HHS Public Access

Author manuscript

Biomed Chromatogr. Author manuscript; available in PMC 2015 March 03.

Published in final edited form as:

Biomed Chromatogr. 2013 August ; 27(8): 994–1002. doi:10.1002/bmc.2893.

Pharmacokinetic application of a bio-analytical LC-MS method developed for 5-fluorouracil and methotrexate in mouse plasma, brain and urine

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Abstract

In the past we have reported significant cognitive deficits in mice receiving 5-fluorouracil in combination with low-dose methotrexate. To explain such interactions, a pharmacokinetic study was designed. A sensitive bio-analytical method was therefore developed and validated for 5-fluorouracil and methotrexate in mouse plasma, brain and urine with liquid chromatography coupled to a single quadrupole mass spectrometer. Chromatographic separation was accomplished by Agilent® Zorbax® SB-C_{18} column, with isocratic elution (5 mM ammonium acetate and methanol, 70:30, %v/v) at a flow rate of 300 μL/min. The limit of quantitation for both drugs was 15.6 ng/mL (plasma and brain) and 78.1 ng/mL (urine), with interday and intraday precision and accuracy ≤15% and a total run time of 6 min. This bio-analytical method was used for the pharmacokinetic characterization of 5-fluorouracil and methotrexate in mouse plasma, brain and urine over a period of 24 h. This method allowed characterization of the brain concentrations of 5-fluorouracil over a period of 24 h.

Keywords

5-fluorouracil; methotrexate; brain; LC-MS; pharmacokinetics

Introduction

5-Fluorouracil (5-FU) and methotrexate (MTX) are popular chemotherapeutic agents, and have been a common treatment option for breast cancer patients. However, recent studies suggest a possible role of such agents in cognitive deficits observed amongst patients (Ahles and Saykin, 2002, 2007). Pre-clinical *in vitro* and *in vivo* studies corroborate these observations (Dietrich *et al*., 2006, 2008; Fardell *et al*., 2010; Han *et al*., 2008). Our group has reported similar findings, wherein mice receiving a combination of 5-FU and MTX exhibited cognitive deficits (Foley *et al*., 2008). Such results demonstrating adverse neurological consequences for commonly used agents such as 5-FU and MTX have

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warranted a closer look at the possibility of pharmacokinetic (PK) interactions between 5-FU and MTX.

For 5-FU (Fig. 1) numerous bio-analytical methods, both quantitative and qualitative, have been reported in the past. Reverse-phase high-performance liquid chromatography (HPLC) has been commonly used with a variety of different detectors such UV–vis, fuorescence, NMR, MS and MS/MS (Heggie *et al*., 1987; Jarugula and Boudinot, 1996; Liu *et al*., 2010, 2012; Peer *et al*., 2012). Of all the methods available for 5-FU detection, HPLC methods with MS detectors are the most sensitive and reliable. Unfortunately most of these methods are fraught with shortcomings, such as complex sample extraction procedures (Joulia *et al*., 1997; Liu *et al*., 2012; Wang *et al*., 1998), complex chromatographic conditions (Pisano *et al*., 2005; Remaud *et al*., 2005), low sensitivity (Chu *et al*., 2003; van Kuilenburg *et al*., 2006) and the requirement for large sample volumes (Coe *et al*., 1996; Li *et al*., 2005; Liu *et al*., 2012; Loos *et al*., 1999; Peer *et al*., 2012). In addition, for 5-FU most reported studies use only plasma, while brain and urine matrices have not been evaluated (Büchel *et al*., 2013; Casale *et al*., 2002; Ciccolini *et al*., 2004; Joulia *et al*., 1997; Kosovec *et al*., 2008; Liu *et al*., 2010, 2012). Analytical methods determining 5-FU concentration in cerebrospinal fluid have been reported (Bourke *et al*., 1973) but were not very successful in tissues such as the brain (Bourke *et al*., 1973; Hao *et al*., 2004). For MTX (Fig. 1), a weak dicarboxylic acid, numerous analytical methods have been reported. HPLC methods with different detectors such as UV–vis, MS and MS/MS have been reported for MTX and its metabolites as well in different biological matrices with acceptable sensitivities (den Boer *et al*., 2012; Guo *et al*., 2007; Lobo and Balthasar, 2003).

