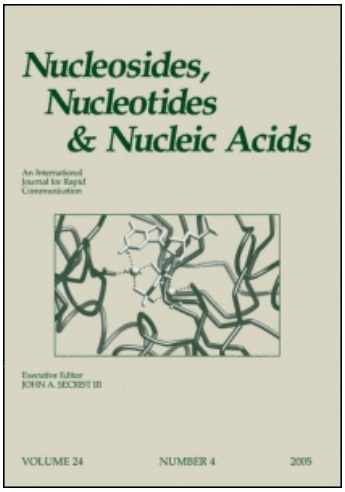

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DETERMINATION OF 5-FLUOROURACIL IN PLASMA WITH HPLC-TANDEM MASS SPECTROMETRY

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□ *In this article, we describe a fast and specific method to measure 5FU with HPLC tandem-mass spectrometry. Reversed-phase HPLC was combined with electrospray ionization tandem mass spectrometry and detection was performed by multiple-reaction monitoring. Stable-isotope-labeled 5FU (1,3–$^{15}N_2$–5FU) was used as an internal standard. 5FU was measured within a single analytical run of 16 min with a lower limit of detection of 0.05 μM. The intra-assay variation and inter-assay variation of plasma with added 5FU (1 μM, 10 μM, 100 μM) was less then 6%. Recoveries of the added 5FU in plasma were > 97%. Analysis of the 5FU levels in plasma samples from patients with the HPLC tandem mass spectrometry method and a HPLC-UV method yielded comparable results ($r^2 = 0.98$). Thus, HPLC with electrospray ionization tandem mass spectrometry allows the rapid analysis of 5FU levels in plasma and could, therefore, be used for therapeutic drug monitoring.*

Keywords 5-Fluorouracil; Dihydropyrimidine dehydrogenase; Pharmacokinetics

INTRODUCTION

5-fluorouracil (5FU) remains one of the most frequently prescribed chemotherapeutic drugs for the treatment of cancers of the gastrointestinal tract, breast, head and neck. To exert its cytotoxic effect against cancer, 5FU must first be anabolized to the nucleotide level. Opposing the activation

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of 5FU to the level of fluoropyrimidine nucleotides are the enzymes of the pyrimidine degradation pathway. Dihydropyrimidine dehydrogenase (DPD) catalyzes the conversion of 5FU to fluoro-5,6-dihydrouracil which is the initial and rate-limiting step in the catabolism of 5FU. A relationship between the 5FU dose intensity and the therapeutic response, as well as toxicity, has been noted. Patients with a DPD deficiency are unable to degrade 5FU and these patients are at risk of developing severe toxicity after the administration of 5FU.[1,2] Therapeutic drug monitoring of the 5FU levels in plasma requires the fast and unambiguous identification and quantification of 5FU. In this study, we describe a fast and specific method to measure 5FU in plasma with HPLC tandem-mass spectrometry.

MATERIALS AND METHODS

Plasma samples were obtained from colorectal patients receiving bolus administration of 5FU (425 mg/m^2) and folinic acid (20 mg/m^2).

Of the Internal standard, 30 μl 1,3-$^{15}N_2$–5FU (Cambridge Isotope Laboratories, Andover, MA, USA) was added to 300 μl of plasma and centrifuged over a Microcon YM-30 filter (Millipore B.V., Amsterdam, the Netherlands) to remove protein. 2 μl of 25% (w/v) HCOOH was added to 70 μl of the deproteinized plasma sample and 50 μl was injected into the HPLC-MS/MS system.

The metabolites were separated on a Phenomenex Aqua analytical column (250×4.6 mm, 5 μm particle size; Bester, Amstelveen, the Netherlands), protected by a guard column (SecurityGuard C18 ODS; 4 × 3.0 mm; Bester, Amstelveen, the Netherlands). Solvent A consisted of 50 mM HCOOH (pH 2.6) and solvent B consisted of methanol. Elution was performed by applying a linear gradient at a flow rate of 1 ml/minute of: 0–8 minutes, 100% solvent A to 40% solvent B; 8–11 minutes, 40% solvent B to 100% solvent B; 11–11.1 minutes, 100% solvent B to 100% solvent A.

A splitter between the HPLC column and the mass spectrometer was used to introduce the eluent at a flow of 50 μl/minutes into the mass spectrometer. The eluent from 5.8 to 8.0 minutes was introduced into the mass spectrometer. A Quattro II tandem mass spectrometer (Micromass, Manchester, United Kingdom) was used in the negative Electrospray ionization (ESI) mode and nitrogen was used as the nebulizing gas. Multiple-reaction monitoring was used to detect the metabolites by the specific m/z transition of precursor ion to fragment.

