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Capillary electrophoretic determination of methotrexate, leucovorin and folic acid in human urine

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Abstract

A simple, rapid and sensitive procedure using capillary zone electrophoresis (CZE) to measure methotrexate, folinic acid and folic acid in human urine has been developed and validated. Optimum separation of methotrexate, folinic acid and folic acid was obtained on a 60 cm × 75 μm capillary using a 15 mM phosphate buffer solution (pH 12.0), temperature and voltage 20 °C and 25 kV, respectively and hydrodynamic injection. Under these conditions the analysis takes approximately 9.0 min. Good results were obtained for different aspects including stability of the solutions, linearity, accuracy and precision. Before CZE determination, the urine samples were purified and enriched by means of a solid phase extraction step with a preconditioned C_{18} cartridge and eluting the compound with a mixture 1:1 of methanol:water. A linear response over the urine concentration range 1.0–6.0 mg L^{-1} for MTX and 0.5–6.0 mg L^{-1} for folinic acid and folic acid was observed. Detection limits for the three compound in urine were 0.35 mg L^{-1}. CZE was shown to be a good method with regard to simplicity, satisfactory precision, and sensitivity.
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Keywords: Capillary zone electrophoresis; Chemotherapeutic agents; Human urine; Methotrexate; Folinic acid; Folic acid

1. Introduction

Methotrexate (MTX), an antimetabolic agent, is an antifolate in a class of folic acid analogs (Scheme 1), that has demonstrated effective antineoplastic activity. MTX is used in the treatment of psoriasis, certain forms of cancer, and certain connective tissues diseases such as rheumatoid arthritis, lupus and scleroderma [1]. The efficacy of antifolate drugs is related to the extent of intracellular polyglutamation [2,3]. In most cells, polyglutamation does not occur until the cell is exposed to 10^{-6} M MTX for at least 6 h [4].

When high doses of MTX are used in patients, they must be carefully monitored for life-threatening toxicity. MTX is nephrotoxic, but its reversible nephrotoxicity may be corrected by administration of leucovorin (folinic acid, LV). MTX induced toxicity can be prevented without diminishing the antitumor activity of the drug, by using LV. This compound is a mixture of two diastereomers of *N*-5-formyl-tetrahydrofolate (Scheme 1) [4]. LV, has become part of antineoplastic combination regimens for several malignant disorders. It has also been used to prevent anemia, after high-and intermediate-dose methotrexate terapy.

Folic acid (FA), 4-(2-amino-4hydroxypteridin-6-yl)methylamino-benzoyl-L-glutamic acid (Scheme 1), part of the vitamin B group (vitamin B_9) is a water soluble vitamin. It is one of the most important components of the haemopoietic system, being the coenzyme that controls the generation of ferrohaeme.

The interest in the determination of this compound lies in recent research which indicates that numerous diseases (for example, macrocytic anaemia associated with leucopenia, psychiatric disorders), especially those concerned with malformations during pregnancy and carcinogenic processes, are related to its deficiency. It is usually employed in the treatment or prevention of megaloblastic anaemia during

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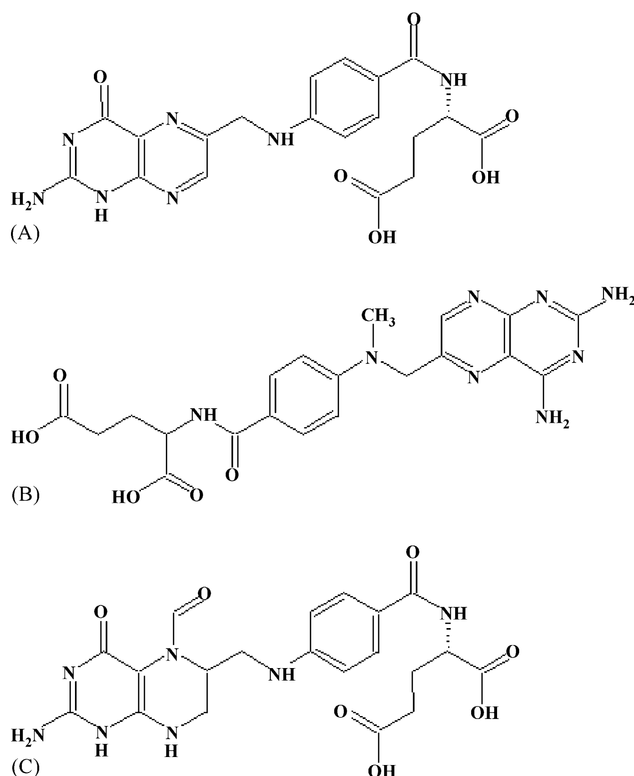

