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Solid-phase extraction of fluoropyrimidine derivatives on a copper-modified strong cation exchanger: determination of doxifluridine, 5-fluorouracil and its main metabolites in serum by high-performance liquid chromatography with ultraviolet detection

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ABSTRACT

A copper-modified strong cation exchange stationary phase was used for the solid phase extraction of the antineoplastic drug doxifluridine (5'-deoxy-5-fluorouridine) and some of its main metabolites from human serum. Sorption of fluoropyrimidine derivatives due to complex formation with copper(II) was maximal at pH values around neutrality. Analytes were eluted by ligand exchange with ammonia. The average recoveries ranged from 70% to 108%. Reversed-phase chromatography with ultraviolet detection was used for the separation and quantitation of the analytes. The overall procedure has been applied to serum samples from patients receiving doxifluridine chemotherapy, and a pharmacokinetic profile has been derived.

INTRODUCTION

Doxifluridine (5'-deoxy-5-fluorouridine, 5'-dFUR) is a pro-drug synthesized [1] in an attempt to improve the therapeutic index of the antineoplastic drug 5-fluorouracil (5-FU). Using the deoxyribofuranosyl moiety as a carrier into neoplastic tissues, 5'-dFUR is cleaved to the active 5-FU preferentially in tumour cells [2–4].

In order to establish the optimal schedule for 5'-dFUR administration, analytical methods capable of a simultaneous determination of (at least) the pro-drug (5'-dFUR) and the drug (5-FU) in biological matrices are essential. Although a large number of analytical procedures have been developed [5], reversed-phase high-performance liquid chromatography (RP-HPLC) is the most widely applied technique in the analysis of fluoropyrimidines. Recently, the possibility of a simultaneous determination of 5'-dFUR, 5-FU, 5-fluorouridine (FUR) and 2'-deoxy-5-fluorouridine (2'-dFUR) in serum has been reported [6].

HPLC analysis of fluoropyrimidines in serum

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requires a preliminary clean-up of the sample, usually performed by protein precipitation followed by liquid–liquid extraction (LLE). These procedures, apart from possible incomplete analyte recovery, are tedious, time-consuming and difficult to automate.

Sample clean-up by solid-phase extraction (SPE) generally ensures good recoveries and reproducibility, is less time-consuming, and can be automated. As far as we know, however, no SPE method has been reported for the simultaneous extraction of 5′-dFUR, 5-FU and other metabolites.

Anion exchange has been used for plasma and serum treatment in the determination of 5-FU by gas chromatography (GC) [7] and HPLC [8]. The same technique was used for the GC determination of 2′-dFUR in plasma [9] and urine [10], and for the GC [11] and HPLC [12,13] determination of 5-FU, 2′-dFUR and FUR. In these last cases, however, sequential cation- and anion-exchange chromatography [11,12] and an additional LLE step [13] were employed to ensure a suitable sample clean-up. Probably, the very different chromatographic properties of 5′-dFUR, 5-FU and their metabolites could explain the difficulty in the development of a SPE procedure capable of their simultaneous coextraction. A different and more specific approach seems then necessary.

Ligand exchange chromatography (LEC) is a well-known technique for separating and isolating compounds that can form complexes or adducts with metal ions [14,15]. A stationary phase, usually a cation-exchange resin, is loaded with a metal ion (typically a transition-metal ion) capable of binding the analyte; although bound to the stationary phase, the metal ion generally retains its coordinating properties so that analyte can be sorbed on the column and then eluted, for example by displacement with another ligand. Such a technique has been employed for the chromatography of nucleotides, nucleosides and nucleic acid bases [16] and, recently, for the trace enrichment of pyrimidine nucleobases, 5-FU, bromacil [17] and of 3′-azido-3′-deoxythymidine (AZT) in human plasma [18].

