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Determination of 5-fluorouracil and its main metabolites in plasma by high-performance liquid chromatography

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ABSTRACT

A relatively simple and sensitive ion-pair high-performance liquid chromatographic method has been developed for measuring the anticancer drug fluorouracil and its main metabolites, fluorouridine, fluorodeoxyuridine and fluorodeoxyuridine monophosphate in human plasma. A reversed-phase C_{18} column (150 mm × 2 mm I.D.) and ultraviolet detection (280 nm) were used. The influence of the tetrabutylammonium phosphate and monopotassium phosphate concentrations and pH of the mobile phase on the various k' values was investigated. The optimal k' values obtained for the four compounds ranged between 0.7 and 5.9. The optimized eluent was (10^{-4} *M* tetrabutylammonium phosphate plus $2 \cdot 10^{-2}$ *M* potassium dihydrogen phosphate, pH 5.9)–methanol (95.5:4.5, v/v). The flow-rate was 0.3 ml/min. The procedure for plasma preparation included solvent extraction using ethyl acetate–methanol (80:20) followed by elution on C_{18} Sep-Pak cartridges to separate the four compounds from constituents normally occurring in plasma. The detection limit of the assay was 2 ng/ml (5-fluorouracil), 10 ng/ml (5-fluorouridine), 10 ng/ml (5-fluoro-2′-deoxyuridine) and 50 ng/ml (5-fluoro-2′-deoxyuridine 5′-monophosphate).

INTRODUCTION

Fluorouracil (FU) is a substance widely used in the chemotherapy of cancers. The biochemical importance of FU and its nucleosides, 5-fluorouridine (FUrd) and 5-fluoro-2′-deoxyuridine (FdUrd), and nucleotide, 5-fluoro-2′-deoxyuridine phosphate (FdUMP), has been demonstrated by Heidelberger [1]. Thereafter, the exploration of their metabolic pathways becomes of great interest. Bases and nucleosides have been chromatographed on reversed-phase and ion-exchange columns [2], and nucleotide separations are performed mostly by anion-exchange chromatography [3]. The reversed-phase mode has also been applied in this field [4], but few systems are available for the simultaneous determination of pyrimidine bases, nucleosides and nucleotides. Until, now, the low FU plasma levels (less than 10 ng/ml) determined have led researchers to use complex techniques, such as mass spectrometry coupled with gas chromatography [5], nuclear magnetic resonance spectroscopy [6,7] or supercritical fluid chromatography with UV detection [6]. According to previous studies [8,11], values lower than 50 ng/ml (FU and FdUrd) could not be determined with liquid chromatography. Only valve-switching brought those limits down to 5 ng/ml for FU [12].

The introduction of an ion-pairing agent into the mobile phase provides an approach to this problem, but this is a complex process involving ion-exchange and ion-pair formation. This paper describes the investigation of several parameters using a reversed-phase chromatographic system in order to obtain optimal separation of FU and its main metabolites FUrd, FdUrd and FdUMP. The method consisted of the use of ion-pair liquid chromatography on a microbore column, and comparable (FU) or even lower (FUrd and FdUrd) determination limits were obtained without the use of the valve-switching technique.

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EXPERIMENTAL

Chemicals

5-Fluorouracil (Roche, Neuilly, France) purchased as an injectable form (50 mg/ml), was diluted in physiological saline. The metabolites, FUrd, FdUrd and FdUMP, as well as iodouracil (IU) for use as an internal standard when the extraction from biological fluids is needed (Sigma, La Verpillére, France) and tetrabutylammonium phosphate (TBA) (Aldrich, Strasbourg, France) were of the highest purity available and used without subsequent purification. All other chemicals were of analytical grade, and the solvents were HPLC quality (Prolabo, Paris, France). The water was deionized using a Water I system (Gelman, Ann Arbor, MI, USA), filtered through a 0.45-μm membrane (Millipore, Molsheim, France), and outgassed under vacuum before use. C_{18} Sep-Pak cartridges were purchased from Millipore. The standard samples were prepared in deionized water. These solutions were filtered through a 0.45-μm membrane (Millipore HV4) before injection.

