ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
28 (2002) 857–866

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

RP–LC determination of 5-fluorouridine in nanoparticulate formulations

P. Arbós [a], M.A. Campanero [b], Juan M. Irache [a,*]

[a] *Centro Galénico, Universidad de Navarra, Aptdo. 177, C/Irunlarrea s/n, 31080 Pamplona, Navarra, Spain*
[b] *Servicio de Farmacología Clínica, Clinica Universitaria de Navarra, 31080 Pamplona, Spain*

Received 1 October 2001; received in revised form 29 October 2001; accepted 31 October 2001

Abstract

5-Fluorouridine (5-FUrd) is an anticancerous drug with a number of side effects due to its high toxicity. One possibility to overcome these drawbacks may consist on the use of polymeric nanoparticles to increase the therapeutic index of this drug. The objective of this study was to develop an analytical high performance liquid chromatography (HPLC) method for the determination of (i) the 5-FUrd content in poly (methyl vinyl ether-co-maleic anhydride) nanoparticles, (ii) its release from these carriers and, its eventual degradation during preparation, storage or release in 5-fluorouracil (5-FU). The chromatography was performed on a reversed-phase encapped column (LiChrospher Select B C_8) with a mobile phase of 0.05 M ammonium acetate (pH 6.5). Ganciclovir (GCV) was used as internal standard and the detection wavelength was 268 nm. The limits of quantification of 5-FUrd and 5-FU were 12 and 5 ng/ml, respectively. Similarly, precision did not exceed 7%. Under our experimental conditions, the maximal drug loading capacity of 5-FUrd was around 105 μg/mg nanoparticle and the drug was released in a biphasic way from these particles. In addition, no degradation of 5-FUrd to 5-FU during either the preparative process or the release studies was observed. In summary, this HPLC method is selective, sensitive, specific and reproducible for the quantification of 5-FUrd in polymeric nanoparticles and release mediums.

Keywords: 5-Fluorouridine; 5-Fluorouracil; HPLC; Nanoparticles; Poly(methyl vinyl ether-co-maleic anhydride)

1. Introduction

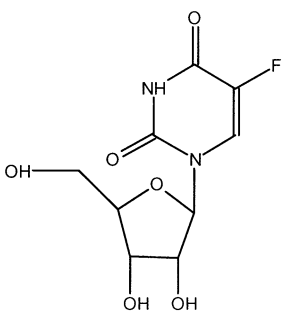
The anticancer compound 5-fluorouridine (5-FUrd) is a riboside of 5-fluoro-2,4(1H, 3H)-pyrimidinedione (Fig. 1A). This fluoropyrimidine nucleoside can be used in clinical oncology in the treatment of malignant neoplasms [1]. Compared with similar drugs, 5-FUrd is around 100-fold more effective than doxifluridine and 5-fluorouracil (5-FU, Fig. 1B) [2]. This increased potency has been related to the fact that this molecule exhibits a faster rate of cellular uptake than other fluoropyrimidines analogs (i.e. 5-FU), and also to the formation of 5-fluoronucleotides and subsequent incorporation into messenger and ribosomal RNA [3]. Nevertheless, this high activity implies an important toxicity.

* Corresponding author. Tel.: +34-948-425-600x6313; fax: +34-948-425-649.
E-mail address: jmirache@unav.es (J.M. Irache).

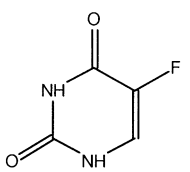
0731-7085/02/$ - see front matter
PII: S0731-7085(01)00683-5

In the last few years, new strategies have been developed in order to decrease the toxicity of active molecules by driving them to the target site, where the drug has to perform its therapeutic effect. In this context, the use of polymeric nanoparticles can be a useful approach to concentrate the loaded drug on the focus of the disease. This fact would result in an increase in the therapeutic index of the loaded molecule by reducing both the given dose and the side effects. Nanoparticles may be defined as submicron drug carrier systems of polymeric nature and are classified in either nanospheres with a matricial structure or nanocapsules formed by a thin polymeric envelope surrounding a vesicular core [4].

In the literature, several analytical procedures using gas chromatography (GC) [5], GC–mass spectrometry [6,7] and high performance liquid chromatography (HPLC) [8–14] have been proposed for the determination of pyrimidine analogs in biological mediums.



(A)



(B)

Fig. 1. Chemical structures of 5-FUrd (A) and 5-FU (B).