The aim of this work is the development of an SPE procedure for the simultaneous extraction of 5′-dFUR, 5-FU, FUR, 2′-dFUR, 5-fluorouridine monophosphate (FUMP) and 2′-deoxy-FUMP (2′-dFUMP) from human serum. The sorption/desorption properties of a copper(II)-loaded strong cation-exchange stationary phase were investigated for this purpose. A simple and effective SPE procedure, ensuring good recovery and reproducibility, has been developed. This procedure, followed by RP-HPLC with UV detection, permits the simultaneous determination of the fluoropyrimidines listed. The overall analytical procedure has been applied to monitor serum 5′-dFUR and 5-FU concentrations in cancer patients treated with doxifluridine.

EXPERIMENTAL

Chemicals

5′-dFUR (Prodotti Roche, Milan, Italy), 5-FU, 5-bromouracil (5-BrU), 2′-dFUMP and 2′-dFUR (Sigma, St. Louis, MO, USA), FUR and FUMP (Calbiochem-Behring, La Jolla, CA, USA) were used as received. Stock solutions were prepared in tridistilled water or the mobile phase, and stored at 4°C in the dark. More dilute solutions were prepared just before use. Methanol (Carlo Erba, Milan, Italy) was HPLC grade. Other chemicals were of analytical-reagent grade. Mobile phases were filtered through 0.45-μm filters (HATF Millipore, France) and vacuum-degassed before use.

Apparatus

The chromatographic system consisted of a Perkin-Elmer (Norwalk, CT, USA) Model 3B dual-pump module, equipped with a Rheodyne (Cotati, CA, USA) 7125 injection valve and a 5 μm Supelcosil LC-18-S, ODS column (250 × 4.6 mm I.D.) (Supelco, Bellefonte, PA, USA). A 5 μm Supelguard LC-18-S pre-column (20 × 4.6 mm I.D.) (Supelco) was used to protect the analytical column. Strong cation-exchange LC-SCX Supelclean solid-phase extraction 1-ml tubes (Supelco) were modified and used for sample clean-up. The detector was an HP 1040A photodiode-array spectrophotometer (Hewlett-Packard,

Palo Alto, CA, USA) interfaced to an HP 85 computer equipped with an HP 9121 dual-disk drive and an HP 7470A plotter. A Perkin-Elmer LCI-100 laboratory computing integrator directly connected to the analogue output of the photodiode-array detector was also used.

Chromatographic and detection conditions

A binary gradient composed of phosphate buffer (0.05 *M*, pH 6.5)–methanol (40:60, v/v) (solvent A) and phosphate buffer (0.05 *M*, pH 6.5) (solvent B) was used. The gradient programme was as described in ref. 6. The flow-rate was 1 ml/min, the injection volume was 20 μl, and the temperature was ambient.

Unless otherwise specified, spectra were acquired in the 210–400 nm range at the apex and on the ascending or descending part of each peak, using a pilot signal at 269 nm (4 nm band-width), a reference signal at 450 nm (80 nm band-width) and a threshold value of 1 mAU. The peak purity was checked by the technique of spectra overlaying after normalization.

Sample collection

Serum samples from healthy donors and from patients with advanced colorectal cancer under chemotherapy with doxifluridine were stored at −50°C. The healthy donors were taking no drugs at the time of sample collection.

Extraction procedure

Before the extraction procedure, the LC-SCX SPE tubes were modified by flushing with 2 ml of methanol, 1 ml of 0.1 *M* copper(II) sulphate solution and 3 ml of phosphate buffer (0.05 *M*, pH 7); tubes were not allowed to dry at these stages.

The serum clean-up procedure was as follows: 300 μl of serum containing the internal standard (5-BrU) were loaded on modified SPE tube, and washed with 2 ml of phosphate buffer (0.05 *M*, pH 7) followed by 2 ml of methanol (tubes were allowed to dry at these stages). The analytes were then eluted with 700 μl of 1.7 *M* ammonia solution. The eluate was collected in a vial containing 70 μl of glacial acetic acid, thoroughly mixed, and 20 μl of the mixture were injected.

