ELSEVIER

Journal of Chromatography A, 916 (2001) 215–224

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Determination of 5-fluorouracil and 5-fluoro-2′-deoxyuridine-5′-monophosphate in pancreatic cancer cell line and other biological materials using capillary electrophoresis

Andrea Procházková[a,b], Shengli Liu[c], Helmut Friess[c], Stefan Aebi[d], Wolfgang Thormann[a,*]

[a]*Department of Clinical Pharmacology, University of Berne, Murtenstrasse 35, 3010 Berne, Switzerland*
[b]*Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic, Veverí 97, 611 42 Brno, Czech Republic*
[c]*Department of Visceral and Transplantation Surgery, University of Berne, Inselspital, 3010 Berne, Switzerland*
[d]*Department of Medical Oncology, University of Berne, Inselspital, 3010 Berne, Switzerland*

Abstract

Capillary zone electrophoresis (CZE) was used for the rapid determination of 5-fluorouracil (5-FU) and 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) in pancreatic cancer cell line (PANC-1), culture medium, plasma and pancreatic tissue. The assay is based upon protein precipitation with acetonitrile followed by a 9-min CZE analysis of the supernatant in an uncoated fused-silica capillary employing a borate buffer and on-column absorbance detection at 265 nm. Using 50 μl of sample, 5-FU levels between 4.12 and 132 μg/ml (31.7–1000 μ*M*) were found to provide linear calibration graphs. Intra-day and inter-day RSD values evaluated from peak height ratios ($n=5$) were <7.6 and <8.8%, respectively. Corresponding RSD values of detection times ($n=7$) were <1 and <1.5%, respectively. The limits of detection for 5-FU and FdUMP were 1.72 and 5.29 μg/ml, respectively. As application, the accumulation of 5-FU by PANC-1 cells over a 4-h time period was investigated. Having a culture medium concentration of 100 μg/ml, the 5-FU cell content was estimated to become equal to that of the surrounding medium (i.e., 100 μg/ml or 3.61 fmol per cell with a volume of 4.7 pl) within that time period. The sensitivity of the assay was sufficient for the determination of 5-FU in all cell samples. FdUMP, however, could not be detected in these samples. Furthermore, the data obtained in uncoated capillaries are compared to those measured in a fused-silica capillary whose inner surface was coated with linear polyacrylamide (about 10-fold reduction of electroosmosis). The latter capillary format was found to be useless for simultaneous analysis of 5-FU and FdUMP in pancreatic cells but could be potentially useful for analysis of these compounds in plasma.

Keywords: Fluorouracil; Fluorodeoxyuridine monophosphate

1. Introduction

5-Fluorouracil (5-FU) is a chemotherapeutic drug, which has been used for many years in the treatment of a wide variety of solid tumors in various organs, including breast, pancreas, stomach, colon and rectum [1,2]. 5-FU is a fluorinated pyrimidine and acts as a prodrug. In the body, a small portion of it is converted into the active metabolites 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) and 5-fluorouridine-5′-triphosphate (FUTP), the rest (ap-

*Corresponding author. Tel.: +41-31-6323-288; fax: +41-31-6324-997.
E-mail address: wolfgang.thormann@ikp.unibe.ch (W. Thormann).

0021-9673/01/$ – see front matter
PII: S0021-9673(00)01171-7

proximately 80%) is degraded (mainly in the liver) via 5,6-dihydro-5-fluorouracil to CO_2, NH_3 and α-fluoro-β-alanine. The antineoplastic effects of 5-FU are caused by the active metabolites. In the presence of a reduced-folate cofactor, FdUMP binds tightly to thymidylate synthase (TS) and inhibits the formation of thymidylate from deoxyuridinemonophosphate (dUMP). FUTP is incorporated in place of uridine triphosphate into RNA and disrupts RNA processing and protein synthesis [1,2]. Little is known about optimized 5-FU application to man. In colorectal cancer, continuous infusion of 5-FU is known to be superior to bolus administration [3]. Furthermore, in breast cancer, the tissue concentration of 5-FU metabolites is reported to predict tumor response [4]. In pancreatic carcinoma and in many other solid tumors, the most effective mode of action, the tissue kinetics of 5-FU and the tissue concentrations required for the inhibition of tumor growth are unknown. Therefore, it is of a great importance to determine 5-FU and its metabolites in tissue cultures, body fluids and tissues because it may ultimately permit the design of more effective schedules of 5-FU administration in these cancers.

Various methods for analysis of 5-FU and its metabolites in different matrices are described in the literature. Assays developed include those based upon gas chromatography (GC) [5], gas chromatography–mass spectrometry (GC–MS) [6] and high-performance liquid chromatography (HPLC) [5,7–14]. Most of them require a relatively high sample volume (hundreds of μl to ml) and laborious and time consuming sample preparation (derivatization [6], extraction [7,10–13]). For analysis of cell cultures, the use of radiolabeled standards has been described [15,16]. This approach is inconvenient as it is associated with high disposal costs and as it cannot be used for human studies. Capillary zone electrophoresis (CZE) [17] is a sensitive technique which was already shown to be capable to separate 5-FU and its metabolites [18] and to determine 5-FU in microdialysates obtained from breast cancer [19]. Furthermore, 5-FU was employed as internal standard for monitoring of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine in plasma [20,21] and urine [21]. CZE is characterized with a high separation efficiency, minimal sample preparation, short analysis time, low operational costs and low sample volumes (nl to μl in absence of sample preparation). These properties make CZE an ideal method for analysis of cell samples.

This work presents a simple and rapid CZE method for determination of 5-FU and FdUMP in pancreatic cancer cell line (PANC-1). Furthermore, it is shown, that the same assay can also be applied to other biological matrices, including culture medium, plasma and pancreatic tissue extracts. Moreover, the comparison of the CZE separation performed in an uncoated capillary and a capillary whose inner surface was coated with linear polyacrylamide is given.