GC has been used to quantify fluoropyrimidine nucleosides. However, this technique is not suitable for the simultaneous determination of 5-FUrd and 5-FU, since, 5-FUrd was partially decomposed to 5-FU at higher temperatures. On the other hand, GC–mass spectrometry methods involve a previous derivatization procedure of the fluoropyrimidine nucleosides with trifluoroacetimide and trifluoroacetic anhydride [6] or *t*-butyldimethylsilane [7]. These derivatization processes allow for the detection of very low drug amounts (around 1 ng/ml), although, the retention time and the long sample preparation procedure make them expensive.

For these reasons, HPLC methods have been applied to the separation of 5-FUrd and other fluoropyrimidines in plasma [8], biological tissues [9] and other body fluids [10]. For the analysis, chemiluminiscence [11], fluorescence [12] or ultraviolet [8–10] detectors have also been used. However, the reported chromatographic methods displayed tailing factors higher than 1, related to the physico–chemical properties of these fluoropyrimidines. In order to overcome this drawback, ion-pair reagents [13] or ion-change chromatography [14] has been used. Nevertheless, these analytical methods imply the use of tedious preparative processes to obtain high-purified samples and, thus, avoid the possibility of irreversible interactions between the components of nanoparticles and the apolar chains of the columns.

The aim of this work was to develop an analytical RP–HPLC method for both the determination of the 5-FUrd content in pharmaceuticals and the evaluation of the drug release properties from the pharmaceutical dosage form. In addition, the technique had to be able to quantify the possibility of 5-FUrd degradation into 5-FU.

2. Materials and methods

2.1. Materials

5-FUrd, 5-FU and 1,3-diaminopropane were supplied by Sigma (Madrid, Spain). Ganciclovir (GCV; Cymevene®) was provided by Roche (Madrid, Spain). Poly (methyl vinyl ether-co-

maleic-anhydride) Gantrez® AN 119; MW 200 000 was kindly gifted by ISP (Barcelona, Spain). Ammonium acetate and all solvents and reagents used were of HPLC analytical grade and were obtained from Merck (Darmstadt, Germany).

2.2. Calibration standards

Stock solutions (1000 μg/ml) of 5-FUrd and 5-FU were prepared in distilled water and stored at 4 °C until use. Both stock solutions were further diluted to obtain 400 μg/ml solutions for the preparation of working standard solutions. For this purpose, aliquots of these last stock solutions were diluted with distilled water to a final volume of 1 ml. The concentration interval for the standard curve samples ranged from 0.5 to 200 μg/ml.

Similarly, an internal standard stock solution of GCV was prepared by dissolving 21.84 mg Cymevene® in water (2 mg/ml). Standard curve samples were prepared by adding 50 μl of the I.S. to 1 ml either 5-FUrd or 5-FU solutions.

2.3. Sample preparation

Poly (methyl vinyl ether-co-maleic anhydride) nanoparticles (PVM/MA) were prepared by a desolvation method and chemical cross-linkage. This method consisted of the preparation of unloaded PVM/MA nanoparticles, which were subsequently incubated, with different amounts of 5-FUrd for 2 h at room temperature. The resulting systems were treated with 1,3-diaminopropane (1,3-DP) as cross-linking agent for 5 min. Then, the batches were purified by centrifugation at 17 000 rpm for 20 min (Rotor 3336, Biofuge Heraeus, Hanau, Germany). Finally, the recovered supernatants (either for drug content or release studies) were diluted to 50 ml on water, spiked with 25 μl I.S. and the amount of 5-FUrd evaluated by injecting an aliquot of this last solution into the HPLC system.

In order to characterise the resulting nanoparticles, the size was determined by photon correlation spectroscopy (PCS) using a Zetamaster analyser system (Malvern Instruments, UK). Samples were always diluted with 0.05 mM phosphate buffered saline (PBS, pH 7.4) and measured at 25 °C with a scattering angle of 90°.

For the release studies, about 50 mg of 5-FUrd-loaded nanoparticles were dispersed in 10 ml PBS (pH 7.4; 0.15 M). The medium was maintained at 37 ± 1 °C under agitation. At determined time intervals, samples were collected, centrifuged and analysed for their 5-FUrd content as described before. Each measurement was made in triplicate.