In both the modification and extraction procedures, the SPE tubes were always flushed at 1 ml/min.

RESULTS AND DISCUSSION

Selection of stationary phase and modifier metal ion

Apparently, the stationary phase employed in LEC acts just as a solid and inert support for the metal ions involved in the ligand-exchange process. To ensure high metal loading and minimal metal losses during chromatographic processes, a strong binding between the stationary phase and the metal ions is necessary; however, if the interaction is too strong, masking or saturating the coordination sites of the metal, the complexation of analytes could be suppressed, leading to a poor chromatographic efficiency. In order to optimize metal immobilization with respect to ligand-exchange efficiency, aminopropyl- (LC-NH_2), diol- (LC-Diol) and sulphonic acid- (LC-SCX) bonded silica stationary phases were tested. A stable metal (*e.g.* copper(II)) immobilization was observed for each stationary phase. However, in the case of copper-loaded LC-NH_2 and LC-Diol stationary phases, poor retention of fluoropyrimidines was observed whatever the metal loading and the pH of the solutions employed. This indicated significant saturation of the coordination sites of the immobilized metal by the amino and hydroxyl groups, respectively. The LC-SCX stationary phase was then selected for the following investigations.

Several metal ions (*e.g.* silver(I), copper(II), mercury(II), lead(II), and nickel(II)) are known to form complexes or adducts with pyrimidine nucleobases, and in particular with fluoropyrimidines [16,19]. However, to be useful in LEC, a metal ion should form, with the analyte, a complex that is stable over a large pH range; moreover, complexation must be reversible. Preliminary experiments involving metal ions immobilized on the LC-SCX stationary phase suggested that some metal ions (*e.g.* nickel(II) and mercu-

ry(II)) cannot be employed because they do not satisfy the above requirements. LC-SCX stationary phases modified with copper(II) and silver(I) ions showed good sorption/desorption properties towards fluoropyrimidine derivatives. However, in the case of the silver(I)-modified stationary phase, the extraction yields (typically 35% for 5-FU and 70% for 5-dFUR) were lower, whatever the sorption pH, than those observed with copper(II) as modifier. To a first approximation, this indicated that the copper(II)–fluoropyrimidine complexes are the most stable and show better sorption behaviour. Previous studies of the homogeneous [19] and heterogeneous [17,18,20] formation of fluoropyrimidine complexes with silver(I) and copper(II) ions have usually revealed the opposite order of stability. This different behaviour can be a consequence of a certain influence of the immobilizing stationary phase on the coordinating properties of the immobilized metal ion.

Modification of the stationary phase with copper(II) was optimal with a 0.1 *M* copper(II) solution (see Experimental): this concentration, in fact, represented a good compromise between maximal loading of the stationary phase and minimal loss of copper(II) from stationary phase during the extractive procedures.

Sorption/desorption studies

Complex formation of pyrimidines with heavy metal ions is strongly dependent on the pH. It involves a Lewis-base type interaction between the metal ion and an electron-donating site of the nucleobase molecule, the nature of donor atom and/or group depending on both the particular nucleobase molecule involved in the complex and the coordinating metal ion [21]. For example, in the case of pyrimidines, coordination can occur through the ring nitrogen atoms and/or the oxygen atoms of carbonyl groups; moreover, in the case of nucleosides, nucleotides and fluoro derivatives, further possible sites, namely the hydroxyl and phosphate groups of the sugar molecule and the halogen atom, could be involved in complex formation [19,22]. The pH, however, governs not only the nature of electron-donating

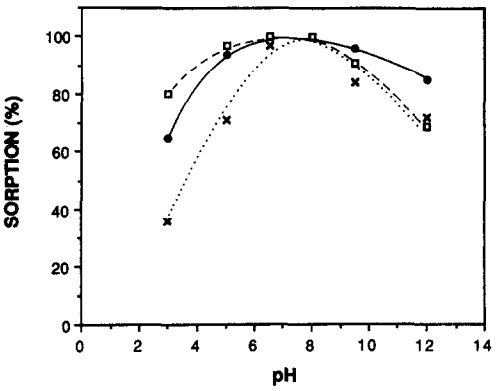

Fig. 1. Percentage sorption, on a copper-modified LC-SCX stationary phase, of 5′-dFUR (□), 5-FU (×) and the internal standard (5-BrU, ●) as a function of sample pH (analyte amounts: 1, 2 and 4 μg for 5-FU, 5′-dFUR and 5-BrU, respectively).

site of the molecule but also the availability of the metal ion.