Equipment

The HPLC system consisted of a computer-monitored Gold PC apparatus (pumps 126, detector 166) (Beckman, Gagny, France), a WISP 512 automatic injector (Waters Millipore, Molsheim, France) and a Beckman Ultrasphere ODS 5 μm microbore column (150 mm × 2 mm I.D.). The mobile phase was pumped at a flow-rate of 0.3 ml/min and not recycled. The detection wavelength was 280 mn.

Optimization of the mobile phase

To optimize the mobile phase, the capacity factors (k') of each compound were adjusted to the polarity (water-to-methanol ratio), the ionic composition (TBA and KH_2PO_4) and the pH of the mobile phase.

Standard eluent. A $2 \cdot 10^{-2}$ *M* KH_2PO_4 (pH 5.0) solution was taken as the standard.

Optimization of the polarity. The separation of non-ionizable compounds (FU, FUrd and FdUrd) was optimized by adjusting the capacity factor (k') values between 1 and 5. Different amounts of methanol (3, 4, 5, 6 and 7%) were added to the standard eluent.

Influence of the TBA concentration. Different amounts of a concentrated TBA solution were added to the standard eluent (pH 5.0) so as to obtain the following TBA concentrations: 10^{-4}, $5 \cdot 10^{-4}$, $10 \cdot 10^{-4}$, $15 \cdot 10^{-4}$ and $25 \cdot 10^{-4}$ *M*.

Influence of the KH_2PO_4 concentration. Different solutions of the standard eluent were prepared containing 10^{-4} *M* TBA and the following concentrations of KH_2PO_4: $2 \cdot 10^{-3}$, $4 \cdot 10^{-3}$, $6 \cdot 10^{-3}$, $8 \cdot 10^{-3}$ and $2 \cdot 10^{-2}$ *M*.

Influence of the pH. The standard eluent containing 10^{-4} *M* TBA and $2 \cdot 10^{-2}$ *M* KH_2PO_4 was adjusted to pH values of 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0, by the addition of small amounts of concentrated NaOH solution.

Bases, nucleosides and nucleotide were chromatographed using the different eluents described, and their respective capacity ratios (k') were calculated.

Plasma extraction

Aliquots of plasma (1 ml) were transferred to glass tubes. After addition of the internal standard IU (100 μl), 50 μl of orthophosphoric acid were added to adjust the pH to *ca.* 2. The tubes were gently mixed and then vortexed after the addition of 3 ml of acetonitrile. Following centrifugation (10 min, 3000 *g*) to separate the two phases, the entire organic layer was pipetted into a 5-ml glass tube and evaporated to dryness at 45°C under a stream of nitrogen for *ca.* 1 h. The dried residue was dissolved in 200 μl of water, vortex-mixed for 30 s, and then applied to a C_{18} Sep-Pak cartridge. The cartridge was eluted with 2 ml of ethyl acetate–methanol (80:20), and the eluate fraction was evaporated to dryness for 30 min as described above. The final residue was dissolved in 200 μl of deionized water, placed in an ultrasonic bath for 2 min, then filtered through a 0.45-μm membrane. A 10-μl volume was injected via the autosampler.

Plasma sample preparation

The method was used for the analysis of plasma sample collected from a cancer patient treated with FU by continuous intravenous infusion (400 mg/m^2 per day). A blood sample was taken during the infusion (two months after the begin-

ning of the treatment), and collected into a heparinized glass tube. The tube was immediately centrifuged at 4°C at 3000 *g* for 10 min and stored at −20°C until analysis.

