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On-line precolumn photochemical generation of pH gradient: micro-high-performance liquid chromatography of methotrexate and its impurities

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ABSTRACT

On-line UV irradiation of a flowing mobile phase that contains formate buffer and hydrogen peroxide results in a significant increase in pH owing to the chain reaction to carbonic acid. This reaction was used for pH gradient formation in micro-high-performance liquid chromatography. The gradient was started by switching on a photoreactor which was inserted in front of the injection valve. The shape of the pH gradient was affected by covering part of the PTFE capillary from UV light. The range between the initial and final pH values corresponds to the range of pK_a values of formic and carbonic acid. The use of photochemical pH control for the separation of methotrexate and its impurities without buffer mixing is illustrated. The reproducibility of retention times was better than 1% and of peak heights better than 25%. The detection limit for methotrexate was 5 pmol at 315 nm.

INTRODUCTION

The popularity of microcolumn high-performance liquid chromatography (HPLC) has increased in recent years [1,2]. However, special requirements are placed on the miniaturization and optimization of the instrumentation.

Generally, the analytical sensitivity and selectivity can be improved by the use of a continuous gradient of the mobile phase composition which leads to a continuous decrease in solute retention on the separation column [3]. The retention of ionizable solutes can be controlled by adjusting the mobile phase pH, ionic strength and concentrations of organic solvents and ion-interacting compounds. In micro-HPLC tubular mixers [4,5], which can generate a gradient of variable profile for microcolumns of 1 mm I.D., splitters [6] with a conventional low-pressure gradient system and small-volume mixing vessel and magnetic stirrer [7,8] have been suggested.

An example of a pH gradient elution programme based on trichloroacetate reduction generated in a coulometric cell by the control of the electrolysis current without buffer mixing was demonstrated by Wright and Evilia [9]. This gradient programme was used for the separation of amino acids in preparative chromatography.

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Baxendale and Wilson [10] described the chain reaction of formic acid with hydrogen peroxide when irradiated by UV light:

$$H_2O_2 \rightarrow HO^{\cdot} + HO^{\cdot}$$
$$HO^{\cdot} + HCO_2H \rightarrow H_2O + {}^{\cdot}CO_2H$$
$${}^{\cdot}CO_2H + H_2O_2 \rightarrow CO_2 + H_2O + HO^{\cdot}$$
$$HO^{\cdot} + {}^{\cdot}CO_2H \rightarrow CO_2 + H_2O$$

Formic acid of pK_a 3.75 is converted into carbon dioxide or at higher pressures, into carbonic acid of pK_a 6.35.

Recently, hydrogen peroxide was used as a mobile phase additive for the post-column on-line photo-oxidative conversion of the cytostatic drug methotrexate to highly fluorescent products [11,12]. These reactions significantly improved sensitivity and selectivity of HPLC determinations in complex biological matrices. On the other hand, such a small amount of hydrogen peroxide in the mobile phase did not affect the retention and stability of solutes.

Methotrexate (MTX, 4-amino-N^{10}-methylpteroylglutamic acid) is an antifolate that has significant antitumour activity in various neoplastic diseases. The parent compound may contain up to 1% of impurities and/or degradation products which can cause febrile reaction after administration. There is a great need for a selective and sensitive determination of aminopterine (AMTP, 25 times more toxic than MTX) and methopterine (METP, degradation product) in MTX (*e.g.*, [13]). A review [14] that included HPLC methods for the determination of methotrexate has been published. The effect of pH on the retention behaviour of pteroylglutamates has also been reported [13].

In this paper, the photoreaction of formate buffer and hydrogen peroxide for pH gradient formation in micro-HPLC is suggested. An example of the separation of methotrexate from its impurities is demonstrated.

EXPERIMENTAL

Methotrexate (99.5% purity by HPLC), aminopterine (95%) and methopterine (93%) were prepared in the Research Institute of Pure Chemicals (Lachema, Brno, Czechoslovakia). Acetonitrile (LiChrosolv grade) was obtained from Merck (Darmstadt, F.R.G.). All other chemicals were of analytical-reagent grade.

Chromatographic separations were performed on a Spectra-Physics (Darmstadt, F.R.G.) Model SP 8700 liquid chromatograph equipped with Rheodyne Model 7125 six-port valve fitted with a 60-μl external loop. A Model SF 769Z UV detector (Kratos, Ramsey, NJ, U.S.A.) was equipped with a 0.5-μl flow cell and set at 315 nm. An OP 208/1 pH meter (Radelkis, Budapest, Hungary) equipped with a 100-μl flow electrode was connected to the outlet of detector to monitor the pH of the column effluent. Chromatograms were recorded on a TZ 4200 dual recorder (Laboratory Instruments, Prague, Czechoslovakia). A CGC 150 mm × 1 mm I.D. glass microcolumn (Laboratory Instruments) was packed with Silasorb SPH C_{18} (d_p = 7.5 μm) (Lachema).