In order to evaluate the effect of pH on fluoropyrimidine sorption, samples with the same analyte content but at different pH were passed at a constant flow-rate through LC-SCX SPE tubes modified with copper(II) ions. The solid phase was allowed to dry so that all the sample could be recovered (the sample volume used, typically 1 ml, was about ten times the void volume of the SPE tube). The chromatographic responses relative to processed and unprocessed samples were then compared. The percentage sorptions so obtained are shown in Fig. 1 for 5-FU, 5-BrU (internal standard) and 5′-dFUR. As can be seen, analyte sorption increased with increasing pH, indicating that complexation with the immobilized metal ion occurred at a deprotonated site of the pyrimidine molecule. Sorption was maximal around neutral pH, and decreased at higher pH values owing to competition between hydroxide ions and analyte molecules for the metal binding sites. This sorption behaviour indicates that the stability constant, K_h, relative to the sorbed copper(II)–L complex formation (where L represents the deprotonated form of the fluoropyrimidine analyte) is greater than the stability of LH, *i.e.* $\log K_h > pK_a$ where K_a is the acidity constant of LH.

Different behaviour has been reported for a

silver(I)-loaded thiol stationary phase [20]. In this case, 5-FU behaved differently, showing maximal sorption at a pH value at least two units higher than its pK_a. In contrast, on the same modified stationary phase, some barbiturates showed maximal sorption at pH values equal to or lower than their pK_a values. These and the previous findings suggest that (a) sorption behaviour cannot be easily predicted by comparing the analyte pK_a and the homogeneous stability constant of metal–analyte complex because the heterogeneously formed metal–analyte complex (*i.e.* the complex involved in ligand-exchange process) could be very different and/or could show a stability constant different from the homogeneous one, and (b) immobilization of metal ion on a stationary phase could result in different coordination behaviour of the metal ion, suggesting the importance of the stationary phase in the overall ligand-exchange process.

Desorption of analytes in LEC can be obtained [17] with a ligand that forms a stronger complex with the loaded metal, by analyte protonation, or by using a different metal ion that will form a complex with the analyte stronger than the one formed with the metal loaded on the stationary phase. In this last case, however, the analytes will be eluted as metal complexes, requiring a chromatographic approach different from the usual RP-HPLC [6].

Desorption via analyte protonation was investigated by using several eluents of different pH in the range 2–7. Unfortunately, recoveries were generally low (typically *ca.* 10%) unless high elution volumes with consequent sample dilution were used. Elution via ligand exchange was found to be more effective. Several ligands were investigated: tartrate was ineffective; EDTA was more effective but required large elution volumes for quantitative recovery; ammonia behaved satisfactorily. An ammonia concentration *ca.* 1.7 *M* (3% v/v) gave the best compromise between analyte displacement and loss of copper(II) ions from the modified SPE tubes (observed at higher ammonia concentration). Using such a concentration, the elution volume required for maximal desorption was 0.7 ml (see Fig. 2).

