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COLUMN LIQUID CHROMATOGRAPHY OF METHOTREXATE AND ITS METABOLITES USING A POST-COLUMN PHOTOCHEMICAL REACTOR AND FLUORESCENCE DETECTION

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SUMMARY

On irradiation with short-wavelength UV light in the presence of hydrogen peroxide, methotrexate and its metabolites 7-hydroxymethotrexate and 2,4-diamino-N^{10}-methylpteroic acid are cleaved into highly fluorescent products. This reaction can be used for the sensitive and selective detection of the compounds in biological fluids, following reversed-phase high-performance liquid chromatographic separation. Study of the effect of the mobile phase composition and irradiation time on fluorescence signal intensity showed that a residence time of ca. 3 s in the on-line photochemical reactor was best. The detection limit for methotrexate was 0.4 ng, for 7-hydroxymethotrexate 1.0 ng and for 2,4-diamino-N^{10}-methylpteroic acid 0.6 ng. The addition of dimethylformamide to the mobile phase enhanced the selectivity of separation.

INTRODUCTION

The disadvantage of the limited applicability of the highly sensitive and selective fluorescence detection can be overcome by a conversion of non-fluorescent products, either before or after separation by high-performance liquid chromatography (HPLC). An extensive review of fluorescence derivatization for HPLC has been published by Lingeman et al. [1].

UV irradiation of the effluent in a post-column reactor is often used to improve the fluorescence detection of various compounds [2–9]. The simplest reactor is a capillary made of a material with good UV transparency (e.g. PTFE). The transparency of PTFE is probably based on a diffuse radiation transfer. Compared with less flexible quartz capillaries, similar or even higher signal intensities are often observed with PTFE capillaries, and the peaks show better symmetry and less tailing. The high efficiency (high signals, rapid decomposition of sol-

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utes) obtained with PTFE capillaries can be explained by the light-tube effect of this material [4].

We have recently published a method for fluorescence detection of methotrexate (MTX) and its metabolites 7-hydroxymethotrexate (7-OH-MTX) and 2,4-diamino-N^{10}-methylpteroic acid (APA) [9]. The method is based on the on-line photooxidative reaction of MTX and its metabolites to highly fluorescent 2,4-diaminopteridine derivatives. The photooxidation is brought about by irradiation of the effluent in the presence of hydrogen peroxide. The oxidant was added directly to the mobile phase. The sensitivity of the method was studied for MTX only with respect to the concentration of hydrogen peroxide in the mobile phase and the length of the capillary (i.e. irradiation time).

In this paper the influence of other factors on the separation and detection limit of MTX and its metabolites is discussed.

EXPERIMENTAL

Chemicals

MTX (99.5% purity by HPLC), 7-OH-MTX (95%) and APA (95%) were prepared in the Research Institute of Pure Chemicals (Lachema, Brno, Czechoslovakia). Acetonitrile LiChrosolv, methanol LiChrosolv, tetrahydrofuran LiChrosolv, dioxan LiChrosolv, N,N-dimethylformamide Uvasol and dimethylsulphoxide Uvasol were from Merck (Darmstadt, F.R.G.). All other chemicals were of analytical grade. Pterine-6-carboxaldehyde and pterine-6-carboxylic acid were gifts from Professor K. Slavik (Prague, Czechoslovakia).

Equipment

Chromatographic separations were performed on a Spectra-Physics Model SP 8100 liquid chromatograph with a Model SP 8110 autosampler (Spectra-Physics, Darmstadt, F.R.G.) fitted with a 10-μl or 80-μl loop, and a Schoeffel Instruments Model FS 970 fluorescence detector (Kratos, Trappenkamp, F.R.G.) with a 5-μl flow-cell. Excitation wavelengths were set at 370 nm (plus filter 7-51) or 282 nm (plus filter 7-54). Fluorescence was measured by using a 417-nm cut-off emission filter. The analytical stainless-steel column (25 cm$\times$0.46 cm I.D.) was packed with LiChrosorb RP-18, d_P 10 μm (Merck), and it was protected by a guard column LC-18, d_P 40 μm (Supelco, Bellefonte, PA, U.S.A.). Chromatographic data were evaluated by a Spectra-Physics 4000 data system. In some analyses peak heights were also measured.