2. Experimental

2.1. Chemicals, standard solutions and origin of samples

All chemicals used were of analytical grade and acetonitrile (ACN) (Biosolve, Walkenswaard, The Netherlands) was of HPLC grade. 5-FU and FdUMP were purchased from Sigma (St. Louis, MO, USA). Theophylline was from the Inselspital Apotheke (Berne, Switzerland), disodium tetraborate decahydrate, sodium dihydrogenphosphate and disodium hydrogenphosphate were from Merck (Darmstadt, Germany). Stock solutions of analytes were prepared in water (5-FU, 2.9 mg/ml and FdUMP, 1.3 mg/ml). The internal standard (I.S.) was dissolved in ACN (theophylline, 25 μg/ml). Plasma samples were drawn from patients which gave their consent before and during selective, intra-arterial 5-FU chemotherapy to the liver (infusion of 600 mg 5-FU/h via a catheter placed into the common hepatic artery). Pancreas tissue stemmed from previously healthy organ donors. Our own plasma was used as blank.

2.2. Preparation of culture medium and cells

The human pancreatic cancer cell line, PANC-1, was cultured in Dulbecco's modified Eagle medium (DMEM) without L-glutamine, supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (Biochrom, Berlin, Germany), 100 units/ml penicillin and 100 μg/ml streptomycin in a 95% air–5%

carbon dioxide humidified incubator at 37°C. 5-FU (25–500 μg/ml) was dissolved in cell culture medium and stored at room temperature. Cells were cultured in 15 cm dishes for 3 days to a total cell number of approximately $2 \cdot 10^7$. If not stated otherwise, 20 ml cell growth medium containing 100 μg/ml 5-FU was added to each dish and cells were incubated at 37°C for 5 min, 10 min, 20 min, 30 min, 1 h, 2 h, 3 h or 4 h, respectively. Cells in medium without 5-FU served as controls. After incubation, cells were rinsed three times with cold phosphate-buffered saline (PBS) solution (pH 7.2) and kept on ice. Residual fluid in the dishes was totally removed. Cells were scraped with a rubber policeman, harvested to tubes and vortex-mixed. Aliquots of 50 μl cell suspension were used for measurement of intracellular 5-FU by CZE and for the determination of cellular protein. Culture medium samples (200 μl) were taken from the dish before and after the incubation with 5-FU.

2.3. Determination of number of cells, 5-FU content per cell, average cell volume and average 5-FU cell concentration

For the determination of the protein content in the PANC-1 cells, 50 μl of cell suspension was mixed with 300 μl of lysis buffer [1% sodium dodecyl sulfate, 20 μ*M* tris(hydroxymethyl)aminomethane hydrochloride, 2.5 μ*M* phosphate buffer (pH 7.4) containing one tablet of proteinase cocktail inhibitor (Boehringer, Mannheim, Germany) per 10 ml]. The mixture was kept on ice for 30 min and centrifuged at 14 000 rpm for 30 min at 4°C. Then, the protein in the supernatant was determined using the bicinchoninc acid (BCA) protein assay (Pierce, Rockford, IL, USA) and a MR 7000 detector (Dynatech, Burlington, MA, USA) at 540 nm. Furthermore, for evaluation of the number of cells, a calibration curve for cell number vs. protein amount was constructed. Cultured PANC-1 cells were trypsinized with 0.25% trypsin. After harvesting, the total volume of cell suspension was adjusted to 10 ml with PBS. Cell number in 1 ml of the cell suspension was counted using a hemocytometer (Leitz, Wetzlar, Germany). Protein content was determined in 0.4–2 ml of cell suspension and the protein content per cell and, together with the 5-FU data obtained by CZE (see below), the average 5-FU content per cell was calculated.

For determination of the average cell volume, cells were removed from the dish surface via trypsinization and stained with 0.4% trypan blue (Sigma–Aldrich, Irvine, UK) on a hemocytometer slide and the cell diameters were randomly measured under the microscope (Leica DMIL, Glattbrugg, Switzerland) via use of a video imaging program (Image Pro 3.1; Medica Cybernetics, Silver Spring, MD, USA). The diameters of 46 randomly selected cells were measured separately. A standard micro-ruler slide (Leitz, Wetzlar, 2 mm, 1 interval 0.001 mm) was used as a distance reference. The volume of a single cell was calculated according to $V=(4/3)\pi r^3$ where r equals half of the determined diameter. The intra-cellular concentration of 5-FU was estimated with the average 5-FU content of a single cell divided by the determined single cell volume.

2.4. Sample preparation for capillary electrophoresis

Protein ultrafiltration was performed by placing 100 μl of sample into Ultrafree-0.5 vials (Millipore, Bedford, MA, USA) and centrifugation at 14 000 rpm for 20 min. Pancreas samples were prepared by cutting a piece of pancreatic tissue (around 0.2 g) to small pieces, mixing with distilled water in the ratio 1:4 (w/v) followed by preparing of a homogenate. If not stated otherwise, samples and calibrators of culture medium, plasma, cell suspension and pancreatic tissue homogenate were prepared by spiking of 50 μl of sample with 5 μl of standard solution (calibrator) or water (sample) and with 100 μl of I.S. in ACN, vortex-mixed for 0.5 min and centrifuged at 9000 *g* for 3 min.