RESULTS AND DISCUSSION

A distinction was made between non-ionizable compounds (FU, FUrd, and FdUrd), whose elution varied only with the polarity, and FdUMP, whose elution varied with the ionization rate (pH) and the concentration of the ions in solution.

In order to optimize the chromatographic resolution, defined as optimal retention per unit time of the three non-ionizable compounds, we tried to obtain capacity factors values (k') ranging from 1 to 5, depending on the polarity of the mobile phase as governed by the water-to-methanol ratio (Fig. 1). Water–methanol (95.5:4.5, v/v) gave results that were close to these limits and was selected for our analysis. However, the elution of the FU–FUrd–FdUrd–FdUMP mixture with this mobile phase composition resulted in a k' value of 0.33 for FdUMP, close to the dead volume of the column and therefore unacceptable.

For this reason we decided to use ion-pair chromatography and to incorporate TBA ions to the mobile phase. Modifying the TBA concentration altered the retention time of FdUMP, its capacity factor increasing with the TBA concentration (Fig. 2). FdUMP can be eluted between the

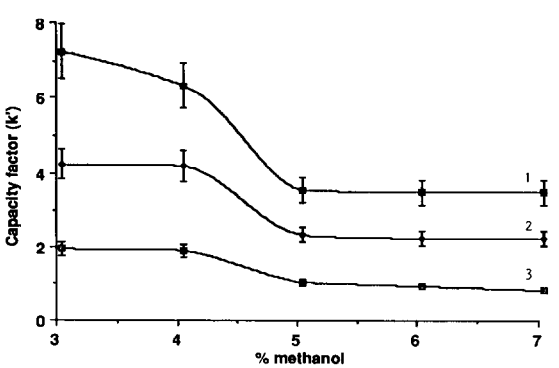

Fig. 1. Optimization of the mobile phase. The separation and elution of each non-ionizable compound were optimized by adjusting the capacity factors ($1 < k' < 5$, error bars = standard deviation of three assays in triplicate). Standard eluent, $2 \cdot 10^{-2}$ *M* KH_2PO_4 (pH 5.0). 1 = FdUrd; 2 = FUrd; 3 = FU.

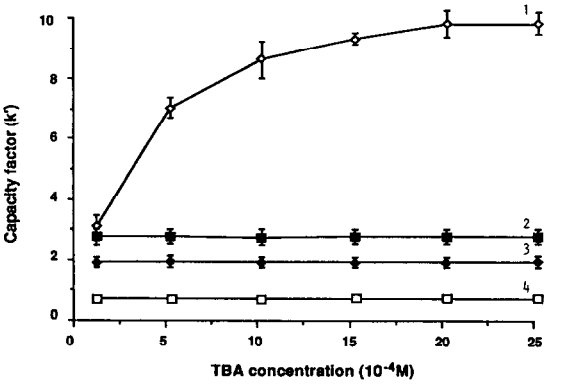

Fig. 2. Influence of TBA concentration. Only the capacity factor of FdUMP was affected by TBA concentration; assays were performed in phosphate buffer, $2 \cdot 10^{-2}$ *M* KH_2PO_4 (pH 5.0) (error bars = standard deviations of three assays in triplicate). 1 = FdUMP; 2 = FdUrd; 3 = FUrd; 4 = FU.

non-ionizable compounds. It should be noted that, for TBA concentrations lower than 10^{-4} *M*, the capacity factor values for the fluorinated base (FU) and the two nucleosides (FUrd and FdUrd) were partly dependent on the TBA concentration (data not shown). These variations remained negligible and did not occur at concentrations above 10^{-4} *M*. When the ionic strength of the aqueous phase increased, ion-pair formation decreased because of the competition between the sample ion and the secondary ion $H_2PO_4^-$ to form an ion-pair with TBA^+. This competition resulted in major variations in the k' value of FdUMP (Fig. 3). The pH was always determined after the addition of secondary ions.