The photooxidation was accomplished in a PTFE capillary (0.25 mm I.D., 1.59 mm O.D.) inserted between the column and the detector. The capillary was coiled around a Sylvania G8T5 germicidal lamp (254 nm); the optimum length of the irradiated section was 110 cm. Aluminium foil was mounted under the lamp housing to increase the reflection of the emitted light. Cooling was effected by sucking air through the reactor housing.

For comparison of UV and fluorescence detection a Spectra-Physics Model SP 8300 UV–VIS detector set at 312.5 nm was used.

The products of decomposition of MTX were separated on a CGC column (15 cm × 0.32 cm I.D.) packed with LiChrosorb RP-18, d_P 5 μm (Merck).

Mobile phase

Mixtures of phosphate buffer of various concentrations and pH, organic polar solvents and hydrogen peroxide were tested. The final mobile phase was 0.05 *M* phosphate buffer (pH 6.2)–acetonitrile–dimethylformamide–30% hydrogen peroxide (500:35:28:0.75). For optimum detection of MTX and APA the mobile phase was sonicated or prepared one day before the separation to achieve constant concentration of dissolved oxygen. For optimum detection of 7-OH-MTX the same mobile phase was degassed by helium purging. The flow-rate of the mobile phase was 1 ml/min. All chromatographic separations were carried out at 45 °C.

Samples

Heparinized blood was centrifuged for 15 min at 16 000 *g* before analysis. The amount of sample injected was 10 or 80 μl for plasma and 10 μl for urine. Samples that could not be chromatographed immediately after collection were stored at −24 °C. Standard samples containing 120 ng of MTX, 120 ng of 7-OH-MTX and 60 ng of APA were freshly prepared before the separation by mixing 0.9 ml of blank plasma or urine with 0.1 ml of an aqueous solution of MTX, 7-OH-MTX and APA.

Limit of detection

The detection limits (LOD) were calculated according to Foley and Dorsey [10]:

$$\mathrm{LOD} = 3s_B/S$$

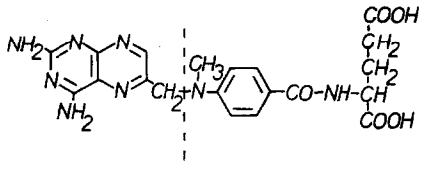
where S is analytical sensitivity slope and s_B is the standard deviation of the baseline. Moreover, the standardized chromatographic detection limits were calculated for chromatographic reference state $k_{ref} = 4$, $N_{ref} = 10\,000$ and $V_{M,ref}$ (ml) = 1.5 according to the equation:

$$q_{L,std} = \frac{\sigma_{V,ref}}{\sigma_{V,exp}} q_{L,exp}$$

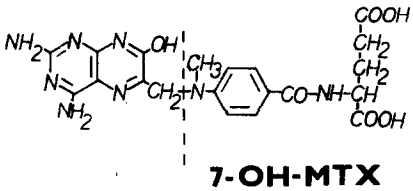
where $q_{L,std}$ is the standardized absolute limit of detection in units of amount, $\sigma_{V,ref}$ is the reference bandwidth calculated from the equation:

$$\sigma_{V,ref} = V_{M,ref}\ (1 + k_{ref})/(N_{ref})^{1/2}$$

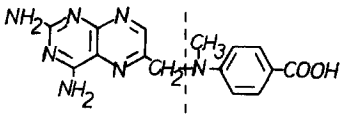
$\sigma_{V,exp}$ is the experimental bandwidth and $q_{L,exp}$ is experimental absolute limit of detection in units of amount.