2.5. Capillary electrophoresis

All measurements were performed on a 270A-HT system (Applied Biosystems, San Jose, CA, USA) that was equipped with fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA). Uncoated capillaries were of 61.8 cm (40.1 cm to the detector)×50 μm I.D.. Capillaries of 60.8 cm (40.0 cm to the detector)×75 μm I.D. were internally coated with linear polyacrylamide (LPA) according

to Hjertén's method [22] which was modified as described in Ref. [23]. Before use, the uncoated capillary was rinsed for 20 min with 1 *M* NaOH, 20 min with 0.1 *M* NaOH and 20 min with water by applying a vacuum of 67.7 kPa at the outlet end. Between runs it was rinsed for 3 min with 0.1 *M* NaOH, 2 min with water and 2 min with background electrolyte (BGE). Coated capillary was rinsed for 15 min with water and for 10 min with BGE before use. Between runs it was rinsed for 4 min with BGE. Sample was introduced by applying a vacuum of 16.9 kPa for 1–2 s. The voltage applied was 25 kV (anode at injection end) and −25 kV (cathode at the injection end) for uncoated and coated capillaries, respectively. The currents were 31–59 μA. The temperature control was set to 31°C. The wavelength used for detection was 265 nm. A personal computer with a PC Integration Pack (Kontron Instruments, Basel, Switzerland) was employed for data registration, evaluation and storage.

3. Results and discussion

3.1. Selection of running buffer for CZE

For the selection of the BGE, the electrophoretic behavior of 5-FU and FdUMP (Fig. 1) was studied in the pH range 4.7–9.3. The dependence of effective mobilities {for definition refer to Ref. [17], the solvent peak was used as a marker of electroosmotic flow (EOF)} vs. pH of BGE is shown in Fig. 2A. It is evident that 5-FU is negatively charged at pH> 5.6. The characteristic change in mobility between pH 7 and 9 is in agreement with the reported pK_a value of 8.0 (the second pK_a value is 13.0) [24]. FdUMP is negatively charged in the whole investigated pH range and possesses a high effective electrophoretic mobility. For the simultaneous determination of both analytes, a BGE pH>6.5 should be employed. Phosphate (0.025 *M*) and tetraborate (0.03 *M*) were chosen as BGE co-ions.

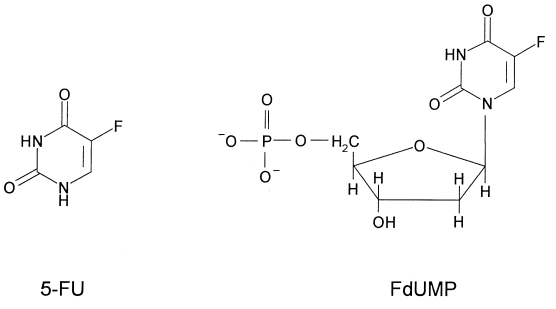

Fig. 1. Chemical structures of 5-FU and FdUMP.

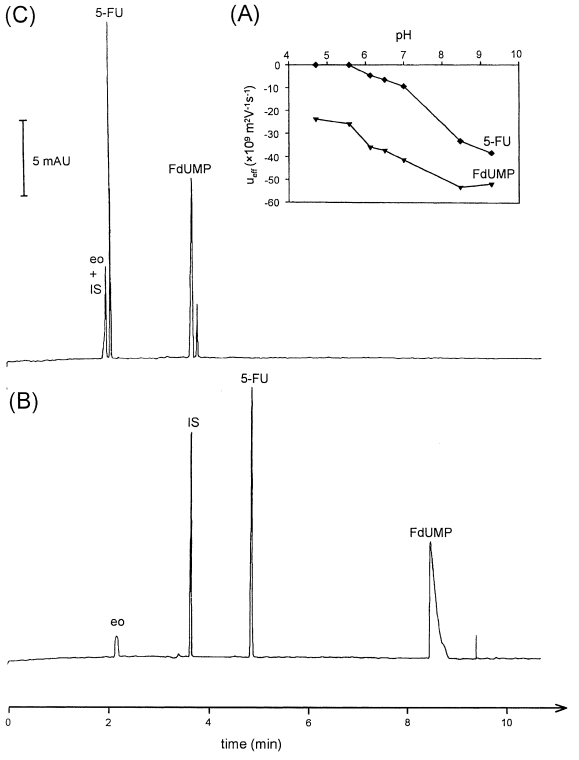

Fig. 2. (A) pH dependence of the effective electrophoretic mobility in 0.023 *M* acetate buffer, pH 4.7–5.6, 0.025 *M* phosphate buffer, pH 6.1–8.5 and 0.03 *M* $Na_2B_4O_7$, pH 9.3. The applied voltage was 25 kV, the currents were 26–51 μA. (B, C) Electropherograms of a standard mixture containing 5-FU (0.61 m*M*), FdUMP (0.51 m*M*) and theophylline (I.S., 0.21 m*M*) separated in an uncoated silica capillary of 61.8 cm (40.1 cm to detector)×50 μm I.D. using BGE: (B) 0.03 *M* $Na_2B_4O_7$, pH 9.2, (C) 0.025 *M* NaH_2PO_4+Na_2HPO_4, pH 6.5. Voltage applied 25 kV, current (B) 46 μA and (C) 31 μA, sample injection 2 s.