Variations in the pH had a major effect on the capacity factors of the samples: the maximum k' values were obtained at pH values where the compounds were fully ionized, and enabled a maximum number of ion-pairs to be formed. At pH 5, the base and nucleosides are present in an undissociated form and cannot form ion-pairs with TBA, but the 5′-phosphate group of the nucleotide FdUMP is ionized and may form ion-pairs with the quaternary ammonium ions. The resulting ion-pairs are electrically neutral and are hardly soluble in the mobile phase. The variation of k' with the pH is illustrated in Fig. 4, the retention time of FdUMP can be altered by adjusting the pH (data not shown).

The composition of the mobile phase was fixed as (10^{-4} *M* TBA plus $2 \cdot 10^{-2}$ *M* KH_2PO_4 buff-

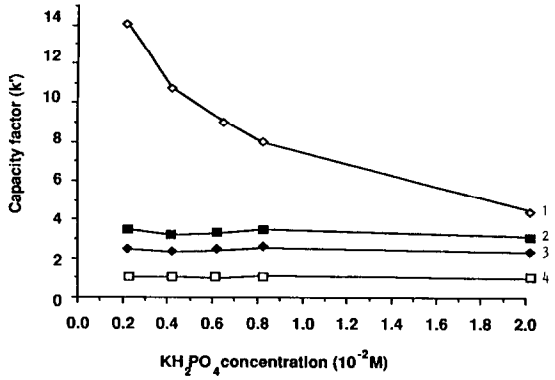

Fig. 3. Influence of KH_2PO_4 concentration. The assays were performed in TBA buffer (10^{-4} *M*, pH 5.0); increasing ionic concentration resulted in competition between sample and phosphate ions to form ion pairs with TBA (error bars = standard deviations of three assays in triplicate are included in graph marks). 1 = FdUMP; 2 = FdUrd; 3 = FUrd; 4 = FU.

er, pH 5.9)–methanol (95.5:4.5, v/v). The retention times for FU, FUrd, FdUrd, IU and FdUMP were 1.9 ± 0.2, 3.6 ± 0.4, 4.6 ± 0.5, 5.2 ± 0.5 and 6.4 ± 0.6 min, respectively (Figs. 5 and 6).

The performance of the extraction and isolation procedure was dependent on the nature of the extraction solvent, the pH and the extraction column. Less satisfactory recoveries were found when diethyl ether or chloroform was used. The pK_a values of the FU, FUrd and FdUrd are *ca.* 8 [11] and FdUMP is much more acidic. Thus pH 2 was chosen for the extraction to ensure that these compounds would be present in the un-ionized

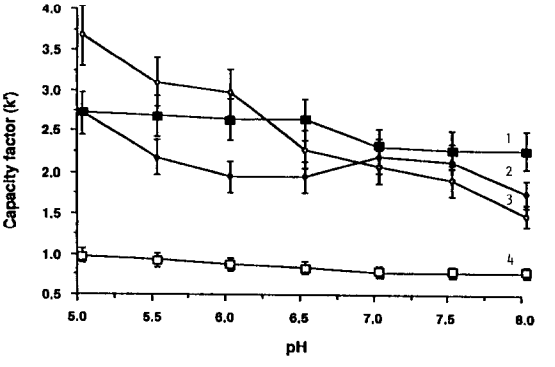

Fig. 4. Influence of pH. Adjusting the pH of the mobile phase allowed FdUMP to elute between the other compounds; assays were performed in phosphate buffer, $2 \cdot 10^{-2}$ *M* KH_2PO_4, 10^{-4} *M* TBA (error bars = standard deviations of three assays in triplicate). 1 = FdUrd; 2 = FUrd; 3 = FdUMP; 4 = FU.

