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Micellar electrokinetic capillary chromatography quantification of cytosine arabinoside and its metabolite, uracil arabinoside, in human serum

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Abstract

Cytosine arabinoside (Ara-C) is widely used to induce remission in adult granulocytic leukemia. High doses can be infused in refractory leukemia or in relapse. After injection, Ara-C is quickly metabolized to uracil arabinoside (Ara-U), the main inactive metabolite. We here described a micellar electrokinetic capillary chromatography (MECC) method to simultaneously determine Ara-C/Ara-U in human serum using 6-*O*-methylguanine as an internal standard. The assay was linear from 6.25 to 200 μg/ml with a quantification limit between 3 and 6 μg/ml. The analytical precision was satisfactory between 2 and 4.3% (within-run) and 3.7 and 7.3% (between-runs). This assay was applied to the analysis of serum from acute granulocytic leukemia patient treated by high doses cytarabine (3 g/m^2 body surface). © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Cytosine arabinoside (Ara-C, cytarabine), a pyrimidine antimetabolite, is an isomer of cytidine, being epimeric in the 2′ position of the sugar moiety [1]. The mechanism of drug cytotoxicity is located in the S phase of the cell cycle. Ara-C is converted by deoxyctidinekinase to 5′-mononucleotide AraCMP, and then phosphorylated to AraCTP which competitively inhibits the DNA polymerase [2]. Ara-C is widely used to induce remission in adult granulocytic leukemia [3]. High doses of Ara-C (>1 g of Ara-C per m^2 body surface) are infused in the treatment of relapsed or refractory leukemia [4]. However, high doses can induce hepatic, gastrointestinal or central nervous system toxicity. No relation is clearly defined between Ara-C serum concentrations and toxic effects although central nervous toxicity is known to be dose- and age-related [5]. After infusion, Ara-C is converted to AraCMP excreted unchanged in urine. The main part is quickly metabolized by cytidine desaminase in uracil arabinoside (Ara-U), the main inactive metabolite [6]. This desamination exits also in vitro and it is necessary to use the tetrahydrouridine (THU) as a specific inhibitor of cytidine

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desaminase to avoid total metabolization of Ara-C to Ara-U [7].

Many assays for Ara-C are described in the literature based on gas chromatography [8], gas chromatography–mass spectrometry [9] and high-performance liquid chromatography (HPLC) [7,10]. Most HPLC methods allow simultaneous determination of Ara-C and Ara-U [3,11]. However, none of these assays are totally satisfactory due to the length of elution time or the presence of endogenous interferants in some patient samples [3]. Capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC) have been developed to determine Ara-C alone or with cytidine in serum plasma [12,13]. To our knowledge, no electrocapillary methods use internal standard and permit simultaneous determination of Ara-C and its metabolite.

The MECC assay we developed has some attractive features, particularly the simultaneous determination of Ara-C and Ara-U within a short analysis time (<12 min). This technique is simple, rapid and specific. We also used an internal standard to improve the assay precision. This method is applicable to pharmacological study to determine pharmakokinetic parameters after cytarabine infusion in adult granulocytic leukemia.

2. Experimental

2.1. Reagents

Ara-C, Ara-U, 6-*O*-methylguanine (6-OMG), 5-methylcytidine (5-MC), 7-methylguanine (7-MG), cytosine, guanine, cytidine, hypoxanthine, xanthine, uric acid, allopurinol, 2′-deoxycytidine, 2′-deoxyadenosine, 2′-deoxyguanosine were obtained from Sigma–Aldrich (Saint Quentin Fallavier, France). THU was purchased from Calbiochem (Meudon, France). Doxorubicine was kindly provided by Dakota Pharm Labs. (Creteil, France). All other chemical reagents, such as sodium decahydrate tetrahydroborate, sodium hydroxide, citric acid, trisodium citrate pentahydrate and sodium dodecyl sulfate (SDS) were of analytical quality and supplied by Sigma–Aldrich. Water for injectable preparations (Frésénius France Pharma, Louviers, France) was used for preparation of different reagents.