Scheme 1. Structures of the molecules. (A) FA: folic acid; (B) MTX: methotrexate; (C) LV: folinic acid.

pregnancy, childhood and other clinical situations often associated with alcoholism and liver diseases [5].

Recently it has been recognized that the concentration of folic acid in the human blood of cancer patients, especially precancerous patients, becomes lower than that of healthy individuals. Therefore, the trend in folic acid concentration, in blood could be a new tumor marker for cancer-screening in the near future [6].

In mammals, folic acid and its derivatives, the folates, serve as acceptors and donors of one carbon units and are involved in amino acid and nucleotide formation [7,8].

Many analytical methods have been reported to analyse MTX in biological fluids, by using enzyme multiplied immunoassay [9], fluorescence polarization immunoassay [10], radioimmunoassay [11], capillary zone electrophoresis [12,13] and HPLC [14–16].

Schleyer et al. [17] proposed the separation of LV and its metabolite 5-methyltetrahydrofolate in serum and urine by HPLC with fluorimetric detection. Several HPLC methods have been developed for the simultaneous determination of the diastereoisomers of LV and 5-methyltetrahydrofolate. All the methods which use photometric detection employ a chiral column for the separation [18–21]. Capillary electrophoresis, with cyclodextrins as chiral selectors, has been developed [22].

A number of methods have consequently been reported for the determination of folic acid in different samples. The most widely used are those based on gas chromatography or high-performance liquid chromatography (HPLC) with absorption [23–28], fluorescence [29–32] or phosphorescence [33] detection, together with bioassays [34,35] and voltamperometry [36,37].

In this paper, we propose a rapid method by capillary zone electrophoresis (CZE) to determine MTX, LV and FA in urine samples after a preconcentration step.

The development of an analytical method for the simultaneous determination of MTX, LV and FA appears convenient due to the use of MTX and LV as drugs antineoplastic and the FA as a possible tumor marker for cancer screening. The method permits the applicability of the MTX and LV determination due to the high doses administered in some patients.

2. Experimental

2.1. Chemical

All chemicals and solvents were of analytical reagent grade. MTX and FA were obtained from Sigma and LV was purchased from Across. Standard solutions of LV, MTX and FA (200 $mg L^{-1}$) were prepared by dissolving the compounds in deionized water. All standards solutions were stored under refrigeration at 4 °C. Working standard solutions were prepared daily by dilution of the stock standard solutions with water.

Electrophoretic separation was performed using a phosphate buffer of pH 12 and ionic strength of 15 mM. The background electrolyte was prepared daily and the set of separation vials was changed after six runs.

2.2. Instrumentation

Analysis was performed with Beckman P/ACE System 5510 capillary electrophoresis equipment with diode-array detection (DAD) and controlled by a Dell Dimension P133V with P/ACE station software. The 60.2 cm (50 to the detector) × 75 μm i.d. fused-silica separation capillary was maintained in a cartridge with a 100 μm × 800 μm detection window.

The use of photodiode detector allowed us to confirm the identity of the peaks, no only by its migration time, but also by the overlay of the UV–vis spectra of the samples with a standard. The wavelength selected for the electropherograms was 305 nm.

The extraction and preconcentration process was achieved with a home-made device composed by Waters manifold Millipore Vacum sep-pack system coupled with a Gilson Minipuls three automatic pump.

2.3. Sample preparation of the urine samples, extraction and preconcentration procedure

Fresh human urine samples were obtained from different volunteers who had not taken the compounds.

Fresh urine samples were directly extracted to solid-phase extraction. The extraction of MTX, LV and FA from the biological samples was performed in a reverse-phase C_{18} cartridge (Waters Sep-Pak Plus, Milford, MA, USA). The cartridge was conditioned prior to use with 5 mL of methanol followed by 5 mL of 10 mM phosphate buffer solution (pH 2.5).