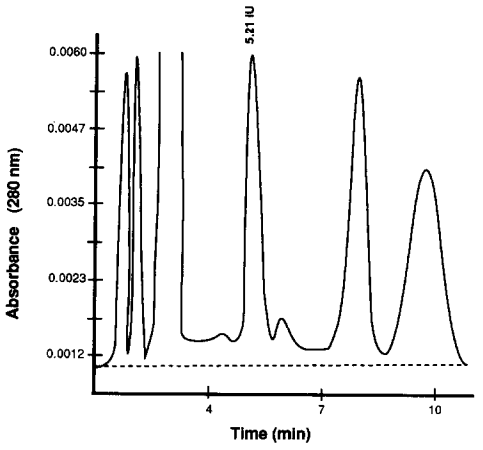

Fig. 5. Representative chromatogram from 1 ml of plasma control extract, supplemented with 500 ng/ml IU as internal standard. The mobile phase was phosphate buffer, $2 \cdot 10^{-2}$ *M* KH_2PO_4, 10^{-4} *M* TBA (pH 5.9)–methanol (95.5:4.5 v/v). For chromatographic conditions, see text.

form but would not result in high extraction rates for FU and FdUMP. In addition, the chromatograms of blank plasma contained additional peaks that interfered with FU.

The mean recoveries from plasma samples of the four compounds were determined by comparing the peak areas measured from the final extracts of plasma containing known concentrations with the peak areas measured from unextracted aqueous solutions supplemented with known concentrations of the compounds. The

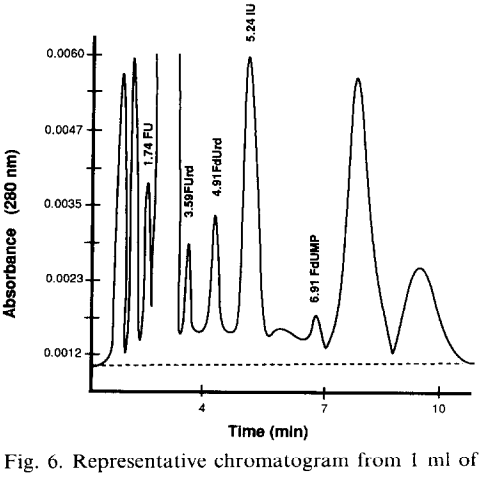

Fig. 6. Representative chromatogram from 1 ml of plasma extract, spiked with 50 ng/ml FU, 100 ng/ml FUrd, 100 ng/ml FdUrd, 500 ng/ml IU as internal standard and 100 ng/ml FdUMP. The mobile phase was as in Fig. 5. For chromatographic conditions, see text.

TABLE I

MEAN RECOVERY RATES AT THREE DIFFERENT CONCENTRATIONS

Compound	Recovery (%)		
	5 μg/ml	0.5 μg/ml	0.05 μg/ml
FU	40	40	43
FUrd	88	83	83
FdUrd	90	88	73
FdUMP	46	19	19

mean recoveries of FU, FUrd, FdUrd and FdUMP were determined in this manner and are listed in Table I. A chromatogram from a plasma sample from a cancer patient, spiked with 500 ng/ml IU (internal standard), is shown in Fig. 7.

At a signal-to-noise ratio of 3, the minimal detectable concentrations after the extraction procedure were 10, 50, 50 and 100 ng/ml, by injecting 10 μl of the 200-μl reconstituted plasma extract at a sensitivity setting of 0.0001 a.u.f.s., for FU, FUrd, FdUrd and FdUMP, respectively. This corresponds to original plasma concentrations of 2, 10, 10 and 50 ng/ml.