However, there are very few sensitive analytical methods reported for a truly simultaneous determination of 5-FU and MTX in complex biological matrices such as the plasma, brain and urine. Sabatini *et al*. (2005) reported an analytical method for simultaneous quantification of 5-FU and MTX; however, the sample matrix used for quantification was Kleenex® tissue used for wiping surfaces. This sample matrix is not as complex as the biological matrices commonly used in PK studies. In this study we present the development and validation of a fast, sensitive and robust LC-MS method for 5-FU and MTX in biological matrices commonly utilized for preclinical as well as clinical PK and pharmacologic evaluation of drugs. The LC-MS parameters we used were the same for both 5-FU and MTX, therefore increasing the ease of analysis; however, we have used separate sample preparation techniques. This sensitive bio-analytical method was applied in the PK analysis for 5-FU and MTX in male Swiss–Webster mice ($n = 3$). This method allowed characterization of the brain concentrations of 5-FU over a period of 24 h, which has not been reported extensively in the past.

Experimental

Chemicals and reagents

5-FU was purchased from GeneraMedix Inc. (Ahmedabad, India); MTX was purchased from Hospira Inc. (Lake Forest, IL, USA). Aminopterin (AMP) and 5-bromouracil (5-BU) were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA) to be used as internal standards (IS) for 5-FU and MTX, respectively. Ammonium acetate, glacial acetic acid and

ethyl acetate (HPLC grade) were purchased from Fisher Scientific (Fairlawn, NJ, USA). HPLC-grade methanol was purchased from EMD chemicals Inc. (Gibbstown, NJ, USA). Deionized nanopure water was obtained from the nanopure de-ionization system (Barnstead/Thermolyne, Dubuque, IA, USA) located in the facility, and was used in all of the experiments. Blank mouse plasma (drug-free) was purchased from Lampire Biological laboratories (Pipersville, PA, USA). On receiving the blank plasma, aliquots of 10 mL were made and stored in a −20°C freezer. Batch number and date of receipt were noted.

Liquid chromatography and mass spectrometry parameters

The liquid chromatography (LC) system used was an Agilent 1100 series HPLC system (Agilent® Technologies, Santa Clara, CA, USA). For chromatographic separation an Agilent® Zorbax® SB-C_{18} (3.5 μm, 150 × 3 mm) analytical column coupled with a C_{18} guard cartridge (4 × 2.0 mm; Phenomenex, Torrance, CA, USA) was used. Columns were maintained at room temperature throughout the analysis. Sample volume injected into the system was 10 μL. Internal standards were 5-BU for 5-FU and AMP for MTX.

The mass spectrometry (MS) system used was an Agilent MSD SL-G1946D (Agilent® Technologies, Santa Clara, CA, USA). For determining appropriate MS parameters, flow injection analysis was performed with the drug solution at a concentration of 1 μg/mL in de-ionized nanopure water. The same mass spectrometer parameters were used for 5-FU and MTX. These parameters were applicable for all matrices (plasma, brain, urine) and were as follows: fragmentor voltage, 100 V; drying gas flow rate, 8 L/min; gas temperature, 250°C; nebulizer pressure, 40 psig; capillary voltage, 2500 V (±). Analysis for 5-FU and 5-BU was done in a negative ion mode with single ion monitoring (SIM) values of 129 and 189, respectively. For MTX and AMP, analysis was performed in a positive ionization mode with SIM values of 455 and 441, respectively. The MS detector described above has the capacity to quantify positive and negative ions simultaneously. The ionization source used for the method was electrospray ionization. All responses obtained were analyzed using the Agilent® ChemStation® software.

LC method development

With the parameters obtained for MS by flow injection analysis, we proceeded with developing appropriate LC parameters. Table 1 explains in detail various protocols evaluated for the determination of 5-FU and MTX in plasma, brain and urine samples.