MTX



7-OH-MTX



APA

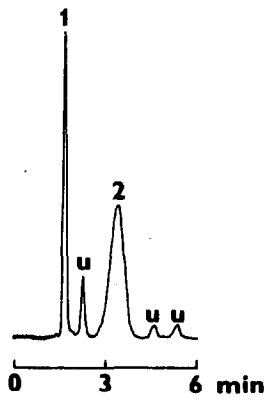


Fig. 1. Structural formulae of MTX and its metabolites 7-OH-MTX and APA. Dashed lines indicate bonds that are cleaved on irradiation.

Fig. 2. Chromatogram of the products of MTX photodecomposition. Column, CGC 15 cm×0.32 cm I.D. packed with LiChrosorb RP-18, d_p 5 μm; mobile phase, 0.05 *M* phosphate buffer (pH 6.2)-acetonitrile (500:40); fluorescence detection at 370 nm excitation wavelength and 418 nm emission wavelength; flow-rate, 1 ml/min; amount of sample, 10 μl; column temperature, 45°C. For other conditions see Experimental. Peaks: 1=2,4-diaminopteridine-6-carboxylic acid; 2=2,4-diaminopteridine-6-carboxaldehyde; u=unidentified.

RESULTS AND DISCUSSION

Fluorescence characteristics

MTX and its metabolites do not exhibit native fluorescence. However, they are cleaved by UV irradiation in the presence of hydrogen peroxide to highly fluorescent compounds (i.e. 2,4-diaminopteridine-6-carboxaldehyde and corresponding carboxylic acid). The extra-ring C–N bond is cleaved, as indicated in Fig. 1. A chromatogram of the photolytic decomposition products is shown in Fig. 2. Peaks of 2,4-diaminopteridine-6-carboxaldehyde and of the corresponding carboxylic acid were identified on the basis of chemical reactivity of both compounds and comparison with the chromatographic behaviour of some analogues [11].

TABLE I

DEPENDENCE OF MTX, 7-OH-MTX AND APA RESPONSE ON MOBILE PHASE COMPOSITION

Mobile phases were mixtures of 0.05 *M* phosphate buffer (pH 6.2) and 30% hydrogen peroxide (500:0.75) with various organic solvents; they were not degassed. For comparison, the fluorescence response in the mobile phase with acetonitrile was set at 100%.

Mobile phase	Organic solvent(s)	Percentage in mobile phase	Response (%)		
			MTX	7-OH-MTX	APA
1	Acetonitrile	10	100	100	100
2	Dioxan	9	45	160	100
3	Dimethylformamide Acetonitrile	5 6	95	90	100
4	Dimethylformamide Acetonitrile	10 2	85	75	100
5	Tetrahydrofuran Acetonitrile	2 10	2	8	5
6	Dimethylsulphoxide	5	2	2	5
7	Methanol	20	1	1	1
8	Ethanol	7	4	5	8
9	2-Propanol	10	2	2	2

Effect of various parameters on the photochemical reaction

Influence of mobile phase composition. The composition of the mobile phase influences both the separation of MTX and its metabolites and also the sensitivity of the fluorescence detection. In our search for the optimum mobile phase eight organic solvents were tested (Table I). The fluorescence response is generally much lower with protic solvents than with aprotic ones. However, this does not hold for all aprotic solvents, e.g. tetrahydrofuran and dimethylsulphoxide. The effect of the solvent on photodecomposition is different for particular derivatives: e.g. with dioxan instead of acetonitrile the fluorescence response is enhanced for 7-OH-MTX and decreased for MTX. The addition of dimethylformamide caused a slight decrease of fluorescence response; the effect is more marked for 7-OH-MTX than for MTX. On the other hand, the response of APA remains virtually the same in mobile phases with acetonitrile, dioxan or dimethylformamide. The behaviour of MTX and 7-OH-MTX cannot be explained yet, since information about the photochemical reaction mechanism is lacking.