2.2. Electrophoresis

CZE or MECC were performed using the P/ACE 5500 system (Beckman, Gagny, France) equipped with a variable-wavelength UV–visible detector. A fused-silica capillary tube was used [57 cm (injector–detector length 50 cm)×75 μm I.D.]. The part of the capillary ensuring separation was maintained at a constant temperature by immersion in a coolant circulating in the capillary cartridge with a rectangular detection window (100×800 μm).

Direct detection was performed at 280 nm with a bandwidth of 10 nm. The frequency for data acquisition was set at 16 Hz. Data were collected and analyzed with the Gold System (Beckman, Gagny, France).

All measurements were performed in normal polarity (input anode, output cathode). The capillary was thermostated at 35°C and a constant voltage of 12 kV was applied. Injection of samples was performed in an hydrodynamic mode with an injection time of 2 s under a pressure of 20 p.s.i. (1 p.s.i.= 6894.76 Pa).

The fused-silica capillary was conditioned before each series of analysis by washing with sodium hydroxide 0.1 *M* (5 min), water (5 min) and finally with the electrolyte solution (5 min). Between each sample, the capillary was washed with sodium hydroxide 0.1 *M* (1 min), water (1 min), before equilibration for 1 min with the electrolyte solution. These washings were intended to improve the reproducibility of the electroosmotic flow and thus that of migration time.

CZE was performed using either a 25 m*M* citrate buffer, pH 3 or a 25 m*M* solution of sodium borate, pH 10 (pH meter; Radiometer, Neuilly-sur-Seine, France). MECC electrolyte solutions were composed of a 25 m*M* solution of sodium borate adjusted to pH 10 and containing 10, 30, 60, 80 or 100 m*M* SDS. All electrolyte solutions were filtered through a 0.45-μm membrane (Polylabo, Strasbourg, France) before use.

2.3. Standard solutions

Stock solutions (1 mg/ml) of Ara-C, Ara-U, internal standards and THU were prepared in water. All stocks solutions were stored at −20°C for up to 8

weeks. Working solutions of internal standard (I.S.) and THU in water were extemporaneously prepared by diluting the stock solutions 1:10 and 1:5, respectively.

Serum standards (calibration standards) were prepared at concentrations of 100 to 6.25 μg/ml for Ara-C and 200 to 12.5 μg/ml for Ara-U. The first serum standard concentration (100 μg/ml Ara-C and 200 μg/ml Ara-U) was obtained by diluting 100 and 200 μl of Ara-C and Ara-U stock solutions in blank serum spiked with 80 μl of THU working solution (200 μg/ml). Other serum standards were obtained by serial twofold dilution in blank serum. A 50-μl volume of I.S. working solution (5 μg) was used for internal standardization.

2.4. Extraction procedure

Serum standards (200 μl) were pipetted into a 1-ml Eppendorf tube containing 50 μl of internal standard. A 400-μl volume of acetonitrile was added to precipitate proteins. Tubes were shaken for 2 min and were centrifuged at 15 000 rpm for 5 min. Supernatant were transferred to glass tubes and evaporated to dryness under nitrogen at 40°C. Residues were dissolved in 120 μl electrolyte solution. Plasma samples were treated in the same manner.

2.5. Biological samples

A 5-ml volume of blood was collected in glass without anticoagulant tubes and containing 1 μg of THU. Samples were kept on ice and the serum was promptly separated. Samples not immediately assayed were stored at −20°C.

2.6. Clinical application

To validate our method, Ara-C and Ara-U concentrations were determined in a 44-year-old female treated for acute granulocytic leukemia (LM2). Cytosine arabinosine was administered in a 2-h infusion (3 g/m^2 body surface) at J1, J3 and J5. Samples were collected to end of infusion, 2 h and 6 h after at J1 and J3. At J5, a single sample was collected 6 h after the last infusion.

3. Results and discussion

3.1. Choice of the method

To obtain a specific and reliable separation for Ara-C, Ara-U and internal standard, we tested CZE and MECC methods. All assays were performed with 1:10 dilution of Ara-C, Ara-U and internal standards stock solutions in water injected 2 s.

3.1.1. Capillary zone electrophoresis

In CZE, electrophoretic separation is generally obtained in pH<3 or pH>8 conditions in which the surface state of silica is easily reproducible.