2.4. Instrumentation and chromatographic conditions

Liquid chromatography analysis were performed using an HP system (Hewlett–Packard, Palo Alto, CA, USA) consisting of an HP 1050 autosampler and a HP 1050 diode-array detector set at 268 nm. The chromatographic system was equipped with a reversed-phase 250×4 mm C_8 lichrospher® Select B column (5 μm) provided by Merck, and a 4×4 mm precolumn filled with the same material.

The mobile phase, pumped at 1 ml/min, was composed of 0.05 M acetate ammonium (pH 6.5) and was filtered and degassed prior to use. The column was termostatized at 30 °C and the volume injection was 30 μl. Under these experimental conditions the run time was 15 min.

2.5. Application of the method

This analytical HPLC method was applied to determine the 5-FUrd content in nanoparticulate dosage forms and its release from these pharmaceutical dosage forms. In addition, this technique enabled us to determine the eventual degradation of 5-FUrd into 5-FU and, therefore, to elucidate the capacity of these pharmaceutical dosage forms to protect the loaded drug against its degradation. In fact, 5-FUrd degrades to 5-FU and ribose in aqueous media by a hydrolysis mechanism [3].

Different 5-FUrd-loaded formulations were prepared to evaluate the influence of 5-FUrd bulk concentration on the payload of PVM/MA. On the other hand, the drug loading was calculated as follows:

$$\text{Drug loading} = \frac{\text{Amount of } 5-\text{FUrd in nanoparticles } (\mu g)}{\text{PVM/MA nanoparticle yield (mg)}} \times 100 \quad (1)$$

The amount of 5-FUrd entrapped into the nanoparticles was calculated as the difference between the initial drug and the amount of the active molecule recovered in the supernatants during the purification step by centrifugation.

The nanoparticle yield was determined by gravimetry. For this purpose, PVM/MA nanoparticles, freshly prepared, were freeze-dried. Then, the yield was calculated as the difference between the initial amount of the polymer used to prepare nanoparticles and the weight of the freeze-dried carriers.

Concerning the release studies, the amount of drug determined in the supernatants was cumulated and the results expressed as the amount of drug released from PVM/MA nanoparticles versus time.

3. Results

The analytical peaks of 5-FUrd, 5-FU and the internal standard GCV were well-resolved one from each other (Fig. 2A).

3.1. Selectivity of the assay

The selectivity of the assay was studied by injecting supernatants of both loaded and unloaded PVM/MA nanoparticles. Under these chromatographic conditions, no interferences were observed and the resolution amount the peaks was satisfactory (Fig. 2B and C).

3.2. Sensitivity of the assay

Detection and quantification limits (LOD and LOQ) of the method were determined by the evaluation of the peak baseline noise in six blank samples. Thus, LOD defined as the lowest drug concentration, which can be determined and calculated as three times the variation in the measured response, was calculated to be 4 and 2 ng/ml for 5-FUrd and 5-FU, respectively. In the same way, LOQ estimated as ten times the variation in the measured response was calculated to be 12 and 5 ng/ml, respectively.

Table 1
Standard curves for HPLC assay in aqueous solutions

	N	Slope	Intercept	*r*
5-Fluorouridine				
Day 1	6	0.0168	0.0020	0.9999
Day 2	6	0.0173	−0.0038	0.9999
Day 3	6	0.0177	−0.0032	0.9999
5-Fluorouracil				
Day 1	6	0.0285	−0.0053	0.9999
Day 2	6	0.0282	−0.0017	0.9997
Day 3	6	0.0288	0.0050	0.9995

3.3. Linearity of the assay

Linearity was determined by plotting a standard curve from the ratio between either 5-FUrd or 5-FU peak areas to the GCV (I.S.) peak area, versus the corresponding drug concentration in the aqueous media. The calibration curves were linear on three different days over the range 0.5–200 μg/ml (see Table 1). For 5-FUrd, the slope value and the intercept were calculated to be 0.0173 ± 0.0004 and -0.0017 ± 0.0032, respectively. For 5-FU, the slope value and the intercept were 0.0285 ± 0.0003 and -0.0007 ± 0.0052, respectively. In all cases, linear regression analysis displayed correlation coefficients greater than 0.999. In addition, relative error in each concentration was calculated in the mean curve (Table 2).

A linearity test, based on the comparison between the calculated and the nominal values, was

Fig. 2. Chromatograms resulting from the analysis of a standard solution of 5-FU, 5-FUrd and GCV (A), a supernatant obtained during the centrifugation of unloaded PVM/MA nanoparticles (B) and a supernatant from the purification step of 5-FUrd-loaded nanoparticles (C). FU, 5-fluorouracil; Furd, 5-fluorouridine; GCV, ganciclovir (internal standard).