Different volumes (between 2 and 10 mL) of urine were slowly loaded into the conditioned cartridge. The cartridge was then washed with 2 mL of 10 mM phosphate buffer, pH 2.5 and 6 mL of 2.5 mM phosphate buffer, pH 2.5.

Finally, MTX, LV and FA were eluted with 1 mL of a solution methanol:water (1:1). This extract was immediately injected into the capillary electrophoresis equipment. The stability of the extract was checked during 12 h with satisfactory electropherogram (corrected peak areas of the three drugs were constant during the stability study, and not new peaks were observed).

2.4. Operating conditions

Before the first use the capillary was conditioned by flushing with 0.1 M NaOH for 20 min, water for 15 min, and the background electrolyte solution for 10 min.

The selected running buffer was a phosphate buffer 15 mM of pH 12.0 for the standards and urine samples. Urine and standards samples were kept at 20 °C inside the capillary electrophoresis equipment. Between measurements the capillary was conditioned for 2 min with 0.1 M NaOH and 4 min with running buffer in order to get repetitive electropherograms.

The injection of the samples was hydrodynamic for 7 s. Separations were performed at 25 kV. Under these conditions the current was 82.6 μA. In all cases the electropherograms were recorded at 305 nm.

Duplicate injections of the solutions were performed and average-corrected peak areas (CPA) (area/migration time) were used for quantitative analysis. All days, the data generated from the first two injections were not used on account of the necessary equilibration system.

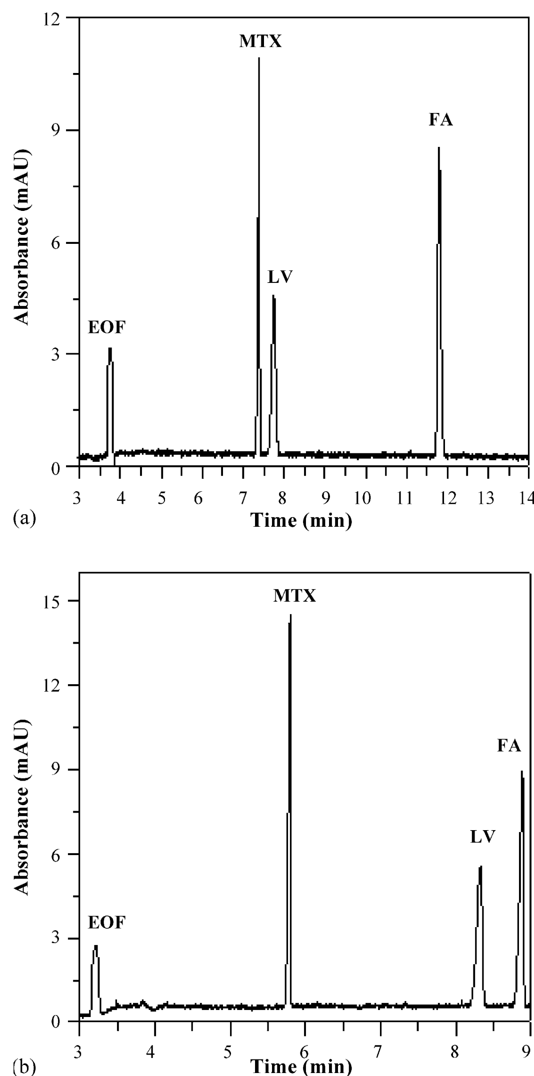

Fig. 1. (a) Electropherogram for a standard mixture of 18 mg L^{-1} of methotrexate, folinic acid and folic acid. Operating conditions: a borate buffer 20 mM pH 9.7, hydrodynamic injection 7 s, 30 kV and 20 °C. (b) Electropherogram for a standard mixture of 18 mg L^{-1} of methotrexate, folinic acid and folic acid. Operating conditions: a phosphate buffer 20 mM pH 12.0, hydrodynamic injection 7 s, 25 kV and 20 °C.