Using 0.03 *M* $Na_2B_4O_7$ (pH 9.2), good resolution between 5-FU, FdUMP and theophylline (internal standard, I.S.) was obtained (Fig. 2B). 5-FU and I.S. were found to create sharp, narrow and symmetric peaks that were detected after 4.9 and 3.8 min, respectively. For FdUMP (detected at 8.6 min), however, a broad and asymmetric peak was moni-

tored. With increasing concentration of the co-ion of the BGE (investigated range: 0.005–0.04 *M* $Na_2B_4O_7$), the FdUMP peak became slightly narrower and better resolved from an unknown component present in the standard and comigrating with FdUMP. Improvement in the peak shape, however, was not significant. Using different BGEs [acetate buffer: 0.023 *M* acetic acid titrated with 0.5 *M* NaOH to pH 4.7–5.6; phosphate buffer: equivolumes of 0.025 *M* NaH_2PO_4 and 0.025 *M* Na_2HPO_4 were mixed and titrated with 0.5 *M* NaOH to pH 7.0–8.5; 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES) buffer: 0.05 *M* CHES titrated with 0.5 *M* NaOH to pH 8.4–9.7; 3-cyclohexylamino-1-propanesulfonic acid (CAPS) buffer: 0.02 *M* CAPS titrated with 0.5 *M* NaOH to pH 11.0] and adding 7 *M* urea, 0.02–0.08% (w/v) hydroxyethylcellulose, 4% (v/v) Triton X-100 and 10% (v/v) acetonitrile to the BGE did not provide improved data either. Only a BGE consisting of 0.025 *M* phosphate buffer (pH 6.1–6.5) prepared by titrating 0.025 *M* NaH_2PO_4 with 0.025 *M* Na_2HPO_4 to the desired pH was found to create a narrow and symmetric peak for FdUMP that was well resolved from the unknown component present in the standard (Fig. 2C). 5-FU, however, at this pH is only slightly negatively charged and thus migrates in close proximity to the EOF marker. Furthermore, theophylline is neutral, migrates with EOF and cannot be used as I.S. Shifting of the pH of the phosphate buffer to higher or lower values resulted in peak broadening of FdUMP. Based on these results, two different BGEs were chosen for experiments with the biological samples: (i) 0.03 *M* $Na_2B_4O_7$ (pH 9.2) for determination of 5-FU using theophylline as I.S. and for monitoring of the presence of FdUMP and (ii) phosphate buffer composed of 0.021 *M* NaH_2PO_4 and 0.004 *M* Na_2HPO_4 (pH 6.5) for quantification of FdUMP. Depending on the type of biological sample analyzed, some additional buffer optimization (fine tuning) was required and will be discussed individually (see below).

3.2. Sample preparation for CZE

To remove proteins from the sample, protein precipitation with ACN and protein ultrafiltration (both described in Section 2.4) were evaluated. Using protein precipitation, sample–ACN ratios of 1:1 and 1:2 (v/v) were investigated. With the pancreatic cancer cell suspension, equal amounts of the fluids were found to be insufficient for complete cell lysis (observation of the precipitate under a microscope). The 1:2 ratio was used for all matrices without any problems. Moreover, electropherograms of cell samples with the higher content of ACN were determined to exhibit sharper and better resolved peaks and shorter detection time intervals. Protein ultrafiltration can be used because of the extremely low protein binding capacity of 5-FU (approximately 4%) [25]. In the case of pancreatic tissue homogenates, the electropherograms obtained with ultrafiltrates were found to be identical to those monitored with the supernatant after ACN precipitation (data not shown). Peak heights, however, were 2.5-fold higher after injection of ultrafiltrates (no dilution with precipitating agent). Compared to ACN precipitation, ultrafiltration is more time consuming and more expensive. It is thus recommended for analysis of samples with low analyte concentration only.

3.3. Analysis of ACN pretreated biological samples in uncoated capillaries

3.3.1. PANC-1 sample

Using the pH 9.2 borate buffer (30 m*M* $Na_2B_4O_7$), several major peaks were obtained. Electropherograms obtained with cells that were exposed for 1 h to culture medium containing 500 μg/ml 5-FU and the same sample fortified with 5-FU and FdUMP are depicted in Fig. 3A and B, respectively. Blank sample was not available here. Both analytes are shown to be well separated. The 5-FU peak detected at 5.5 min (Fig. 3A) was determined to correspond to a concentration of 140.9 μg/ml. FdUMP was not detected in this sample as well as in any other cell samples that were analyzed in that work. For quantification of analytes, theophylline was used as I.S. In some samples, however, comigration of the I.S. with an unknown sample component was observed. With the BGE pH shifted to 9.4 (adjusted with 0.5 *M* NaOH), however, no interferences were observed.

3.3.2. Culture medium

In the culture medium, the 5-FU concentration prior and after the incubation of cells was evaluated.

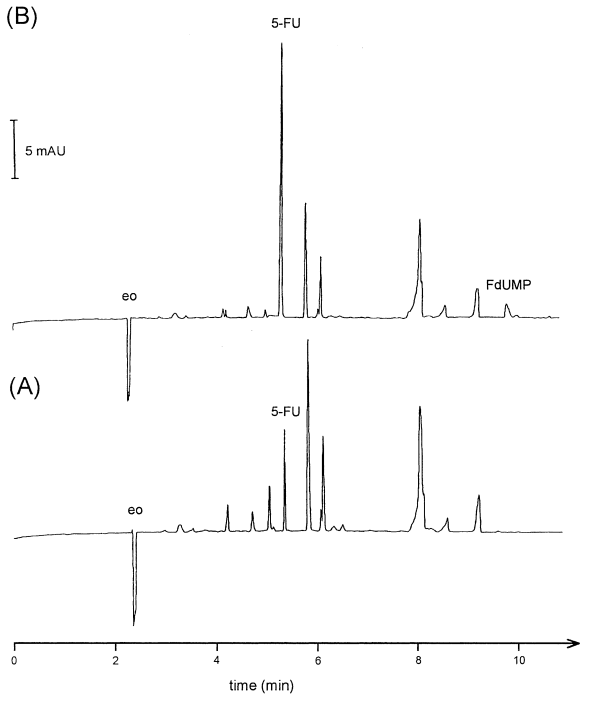

Fig. 3. Electropherograms of (A) PANC-1 cells incubated for 1 h in a culture medium containing 3.84 m*M* (500 μg/ml) 5-FU and (B) sample fortified with 1.43 m*M* 5-FU and 0.11 m*M* FdUMP. BGE: 0.03 *M* $Na_2B_4O_7$, pH 9.2. Voltage used 25 kV, current 47 μA. Sample injection 2 s. Uncoated silica capillary 61.8 cm (40.1 cm to detector)×50 μm I.D.