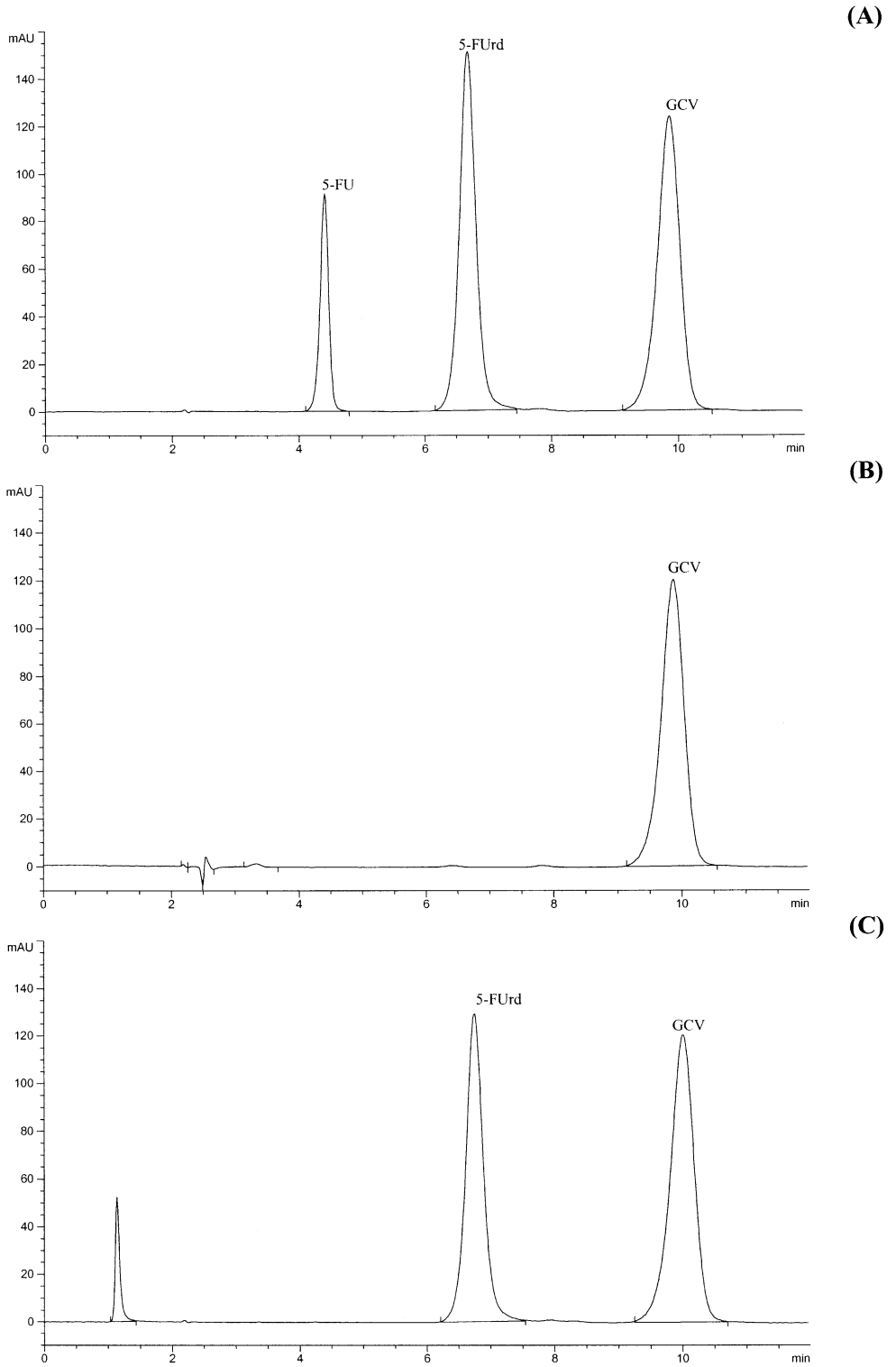


Fig. 2. (*Continued*)

Table 2
Statistical evaluation of the analysis results in standard curves over 3 days