3. Results and discussion

3.1. Optimisation of pH and ionic strength of electrolyte

The pH of the running electrolyte had a significant impact both on the ionization of the acidic silanols on the capillary wall and on the electrophoretic mobilities of the studied compounds. Due to the p*K*s of MTX are 3.5, 4.8 and 5.1, p*K*s of LV 3.6 and 4.8 and FA p*K* 3.5 and 4.8, the anionic form in our three compounds are upper to pH 5.1. Because of the structures of the analytes (Scheme 1), basic buffers could be used to promote their possible ionization.

The buffers tested were borate (20 mM, pH 8.5–10.2) and phosphate (20 mM, pH 11.3–12.3). The values of pH between 5.1 and 8.0 were not considered due to the irreproducibility of the EOF in this range of pH. In the borate and phosphate buffers the electrophoretic migration velocities of the three drugs were lower than the electro-osmotic velocity due to the fact that the three compounds were present in the anionic form. The borate buffer (20 mM, pH 9.7) showed that the elution order is MTX, LV and FA and the resolution between the MTX and LV is the most critical parameter as can be seen in Fig. 1a. The phosphate buffer (20 mM, pH 12.0) showed that the elution order was the same (MTX, LV and FA) but the resolution between the MTX and LV compounds was not under this pH a critical parameter as can be seen in Fig. 1b. The best separation and resolution of the compounds from the human urine extracted was achieved again at pH 12.0. At pH 12.0, the compounds studied (MTX, LV and FA)

have no interferences of the unknown components from the urine. The phosphate buffer allows the analysis in a shorter period of time than borate buffer, i.e. from 12 min (borate buffer pH 9.7, 20 mM) to 9 min (phosphate buffer pH 12.0, 15 mM). Under these conditions the migration times for MTX, LV and FA were 5.8, 8.3 and 8.9 min, respectively (Fig. 1b).

The effect of phosphate buffer solution concentration (5–20 mM) in urine extracted samples on the migration time of the compounds was studied. When the concentration of buffer was increased the migration times also increased. A phosphate buffer solution 15 mM was selected as optimum for the best resolution in the urine extracted samples.

3.2. Effect of applied voltage

The effect of the voltage applied from 5 to 30 kV was investigated. When the voltage increases, the migration time of all the drugs decrease as well as the resolution between peaks. A voltage of 25 kV selected as optimum value as a compromise between run time, generated current and resolution.

3.3. Effect of temperature

Capillary temperature control is important in capillary electrophoresis. Changes in capillary temperature can cause variations in efficiency, viscosity, migration times, injection volumes and detector response. The effect of temperature on the separation was investigated in the range 20–35 °C. Solute migration times increased because of changes in the viscosity the medium. The temperature giving the best compromise between resolution and run time, with a good level of baseline noise, was 20 °C, and it was selected as optimum.

3.4. Optimization of injection time

In order to decrease the detection limits, the injection time was varied between 1 and 20 s. The corrected peak area (CPA) increased with increasing injection time but poor resolution between the peaks (FA and LV) was also observed. For this reason the optimum value chosen was 7 s. The pressure used for injection was always 0.5 psi.

3.5. Optimization of the washing step

Sample components can become adsorbed onto the capillary surface and change the effective charge on the wall. So, the capillary is flushed between injections with the electrolyte during 2 min (when MTX, LV and FA compounds were dissolved in water). But, with urine samples extracts, it was necessary a rinse step of 2 min with NaOH 0.1 M and 4 min with separation electrolyte in order to obtain an unchanging EOF.

3.6. Optimized capillary electrophoretic conditions

From these studies, the following electrophoretic conditions were selected as optima:

- electrolyte: phosphate buffer 15 mM pH 12.0;
- voltage: 25 kV;
- capillary: fused-silica (60.2 cm × 75 μm i.d.);
- injection: hydrodynamic, 7 s (0.5 psi);
- temperature: 20 °C;
- detection wavelength: 305 nm.

4. Solid-phase extraction of the human urine samples (SPE)

Due to the presence of a large quantity of various interfering compounds and the low concentration of the drugs under study, it was necessary to extract the compounds of interest in order to obtain a cleaner electropherogram. C_{18} cartridges were used to extract the studied drugs from the human urine. Variables such as organic solvent, washing steps using different solvents, and final volume of the extract, were studied.