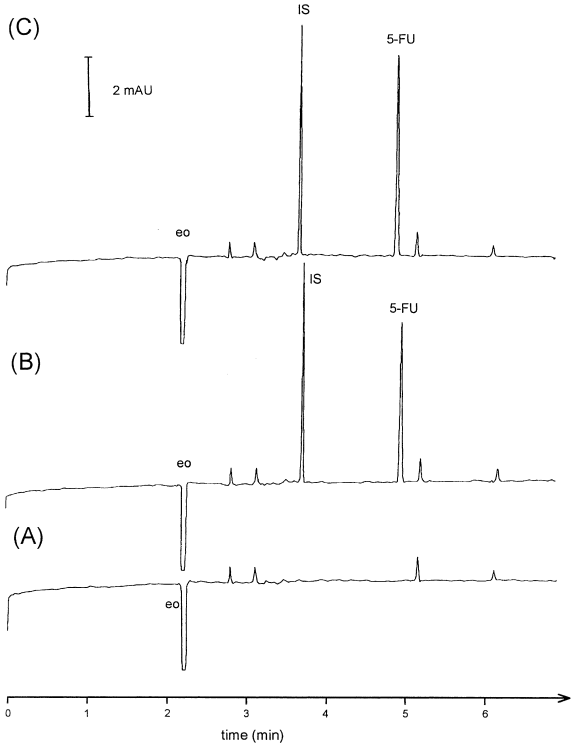

Fig. 4. Electropherograms of (A) blank culture medium, (B) blank culture medium fortified with 5-FU (0.51 m*M*) and I.S. (0.14 m*M*) and (C) medium taken at the beginning of the cell incubation. Composition of BGE and conditions as in Fig. 3.

Electropherograms of typical blank culture medium, culture medium spiked with 5-FU and I.S. and the medium at the beginning of the cell incubation are presented in Fig. 4. For the blank medium, four minor peaks were detected which do not interfere with the analytes of interest. In these samples, 5-FU and I.S. are shown to be well recovered and separated. Furthermore, as the culture medium was stored at room temperature and incubation took place at 37°C, 5-FU dissolved in the culture medium was determined to be stable at these temperatures and over a time period of at least 4 days.

3.3.3. Human plasma

In blank human plasma, two peaks of unknown endogenous compounds which do not interfere with the determination of 5-FU and FdUMP were detected (Fig. 5A). A typical electropherogram obtained with a fortified human plasma (0.28 m*M*) and data from a patient under 5-FU treatment are presented in Fig. 5B and C, respectively. The plasma 5-FU level of all patients investigated was found to be below the limit of quantification (<4.12 μg/ml, see below). This did not come as a surprise as these patients received the drug directly into the arteria hepatica communis (cf. Section 2.1) and 5-FU became rapidly metabolized in the liver. For construction of calibration curves, calibrators were prepared with bovine plasma which is less expensive than human plasma. Electropherograms monitored with bovine plasma (data not shown) were similar to those obtained with human plasma (peak at 4.6 min was not detected).

3.3.4. Pancreatic tissue

In the electropherogram obtained with blank human pancreatic tissue several major peaks were detected (Fig. 6A). All of them, however, are well separated from 5-FU and FdUMP (Fig. 6B). No

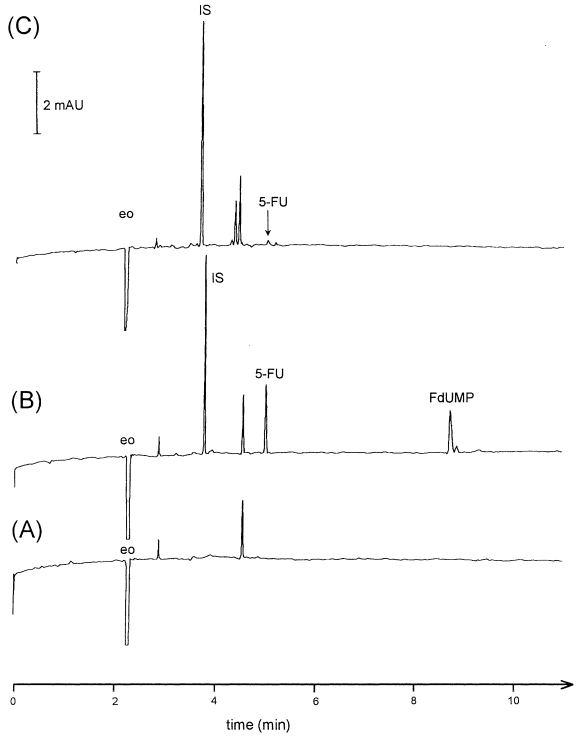

Fig. 5. Electropherograms of (A) blank human plasma, (B) blank human plasma fortified with 5-FU (0.28 m*M*), FdUMP (0.23 m*M*) and I.S. (0.14 m*M*), and (C) plasma of patient under the treatment with 5-FU. Composition of BGE and conditions as in Fig. 3 except for sample injection 1.5 s.