Analysis of 5FU levels in plasma was also performed using a reversed-phase HPLC-UV method, as described before.[3]

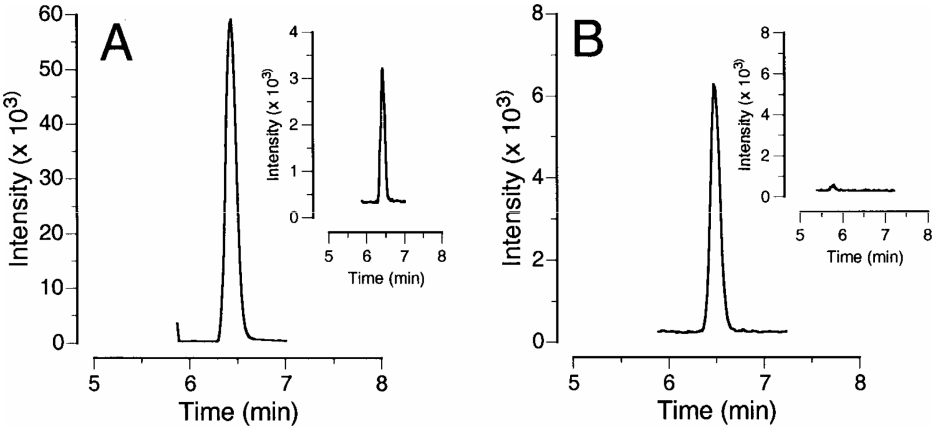

FIGURE 1 HPLC-ESI tandem MS chromatograms of 5FU. Panel A shows the chromatogram of a standard of 5FU (100 μM). The insert shows the chromatogram of the internal standard 1,3-$^{15}N_2$-5FU (5 μM). Panel B shows a chromatogram of 5FU (8.4 μM) in plasma of a patient. The insert shows the chromatogram of a plasma sample obtained prior to the administration of 5FU.

RESULTS

The detection of 5FU and the internal standard 1,3-$^{15}N_2$–5FU was performed using multiple-reaction monitoring with an *m/z* 129→42 and *m/z* 131→43, respectively. The optimal settings of the mass spectrometer for the detection of 5FU and 1,3-$^{15}N_2$–5FU were a cone voltage of 35V and a collision energy of 15 eV. The intra-assay variation and inter-assay variation of plasma with added 5FU (1 μM, 10 μM, 100 μM) was less then 6%. Recoveries of the added 5FU (1 μM, 10 μM, 100 μM) were >97% and the detection limit of 5FU was 0.05 μM. Figure 1 shows the HPLC-ESI tandem MS chromatograms of standards of 5FU and 1,3-$^{15}N_2$–5FU as well as the analysis of 5FU in plasma obtained from a patient receiving bolus administration of 5FU. Figure 1 shows that 5FU readily was detectable in plasma from the patient whereas no 5FU could be detected in plasma obtained prior to the treatment of the patient with 5FU. Figure 2 shows the comparison of the analysis of the 5FU levels in plasma samples from patients with the HPLC tandem mass spectrometry method and a reversed phase HPLC-UV method. A linear relationship between the two methods was observed ($r^2 = 0.98$, $y = 0.98$ x) and the Bland-Altman plot showed that both analysis yielded comparable results.

DISCUSSION

5FU has a relatively narrow therapeutic index and a strong correlation has been described between exposure to 5FU and both hematological and gastrointestinal toxicity. Despite the many different treatment schedules that

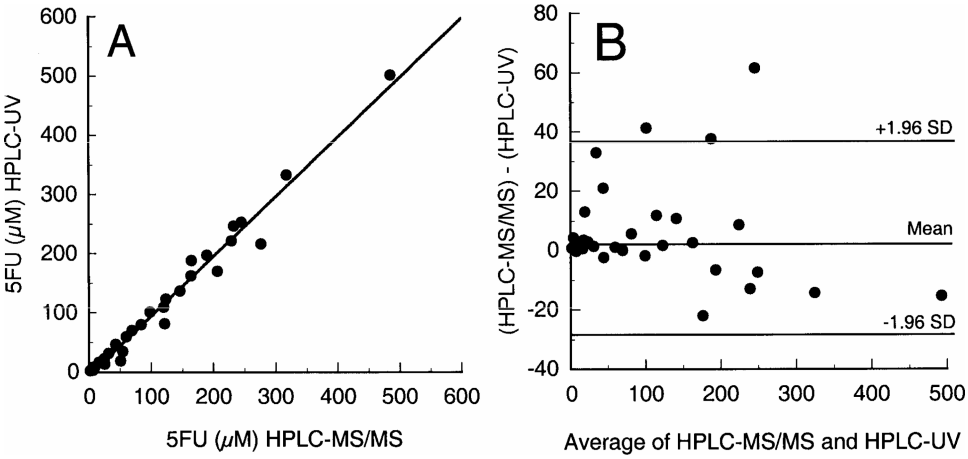

FIGURE 2 Comparison between the HPLC-MS/MS method and a HPLC-UV method. Panel A shows the linear correlation between the two methods ($R^2 = 0.98$, $y = 0.98\ x$). Panel B shows a Bland-Altman plot in which the differences between the two methods are plotted against the average of the 2 methods. The mean difference ± SD = 5±17.

exist for 5FU, comparable AUC thresholds have been observed for the onset of severe toxicity. In the case of a deficiency of DPD, profound alterations in the metabolism of 5FU can be expected with an increased likelihood of developing severe toxicity.[1,2] Controlling the AUC of 5FU might therefore be an attractive approach.

In this study, we demonstrated that with HPLC-tandem mass spectrometry, 5FU could be measured within 16 min with a detection limit of 0.05 μM, which is at least 6 times more sensitive than the HPLC-UV method.[3] The use of stable-isotope-labeled 5FU enabled the correction of the signal for quenching by coeluting compounds, resulting in high recoveries of >97%. The reproducibility of our method is demonstrated by the low intra- and inter-assay variation (<6%). Thus, the highly specific and sensitive HPLC tandem mass spectrometry method allows the rapid and unambigious analysis of 5FU levels in plasma and, therefore, could be used for therapeutic drug monitoring.

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