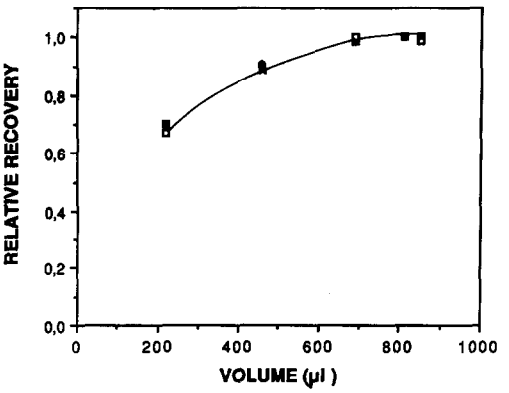

Fig. 2. Relative recovery, on a copper-modified LC-SCX stationary phase, of 5′-dFUR (□), 5-FU (×) and the internal standard (5-BrU, ●) as a function of the elution volume (analyte amounts: 0.5, 1 and 2 µg for 5-FU, 5′-dFUR and 5-BrU, respectively).

Analytical application

The effectiveness of the SPE procedure described here can be gathered from Fig. 3, which shows a typical chromatogram of an extract of serum spiked with known amounts of fluoropyrimidines. As can be seen, it is possible to coextract 5′-dFUR, 5-FU and some of the fluorinated nucleosides (*i.e.* FUR and 2′-dFUR) and nucleotides (*i.e.* FUMP and 2′-dFUMP) involved in the metabolism of 5-FU. Direct comparison with chromatograms of a blank serum, as well as a

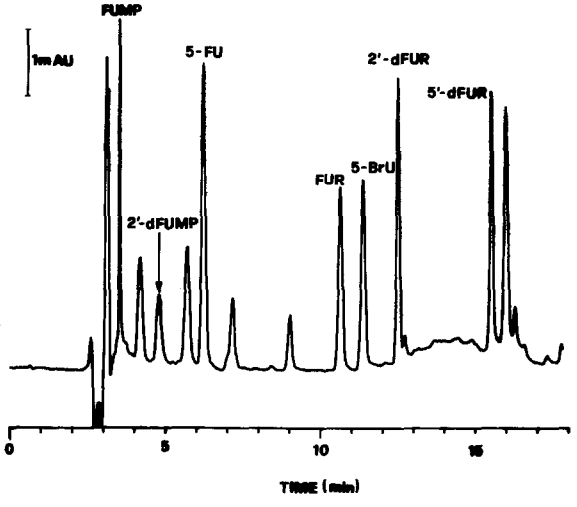

Fig. 3. Chromatogram of the extract of a drug-free serum sample spiked with the indicated fluoropyrimidine derivatives (5 µg/ml each).

TABLE I

PERCENTAGE RECOVERIES FROM SPIKED SERUM SAMPLES AND LIMITS OF DETECTION FOR FLUORINATED PYRIMIDINES

Analyte	Level (μg/ml)	Mean recovery (%)	R.S.D. ($n = 5$) (%)	LOD[a] (μg/ml)
5'-dFUR	0.5	95	4.0	0.06
	5	103	3.0	
	50	101	2.9	
5-FU	0.5	84	4.2	0.2
	5	90	6.3	
	50	88	4.5	
5-BrU	5	100	4.2	–
FUR	5	108	3.5	0.1
2'-dFUR	5	106	2.8	0.07
FUMP	5	70	4.5	0.07
2'-dFUMP	5	85	3.3	0.4

[a] Limits of detection at a signal-to-noise ratio of 3; noise calculated peak-to-peak in a blank chromatogram at the elution time of the analyte of interest.

peak purity test, by spectra overlaying techniques gave no evidence of significant interferences from endogenous serum components.

Recoveries of the investigated fluoropyrimidines from spiked serum samples (calculated by comparison with standards dissolved in mobile phase) are shown in Table I. As can be seen, the recovery (at least for 5'-dFUR and 5-FU, which are the most relevant compounds in the pharmacokinetic study) is independent of the analyte concentration (mean values not significantly different according to one-way ANOVA at $p = 0.05$). Calibration curves for 5'-dFUR and 5-FU in serum were linear over almost four concentration decades; the correlation coefficients were better than 0.999, and the intercepts of the calibration lines were not significantly different from zero at the 95% confidence level. A typical calibration curve for 5'-dFUR gave a slope of 0.771 ± 0.003 mAU ml/μg and an intercept of -0.02 ± 0.05 mAU. The within-day ($n = 5$) and between-day ($n = 6$) coefficients of variations