3.1.1.1. Separation in acidic media. Ara-C and Ara-U separation was tested in 25 m*M* citrate buffer, pH 3. With this electrolyte solution, Ohm's law was linear until 12 V and current strength was 15 μA. Under these conditions, Ara-C and Ara-U were positively charged (Ara-C, pK_a 4.1; Ara-U: pK_a 9.2) [14] and migrate quickly (retention time Ara-C: 4.89 min; Ara-U: 4.92 min) towards the cathode with a very good efficiency (plates per meter of capillary Ara-C: 311 400; Ara-U: 687 900). However, the simultaneous analysis of Ara-C and Ara-U was not possible because both substances migrated with the same apparent mobility (Ara-C: μ_e 0.00080892; Ara-U: μ_e 0.00080399). Our results for Ara-C are similar those reported to Lloyd et al. [13]. These analytical conditions does not allow the separation of structural similar compounds as reported by Krivankova et al. [12] for the simultaneous separation of Ara-C and cytidine.

3.1.1.2. Separation in alkaline media. We used a 25 m*M* solution of sodium borate adjusted with HCl or NaOH to pH 8 to 12. For all pH values, the Ohm's law linearity was respected until 12 V and current strength ranged from 18 μA (pH 8) to 30 μA (pH 12). Fig. 1 presents retention times of Ara-C, Ara-U and three compounds tested as potential internal standard (6-OMG, 7-MG and 5-MC). We choose purine (6-OMG, 7-MG) or pyrimide (5-MC) analogues as a potential internal standard. 5-Methylcytidine has been already described as internal standard in the HLPC method [3]. At pH 8 or 9,

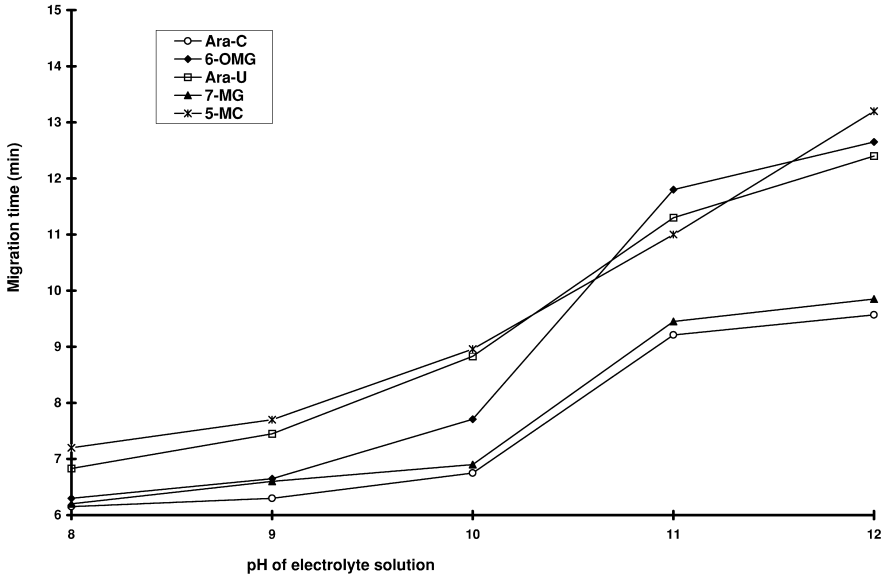

Fig. 1. Influence of pH electrolyte solutions on retention times of various compounds. Electrophoretic conditions: fused-silica capillary [57 cm (effective length 50 cm)×75 μm I.D.]; electrolyte, 25 m*M* sodium borate buffer; voltage applied, 12 kV, temperature, 35°C; injection time, 2 s.

all compounds were poorly separated. Between pH 10 and 12, Ara-C and Ara-U were correctly separated. In that all pH range, 7-MG was simultaneously eluted with Ara-C and 5-MC with Ara-U. Only 6-OMG could be separated from Ara-C and Ara-U at pH 10 and pH 11. As shorter retention times were obtained at pH 10, we selected this pH.