Influence of pH. The influence of pH on the yield of the photochemical reaction response was studied by using non-degassed mobile phase with 0.05 *M* phosphate buffer of pH 2.5–7.5. The relative fluorescence responses observed for MTX and its metabolites at various pH values are shown in Fig. 3. For MTX the maximum response was observed at pH 6.75, and for 7-OH-MTX at pH 4.6. The response for APA showed a plateau at pH values above 6.2. In the case of MTX the response decreased as the pH value was lowered, and for 7-OH-MTX the response significantly decreased above pH 5. The optimum pH value of the phosphate buffer for all compounds is 6.2, where the sum of the responses is at a maximum.

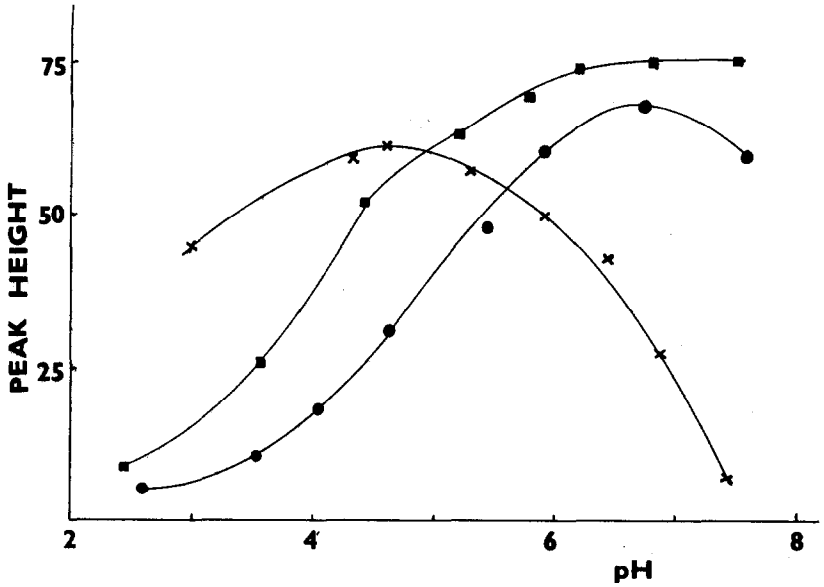


Fig. 3. Influence of mobile phase pH on fluorescence signals of MTX (●), 7-OH-MTX (×) and APA (■). The pH value of phosphate buffer was varied from 2.5 to 7.5. For other conditions see Experimental.

Influence of phosphate buffer concentration. The dependence of the fluorescence response on the concentration of phosphate buffer in the mobile phase is shown in Table II. As expected, the fluorescence is quenched in mobile phases with a high buffer concentration. In 0.4 *M* phosphate buffer the response was only 60–75% of that in water. It is important that quenching is negligible for buffer concentrations ranging from 0.015 to 0.05 *M*, which are needed for satisfactory chromatographic separation.

Influence of hydrogen peroxide concentration. The dependence of the fluorescence response of MTX on the concentration of hydrogen peroxide was described in our previous paper [9]. The same concentration (12 m*M*) was found to be optimum for the fluorescence detection of 7-OH-MTX and APA.

TABLE II

DEPENDENCE OF FLUORESCENCE RESPONSE ON THE CONCENTRATION OF PHOSPHATE BUFFER (pH 6.2)

For conditions see Experimental.