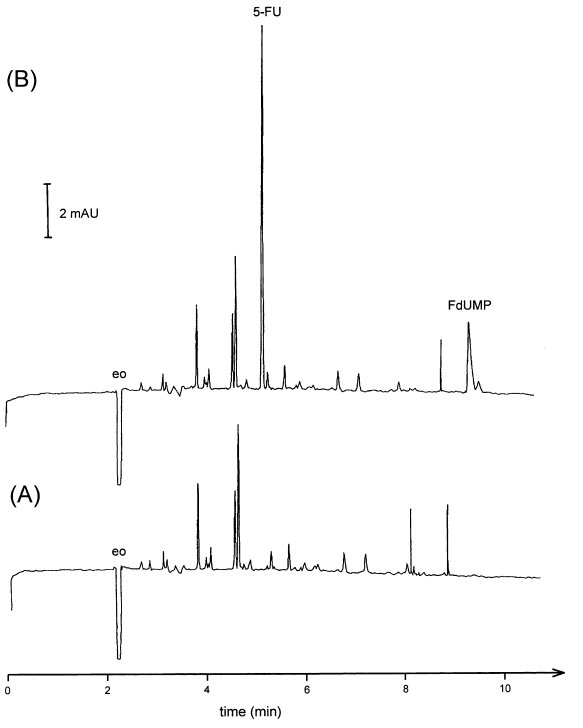

Fig. 6. Electropherograms of (A) blank human pancreas and (B) blank human pancreas fortified with 5-FU (0.47 m*M*) and FdUMP (0.14 m*M*). Composition of BGE and conditions as in Fig. 3.

patient samples were available yet. Because of the difficulty in the availability of human pancreatic tissue, rat pancreas was analyzed as a potential material for construction of calibration graphs. The CZE pattern, however, was determined to be substantially different from that of human pancreas. It was found to be more complex (data not shown) and can thus not be employed for the preparation of calibrators.

3.3.5. Qualitative and quantitative evaluation

Identification of the compounds was based on the evaluation of the detection times of analytes. The imprecision of migration times within 1 day was very good. For both (5-FU and FdUMP) analytes, relative standard deviations (RSDs) were found to be $<1.0\%$ ($n=7$). Inter-day RSDs were $<1.5\%$ ($n=7$). The electroosmotic mobility (evaluation based upon the peak produced by the sample solvent, for examples see Figs. 3–6) was determined to vary in the range $6.80\cdot10^{-8}-7.22\cdot10^{-8}$ $m^2\ V^{-1}\ s^{-1}$ [mean: $6.94\cdot10^{-8}$ $m^2\ V^{-1}\ s^{-1}$; RSD: 2.41% ($n=6$)]. For the assessment of intra-day and inter-day imprecisions, cell samples spiked with 5-FU (4.12–33.0–132 μg/ml) were analyzed repeatedly. Intra-day RSDs ($n=5$) evaluated from the peak height and area ratios of the compound to the respective properties of the I.S. were 7.64–4.28–3.60% and 28.39–5.02–3.15%, respectively. Inter-day RSDs ($n=4$) were 8.79–2.38–5.12% and 33.6–6.03–4.36%, respectively.

Quantitation of 5-FU was based upon internal, six-level calibration (4.12–132 μg/ml concentration range) using both peak height and area ratios of the compound to the respective properties of the I.S. Five calibrations were undertaken for the determination of 5-FU in cell samples. Calibration regression lines [stated as $y=$(mean of slope$\pm$standard deviation)$x+$(mean of y-intercept$\pm$standard deviation)] and regression coefficients were determined to be $y=(107.74\pm3.98)x+(0.386\pm0.946)$, $r^2=0.999$

and $y=(69.20\pm2.101)x+(0.620\pm1.596)$, $r^2=0.997$ for concentration (μg/ml) vs. peak height ratio and peak area ratio, respectively. In all cases, the regression lines were linear (F values>660) over the concentration range examined, the y-intercepts were observed to be significantly smaller than the lowest calibrator values and were thus negligible. The limit of detection (LOD, defined as signal:noise=3:1) was found to be 1.72 μg/ml ($1.32\cdot10^{-5}$ M) for 5-FU and 5.29 μg/ml ($1.62\cdot10^{-5}$ M) for FdUMP. The sensitivity was sufficient for the determination of the 5-FU levels in PANC-1 cell samples whereas FdUMP was undetectable in all analyzed samples.

3.4. Analysis of 5-FU and FdUMP in LPA-coated capillaries

In an attempt to avoid broadening of the FdUMP peak, electrophoresis in a capillary with the inner surface coated with linear polyacrylamide was investigated. Due to a strong decrease of electroosmosis compared to the uncoated capillary (electroosmotic velocity after 57 analyses was $0.74\cdot10^{-8}$ m^2 V^{-1} s^{-1}), polarity had to be reversed (cathode at injection end) and compounds of interest were detected in reversed order (Fig. 7A). Significant improvement in the shape of the FdUMP peak was observed. All peaks were symmetric and narrow and the time interval required for analysis of the three compounds was short (<6 min).

For the analysis of plasma using the pH 9.2 buffer (0.015 M $Na_2B_4O_7$), stacking of FdUMP induced by the sample matrix was observed (data not shown). This effect makes quantification more difficult [26]. It could be eliminated by shifting the pH of the BGE to 9.4 (addition of NaOH) and good separation of all components was achieved (Fig. 7B). This system was found to be suitable for the simultaneous quantification of 5-FU and FdUMP in plasma. The calibration equations (stated as y=slope·x+y-intercept) and regression coefficients for 5-FU were $y=168.30x-3.03$ and $r^2=0.999$ and $y=240.13x-1.76$ and $r^2=0.999$ for concentration (μg/ml) vs. peak height ratio and peak area ratio, respectively. Corresponding equations for FdUMP were $y=305.08x-4.33$ and $r^2=0.991$ and $y=594.63x-0.93$ and $r^2=0.999$, respectively. LODs were 0.80 μg/ml ($9.84\cdot10^{-6}$ M) for 5-FU and 2.62 μg/ml ($8.02\cdot10^{-6}$ M) for FdUMP. These values refer to sample preparation based upon protein precipitation with ACN (cf. Section 2.4).