Concentration added (μg/ml)	Concentration found (mean ± S.D.; μg/ml)		Relative error (%)	
	5-Fluorourdine	5-Fluorouracil	5-Fluorouridine	5-Fluorouracil
Accuracy ($n = 9$)				
0.5	0.55 ± 0.04	0.56 ± 0.03	10.00	12.00
1	1.01 ± 0.04	1.10 ± 0.04	1.00	10.00
5	4.80 ± 0.26	5.26 ± 0.27	−4.00	5.20
10	9.54 ± 0.45	10.15 ± 0.36	−4.60	1.50
25	25.41 ± 1.21	25.50 ± 1.57	1.64	2.00
50	50.59 ± 0.90	50.63 ± 0.41	1.18	1.26
75	75.25 ± 1.64	72.92 ± 1.56	0.33	−2.77
100	99.82 ± 2.02	99.68 ± 2.49	−0.18	−0.32
200	200.36 ± 5.05	200.71 ± 3.48	0.18	0.35

The calculated calibration curve for 5-FUrd and 5-FU were $y = 0.0172 \times -0.0016$ ($n = 6$; $r > 0.999$) and $y = 0.0285 \times -0.0007$ ($n = 6$; $r > 0.999$), respectively.

carried out to confirm the linearity. For both the compounds, 5-FUrd and 5-FU, the assay exhibited linearity ($r > 0.999$) with slopes near to the unit. For 5-FUrd, the t value was calculated to be 383.79 and the intercept (calculated value of $-2.995E-06$) was not found to be statistically different from zero. For 5-FU, the slope was also near to the unit (t value was 309.15) and the intercept close to zero (t value 4.15E-06).

3.4. Accuracy of the assay

Accuracy of the analytical assay was determined as the percentage of the systematic error, which was estimated as the difference between the experimental value and the theoretical value.

Accuracy studies during the same day (intraday assay) at low, medium and high concentrations of both drugs were within the acceptable limits (< 15%) [15] at all the concentrations tested (Table 3).

3.5. Precision of the method

To calculate the precision of the method, within-day and between-day tests were performed. The precision was expressed as coefficient of variation (C.V.). Within-day variability assay was determined by measuring five control samples at four concentrations on the same day. The values are summarised in Table 4. These data clearly demonstrate that the analytical method is reproducible within the same day.

Between-day variability of the method was tested by the determination of five control samples at four concentrations on 3 different days. The samples were prepared on the same day, fractionated in aliquots and stored at 4 °C. From these results (Table 4), it was clear that the analytical method is reproducible between different days.

3.6. Robustness

For the robustness study, the following analytical columns were assayed: Nucleosil-120 C_8 (5 μm particle size; 12.5 × 0.40 cm; Scharlau, Barcelona, Spain), Ultrabase C_8 (5 μm particle size; 25 × 0.40 cm; Scharlau), Kromasil-100 C_8 (5 μm particle size; 12.5 × 0.40 cm; Teknokroma, Barcelona, Spain) and Spherisorb C_8 (5 μm 15 × 0.46 cm; Teknokroma). In all cases, the columns well resolved the peaks and no significant variations in the chromatographic results were found.

Similarly, the modification of the column temperature did not significantly modify the elution times. However, modifications in the flow-rate induced changes in the retention times.

3.7. Application of the method

The reported method was used for the determination of the 5-FUrd content in PVM/MA nanoparticles, and to study the release profile of this drug from these pharmaceutical dosage forms. In addition, this method was also used to determine the degradation of 5-FUrd to 5-FU.

The desolvation technique used here to prepare PVM/MA carriers displayed a yield of 75.5 ± 3.0% of the bulk polymer transformed in nanoparticles. In addition, the size of PVM/MA carriers was typically about 200 nm. The drug loading was calculated by means of Eq. (1) (see experimental section) and this parameter was plotted versus the ratio between the initial amount of 5-FUrd and the initial amount of PVM/MA. As can be observed in Fig. 3, the drug loading increased in a rectilinear way with the concentration of the drug until a 5-FUrd/polymer ratio of around 800 μg/mg, where a plateau was reached. Under these conditions, the maximal loading capacity of 5-FUrd to PVM/MA nanoparticles was calculated to be around 105 μg drug per mg nanoparticle.

On the other hand, the described method was used for the determination of the in vitro drug release from nanoparticles. For this purpose, two formulations were prepared with a different drug/polymer ratio of either 100 (formulation NP A) or 200 μg/mg (Np B). Fig. 4 displays the release profiles of 5-FUrd from the two different formulations in PBS (pH 7.4; ionic strength 0.15 M at 37 ± 1 °C). From these results it was clear that a fraction of this drug was rapidly released during the first 2 h, whereas, a significant amount of the loaded drug remained associated to the nanoparticles.