Phosphate buffer concentration (mol/l)	Relative response		
	MTX	7-OH-MTX	APA
0	88	75	60
0.015–0.05	75	65	55
0.075	70	63	54
0.4	60	56	35

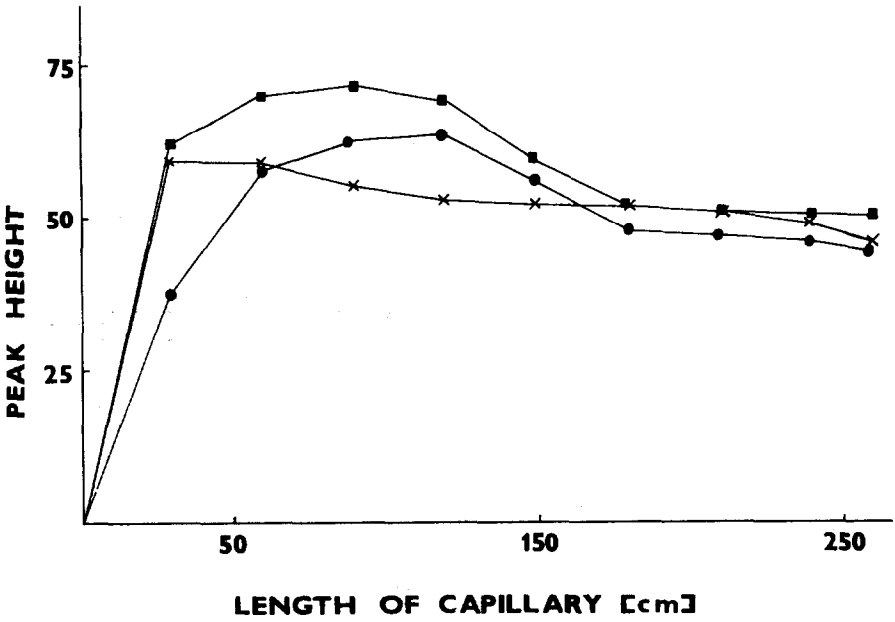


Fig. 4. Dependence of the fluorescence response of MTX (●), 7-OH-MTX (×) and APA (■) on the length of the irradiated section of the PTFE capillary. At a given flow-rate, 1 cm of the capillary corresponds to 0.030 s of irradiation time. For other conditions see Experimental.

Influence of irradiation time. At a given flow-rate, the irradiation varied with the length of the capillary (Fig. 4). Maximum response was found with the irradiation times of 3.2 s for MTX, 2.4 s for APA and 0.9 s for 7-OH-MTX. In all cases, prolonged irradiation resulted in a slight decrease of the fluorescence response.

Dependence of the detection limit on the presence of oxygen in mobile phase. In Table III the detection limits for MTX and its metabolites are given for a mobile phase that was not degassed and one that was thoroughly purged with helium for

TABLE III

LIMITS OF DETECTION OF PARTICULAR DERIVATIVES IN DEGASSED AND NON-DEGASSED MOBILE PHASE AT DIFFERENT EXCITATION WAVELENGTHS

For conditions see Experimental.

Compound	Limit of detection (ng)				
	Excitation wavelength 282 nm		Excitation wavelength 370 nm		$q_{L,std}$
	Non-degassed	Degassed	Non-degassed	Degassed	
MTX	0.4	0.8	0.4	0.9	0.4★
7-OH-MTX	4.2	2.7	1.5	1.0	1.0★★
APA	1.0	1.9	0.6	1.2	0.4★

★Not degassed.
★★Degassed.

15 min. The excitation wavelengths were 282 and 370 nm. The results show that the presence of oxygen in the mobile phase strongly influences the detection of the various compounds. In the mobile phase that was not degassed the detection limits of MTX and APA were half those in the degassed mobile phase. The opposite was observed for 7-OH-MTX: in the presence of oxygen in the mobile phase the detection limit is increased 1.5 fold. In practice it is rather difficult to prevent the mobile phase coming into contact with atmospheric oxygen; to achieve this it is necessary to keep the mobile phase under helium.

Using the fluorescence method described, limits of detection were lower than with UV detection: twenty times lower for MTX and APA in the mobile phase that was not degassed and eighteen times lower for 7-OH-MTX in the degassed mobile phase, at an excitation wavelength of 370 nm.

