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Short communication

Hydrophilic interaction liquid chromatography–APCI–mass spectrometry determination of 5-fluorouracil in plasma and tissues

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Abstract

A simple and fast analytical method using hydrophilic interaction liquid chromatography (HILIC) coupled with mass spectrometry was developed to analyse 5-fluorouracil (5-FU) in plasma and tissues. The HILIC system overcomes problems reported in obtaining satisfactory retention of 5-FU with other types of HPLC systems. After addition of internal standard (IS) (5-Chlorouracil (5-CU)), plasma proteins were precipitated with acetonitrile, and tissue samples homogenised with a micro-dismembrator. The analysis was performed using a polymer-based column (Ashaipak NH_2) and the compounds were eluted under gradient conditions at 1 ml/min using a mobile phase containing a mixture of ammonium formate and acetonitrile. MS detection used a API 4000 mass spectrometry with heated nebulizer source and multiple reaction monitoring operated in the negative ion mode. The mass transitions of 5-FU and its internal standard were 129 $m/z \rightarrow 42$ m/z and 145 $m/z \rightarrow 42$ m/z, respectively. The lower limits of quantitation in plasma and tissues were about 5 ng/ml and 10 ng/g, respectively, using 25 μl of plasma and 50 mg of tissue. Good linearity, accuracy and precision were obtained in all matrices tested. The suitability and robustness of the method for in vivo samples were confirmed by analysis of mouse plasma, muscle and tumour from animals dosed with 5-FU.

Keywords: 5-Fluorouracil; HILIC–MS/MS; Mouse plasma; Tissue

1. Introduction

5-Fluorouracil (5-FU), a pyrimidine analog with a stable fluorine atom in position 5 of the uracil ring (Fig. 1), is a cytostatic agent, which has been widely used in the treatment of various solid tumours for more than 20 years, and is still one of the most commonly used anticancer drugs [1–3]. Several techniques have been developed in the last 25 years for the quantitation of 5-FU, related pro-drugs, and their metabolites, in biological matrices. Among many published analytical methods, HPLC-UV is the most frequently reported technique for the determination of 5-FU in plasma, serum, tissue and urine [4–19]. Methods using HPLC methods with fluorescence detection [20,21] have also been described although derivatisation is required and sample pre-treatment procedures are more complex. Several other methods, which include GC [22,23], have been described to analyse 5-FU in biological samples. Increased sensitivity was reported using GC–MS [24–26] and LC–MS [27] methods, although these methods required derivatisation resulting in a more complex and time-consuming procedure. The chromatographic separation of 5-FU in extracts of biological samples has usually been performed by reversed-phase [9,11–19,27] and reversed-phase ion pair methods [4–6,8]. The disadvantage of the majority of these techniques is the poor retention of 5-FU, due to its high polarity, and the compound typically elutes close to the solvent front, even when the mobile phase contains little or no organic solvent (methanol or acetonitrile). Some authors have tried to circumvent this problem by using two columns in line [4,7], by valve switching [5,20], or by gradient elution [11,19]. Other authors have tried to use normal-phase [21] or ion exchange chromatography [10]. Several methods are

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preceded by time-consuming solid-phase extraction (SPE) [13], or liquid–liquid extraction (LLE) with, for example, isopropanol/diethyether [22], *n*-propanaol/diethylether [12,18], ethylacetate [11,17,26], ethylacetate/methanol [8,9] or petroleum/propanol [6]. Other papers describe protein precipitation (PP) using trichloroacetic acid [10,16], ethanol [7], perchloric acid [15] or acetonitrile [21,25,27] to provide faster sample pre-treatment. Other authors also report a PP combined with LLE [14,24] or LLE combined with SPE [8]. In the methods reported in literature, no less than 0.25 ml of plasma has been used to extract 5-FU; this is a limitation when a pharmacokinetic experiment is performed in small animals such as the mouse, where limited volume of plasma (normally less than 50 μl) is available.

In order to obtain information quickly for research studies, the development of a practical and rapid analytical method for determination of mouse plasma and tumour concentration of 5-FU was undertaken. In spite of the many methods already published, improved procedures for the analysis of 5-FU may also be useful for analysis of clinical samples.

In order to obtain good chromatographic retention, hydrophilic interaction liquid chromatography (HILIC) represents an alternative to reversed-phase HPLC; in the HILIC mode an aqueous–organic mobile phase is used with a polar stationary phase to provide a "normal-phase" type of retention behaviour [28]. Recently, HILIC–MS/MS bioanalytical methods have been published indicating that this methodology can be ideal for analysing polar compounds in biological fluids [29–31].