In order to determine the nature of the association between the drug and PVM/MA nanoparticles, different sets of experiments, consisting of the measurement of the immediately released fraction of 5-FUrd, were performed. Fig. 5 clearly shows that the immediately released drug increased with the initial drug bulk concentration.

Finally, it is interesting to note that no significant signs of 5-FUrd degradation, either during the preparative process or the release studies, were observed.

4. Discussion and conclusions

Pyrimidine base and nucleosides, such as 5-FUrd and 5-FU, have been traditionally chromatographed on reversed-phase or ion-exchange columns, since, they are two slightly ionisable compounds whose elution varies with the polarity of the mobile phase and the ionisation rate [13]. For these reasons, the development of HPLC method for the determination of these drugs must involve variations of these factors. 5-FUrd and 5-FU are two strongly polar compounds with poor solubility in apolar mediums, such as the stationary chromatographic phases previously used. Therefore, these molecules are eluted with low capacity factors (less than 1) when the methanol–water ratio of the mobile phase is higher than 95.5:4.5 v/v. Under these analytical conditions, the estimated k values are close to the dead volume of the column and, obviously, are unacceptable.

Table 3
Accuracy of the method, expressed as relative error in %, for determining both 5-FUrd and 5-FU concentrations

Concentration added (μg/ml)	Concentration found (mean ± S.D.; μg/ml)		Relative error (%)	
	5-Fluorouridine	5-Fluorouracil	5-Fluorouridine	5-Fluorouracil
Accuracy ($n = 5$)				
1	1.07 ± 0.02	1.08 ± 0.01	7.40	8.30
50	52.06 ± 0.24	48.52 ± 0.20	4.12	2.96
100	108.05 ± 0.22	101.27 ± 0.18	8.05	0.27

Table 4
Between and within-day variability of the HPLC method for determining 5-FUrd and 5-FU concentrations

Concentration added (μg/ml)	Between-day variability ($n = 5$)		Within-day variability ($n = 5$)	
	Concentration found (mean ± S.D.; μg/ml)		Concentration found (mean ± S.D.; μg/ml)	
	5-Fluorouridine	5-Fluorouracil	5-Fluorouridine	5-Fluorouracil
1	1.03 ± 0.03 (2.87)[a]	1.09 ± 0.04 (3.46)	1.07 ± 0.02 (2.28)	1.08 ± 0.01 (0.57)
10	10.03 ± 0.68 (6.80)	10.29 ± 0.30 (2.96)	10.89 ± 0.24 (2.20)	10.59 ± 0.06 (0.54)
50	51.24 ± 1.00 (1.96)	49.87 ± 1.23 (2.47)	52.06 ± 0.24 (0.46)	48.52 ± 0.20 (0.42)
100	100.24 ± 5.12 (5.11)	97.64 ± 5.58 (5.71)	108.05 ± 0.22 (0.20)	101.27 ± 0.18 (0.18)

[a] C.V. expressed in percentage.

On the other hand, variations in the pH had a major effect on the capacity factors of these compounds. Under pH conditions between 5.0 and 8.0, 5-FU can be found in its undissociated form (pK_a 8). This fact explains the absence of modification in its retention time by adjusting the pH conditions of the mobile phase. For this reason, adjusting the pH can not alter its retention time. However, 5-FUrd is ionised in this pH medium, due to its pK_a. Barberi-Heyob et al. [13], clearly demonstrated that values of the mobile phase higher than 6.0 dramatically increased the 5-FUrd capacity factor (k').

In order to solve these problems, we have replaced the C_{18} reversed phase column with a slightly more polar encapped stationary phase such as the Lichrospher C_8 60 RP-select B column. This column is a pH stable column packed with a monofunctional phase bonded to extremely pure spherical silica particles that exhibit good peak shape. In addition, we have included ammonium acetate in the mobile phase. Ammonium acetate is a good agent for improving the nucleotide retention in the stationary phases usually employed in reversed-phase HPLC methods [16]. This chemical has a high buffering capacity in the chosen pH range and, therefore, is a good agent for providing greatly improved chromatographic separations without column deterioration, such as usually happens when ion-pair agents are used.