3.1.2. Micellar electrokinetic capillary chromatography

To improve resolution between Ara-C, Ara-U and 6-OMG, we tested the MECC method using 25 m*M* sodium borate with SDS concentrations ranging from 10 m*M* (critical micelle concentration) to 100 m*M* (maximal micelle concentration). Fig. 2a shows retention times of Ara-C, Ara-U and 6-OMG according to SDS concentrations. Ara-C and Ara-U are hydrophyl compounds and their retentions times are poorly influenced by SDS concentration. In contrast, 6-OMG is a more lipophyl substance and we observed a linear relation between SDS concentration and retention time. The optimal separation (Fig. 2a) and efficiency (Fig. 2b) for Ara-C, Ara-U and 6-OMG was obtained with 30 m*M* of SDS.

3.2. Optimization of the method

3.2.1. Influence of ionic strength

Using 30 m*M* SDS, we adjusted the ionic strength of the borate sodium buffer between 25 and 100 m*M*. Fig. 3a shows the evolution of retention times. Between 25 and 75 m*M*, we observed a linear relation between molarity and retention times which were strongly increased. Separation between compounds was not improved by the increase in ionic strength. As shorter retention times were obtained with 25 m*M* borate buffer, we selected this ionic strength.

3.2.2. Influence of temperature

Using an electrolyte of 25 m*M* sodium borate and 30 m*M* SDS, we studied temperature influence between 15 and 40°C. The best separation was obtained at 35°C (Fig. 3b).

3.3. Validation of the method

The optimized method (electrolyte: 25 m*M* sodium borate buffer, 30 m*M* SDS, pH 10; temperature

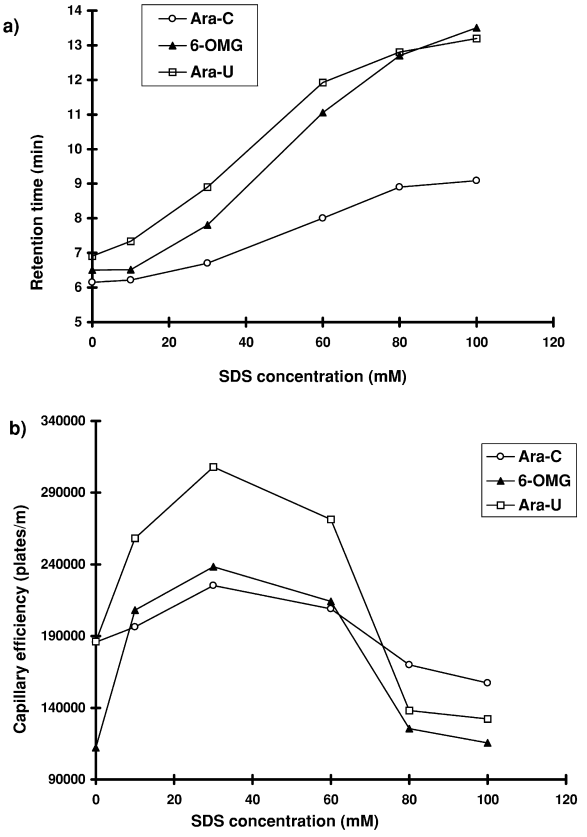

Fig. 2. Influence of SDS concentrations on retention times (a) and capillary efficiency (b). Electrophoretic conditions as in Fig. 1.