In this paper, a simple and fast LC–MS/MS method using HILIC is described for the assay of 5-FU in mouse plasma and tissues; application of the method to support pharmacokinetic evaluation during efficacy and PK/PD studies is also described.

2. Experimental

2.1. Materials

5-FU and 5-Chlorouracil (5-CU, Fig. 1) were obtained from Sigma–Aldrich, Milan, Italy. All other chemicals and solvents were of analytical grade from Carlo Erba Reagents (Milan, Italy).

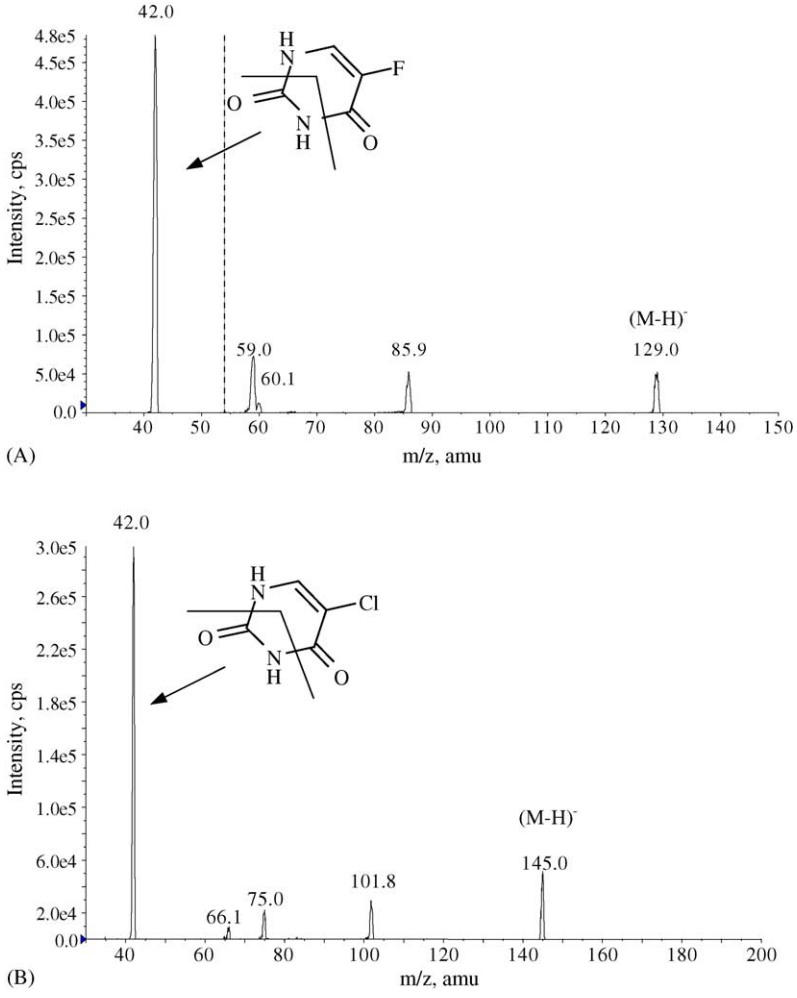

Fig. 1. Product ion scan mass spectra of the deprotonated molecule of 5-FU (A) and 5-CU (B).

Water was prepared in house using a Milli-Q Plus 185 system (Millipore, Vimodrone, Italy). Nude mice bearing HT29 tumours, a mammalian implanted carcinoma, were used for the study. Blood was collected through orbital bleeding via heparinized capillary tubes and then centrifuged for 10 min at 1500 × *g* in order to obtain plasma.

Dissected tissues and plasma samples were frozen and stored at −80 °C until analysis.

2.2. Preparation of standard solutions

Standard and QC samples were made from two separate stock solutions (1 mg/ml in methanol) of 5-FU. Working standard solutions were prepared by dilution of the stock solutions, and aliquots (10 or 20 μl) were spiked into 200 μl of mouse plasma or 50 mg of ground tissue to provide plasma concentrations of 5-FU equivalent to 18,000, 9000, 1200, 600, 120, 10 and 5 ng/ml and tissue concentrations of 20,000, 10,000, 1000, 500, 100, 20 and 10 ng/g.

QC samples at concentrations of about 20, 900 and 5000 ng/ml were prepared by adding aliquots of the working solutions to 200 μl of mouse plasma. Aliquots of appropriate working solutions were added to 50 mg of ground tissue to provide QC concentrations of 12,000, 4000 and 40 ng/g in muscle and tumour, and of 10,500, 2990 and 30 ng/g in liver and brain.