The photo-oxidation was accomplished in a PTFE capillary (0.35 mm I.D., 1.59 mm O.D.) which was coiled around the moving quartz tube inside which was placed a

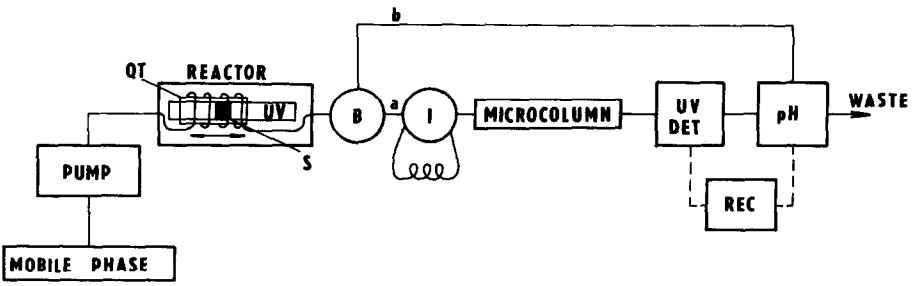

Fig. 1. Schematic diagram of the chromatographic apparatus. Solid lines, liquid connections, dashed lines, electrical connections. I = injection valve; B = bypass-valve; UV DET = UV detector; UV = UV lamp; pH = pH detector; S = strip of black paper; QT = quartz tube.

tubular 8-W low-pressure mercury lamp (GTE, Sylvania G8T5); the length of the irradiated capillary was 500 cm, representing a volume of 480 μl. A strip of black paper served to shield the central part of the capillary from UV illumination. A schematic diagram of the apparatus is shown in Fig. 1.

The mobile phase for the separation of methotrexate and its derivatives was 0.05 *M* formate buffer (initial pH 3.8)–acetonitrile–30% hydrogen peroxide (90:10:0.5, v/v/v) at a flow-rate of 50 μl/min.

RESULTS AND DISCUSSION

Formation of gradient

The typical course of the photoreaction which takes place in the stream of the mobile phase as detected by a pH meter is shown in Fig. 2, which also illustrates the dependence of pH on the irradiation time and shows that the photoreaction is completed in 120 s.

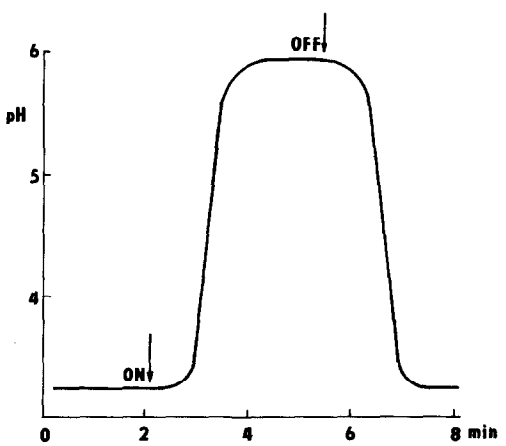

Fig. 2. Time dependence of the pH of the mobile phase after switching on the UV lamp. Configuration of the apparatus as in Fig. 1 (bypass in position b). Mobile phase, 0.05 *M* formate buffer (pH 3.3) with 0.5% of 30% hydrogen peroxide; volume of the PTFE capillary, 480 μl; flow-rate, 200 μl/min.

In order to create different gradient profiles, part of the PTFE capillary was shielded from UV light as shown in Fig. 1. An example of such a profiled gradient is shown in Fig. 3. The part of the curve preceding point 0 has a constant pH determined by the pumped mobile phase. The UV lamp is switched on at time 0 and the gradient is started. The section a represents the volume of the mobile phase, which is illuminated from time 0 to t_1 and the first part of the gradient takes place; t_1 is the time period which the mobile phase needs to pass from the shielded part through the illuminated part of the capillary a (100 μl) to the end of the capillary. In the next segment, b, the mobile phase is irradiated for a time period t_1 and an isocratic part corresponding to the volume of the shielded part of the capillary b (225 μl) is obtained. Part c corresponds to the mobile phase volume illuminated for a time period of t_1 up to time t_2, where a further pH change occurs; t_2 is the time period which the mobile phase needs to pass from entering the irradiated part of the capillary to its shielded part, *i.e.*, 80 μl. The last section, d is where the mobile phase is irradiated for a time $t_1 + t_2$; the photo-oxidation is completed and the pH value remains constant. After the lamp has been switched off, the curve slopes down in two steps. The first one is longer than c and its length is in accord with the larger volume of the first part of the capillary. The middle section, f, is the isocratic part of the previously irradiated part as in b. Broadening of the last section, g, is caused by the dispersion of the corresponding pH gradient band in the capillary. In this a way a variable gradient profile can be formed. Bubbles arising at a formate buffer concentration higher than 0.1 *M* (see below) indicated that some additional reactions can occur. Anyway, the initial gradient shape is rather distorted by mixing in the analytical column, so that a nearly exponential shape is characteristic.