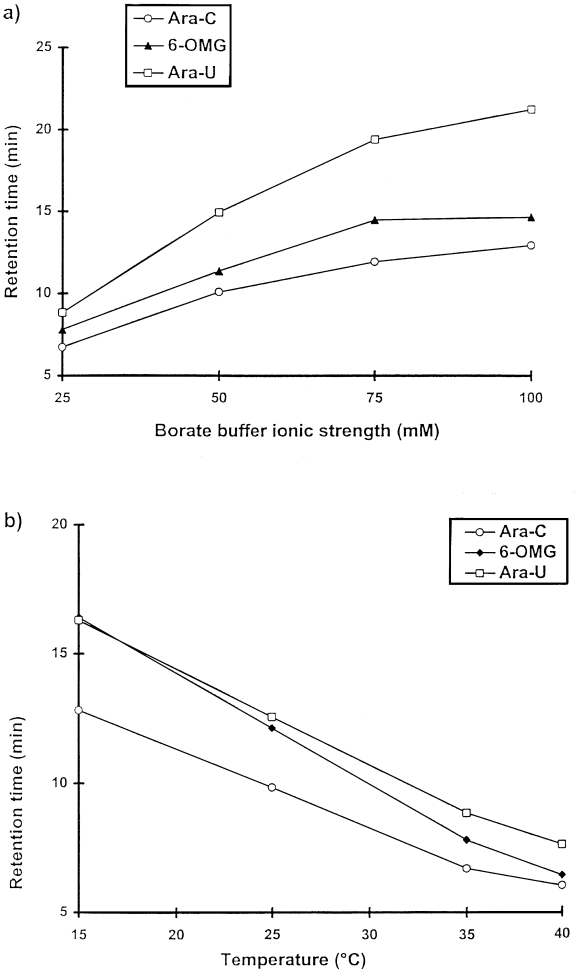

Fig. 3. Influence of ionic strength (a) and temperature (b) on retention times. Electrophoretic conditions: fused-silica capillary [57 cm (effective length 50 cm)×75 μm I.D.]; electrolyte, 30 m*M* SDS; voltage applied, 12 kV; injection time, 2 s.

35°C) was validated for routine Ara-C and Ara-U assays in human serum. Criteria retained for this optimization are similar to those generally used for HPLC and now applied to capillary electrophoresis [15]: specificity, reproducibility of migration time, linearity, sensitivity and percentage recovery as well as intra- and inter-day accuracy and precision.

3.3.1. Specificity

Fig. 4 shows the separation and determination of Ara-C and Ara-U in human serum using 6-OMG as internal standard. In the electropherogram obtained after extraction of blank serum, no additional peaks interfere with the measured compounds and I.S. (Fig. 4a). All compounds were well separated with migration time: Ara-C (6.75 min, μ_e 0.00048598), I.S. (7.78 min, μ_e 0.00044975) and Ara-U (8.83 min, μ_e 0.00035321) (Fig. 4b). Retention times were similar as those described by the HPLC method [3] but with improved resolution. The peak shapes had no evidence of tailing. The electropherogram from a patient treated with cytarabine had a similar appearance (Fig. 4c).

3.3.2. Linearity

The linearity of the method (corrected peak areas ratio of the drugs to the I.S. versus concentrations) was evaluated over a concentration range of 6.25 to 100 μg/ml for Ara-C and 12.5 to 200 μg/ml for Ara-U. The data for regression analysis performed