When the excitation wavelengths of 370 and 282 nm were compared, a higher response was found only for MTX in the latter case. As various components of biological samples, especially urine, show a weaker fluorescence response at 370 nm, this excitation wavelength is selected as more suitable for the analysis.

For the mobile phase that was not degassed the relative standard deviation for five injections was 1.02% for 22 ng of MTX, 2.10% for 20 ng of 7-OH-MTX and 0.65% for 10 ng of APA.

A linear relationship between peak height and sample amount was observed up to 75 ng for MTX, 70 ng for 7-OH-MTX and 50 ng for APA. Coefficients of variation of less than 2.5% were found for sample amounts ranging from 5 ng to the upper limit of linearity for all three compounds.

Separation

In order to achieve sufficient separation of MTX and its metabolites in the shortest time possible, the influence of mobile phase composition on resolution and separation efficiency was studied. Fig. 5 shows the dependence of log k of all three derivatives on the pH value of the phosphate buffer in mobile phase 1 (Table I).

The values of resolution (R_s) and theoretical plate number (N) found for MTX and 7-OH-MTX in mobile phases containing various amounts of dimethylformamide are given in Table IV.

In mobile phase 1 containing only acetonitrile and no dimethylformamide, the resolution of MTX and 7-OH-MTX is only 0.7. As the separation factor α of both compounds is only 1.05, a column efficiency of 16 000 theoretical plates should be necessary to achieve an R_s value of 1.5. The addition of 5% of dimethylformamide improves both the resolution and the efficiency, so that a perfect baseline separation of MTX and 7-OH-MTX can be obtained (see Fig. 6A). Partial substitution of acetonitrile by dimethylformamide in the mobile phase also helped to prolong the life of the guard column.

Even better results were achieved for the separation of MTX and its metabolites when the content of dimethylformamide was increased to 10%; however, the fluorescence diminished at the same time (Table I). Determination of 7-OH-MTX is also possible in mobile phase 2 (Table I) with dioxan, where for peaks MTX and 7-OH-MTX an R_s value of 1.0 was achieved.

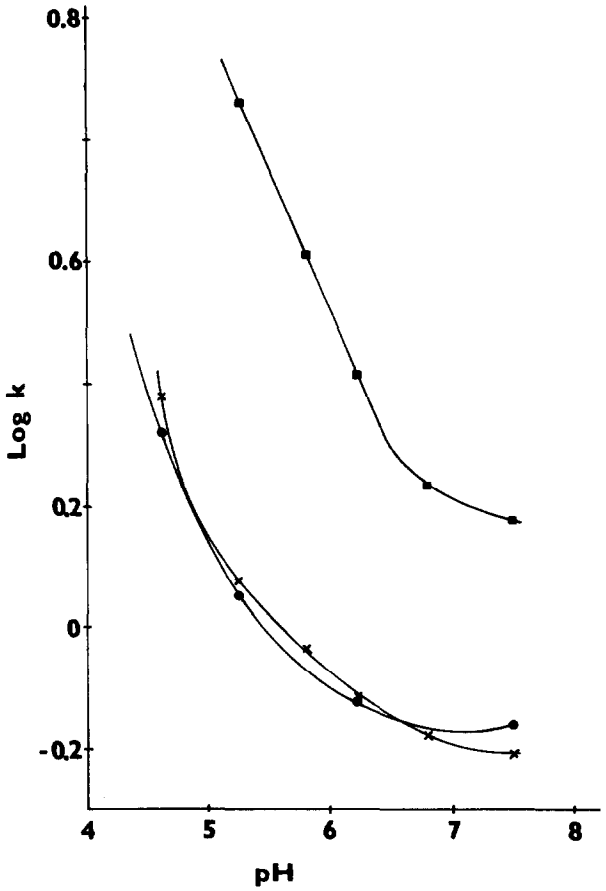


Fig. 5. Dependence of log k of MTX (●), 7-OH-MTX (×) and APA (■) on mobile phase pH. Mobile phase 1 (Table I) was used. For other conditions see Experimental.