2.3. Plasma sample preparation

Unknown and standard samples were prepared by pipetting 25 μl of mouse plasma into 96-well plate. Plasma proteins were precipitated by adding 200 μl of acetonitrile containing 5-CU as internal standard (IS) at a concentration of 900 ng/ml. This mixture was vortex mixed for 10 s and centrifuged for 20 min at 4000 × *g*. An aliquot (10 μl) was injected into the LC system.

2.4. Tissue sample preparation

An amount of each tissue sample (maximum was typically 400 mg) was frozen in liquid nitrogen and pulverized using a micro-dismembrator (Braun Instruments, Germany) [32]. A portion (50 mg) of the pulverized powder was transferred into an Eppendorf tube, and 510 μl of methanol containing IS at a concentration of 900 ng/ml was added. After agitation and ultracentrifugation at 49,000 × *g* for 15 min, an aliquot (10 μl) of supernatant was injected into HPLC system.

2.5. HILIC conditions

The liquid chromatographic system consisted of a HP-1100 solvent-delivery system (Agilent, Cernusco s/n, Italy) and a CTC-PAL autosampler (Alfatech, Genova, Italy).

2.5.1. Method 1

The analytical Adsorbosphere XL $NH_2$90A° column (150 mm × 4.6 mm i.d., 3 μm), was obtained from Alltech Italia S.r.l, Sedriano, Italy. The mobile phase contained of acetonitrile and 2 mM ammonium acetate buffer at pH 6.5. The compounds were eluted under gradient conditions with a 3 min linear gradient running from 95:5 (acetonitrile:buffer) to 75:25 (acetonitrile:buffer), followed by 0.8 min of isocratic elution, before return to the initial conditions in 0.1 min; after a further 2.6 min for re-equilibration, the next injection was made. The LC flow rate was 1 ml/min directed towards the mass spectrometry interface. The column was maintained at 45 °C throughout the analysis.

2.5.2. Method 2

The analytical Phenomenex Ashaipak $NH_2$50D column (150 mm × 4.6 mm i.d.) was obtained from Chemtek Analytical, Bologna, Italy. The column was maintained at 45 °C and the compounds were eluted using a mobile phase containing acetonitrile and 10 mM ammonium formate buffer at pH 3.5. The compounds were eluted under gradient condition, as follows: a 2 min isocratic elution (90:10, v/v, acetonitrile:buffer) followed by a 1.30 min linear gradient to 75:25 (v/v, acetonitrile:buffer), followed by 0.8 min of isocratic elution before return to the initial conditions in 0.1 min; after 2.6 min of re-equilibration, the next injection was made. The LC flow rate was 1 ml/min.

2.6. MS conditions

MS was performed on a PE-Sciex (Applied Biosystems, Monza, Italy) Model API 4000 triple-quadrupole mass spectrometer. The instrument was operated using atmosphere pressure chemical ionisation (APCI) utilising the PE-Sciex heated nebulizer source. Curtain gas (nitrogen) was 30 psig, while the nebulizer gas (air) pressure was set at 60 psig. Multiple reaction monitoring (MRM) was used in the negative ion mode. The mass spectrometer was programmed to admit the pseudo-molecular ion $[M-H]^-$ at *m*/*z* 129 and 145 for 5-FU and internal standard, respectively, via the first quadrupole mass filter (Q_1) followed by collision-induced fragmentation in Q_2 and monitoring via Q_3 of the product ions at *m*/*z* 42 for both compounds. The orifice potential and the collision energy were set at −32 and −28 V, respectively. The dwell time for each transition was 400 ms. Peak area ratios obtained from the MRM chromatograms of the parent compound (*m*/*z* 129 → 42) and the internal standard (*m*/*z* 129 → 42) was used for quantitation. Both quadrupoles were maintained at unit mass resolution. Sciex Analyst software (Version 1.2) was used for instrumentation control, and data collection.

2.7. Calculations

Calibration curves were plotted using weighted linear least-squares regression analysis (weighting factor $1/x^2$) according to the equation $y = a + bx$, where y is the peak area

ratio, x the concentration of the calibration samples, a the intercept and b is the slope of the regression line. The weighting factor was chosen to minimise deviation of back-calculated values from theoretical concentrations. Subsequently, concentrations of the QC and unknown samples were calculated from the regression equation of the calibration curve.