Different volumes of urine were charged to the cartridge with the object to evaluate its maximal capacity. A value of 6 mL of urine was selected as the best results. It was possible with this volume, to preconcentrate six times the original urine samples (a urine sample with 0.5 mg L^{-1} of each compunds allows to get a methanol extract of 3.0 mg L^{-1}).

Fig. 2 shows the electropherograms corresponding to the methanolic extract from a blank and a urine spiked with 3 mg L^{-1} of each compound.

5. Validation of the electrophoretic procedure

5.1. Stability

The stability of spiked urine extract containing methotrexate, folinic acid and folic acid was evaluated by comparing corrected peak areas obtained at different time intervals. It was found that the extract was stable for at least 12 h.

5.2. Linearity

The linearity of the assay was checked by different urine samples after SPE treatment spiked with the three drugs at concentrations ranged from 1 to 6 mg L^{-1} by methotrexate, 0.5 to 6 mg L^{-1} by folinic acid and folic acid ($n = 7$). In all the cases a volume of 6 ml of urine was loaded to the conditioned cartridge and extracted with 1 mL of a solution of methanol:water 1:1. The results were given in terms of peak height and corrected peak areas and the linear regression equations obtained, correlation coefficients and detection limits had been presented in Tables 1 and 2, respectively. The satisfactory correlation coefficients confirm that

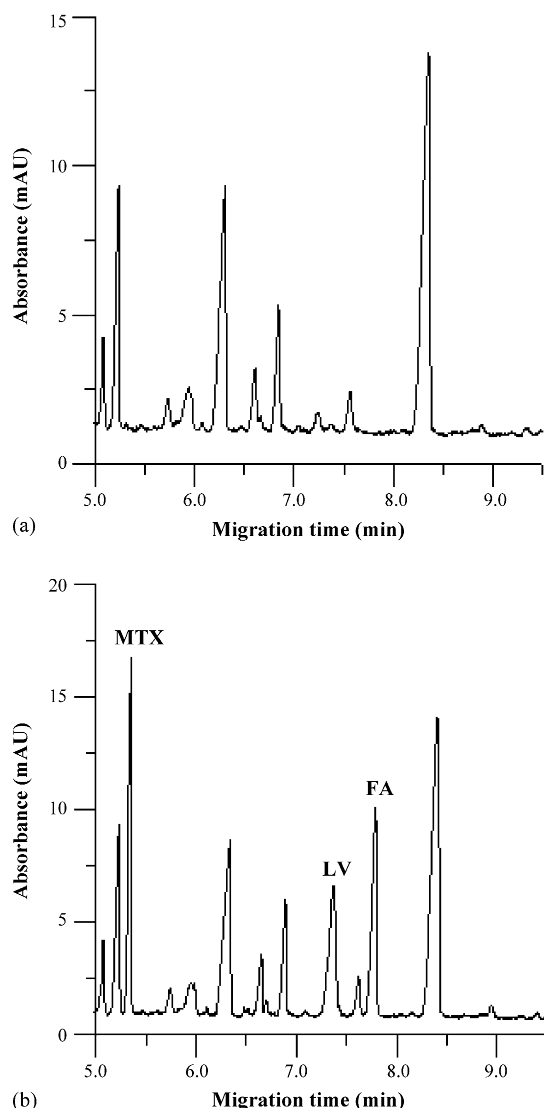

Fig. 2. Electropherograms obtained from (a) urine and (b) urine spiked with 3 mg L^{-1} of methotrexate, folinic acid and folic acid, respectively (λ = 305 nm).

the three compounds responses were linear over the concentration range studied for biological applications. The values of the detection limits confirm its real possible determination over clinical samples from patients under MTX treatment.

No significant differences were found between the calibration graphs performed in fresh urine (taken directly from a healthy man or woman) and frozen and thawed urine (urine conserved during two weeks in the freezer at −18 °C and was lastly unfrozen the same day of the analysis).

In this paper the techniques used to validate the peak purity of the studied compounds (*specificity*) present in urine samples were:

- Normalization and comparison of spectra from different peak sections.
- Absorbance at two wavelengths.

Both techniques showed that the purity of the peaks corresponding to the compounds studied in urine present a high level of purity. Therefore, no interference by matrix effect was observed.