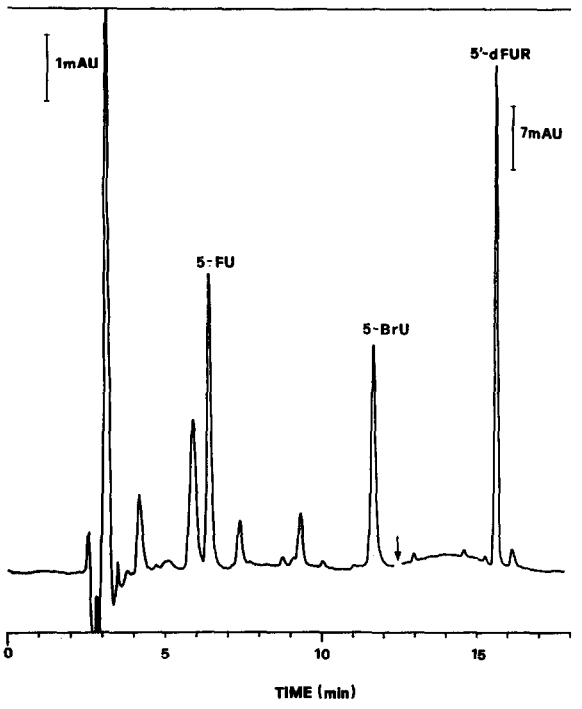

Fig. 4. Chromatogram of the extract of a serum sample taken from a patient under 5'-dFUR treatment (4 g/m² by i.v. infusion over 60 min). Sampling time, 120 min from the start of drug infusion. 5'-dFUR and 5-FU calculated serum concentrations were 74.4 and 3.8 μg/ml, respectively. (The arrow indicates a sensitivity change.)

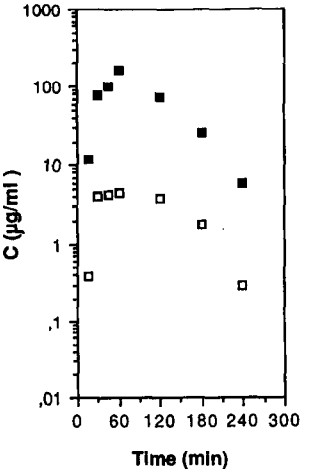

Fig. 5. Time-course of 5'-dFUR (■) and 5-FU (□) in a patient under 5'-dFUR chemotherapy (4 g/m² by i.v. infusion over 60 min).

(C.V.) for all analytes ranged from 2.4% to 4.1% and from 8.6% to 11.8%, respectively. Detection limits are shown in Table I.

The overall analytical procedure has been tested on serum samples from cancerous patients receiving doxifluridine therapy (4 g/m^2 by i.v. infusion over 60 min). Fig. 4 shows a typical chromatogram. Whatever the serum sampling time, only the peaks relevant to 5′-dFUR and 5-FU were detected, as already reported by Palmisano *et al.* [6]. Furthermore, considering that the SPE procedure also permits the extraction of FUMP and 2′-dFUMP with respect to the LLE procedure described by Palmisano *et al.* [6], these results suggest that FUMP and 2′-dFUMP are well below the detection limits of the analytical method discussed here.

Using the proposed method, the time profiles of 5′-dFUR and 5-FU concentrations in the serum of a patient under doxifluridine chemotherapy have been derived (Fig. 5). As can be seen, the serum 5′-dFUR elimination follows first-order kinetics. From the analysis of this time profile, a 5′-dFUR elimination half-life of 43 min was calculated, with a peak concentration of 169.4 μg/ml; moreover, 1.07 μmol · h/ml was the area under the curve (AUC) parameter, including the end correction, and the plasma clearance rate was 256 ml/min. Similarly, for 5-FU, the peak concentration was 4.5 μg/ml and the AUC was 81.5 nmol · h/ml.

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