Effect of various parameters on photoreaction

Photochemical reactions are functions of many variables and experimental approaches, and all these variables should be considered whenever one wishes to utilize photochemical reactions in HPLC.

Even the addition of small amounts of methanol limited the photoreaction. On the other hand, acetonitrile did not seriously affect the pH jump, but it was slightly

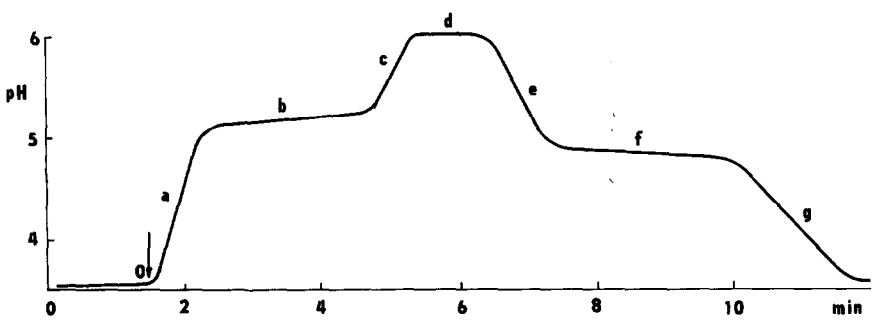

Fig. 3. Formation of the profiled gradient with the aid of a strip of paper shielding part of the PTFE capillary. Configuration of the apparatus as in Fig. 1 (bypass in position b). Mobile phase, 0.05 *M* formate buffer (pH 3.6) with 0.5% of 30% hydrogen peroxide; volume of the PTFE capillary, 480 μl; flow-rate, 100 μl/min; volumes of the parts of the PTFE capillary, a = 100 μl, b = 225 μl, c = 80 μl.

lower. An additional amount of hydrogen peroxide suppressed this decrease. The small concentration of acetonitrile present in the mobile phase did not cause any damage to the PTFE capillary wall or any cracking even at a pressure of 40 bar. The possibility of using small amounts of acetonitrile is also supported by other workers [15,16].

As stated by Baxendale and Wilson [10], in the presence of air the rate of hydrogen peroxide decomposition in the presence of formic acid decreased considerably. However, neither the formate decomposition not the pH change was significantly dependent on the presence of air.

One problem associated with this method of pH variation is the formation of bubbles at formate concentrations higher than 0.1 *M*. This drawback was eliminated at concentrations below than 0.1 *M* and by using a back-pressure source, *e.g.*, a bent PTFE capillary inserted behind the UV detector.

Measurements of the stability of the pH of the mobile phase showed that the pH increased by not more the 0.1 after 24 h.

The pH change is dependent on the initial pH of the formate buffer. At pH $\leq$ 3.0 the pH jump decreased considerably. The negligible change in the presence of an excess of formic acid occurs because only a small amount of formate is present. The formic acid is thus converted into carbonic acid without a significant pH change. A similar effect takes place at high pH values. The optimum region of the initial pH of the formate buffer is 3.0–4.5. However, the method is applicable with pH varying over the range 3–7.5, which covers the range of pH used with modified silica packings in practical HPLC.