5.3. Precision

The precision of the proposed method was expressed in terms of relative standard deviation (R.S.D.).

In order to test the precision of the procedure, 10 injections of a standard of 10 mg L^{-1} of methotrexate, folinic acid and folic acid were carried out sequentially in 2 different days. The precision of the migration times, corrected peak area and height were good with a R.S.D. between 0.45 and 0.70% for migration time, 2.0 and 3.9% for corrected peak area and 1.2 and 1.7% for peak height.

To evaluate the precision of the solid phase extraction process, eight different cartridges were conditioned and loaded with 6 mL of an urine sample spiked with 3 mg L^{-1} of each compound and were carried out sequentially. The precision of the migration times, corrected peak area and peak height were satisfactory with a R.S.D. between 0.78 and 1.27% for migration time, 3.2 and 6.4% for corrected peak area and 2.3 and 4.1% for height.

6. Applications

To demonstrate the usefulness of the proposed method, several aliquots of MTX, LV and FA solutions were added into a spiked urine (2 mg L^{-1} of each compound). In all cases the standard addition method was used for the drugs determination, due to the possible matrix effect from the urine (n = 5). To determine the recoveries of MTX, LV and FA in human

Table 1
Statistical data for determination of MTX, LV and FA in extracts of urine samples in terms of corrected peak areas

	MTX	LV	FA
LOD* (mg L^{-1})	0.39	0.36	0.30
LOD** (mg L^{-1})	0.77	0.83	0.66
Intercept	$-2.15 \times 10^2 \pm 3.11 \times 10^2$	$-3.87 \times 10^2 \pm 1.35 \times 10^2$	$-3.05 \times 10^2 \pm 1.58 \times 10^2$
Slope	$2.41 \times 10^3 \pm 7.98 \times 10^1$	$1.12 \times 10^3 \pm 4.06 \times 10^1$	$1.57 \times 10^3 \pm 4.36 \times 10^1$
R^2	0.9978	0.9974	0.9981
Linear range (mg L^{-1})	1.0–6.0	0.5–6.0	0.5–6.0

* Winnerforned and Long.
** Clayton.

Table 2
Statistical data for determination of MTX, LV and FA in extracts of urine samples in terms of height peak

	MTX	LV	FA
LOD* ($mg L^{-1}$)	0.44	0.35	0.18
LOD** ($mg L^{-1}$)	0.87	0.81	0.41
Intercept	$9.76 \times 10^2 \pm 6.58 \times 10^2$	$-1.12 \times 10^2 \pm 1.85 \times 10^2$	$-4.26 \times 10^2 \pm 9.23 \times 10^1$
Slope	$4.52 \times 10^3 \pm 1.69 \times 10^2$	$1.56 \times 10^3 \pm 4.99 \times 10^1$	$1.57 \times 10^3 \pm 2.61 \times 10^1$
R^2	0.9972	0.9980	0.9994
Linear range ($mg L^{-1}$)	1.0–6.0	0.5–6.0	0.5–6.0

* Winnerforned and Long.
** Clayton.

Table 3
Recoveries obtained by each compound in urine samples

Compound	Added concentration ($mg L^{-}$)	Found concentration (CPA)	% Recovery (CPA)	Found concentration (height)	% Recovery (height)
MTX	2.00	1.71	85.5	1.86	93.0
LV	2.00	2.22	111.0	2.14	107.0
FA	2.00	1.74	87.0	2.27	113.5

urine the corrected peak area and the peak height were used for the drugs determination in the urine. Good recoveries were obtained for the three analyzed compounds. In Table 3, the obtained recoveries have been presented.

7. Conclusions

A simple and rapid method has been developed for the analysis of methotrexate, folinic acid and folic acid in urine samples by capillary zone electrophoresis. The electrophoretic method has been validated for the analysis of the three compounds in urine samples; it has been shown that the experimental results with respect to linearity, accuracy, specificity, sensitivity and precision demonstrate the reliability of the electrophoretic procedure for its intended application.

As may be observed, a good resolution was obtained between the interfering compounds of the urine and the analyzed drugs. In all cases the standard addition method was used for the determination drugs in the real samples. The results obtained concerning linearity, recovery, precision and sensitivity were highly satisfactory

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