The UV detector was used to study the contribution of the photoreactor capillary to extra-column peak broadening, and a reduction of the system efficiency from 4300 to 3600 theoretical plates was observed for MTX.

TABLE IV

DEPENDENCE OF SEPARATION EFFICIENCY AND RESOLUTION OF MTX AND 7-OH-MTX PEAKS ON THE CONTENT OF DIMETHYLFORMAMIDE IN MOBILE PHASE

Sample volume, 10 μl; for other conditions see Experimental.

Dimethylformamide (%)	R_s	N	
		MTX	7-OH-MTX
0	0.7	2900	3000
3	1.3	3100	3700
5	1.5	3500	4100
10	1.8	3500	4000

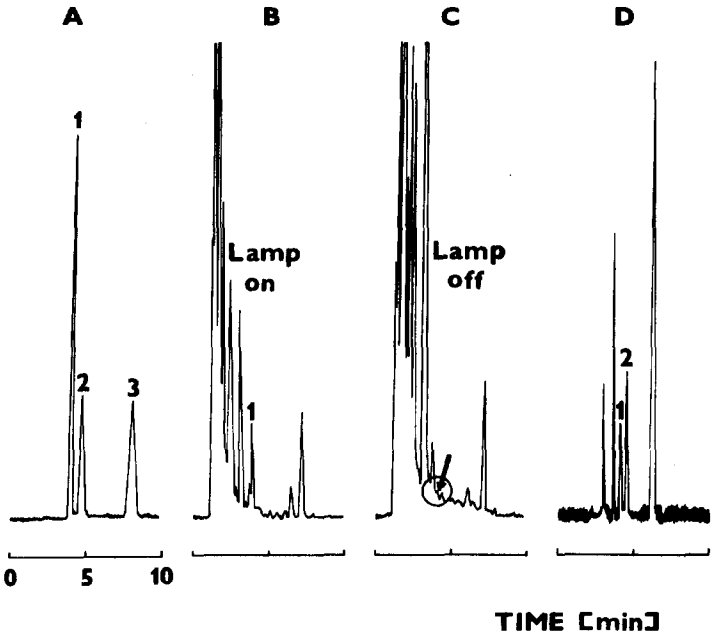


Fig. 6. Chromatograms of urine and plasma samples of a patient treated with MTX. (A) Standard mixture containing 120 ng of MTX (1), 120 ng of 7-OH-MTX (2) and 60 ng of APA (3); (B) chromatogram of a urine sample collected 8 h after the administration of 20 mg of MTX (1) (concentration of MTX in the sample, 240 ng/ml); (C) chromatogram of the same sample as in B repeated with UV lamp switched off (the arrow indicates the position of the fluorescent product of MTX photooxidation); (D) chromatogram of plasma sample collected 8 h after administration of 20 mg of MTX [concentrations found: 12.5 ng/ml MTX (1) and 44 ng/ml 7-OH-MTX (2)].

Application of the method

The method was used to monitor the concentration of MTX, 7-OH-MTX and APA in plasma and urine. Fig. 6 shows a chromatogram of a standard mixture of MTX, 7-OH-MTX and APA, and chromatograms of urine and plasma samples collected 8 h after the administration of 20 mg of MTX. The sample of urine was chromatographed twice: with (B) and without (C) UV irradiation of the column effluent. Comparison of the two chromatograms allows the peaks of naturally fluorescent products to be eliminated.

CONCLUSIONS

Compared with other post-column derivatization techniques, the method described here has several advantages: (i) in contrast to the addition of a reagent solution, there is no extra peak dilution, no mixing problems, less pump noise and no need for an extra pump; (ii) the cleavage is achieved in a fairly short time (1–4 s), so that the photochemical reactor has only a small effect on the resolution and system efficiency; (iii) the fluorescence detection is ca. twenty times more sensitive than UV detection.

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