3. Results and discussion

An HILIC–MS/MS method has been developed for the determination of 5-FU in mouse plasma and tissues. Due to the relatively small number of samples available and as support was being provided for drug discovery experiments, it was not the goal of this work to perform extensive validation of the method, but to provide sample analysis with sufficient accuracy, precision and robustness to meet the needs of the study. Tissue samples were prepared by "pulverization" using a micro-dismembrator [32]. A particular advantage of the micro-dismembrator procedure is that the tissues remain frozen during processing. This should help to prevent any degradation of nucleotides and/or nucleotides to 5-FU as already described by Peters et al. [33].

Full scan mass spectrometry of 5-FU and 5-CU revealed the deprotonated parent molecule ions $[M-H]^-$ to be in abundance with a mass-to-charge ratio (m/z) of 129 and 145, respectively. The product ion spectra using collision energies of −32 and −28 V, respectively, resulted in major fragment ions at 42.0 m/z for both compounds (Fig. 1). The fragmentation pattern is shown in Fig. 1A (5-FU) and Fig. 1B (5-CU).

5-FU is a low molecular weight and very hydrophilic compound, which makes chromatographic separation very challenging, when using traditional reversed-phase HPLC conditions. The selection of HILIC conditions (i.e. a silica column and aqueous-organic phase) for this analysis was based on reports in the literature of analysis of other polar compounds. HILIC is a versatile and effective alternative to increase retention of hydrophilic molecules that are difficult to retain by reversed-phase chromatography. Hydrophilic molecules such as 5-FU are well retained on the normal-phase columns used for HILIC. Moreover, this technique requires a high percentage of organic solvent in the mobile phase and these conditions are favorable to obtain good sensitivity with LC–MS.

A silica-based amino column was initially used and under the chromatographic conditions utilised, 5-FU was well retained with a retention time (RT) of about 4 min. This column was tested using extracts of mouse plasma samples. The calibration curve was linear over the range 5–20,000 ng/ml and the coefficient of linear regression was higher than 0.99. The back-calculated concentration values for the calibration standards showed a bias ranging from −1.3 to 1.0%. Intra-assay precision (R.S.D.), evaluated in triplicate with three different concentrations of QC samples, ranged from 2.8 to 3.7%. The intra-day accuracy showed a bias ranging from 7.2 to 13.8%.

In spite of these good initial results, under the conditions tested for 5-FU, chromatographic reproducibility appeared to be a big limitation of this column. After 60 injections, the retention time showed a shift of about 1.34 min compared with the first injection. Similar results were seen with several examples of the same column type. The change of the retention time and, consequently, a very short column life-time was probably caused by self-hydrolysis of the bonded phases by the basic amino group linked to silica; apparent deposition of silica material was also observed on the mass-spectrometry voltage needle and on the curtain plate. This was observed after just 4–5 h of analysis, or even simply after passage of mobile phase through the column for a similar time.

Considering these results, the use of a polymer-based amino column was explored. Under the same chromatographic conditions, 5-FU was eluted with a retention time

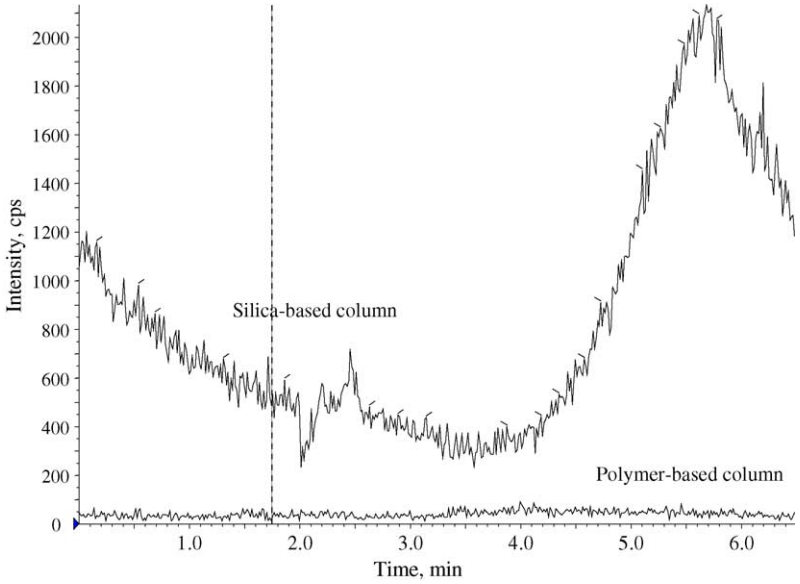

Fig. 2. Column stability: MRM traces of 5-FU after methanol injection on amino silica-based and amino polymer-based column.