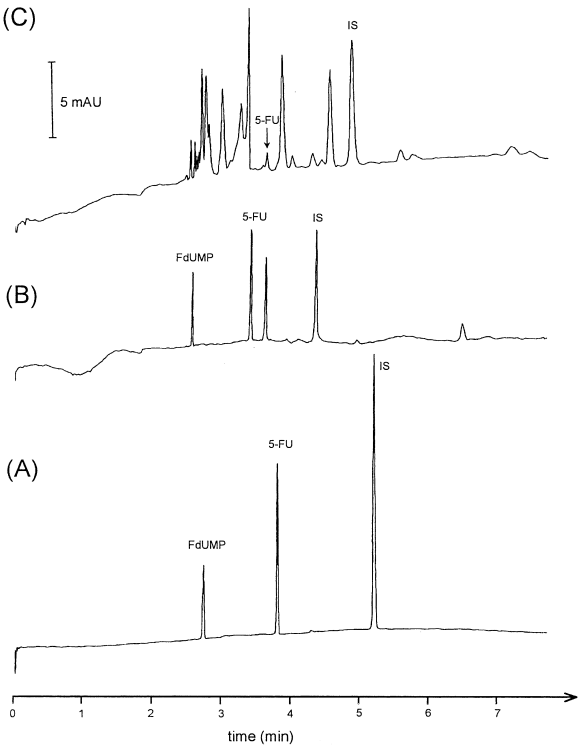

Fig. 7. Separation in an LPA-coated capillary. Electropherograms of (A) standard mixture containing 5-FU (0.17 mM), FdUMP (0.08 mM) and theophylline (I.S., 0.07 mM), (B) human plasma fortified with 5-FU (0.51 mM), FdUMP (0.23 mM) and I.S. (0.04 mM), and (C) cell line sample, determined concentration of 5-FU was $1.14\cdot10^{-4}$ M. BGE: 0.015 M $Na_2B_4O_7$ of (A) pH 9.2 and (B, C) pH 9.4. Voltage −25 kV, current 79 μA (A) and 86 μA (B, C), sample injection 2 s (A, B) and 1 s (C).

For the analysis of cells, a large amount of unknown components was found to migrate unresolved from FdUMP (Fig. 7C). Furthermore, all peaks were observed to be broader compared to those detected for analysis in the uncoated capillary format. Thus, for analysis of cell samples, the use of a coated capillary was not further investigated.

3.5. Accumulation of 5-FU by PANC-1 cells

The developed CZE method was applied to the study of the 5-FU accumulation by PANC-1 cells. To

set the concentration of the drug in the culture medium, cells were first exposed for 1 h to media containing 500, 100, 50 and 25 μg/ml 5-FU. Analysis of the cells revealed that the concentration of the drug in the culture medium has to be at least 30 μg/ml. For further experiments, culture media containing 100 μg/ml 5-FU were used. Stability of 5-FU in such a medium was verified by incubation at 37°C for 84 h. Drug concentrations were evaluated before and after the incubation and the difference was found to be within the range of deviation of the assay [RSDs were 5.64% and 2.48% ($n=2$) for the concentration evaluated using peak height and area ratios, respectively].

For the determination of the intracellular 5-FU concentration in PANC-1 cells, the following steps were undertaken. Firstly, the 5-FU drug content in 50 μl of cell suspension was determined by CZE (see above). Then, the protein content in the same volume of cell suspension was measured with the BCA protein assay as described in Section 2.3. The number of cells was determined using the linear calibration graph for protein amount (μg) vs. number of cells ($\cdot 10^{-6}$), namely $y=149.5x+11.2$ ($r^2=0.9995$). Consequently, the average drug content in a single cell could be calculated. Finally, the intracellular 5-FU concentration was estimated using the determined average volume±SD of a single cell, namely 4.704±1.092 pl (for details refer to Section 2.3). The intracellular concentration of 5-FU was determined as function of the time of exposure to the drug in the time interval between 5 min and 4 h (Fig. 8). From the obtained graph it is evident that the intracellular concentration of 5-FU increased significantly within the first 20 min of drug exposure. Thereafter, further drug accumulation was found to become moderate. Overall, the data reveal that the intracellular 5-FU concentration reaches the 5-FU concentration level of the surrounding incubation medium. For that experiment, the average concentration of 5-FU within a PANC-1 cell is expressed in μg/ml (pg/nl) and can thus easily be compared to the drug concentration of the medium (100 μg/ml) to which the cells were exposed to. It is important to note that the performed conversion is invariant to differences in volumes of cell suspension and amounts of cells in the sample. For this purpose, equivalent volumes of cell suspension were used for the determination of 5-FU by CZE and for protein determination.

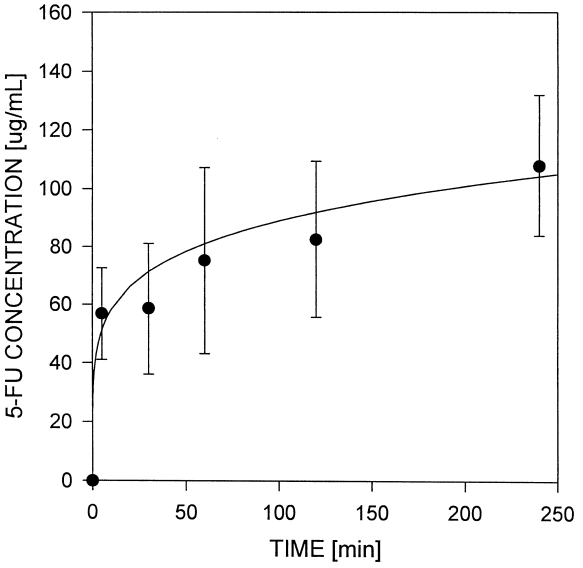

Fig. 8. Intracellular concentration of 5-FU in PANC-1 cells vs. exposure time to a medium containing 100 μg/ml 5-FU. Each data point represents the mean±1SD ($n=3$) and the solid line was determined using non-linear regression analysis. CZE analyses were performed in an uncoated capillary of 61.8 cm (40.1 cm to detector)×50 μm I.D. and using a pH 9.4 BGE. Other conditions as in Fig. 3.