Preparation of stock solutions, calibration standards and quality controls

5-FU and MTX were commercially available as 50 mg/mL solutions in saline. For AMP, stock solutions were prepared in methanol with 4% DMSO at 200 μg/mL final concentration, and were stored in amber colored bottles at −20°C. For 5-BU, stock solutions were prepared in 100% methanol at 200 μg/mL final concentration, and were stored in amber colored bottles at −20°C. On the day of the experiment fresh stock solutions at 40 μg/mL concentration were prepared in de-ionized water for both the drugs. For the standard curve, calibration standards were prepared by adding appropriate aliquots from the stock solutions of 5-FU and MTX to the blank murine matrix (plasma, brain or urine), after which serial dilution was performed with the appropriate matrix to obtain a standard curve. The

standard curve comprised seven nonzero concentrations ranging from 15.6 ng/mL to 1 μg/mL (plasma and brain) and from 78 ng/mL to 5μg/mL (urine). A working solution of AMP was prepared by diluting stock solutions with 100% methanol to make a final concentration of 15.6 ng/mL. A working solution of 5-BU was prepared by diluting stock solution with 100% ethyl acetate to make a final solution of 15.6 ng/mL. Separate stock solutions were prepared for quality control (QC) samples, and were used to validate the assay. For plasma and brain samples, three QC samples were chosen for each drug at the following concentrations to cover the entire standard curve range: 31.3 ng/mL (low), 125 ng/mL (medium) and 500 ng/mL (high). For urine samples three QC samples were chosen for each drug at the following concentrations: 156 ng/mL (low), 625 ng/mL (medium) and 2500 ng/mL (high).

Method validation

Calibration curve—Seven nonzero concentrations plus one blank sample comprised the calibration curve with concentrations 15.6, 31.3, 62.5, 125, 250, 500 and1000 ng/mL for plasma and brain samples and 78.1, 156.3, 312.5, 625, 1250, 2500 and 5000 ng/mL for urine samples. The standard curve was constructed by plotting the peak area ratios of drug and IS against theoretical concentrations of calibration standards. Linear regression analysis of the standard curve was performed using Graph Pad software (version 4.0), with a weighting factor of $1/x$ (where x = concentration). Back-calculation of standards and QCs was performed using the formula $y = mx + c$ for evaluation of accuracy and precision on five separate occasions.

Accuracy and precision—Accuracy and precision of the given assay were determined for the three matrices containing known concentration of the drugs. Intraday accuracy and precision were determined by analyzing six replicates of QC samples on the same day. Interday accuracy and precision were determined by analyzing five replicates of QC samples, with the procedure performed on five separate occasions. At each concentration of the QC samples, precision was determined by calculating the percentage coefficient of variation, which was defined as:

$$\mathrm{CV}\% = (\text{standard deviation}/\text{mean}) \times 100$$

Accuracy was determined by calculating the percentage relative error (RE), which was defined as: RE% = [(mean observed − theoretical)/ theoretical] × 100. The samples passed validation if the accuracy and precision at each QC concentration were ≤15% for all the conditions tested.

Extraction efficiency and matrix effect—Extraction efficiency and matrix effect were evaluated for plasma, brain and urine samples (n = 3) at concentrations of 31.3, 125 and 500 ng/mL for both 5FU and MTX. For this purpose, three sets of samples were prepared. In set 1, plasma, brain or urine samples were spiked with drug + IS prior to sample extraction process. In set 2, plasma, brain or urine samples were spiked with drug + IS post sample extraction. In set 3, drug and IS concentrations were prepared in a solution of 50:50% v/v ammonium acetate–methanol solution. Extraction efficiency (EE) was defined as:

$$\mathrm{EE}\% = (\text{peak area ratio of drug+IS in set 1} / \text{peak area ratio of drug+IS in set 2}) \times 100.$$

Matrix Effect (ME) was calculated as:

$$\mathrm{ME}\% = 100 - [(\text{peak area ratio of drug+IS in set 2} / \text{peak area ratio of drug+IS in set 3}) \times 100]$$

Freeze–thaw stability—Freeze–thaw cycles for samples stored at −80°C were performed at 4 and 22 h for both 5-FU and MTX in plasma and brain (n = 2) at QC concentrations of 31.3, 125 and 500 ng/mL. Urine samples were analyzed immediately upon collection for the calculation of f_e. Therefore freeze–thaw stability in urine was not tested. Relative error was calculated as:

$$\mathrm{RE}\% = [(\text{mean observed} - \text{theoretical value}) / \text{theoretical value}] \times 100$$

Application to PK study

Male Swiss–Webster mice (5–6 weeks old; 25–30 g) were used for all PK studies. All study protocols were approved by the Institution of Animal Care and Use Committee (Temple University, Philadelphia, PA, USA). Animals were housed in well-ventilated cages, exposed to a regular light–dark cycle of 12 h each. Food and water were available *ad-libitum*.