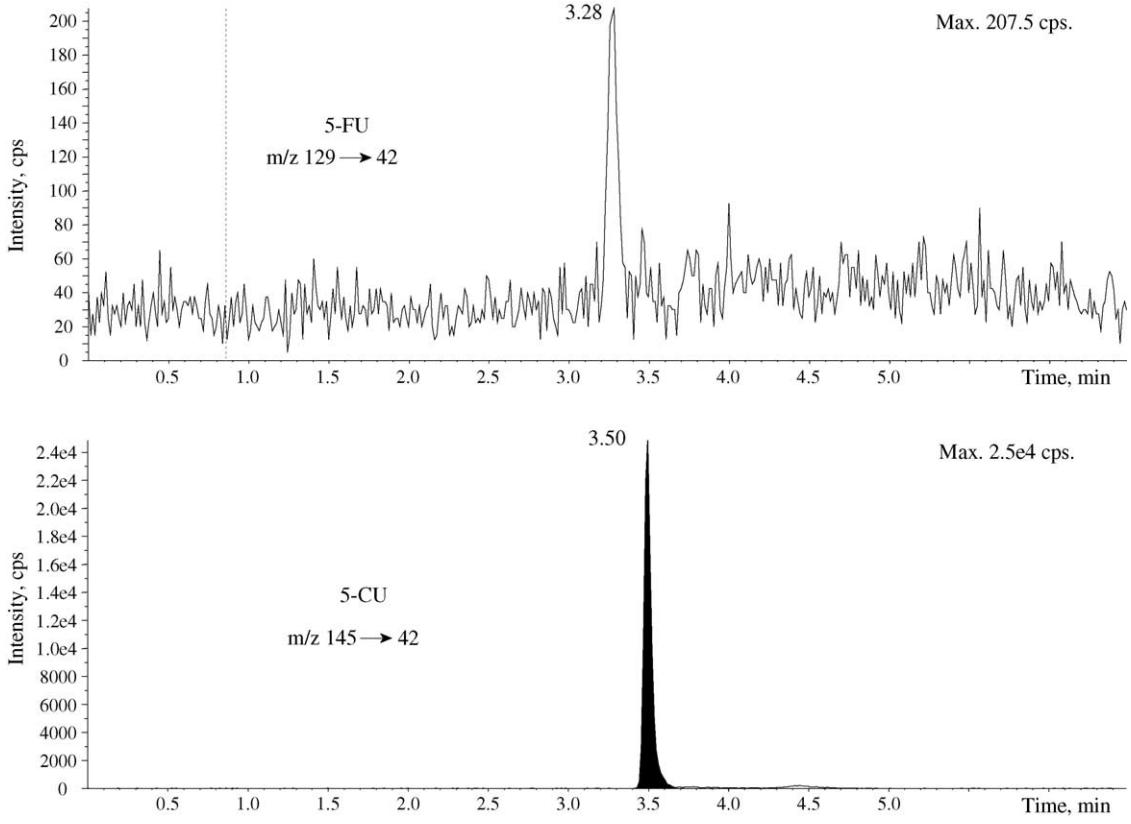

Fig. 3. Extracted ion chromatograms of extracted blank mouse plasma spiked with IS (5-CU).

of 3.46 min. This type of hydrophilic column has different chemistry linking the amino group to the stationary phase support and the absence of silica provides a wide pH stability range. No evidence of deposition of material on the mass spectrometry needle was observed after several days of use of this polymer-based column. Retention time reproducibility was much improved with a R.S.D. of the RT of 0.27% after 45 injections (intra-run) and 0.20% after more than 135 injections (inter-run). A more stable background signal, acquired at the transition of 5-FU, was obtained using this column in comparison with that obtained with silica-based column under the same chromatographic conditions (Fig. 2). A further advantage of this polymer-based column was that it is shipped containing acetonitrile, and this saves time and the complication of changing between immiscible solvents; this is required for the silica columns that are received containing hexane.

A partial method validation was performed for mouse plasma and tissues. Blank plasma extracted showed an interference peak with a retention time close to, but resolved from the 5-FU peak (Fig. 3). Good sensitivity was observed after the injection of 10 μl of the final extract corresponding to 25 pg on column with a S/N higher than 5. The lower limit of quantitation was 5 ng/ml of 5-FU in plasma (Fig. 4) and 10 ng/g in tissues. No carry-over effect was observed after the injection of an extracted blank sample following the highest calibration standard.