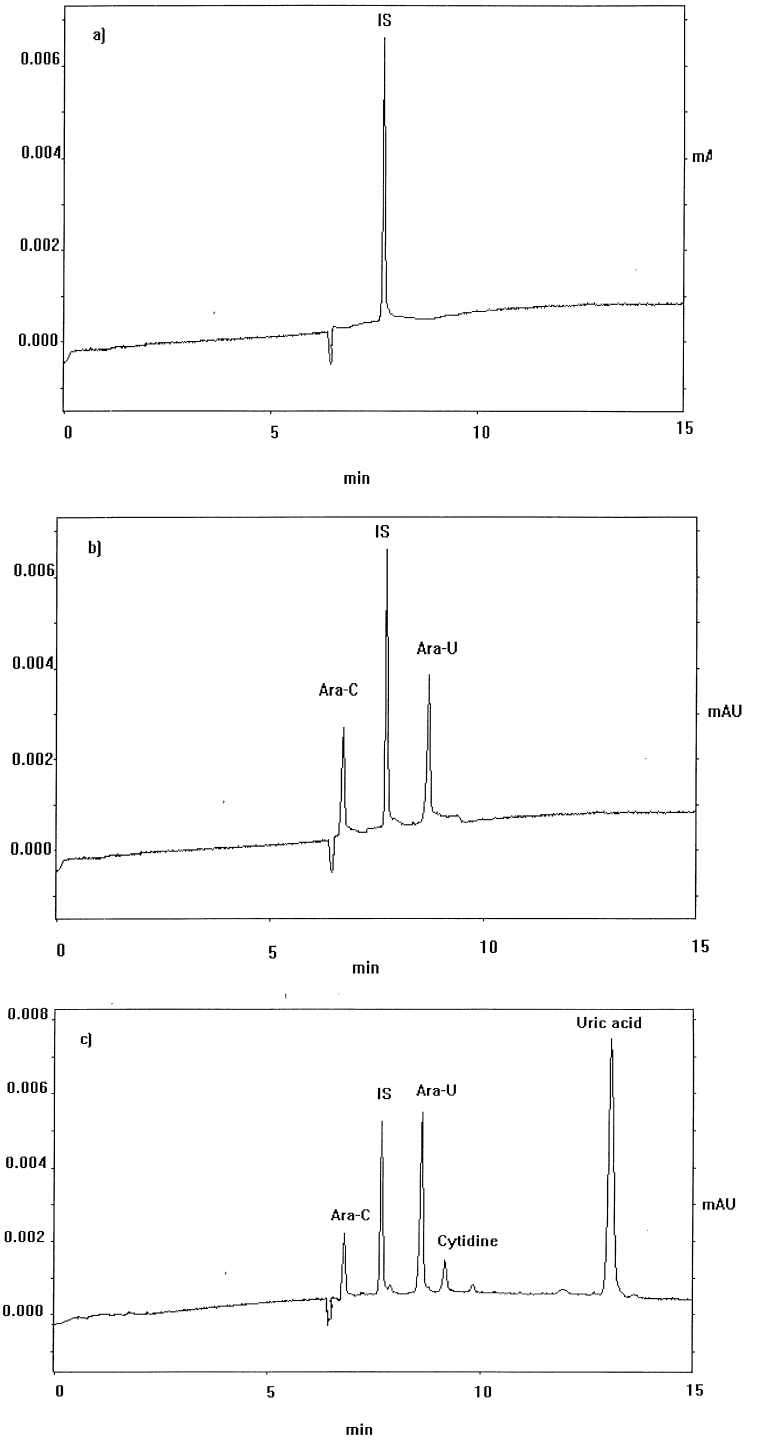

Fig. 4. Electropherograms of extracted blank serum containing 25 μg/ml 6-OMG (a), spiked human serum containing 25 μg/ml Ara-C, 50 μg/ml Ara-U and 25 μg/ml 6-OMG (b) and human serum of a patient treated with cytarabine (3 g/m^2, J3 end of infusion) (c). Electrophoretic conditions as in Fig. 3. Detection was performed at 280 nm with a bandwidth of 10 nm (mAU: milliabsorbance units).

by the least-squares method gave the following formulae: $y=0.0428x-0.0549$ (Ara-C) and $y=0.0154x-0.0231$ (Ara-U). These equations were determined by three calibrations obtained on different days. Correlation coefficients ($r>0.999$) confirm the excellent linearity of the method.

3.3.3. Precision

To determine between- and within-run precision, injections were realized 20 times for two levels of concentration (12.5 and 50 μg/ml Ara-C; 25 and 100 μg/ml Ara-U). Table 1 indicates mean values, standard deviations (SD) and coefficients of variation (C.V.s) obtained. In all cases, precision was excellent, with C.V.s below 5% for within runs and below 10% for between runs. Coefficients of variation are comparable to those described without I.S. [12,13]. However, I.S. use permits one to control the extraction procedure.

3.3.4. Detection (DL) and quantification (QL) limits

Detection limits, defined as the smallest quantities of Ara-C and Ara-U clearly distinguishable from baseline, were estimated to be three-times the signal-to-background ratio. DL was determined by analysis of solutions with decreasing concentrations of Ara-C or Ara-U. For Ara-C, the detection limit was estimated to be 1 μg/ml, which gave a QL of 3 μg/ml. This QL was similar to that described in a HPLC procedure [3] or a capillary method [12]. However under our conditions, this Ara-C detection limit was poor compared to that determined in an acidic media CZE report [13]. For Ara-U, DL and QL were, respectively, 2 and 6 μg/ml.