Under these conditions the selectivity of the chromatographic method was optimum to allow us the quantification of both 5-FUrd and 5-FU in the mediums described above. Moreover, these analytical conditions offered a clear resolution of the compounds of interest ($R_s = 9.17 \pm 0.54$, $n = 20$) without using organic solvents (i.e. acetonitrile or methanol) in the mobile phase.

The limits of quantification and detection were found to be of the same order of magnitude as that reported by other authors [8,9]. However, lower detection limits have been reported by Baberi-Heyob et al. [13]. Nevertheless, all these values have been reached after either extraction and isolation treatment of the samples [9] or using a column switching method after precolumn derivatization [8]. Both procedures resulted in a concentration of the sample. In our case, we did not previously treat the samples and directly injected the sample in the column.

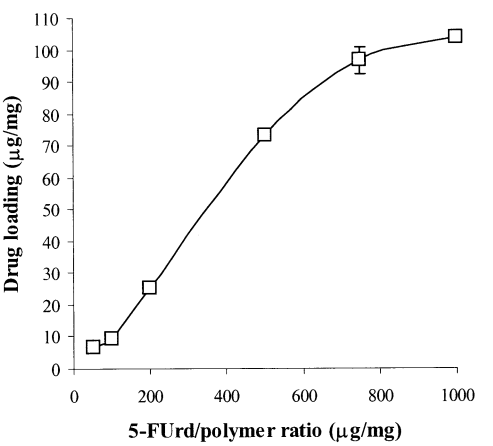


Fig. 3. Application of the method. Influence of the 5-FUrd/polymer ratio on the drug loading (μg/mg nanoparticles). Nanoparticulate batches were prepared with a drug/polymer ratio range from 50 to 1000 μg/mg.

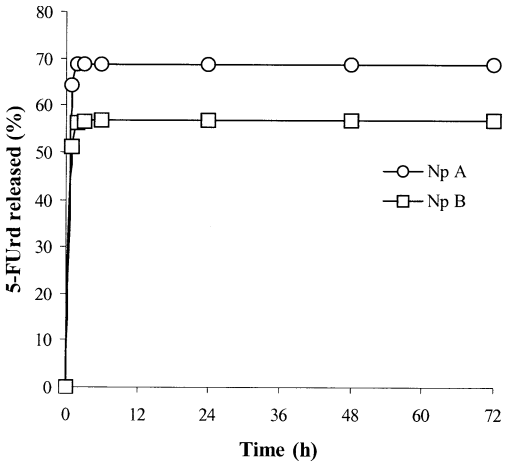


Fig. 4. Application of the method. Release study of 5-FUrd from PVM/MA nanoparticles in PBS (pH 7.4) at 37 ± 1 °C. Nanoparticulate batches were prepared either with a drug/polymer ratio of 100 μg/mg (Np A) or 200 μg/mg (Np B).

The technique described here was successfully applied to both the quantification of the drug loading in PVM/MA nanoparticles and the release profile of this drug from these pharmaceutical dosage forms. All of these results suggest that the nanoparticulate systems permit the designing of pharmaceutical dosage forms containing two drug fractions that may be released in a different way. First of all, the immediate release or burst effect may be due to a weak interaction of the drug with the surface of the carriers. On the other hand, the fraction strongly bound to the nanoparticles would be released slowly and in a more sustained way.

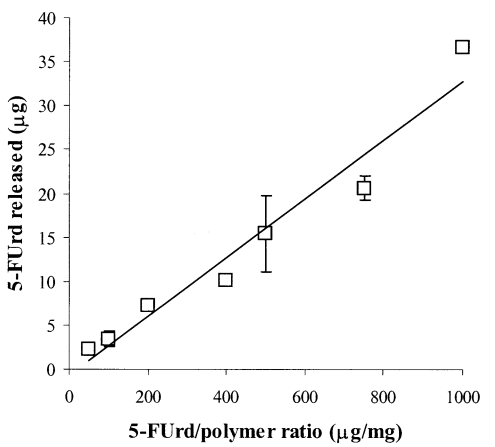


Fig. 5. Application of the method. Influence of the 5-FUrd/polymer ratio on the 5-FUrd immediately released fraction from nanoparticles. Nanoparticulate batches were prepared with a drug/polymer ratio range from 50 to 1000 μg/mg.

In summary, the HPLC method described here is rapid, sensitive, specific and robust. The precision and accuracy of the method are within acceptable ranges. The simplicity of the technique, the minimal volume requirement and the high sensitivity make this technique particularly attractive for the quantification of 5-FUrd in pharmaceuticals.