Table 1
Precision and accuracy of calibration standards for 5-FU in mouse plasma

	4.74 (ng/ml)	9.05 (ng/ml)	121 (ng/ml)	633 (ng/ml)	1,210 (ng/ml)	9,050 (ng/ml)	18,100 (ng/ml)
Batch 1	4.65	8.25	119	619	1,220	9,820	18,500
	5.17	8.66	116	619	1,180	9,830	18,200
Batch 2	4.37	[a]	103	550	1,120	9,800	19,100
	5.28	8.48	133	571	1,340	10,300	[a]
Batch 3	5.18	8.21	115	595	1,180	9,860	17,500
	4.45	9.38	121	600	1,230	10,300	[a]
Mean	4.78	8.90	118	587	1,200	9,760	18,600
R.S.D. (%)	8.4	9.6	8.8	4.9	5.9	6.0	6.7
Bias (%)	0.8	−1.7	−2.5	−7.3	−0.8	7.8	2.8

[a] Concentration >15% of the original value; not included in the calculation.

Table 2
Precision and accuracy of quality control samples for 5-FU in mouse plasma

	Intra-day ($n = 5$)			Inter-day ($n = 15$)		
	18.1 (ng/ml)	928 (ng/ml)	4740 (ng/ml)	18.1 (ng/ml)	928 (ng/ml)	4740 (ng/ml)
Mean	17.6	957	5180	18.1	891	4720
R.S.D. (%)	4.8	4.6	3.6	6.7	7.0	8.8
Bias (%)	−2.8	3.1	9.3	0.0	−4.0	−0.4

No cross-talk between 5-FU and 5-CU mass spectrometry signals was found. Fig. 5 shows the chromatograms obtained from the analysis of plasma samples spiked with 5-FU (A) or 5-CU (B). Matrix effects from co-eluting endogenous components in biological matrices have been well documented in the literature as having the potential to effect the reproducibility and accuracy of the analysis, even if these effects are generally less evident with methods using the APCI interface compared to ESI. In method development experiments performed with 5-FU no significant matrix effects were observed in any of all matrices tested, and similar responses were always seen with standard solutions and matrix extracts.

The method was linear over the concentration range 5–18,000 ng/ml in plasma and 10–20,000 ng/g in mouse muscle, tumour, liver and brain. Linear coefficient of correlation was higher than 0.99 in all matrices tested. Table 1 shows the data obtained from three different calibration curves of 5-FU in mouse plasma. The back-calculated calibration standard points showed a R.S.D. ranging from 0 to 13.1% in all tissues tested. The percent difference between the standard theoretical concentrations and those derived from the calibration curves in all tissues tested range from −14.8 to 10.5%. Table 2 summarises the intra- and inter-assay accuracy and precision results for QC samples prepared in mouse plasma. Table 3 shows the intra-assay data results in four different mouse tissues: tumour, muscle, brain and liver. The data indicate that this method is consistent and reliable with low R.S.D.s and bias values in all matrices tested.

The current method was applied for the determination of 5-FU in plasma, muscle and tumour in mice treated with an i.v. dose of 50 mg/kg of 5-FU. Fig. 6 shows the pharmacokinetic profiles obtained. The results are generally consistent with those already reported in literature [33], giving a further