Application

In order to demonstrate the utilization of this method, methotrexate and its impurities were separated. The effect of the pH gradient applied in micro-HPLC is shown in Figs. 4 and 5. The sample was first chromatographed isocratically at the initial pH of the formate buffer (Fig. 4a). Only AMPT was eluted, after 16 min; MTX

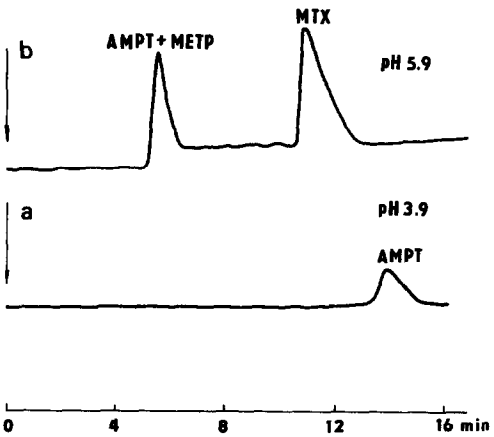

Fig. 4. Chromatograms of a mixture of MTX and its impurities separated with the UV lamp (a) switched off (pH 3.9) or (b) switched on (pH = 5.9). For other conditions, see Experimental.

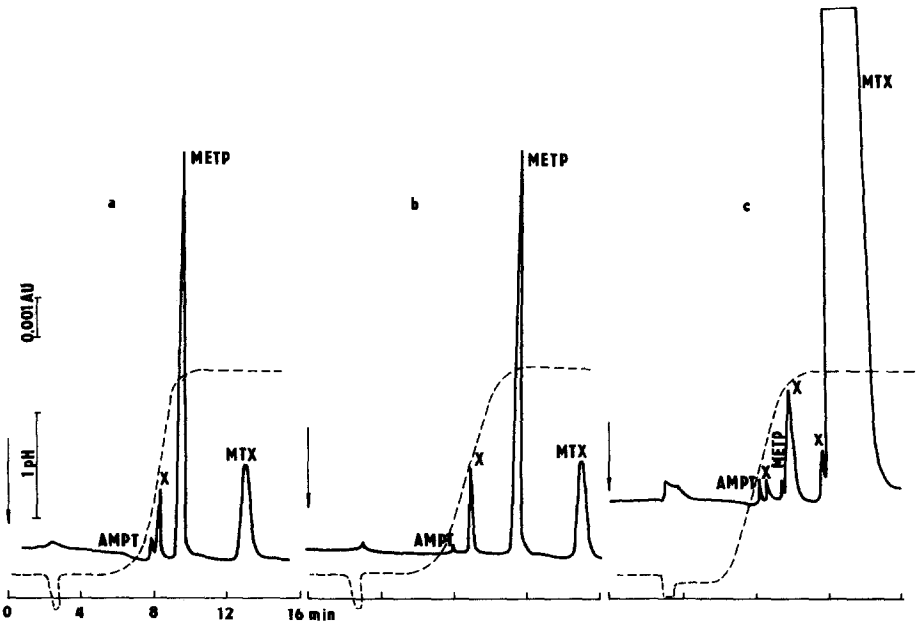

Fig. 5. Chromatograms of MTX and its impurities at (a) the unshielded and (b) and (c) the shielded part (150 μl) of the PTFE capillary. Samples: (a) and (b) mixture of standards; (c) real sample of MTX containing 0.2% of AMPT and 0.15% of METP. X = unknown impurities. For other conditions, see Experimental.

and METP were strongly adsorbed on the reversed-phase material and did not appear even after elution for 35 min. With the lamp switched on at the final pH value, AMPT and METP were coeluted as a broad peak (Fig. 4b). The retention order of compounds is in accord with the results reported by Feyns *et al.* [13].

The influence of the slope of the pH gradient on retention or resolution of solutes is shown in Fig. 5a and b. The unsatisfactory separation of AMPT and an unknown impurity is observed when the pH gradient is too sharp (Fig. 5a). Fig. 5b shows the separation of the same mixture with part of the PTFE capillary shielded from UV light. The resolution of all the peaks in this model mixture was good. Compounds in real MTX sample were also sufficiently separated (Fig. 5c). In addition to the three known compounds, other unidentified peaks were present. The 150-μl volume of the shielded part of the capillary should result in a gradient that is 3 min longer and should also involve two steps. It is possible to explain this smooth gradient by the buffering capacity of silanol groups on Silasorb C_{18} [17].

The jump height decreased to 2 pH units owing to the presence of acetonitrile, as discussed above. The reproducibility of retention times was better than 1% and of peak heights better than 2.5%, as calculated of four injections. The detection limit (signal-to-noise ratio = 3) for MTX was 5 pmol at 315 nm.

CONCLUSIONS

The purpose of these experiments was to demonstrate the feasibility of using a photochemically generated reagent for elution gradient programming. Formation of the gradient during on-line pre-column photo-oxidative reactions is a novel technique in HPLC. It permits variable gradient formation in both directions, *i.e.*, to an upper pH value and also in the opposite direction. The gradient is independent of the injection of the sample and can be started when needed. The method can be applied in micro-HPLC.

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