Both 5-FU and MTX were available as 50 mg/mL solutions for injection. Suitable dilutions were made in sterile saline on the day of the study. A single dose of 75 mg/kg for 5-FU or 32 mg/kg for MTX was administered via intravenous (i.v.) bolus injection through the lateral tail vein using a 0.5 mL insulin syringe (EXELINT® International Co., Los Angeles, CA, USA) with a 29½ gauge needle.

A serial sacrifice sampling protocol was followed for the collection of blood and tissues. Under anesthesia, blood samples were collected in heparinized tubes from the inferior vena cava of mice at time points 5, 10, 15, 30, 45, 60 min and 2, 4, 8, 16, and 24 h. Plasma samples were obtained by collecting the supernatant obtained by centrifuging blood samples at 12,000 rpm for three min at 4°C. For collection of brain tissue samples, animals were sacrificed by cervical dislocation under anesthesia. Collected samples were cleaned, weighed and stored in −80°C until further analysis. For urine collection, animals were placed in individual metabolic cages (Nalgene; Braintree Scientific Inc., Braintree, MA, USA) that had discrete collection counters for feces and urine. Metabolic cages were inspected frequently, on average once every hour. Any urine sample present in the collection counter was removed and placed in a vial assigned specifically for the cumulative collection of urine samples. These samples were obtained over a period of 24 h, and each collected sample was placed immediately at −20°C until analysis.

Noncompartmental analysis (NCA) for 5-FU and MTX was performed to obtain primary and secondary PK parameters. Average (n = 3) plasma and tissue concentration data were used for the NCA performed by WinNonlin® version 6.0 (Phoenix). Area under the curve

($AUC_{0-\infty}$) and area under the first moment curve ($AUMC_{0-\infty}$) were obtained by integrating concentration–time (*C–t*) data in plasma and brain from time zero to infinity. Systemic clearance was defined as CL_s = dose/$AUC_{0-\infty}$. For the parameters mean residence time (MRT) and volume of distribution at steady state (V_{ss}), estimates were obtained using the statistical moment theory (Kong and Jusko, 1988), where MRT = $AUMC_{0-\infty}/AUC_{0-\infty}$, V_{ss} = CL_s × MRT. C_{max} and T_{max} represent maximal concentration and the time to reach maximal concentration respectively. The elimination rate constant (k_{el}) was calculated as k_{el} = 1/MRT, and the half-life ($t_{1/2}$) was calculated as $t_{1/2} = 0.693/k_{el}$. The fraction of drug eliminated unchanged in urine (f_e) was calculated as amount excreted unchanged in urine (0–24 h)/dose amount administered.

Results and discussion

LC method development

Several methods were examined to establish a sensitive, robust and simple bio-analytical method for 5-FU and MTX (Table 1). Of all the methods tested, the most important criteria for selecting the final LC-MS method were sensitivity, short run time and ease of analysis. Machine parameters chosen for LC and MS as reported here and also in the Experimental section above, were amenable for both 5-FU and MTX. The mobile phase for both 5-FU and MTX consisted of ammonium acetate (5 mM, pH 4) and 100% methanol (70:30%, v/v) with an isocratic elution at a flow rate of 300 μL/min. Retention times for the analytes were: 2.6 min (5-FU), 3.2 min (5-BU), 5.7 min (MTX) and 3.5 min (AMP). Representative chromatograms for 5-FU and MTX are shown in Figs 3 and 4.

In the past, 5-FU analytical methods have commonly involved HPLC methods with UV detectors (Coe *et al*., 1996; Heggie *et al*., 1987; Jarugula and Boudinot, 1996; Joulia *et al*., 1997). Recently MS detectors have been used, with reported limit of quantitation (LOQ) values of 130 ng/mL (van Kuilenburg *et al*., 2006), 10 ng/mL (Kosovec *et al*. 2008; Woo *et al*. 2008) and 5 ng/mL (Licea-Perez *et al*., 2009), which are comparable to our reported value (15.6 ng/mL). However these methods have some drawbacks, such as complex derivitization procedures for sample preparation (Licea-Perez *et al*., 2007; Liu *et al*., 2010), absence of a validated method in matrices other than plasma (Kosovec *et al*., 2008) and longer run times (van Kuilenburg *et al*., 2006). Similarly for MTX, HPLC methods with UV (Aboleneen *et al*., 1996; Sartori *et al*., 2008; Sparreboom *et al*., 1999) or fuorescence detectors (Lobo and Balthasar, 2003) have been reported. Recent studies have reported LOQ values of 3.9 ng/mL (Blakeley *et al*., 2009), which are comparable to our LOQ of 15.6 ng/mL; however, concentrations as low as 3.9 ng/mL are rarely observed for MTX in a PK study spread over 24 h (den Boer *et al*., 2012). Also, there are very few analytical methods that deal with a truly simultaneous determination of 5-FU and MTX. In this study we applied discrete sample preparation methods for the two drugs. However, we established common LC-MS machine parameters for both 5-FU and MTX in complex biological matrices such as the plasma, brain and urine. In doing so, we have improved the efficiency of analysis of these two drugs together as opposed to developing completely separate analytical methods as was previously performed.