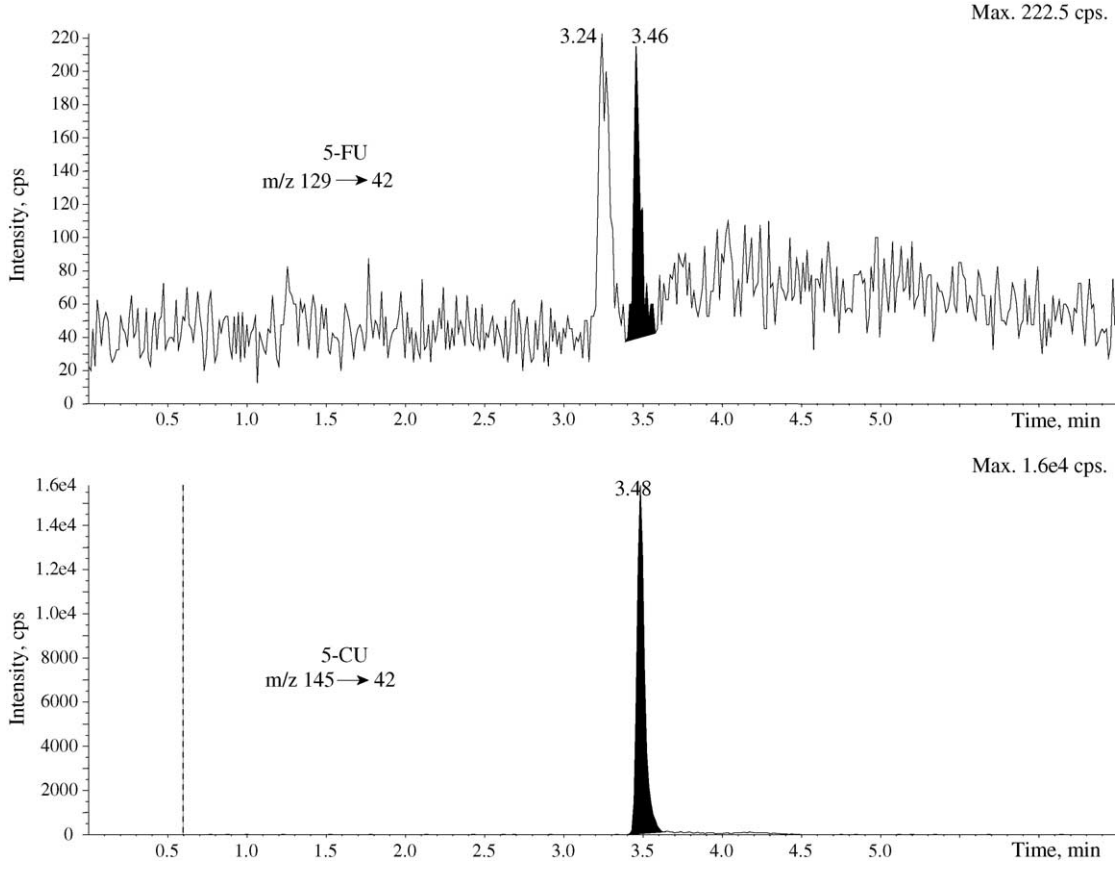

Fig. 4. Extracted ion chromatograms of extracted blank mouse plasma spiked with 5 ng/ml (LLOQ) of 5-FU and IS (900 ng/ml).

