conditions above, blank and spiked plasma samples were directly injected into the system. As demonstrated in Fig. 9, a relatively clean blank chromatogram was obtained.

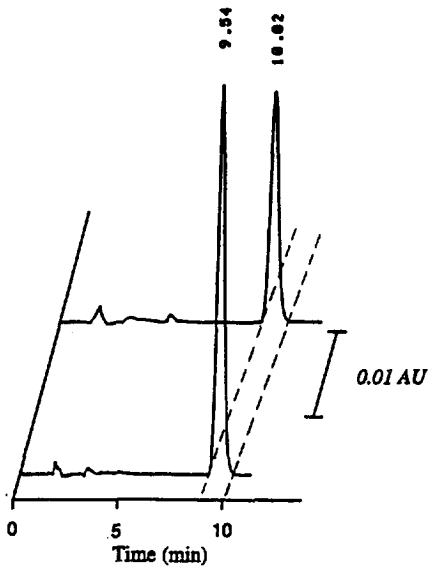

Fig. 8. Chromatograms obtained from the analytical column with (front) single-column system and (back) coupled-column system. Different concentrations of MTX were injected.

The precolumn was stable without noticeable pressure change during the whole analysis period with ≥300 plasma injections. The detection limit for analysing MTX was about 10 ng/ml, based on a signal-to-noise ratio of three. The analysis capacity was 90 samples per 24 h. No studies on repeatability and recovery were performed since a more sensitive assay utilizing fluorimetric detection was developed based on the column-switching system described here [24].

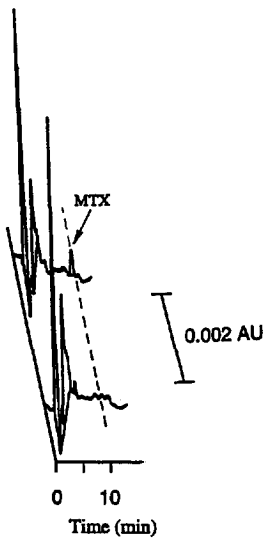

Fig. 9. Determination of MTX in human plasma. (Front) Blank chromatogram; (back) plasma spiked with 22 ng/ml of MTX. Conditions as described in Section 2.

4. Conclusions

Methotrexate (MTX) is present as a divalent anion in the mobile phase of pH 7.4. However, the retention as an ion-pair using tetrabutylammonium (TBA) as the counter ion is consistent with the distribution of a 1+1 ion-pair to the solid phase LiChrosphere RP 18. The retention of MTX can also be regulated by the acetonitrile concentration and there is a linear relationship between the logarithm of the retention factor and the acetonitrile concentration.

A column-switching system for the direct injection of plasma was designed, based on ion-pair adsorption chromatography with TBA as the counter ion. The precolumn was an alkyl-diol silica (ADS) based restricted access medium with C_8-ligands in the pores, coupled to a C_{18} silica gel analytical column. The analytes were trapped on the precolumn and transferred by back-flush to the C_{18} column. Principles to obtain adequate enrichment of the analytes on the analytical column were evaluated and a new relationship for this effect in coupled-column systems of this kind is presented. Applying UV at 307 nm, the limit of detection for MTX was 10 ng/ml based on a signal-to-noise ratio of three, and 90 samples per 24-h period could be analysed.

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