To investigate the linearity of the procedure, blank plasma samples were spiked with amounts

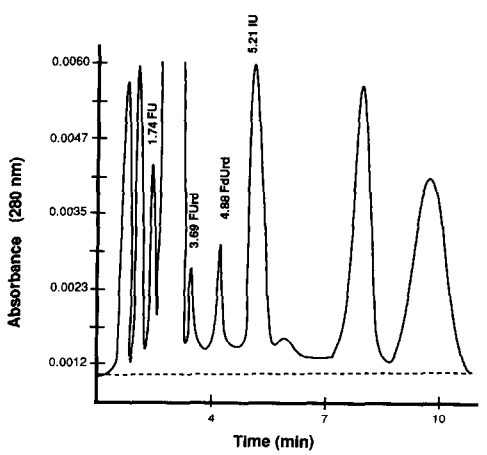

Fig. 7. Representative chromatogram of a plasma sample spiked with 500 ng/ml IU as internal standard obtained from a cancer patient treated with 400 mg/m² FU per day by continuous intravenous infusion. Concentrations of FU, FUrd and FdUrd were 46, 35 and 92 ng/ml respectively. The mobile phase was as in Fig. 5. For chromatographic conditions, see text.

varying from 0.01 to 5 μg/ml for FU and from 0.05 to 5 μg/ml for FUrd, FdUrd and FdUMP. The calibration curves showed good linearity, as can be seen from the following equations: FU, $y = 1.591\ (\pm 0.003)x - 0.3\ (\pm 0.2)$ ($r > 0.998$); FUrd, $y = 0.636\ (\pm 0.005)x - 0.4\ (\pm 0.1)$ ($r > 0.998$); FdUrd, $y = 1.383\ (\pm 0.002)x - 0.3\ (\pm 0.2)$ ($r > 0.998$); FdUMP, $y = 0.842\ (\pm 0.005)x - 0.8\ (\pm 0.4)$ ($r > 0.990$). The calibration curves were obtained after analysing five samples; x and y are the concentration of the compounds (ng/ml) and the peak area, respectively, and r is the correlation coefficient.

Previous reports [8,6,10,11] did not indicate any possibility of increasing the sensitivity of an HPLC assessment for fluoropyrimidines or of detecting concentrations lower than 50 ng/ml unless valve-switching was used [12]. Detailed studies investigating the disposition kinetics of FU, FUrd and FdUrd are limited [13–15]. This may be due to the lack of a simple and sensitive method to quantitate the three compounds in biological fluids. Methods have been described for the determination of FdUrd in plasma using HPLC in conjunction with other labelled drugs [16]. The HPLC method of Sommadossi and Cano [17], based on spectrophotometric detection, was highly specific, but the simultaneous quantitation of FU was not possible according to the authors. Gustavsson *et al.* [18] reported a method for the simultaneous analysis of FU and FdUrd in plasma. The procedure involved deproteinization with picric acid followed by ion-exchange chromatography and analytical isotachophoresis. The complexity of the procedure and the instrumentation involved will probably result in a method having limited utility.

The use of ion-pair chromatography is obviously advantageous as far as the assessment of FdUMP is concerned, since it is possible to adjust the retention time so that it elutes between the peaks of the other fluoropyrimidines. Such an alteration should be particularly useful in the presence of interfering peaks from the plasma.

It should be specified that the sensitivity limit reported for FdUMP (50 ng/ml) was obtained with a retention time of 6.4 ± 0.6 min. Finally, even though the use of a microbore column (2 mm I.D.) improved the sensitivity, the extraction

procedure should be improved in order to reduce as much as possible the presence of unwanted peaks and of substances adsorbing on the top of the column, which is one of the main drawbacks of that microbore method.

CONCLUSION

The present method enables the quantitation of FU, FUrd and FdUrd in human plasma. The sensitivity of the method is adequate for the analysis of FU, FUrd and FdUrd following the administration of FU [19]. This method will therefore enable dosages to be adjusted, following monitoring the metabolites, and it may be possible to define the metabolic pathway. The procedure should thus permit more detailed pharmacokinetic investigations of fluorouracil. FdUMP could probably be detected in tissue samples. Furthermore, this method could be useful in *in vitro* studies or in animal studies if radiolabelled compounds are used, when the low sensitivity level with UV detection for FdUMP would not represent a major problem.

ACKNOWLEDGEMENTS

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