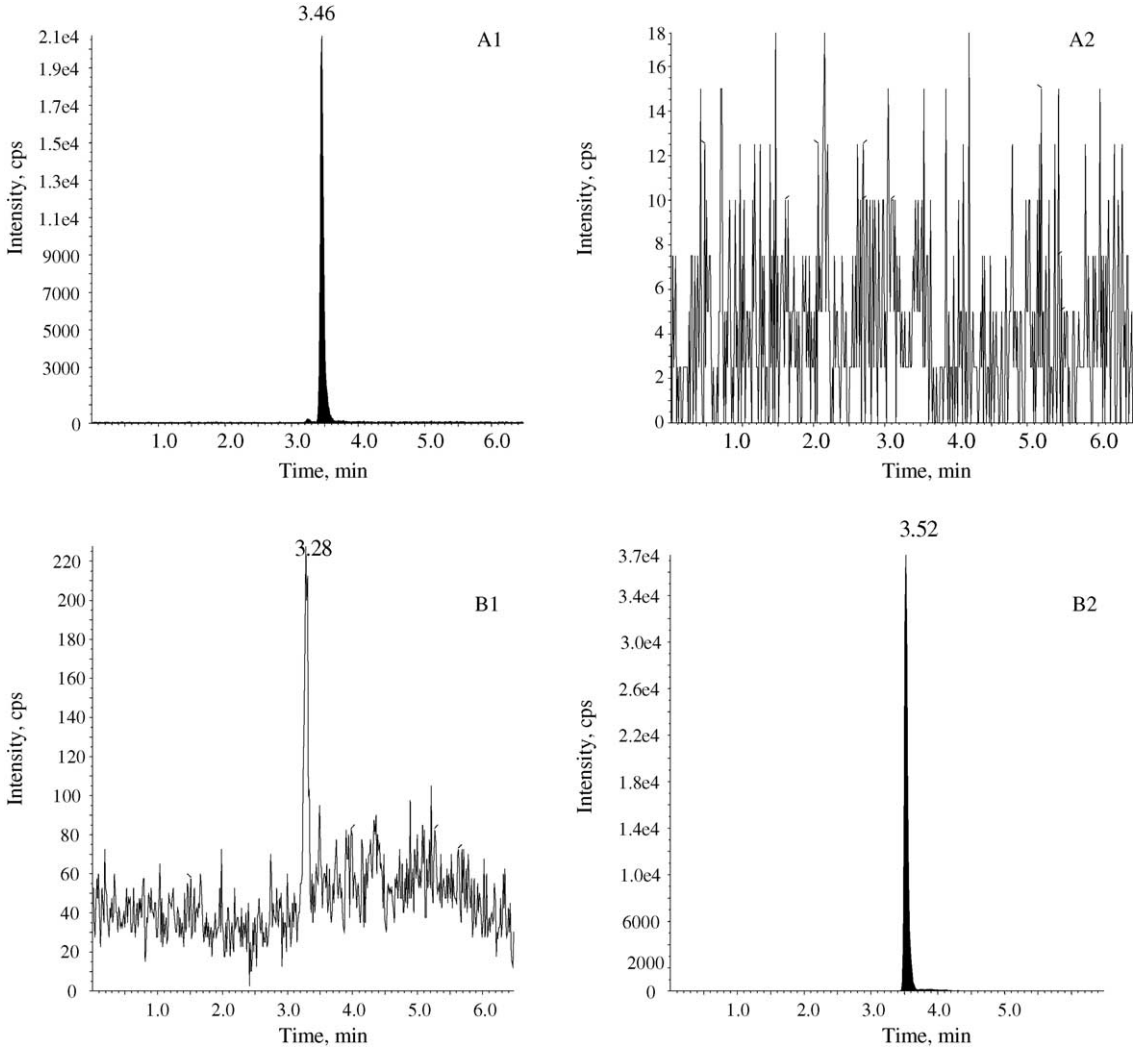

Fig. 5. Extracted ion chromatograms of extracted mouse plasma spiked with 1000 ng/ml of 5-FU (A1 extract ion of 5-FU, A2 extract ion of 5-CU) and 900 ng/ml of 5-CU (B1 extract ion of 5-FU, B2 extract ion of 5-CU).

Table 3
Accuracy and precision intra-day of the QC sample for 5-FU in mouse tissues

Mouse tissue	Theoretical concentration (ng/g)	Measured concentration (ng/g)		
		Mean	R.S.D. (%)	Bias (%)
Muscle	40.0	43.8	7.68	9.5
	4,000	4,020	7.11	0.5
	12,000	11,200	8.46	−6.7
Tumour	40.0	37.3	7.90	−6.8
	4,000	4,270	8.69	−6.8
	12,000	12,200	11.9	1.7
Liver	30.0	32.5	5.55	8.3
	2,990	2,570	3.40	−14.0
	10,500	10,400	2.88	−1.0
Brain	30.0	30.3	9.03	1.0
	2,990	2,920	4.10	−2.3
	10,500	10,300	8.83	−1.9

$N = 3$ for intra-day measurements.
%Bias = [(observed value − nominal value)/nominal value] × 100.

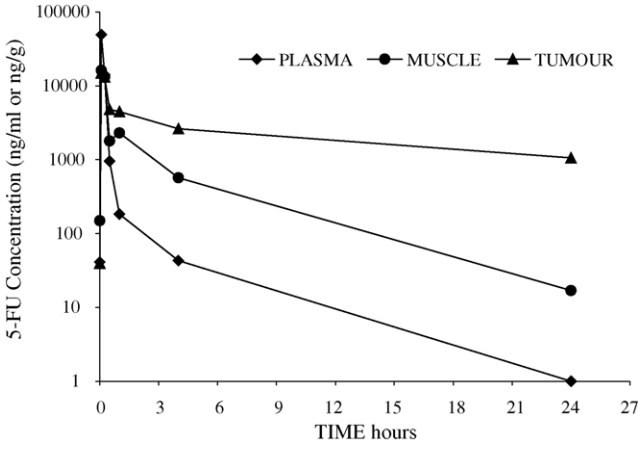

Fig. 6. Plasma, muscle and tumour (HT29, mammalian implanted carcinoma) profile of 5-FU in nude mice treated with 50 mg/kg i.v. of 5-FU in 5% glucosate.

confirmation of the validity of the method presented in this paper.

4. Conclusions

In conclusion, the LC–MS/MS method presented for 5-FU quantitative analysis in mouse plasma and tissues, using an HILIC, can be considered a valid alternative to the previous analytical methods. A simple protein precipitation procedure was used to extract the analyte and its internal standard from plasma and pulverized portion of tissues. Limitations of stability observed with an amino silica-based column were overcome by using a polymeric-based amino column. The method proved to be robust, precise and capable of accurately determining 5-FU in mouse plasma and tissues. The LLOQ is 5 ng/ml following extraction of only 25 μl of mouse plasma, and 10 ng/g using only 50 mg of tissue. This method is suitable to evaluate the pharmacokinetics of 5-FU during preclinical efficacy and PK/PD studies, and the method should be suitable for analysis of clinical samples.

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