4. Conclusions

A simple CZE assay for the monitoring of 5-FU and FdUMP in different biological matrices was developed. The technique involves deproteinization with ACN followed by CZE analysis of the supernatant within several minutes. It was successfully applied for quantification of 5-FU in in vitro studies with pancreatic cancer cells and was extended to other matrices, such as culture medium, plasma and pancreatic tissue. The data reveal that the intracellular 5-FU concentration reaches the 5-FU concentration level of the surrounding incubation medium. Having cell cultures with 100 μg/ml 5-FU in the medium, the 5-FU concentration within a PANC-1 cell is shown to become equal to that of the surrounding medium within about 4 h of exposure time. The developed technique will be used for

monitoring of 5-FU in other in vitro experiments and later in samples of patients with pancreatic cancer in order to optimize the chemotherapy with that drug. For determination of the levels of the active metabolite FdUMP, however, the sensitivity of the assay is not sufficient. It would have to be increased by using preconcentration techniques (e.g., extraction, on-line combination of capillary isotachophoresis with zone electrophoresis) and/or improved detection schemes.

Acknowledgements

The authors would like to thank Dr. A. Tempia-Caliera for his help in the collection of the patient's blood samples. This work was supported by the Swiss National Science Foundation, by the Grant Agency of the Czech Republic, grant No. 203/99/0044 and by the Grant Agency of the Academy of Sciences of the Czech Republic, grant No. A 4031703.

References

[1] C. Heidelberger, in: J.F. Holland (Ed.), Cancer Medicine, Lea and Febiger, Philadelphia, PA, 1993, p. 801.
[2] H.M. Pinedo, G.F.J. Peters, J. Clin. Oncol. 6 (1988) 1653.
[3] Meta-Analysis Group in Cancer, J. Clin. Oncol. 16 (1998) 301.
[4] M. Müller, R.M. Mader, B. Steiner, G.G. Steger, B. Jansen, M. Gnant, T. Helbich, R. Jakesz, H.G. Eichler, B. Blochl-Daum, Cancer Res. 57 (1997) 2598.
[5] U.R. Tjaden, E.A. de Bruin, J. Chromatogr. 531 (1990) 235.
[6] L.W. Anderson, R.J. Parker, J.M. Collins, J.D. Ahlgren, D. Wilkinson, J.M. Strong, J. Chromatogr. 581 (1992) 195.
[7] T. Stein, G.P. Burns, B. Bailey, L. Wise, J. Chromatogr. 507 (1990) 259.
[8] W. Jäger, M.J. Czejka, J. Schüller, U. Fogl, E. Czejka, H. Lackner, J. Chromatogr. 532 (1990) 411.
[9] J.A. Smith-Rogers, W.P. Tong, M.E. Duafala, M. Markman, J.R. Bertino, J. Chromatogr. 566 (1991) 147.
[10] M. Barberi-Heyob, J.L. Merlin, B. Weber, J. Chromatogr. 573 (1992) 247.
[11] M. Barberi-Heyob, J.L. Merlin, B. Weber, J. Chromatogr. 581 (1992) 281.
[12] M.J. Del Nozal, J.L. Bernal, A. Pampliega, J.C. Pastor, M.I. Lopez, J. Chromatogr. 607 (1992) 183.
[13] M.J. Del Nozal, J.L. Bernal, A. Pampliega, P. Marinero, M. Pozuelo, J. Chromatogr. B 656 (1994) 397.
[14] P. Compagnon, L. Thiberville, N. Moore, C. Thuillez, C. Lacroix, J. Chromatogr. B 677 (1996) 380.
[15] A.L. Pogolotti, P.A. Nolan, D.V. Santi, Anal. Biochem. 117 (1981) 178.
[16] J.P. Sommadossi, D.A. Gewirtz, R.B. Diasio, C. Aubert, J.P. Cano, I.D. Goldman, J. Biol. Chem. 257 (1982) 8171.
[17] F. Foret, L. Křivánková, P. Boček, Capillary Zone Electrophoresis, VCH, Weinheim, 1993.
[18] E.A. de Bruijn, G. Pattyn, F. David, P. Sandra, J. High Resolut. Chromatogr. 14 (1991) 627.
[19] R.M. Mader, M. Brunner, B. Rizovski, C. Mensik, G.G. Steger, H.G. Eichler, M. Müller, Electrophoresis 19 (1998) 2981.
[20] J. Olgemöller, G. Hempel, J. Boos, G. Blaschke, J. Chromatogr. B 726 (1999) 261.
[21] H.J.E.M. Reeuwijk, U.R. Tjaden, J. van der Greef, J. Chromatogr. B 726 (1999) 269.
[22] S. Hjertén, J. Chromatogr. 347 (1985) 191.
[23] S. Krásenský, S. Fanali, L. Křivánková, P. Boček, Electrophoresis 16 (1995) 968.
[24] A.C. Moffat, J.V. Jackson, M.S. Moss, B. Widdop (Eds.), Clarke's Isolation and Identification of Drugs, Pharmaceutical Press, London, 1986, p. 627.
[25] M. Czejka, J. Schüller, Arch. Pharm. 325 (1992) 69.
[26] L. Křivánková, P. Pantůčková, P. Boček, J. Chromatogr. A 838 (1999) 55.