3.3.5. Overload tests

These tests were performed by overloading a serum containing 20 μg/ml (Ara-C) and 40 μg/ml (Ara-U) with increasing volumes (25 to 100 μl) of a 50 μg/ml Ara-C/Ara-U aqueous solution. The percentages of recovery indicated in Table 2 are within the limits of the recovery test (generally between 80 and 120%).

3.3.6. Interferents substances

We tested physiologic and pharmacologic substances. Uracil, cytidine, thymidine and adenosine, 2′-deoxyuridine, 2′-deoxyadenosine, hypoxanthine, xanthine and uric acid did not interfere (Fig. 4c). Cytosine and 2′-deoxycytidine co-eluted and were not completely separated from Ara-C; however no endogenous peak was found in serum samples. Our method allows a good separation between Ara-C and cytidine, which can be used in cytarabine synthesis [12]. Allopurinol, doxorubicine generally associated to Ara-C during chemotherapy and THU displayed no peaks in the electropherogram field.

3.4. Clinical application

In a patient treated with cytarabine (3 g/m^2 body surface), we determined serum concentrations of Ara-C and Ara-U (Table 3). Values obtained were

Table 1
Precision of the MECC technique relative to concentrations for Ara-C (12.5 and 50 μg/ml) and Ara-U (25 and 100 μg/ml) solutions[a]

Compound (concentration)	Mean concentration	SD	C.V. (%)
Within-run precision ($n=20$)			
Ara-C (12.5 μg/ml)	12.88	0.56	4.3
Ara-U (25 μg/ml)	24.08	0.89	3.7
Ara-C (50 μg/ml)	48.65	1.95	4.0
Ara-U (100 μg/ml)	101.25	2.1	2.0
Between-run precision ($n=20$)			
Ara-C (12.5 μg/ml)	13.02	0.85	7.3
Ara-U (25 μg/ml)	25.10	1.18	4.7
Ara-C (50 μg/ml)	49.66	3.20	6.4
Ara-U (100 μg/ml)	101.30	3.85	3.7

[a] Electrophoretic conditions: fused-silica capillary [57 cm (effective length 50 cm)×75 μm I.D.]; electrolyte, 30 m*M* SDS; voltage applied, 12 kV, temperature, 35°C; injection time, 2 s.

Table 2
Recovery study[a]

50 μg/ml Ara-C/Ara-U solution (μl)	Expected values Ara-C/Ara-U (μg/ml)	Measured values Ara-C/Ara-U (μg/ml)	Recovery Ara-C/Ara-U (%)
0	20/40	22/41.5	110/104
25	32.5/52.5	31.2/52.8	96/100
50	45/65	48/64.3	107/99
75	57.5/77.5	59.2/78.6	103/101
100	70/90	68.6/92.1	98/102

[a] Electrophoretic conditions as in Table 1.

Table 3
Ara-C and Ara-U concentrations determined in a patient treated with 3 g/m^2 cytarabine during 3 days

Day	Time of sample	Ara-C (μg/ml)	Ara-U (μg/ml)
J1	End of infusion	7.9	40
	2 h post-infusion	9.1	92
	6 h post-infusion	8.2	66
J3	End of infusion	6.9	45
	2 h post-infusion	12.3	121
	6 h post-infusion	6.6	85
J5	2 h post-infusion	23	89

similar as those reported by an HPLC procedure in patients receiving high dose [3]. Concentrations were maximal at 2 h post-infusion. In these samples, we observed an increase in Ara-C concentrations between J1 and J5. Assuming that Ara-U is the main inactive metabolite [6], Ara-U concentration measurements evaluate cytidine desaminase activity and chemotherapy efficacy.

4. Conclusions

The MECC method described here allows the simultaneous determination of Ara-C and Ara-U in serum. This technique is rapid, simple with a good precision. It is directly applicable to the routine assays in pharmacokinetic studies or to evaluate toxic effects observed after Ara-C high dose infusion. This method also indirectly evaluates the activity of cytidine desaminase by measurement of Ara-U concentrations. In a future study, it could be interesting to compare evolution of Ara-U kinetic as a potential indicator of cytarabine chemoresistance.

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