References

[1] D. Song, M.G. Wientjes, Y. Gan, J.L. Au, Bladder tissue pharmacokinetics and antitumor effect of intravesical 5-fluorouridine, Clin. Cancer Res. 3 (1997) 901–909.
[2] P. Brusa, F. Dosio, S. Coppo, D. Pacchioni, S. Arpicco, P. Crosasso, L. Cattel, In vitro and in vivo anitumor activity of immunoconjugates prepared by linking 5-fluorouridine to antiadenocarcinoma monoclonal antibody, Farmaco 52 (1997) 71–81.
[3] M.J. Dorta, O. Munguia, J.B. Fariña, V.S. Martin, M. Llabrés, Stability indicating high performance liquid chromatography methods for 5-fluorouridine in aqueous solution, Drug Res. 47 (1997) 1388–1392.
[4] F. Puisieux, G. Barratt, G. Courarraze, P. Couvreur, J.P. Devissaguet, C. Dubernet, E. Fattal, H. Fessi, C. Vauthier, Polymeric micro and nanoparticles as drug carriers, in: M. Dekker (Eds.), Polymeric Biomaterials New York, USA, 1994, pp. 749–794.
[5] W.M. Williams, B.S. Warren, F.H. Lin, Gas–liquid chromatographic analysis of fluoropyrimidine nucleosides and fluorouracil in plasma and urine, Anal. Biochem. 147 (1985) 478–486.
[6] C.G. Zambonin, S. Mastrolitti, F. Palmisano, Derivatization reactions for gas chromatography/mass spectrometry determination of *N*3-methyl-5′-deoxy-5-fluorouridine, Rapid Commun. Mass Spectrom. 14 (1997) 1529–1535.
[7] M. Kubo, H. Sasabe, T. Shimizu, Highly sensitive method for the determination of 5-fluorouracil in biological samples in the presence of 2′-deoxy-5-fluorouridine by gas chromatography–mass spectrometry, J. Chromatogr. 564 (1991) 137–145.
[8] J.M. Joulia, F. Pinguet, P.Y. Grosse, C. Astre, F. Bressolle, Determination of 5-fluorouracil and its main metabolites in plasma by high performance liquid chromatography: application to a pharmacokinetic study, J. Chromatogr. B 692 (1997) 427–435.
[9] M.Z. Del Nozal, J.L. Bernal, A. Pampliega, P. Marinero, M. Pozuelo, Determination of the concentrations of

fluorouracil and its metabolites in rabbit plasma and tissues by high performance liquid chromatography, J. Chromatogr. B 656 (1994) 397–405.

[10] F. Palmisano, F. Berardi, M. De Lena, A. Guerrieri, V. Lorusso, P.G. Zambonin, Simultaneous detrmination of 5′-deoxy-fluorouridine, 5-fluorouracil and its main metabolites in body fluids by HPLC, Chromatographia 33 (1992) 413–417.

[11] S. Yoshida, K. Uraami, M. Kito, S. Takeshima, S. Hirose, High-performance liquid chromatography with chemiluminiscence detection of serum levels of pre-column derivatized fluoropyrimidine compounds, J. Chromatogr. 57 (1990) 57–64.

[12] M. Yamaguchi, M. Nakamura, N. Kuroda, Y. Ohkura, Determination of fluorouracil and 2′-deoxy-fluorouridine (floxuridine) in human serum by high-performance liquid chromatography with fluorescence detection, Anal. Sci. 3 (1987) 75–79.

[13] M. Barberi-Heyob, J.L. Merlin, B. Weber, Determination of 5-fluorouracil and its main metabolites in plasma by high-performance liquid chromatography, J. Chromatogr. 225 (1992) 395–401.

[14] B. Gustavsson, O. Almesjö, M. Berne, J. Wadenström, Simultaneous analysis of 5′-depxy-5-fluorouridine and fluorouracil in plasma by analytical isothacophoresis, J. Chromatogr. 225 (1983) 395–401.

[15] D.R. Jenke, Chromatographic method validation: a review of current practices and procedures. II. Guidelines for primary validation parameters, J. Liq. Chromatogr. Rel. Technol. 19 (1996) 719–736.

[16] M. Merodio, M.A. Campanero, T. Mirshashi, M. Mirshashi, J.M. Irache, Development of a sensitive method for the determination of ganciclovir by reversed-phase high-performance liquid chromatography, J. Chromatogr. A 870 (2000) 159–167.