Sample preparation

Of all the sample preparation protocols that were tested (Table 1), the following sample preparation methods provided the best results analytically – in terms of sensitivity, short run time and ease of analysis. For 5-FU, to 50 μL (plasma) or 20 μL (brain or urine samples) plus drug, a 10*x* volume of ethyl acetate + IS (EA + IS) solution was added. This was then placed in a shaker for 10 min, after which it was centrifuged at 12,000 rpm for 10 min at 4°C. The upper layer was removed completely and collected in a fresh Eppendorf tube. This process was repeated by adding a 10*x* volume of fresh EA + IS solution to the remaining plasma or brain tissue sample. The upper layer was removed completely and collected again. This collected mixture was dried under N_2 for 15 min. The dried residue was reconstituted with 50 μL of mobile phase (plasma sample) or 20 μL of mobile phase (brain or urine samples). Ten microliters of this solution was injected in the LC/MS system for analysis.

For MTX, to 50 μL (plasma) or 20 μL (brain, or urine samples) plus drug, a 3*x* volume of methanol + IS (M + IS) solution was added. This was then vortexed for 10 s and later centrifuged at 12,000 rpm at 4°C for 10 min. The supernatant was collected and dried under N_2 for 30 min. Dried residue was reconstituted with 50 μL of mobile phase (plasma sample) or 20 μL of mobile phase (brain or urine samples). This solution (10 μL) was injected in the LC/MS systems for analysis.

Methods that have reported a truly simultaneous determination of 5-FU and MTX have done so in ‘neat samples’ and not complex matrices such as plasma, brain or urine (Sabatini *et al*., 2005). Although we report two separate sample preparation methods for 5-FU and MTX, machine parameters for LC and MS were the same for both 5-FU and MTX in all matrices. Having common LC and MS machine parameters makes the analytical method consistent across samples and also more efficient and user friendly. Combination studies for 5-FU and MTX performed in the past reported distinctly separate analytical methods for each drug (Batey *et al*., 2002; de Bruijn *et al*., 1987; Moore *et al*., 1994), unlike our method, where only the sample preparation is different for the two drugs. In addition our analytical method is sensitive (LOQ 15.6 ng/mL), has simple sample extraction methods (protein precipitation/ liquid–liquid extraction), a short run time (6 min) and uncomplicated liquid chromatography conditions (isocratic elution), and is robust (Table 2).

Calibration curve

Seven nonzero concentrations over a range of 15.6 ng/mL to 1μg/mL for mouse plasma and brain and 78.1 ng/mL to 5μg/mL in mouse urine were used to establish a calibration curve. The standard curve was constructed by plotting the peak area ratios of drug and IS against theoretical concentrations of calibration standards and fitted to the linear equation: $y = mx + c$ using a weighing factor ($1/x$). For five replicates, average r^2 was ≥0.99. Accuracy and precision of back-calculated concentrations for all QCs were ≤15%. The LOQ represented by the lowest concentration on the standard curve demonstrated appropriate accuracy and precision of ≤20% (Table 2).

Accuracy and precision

Method validation was performed according to the guidelines provided by the US Food and Drug Administration (Shah *et al*., 1991). The LOQ was defined as the lowest concentration on the standard curve for which the accuracy and precision was ≤20% on five separate occasions. The LOQ for 5-FU and MTX in plasma and brain was 15.6 ng/mL and LOQ for 5-FU and MTX was 78 ng/mL in urine. The interday and intraday accuracy and precision for each QC was ≤15%. Assay validation for both 5-FU and MTX is reported in Table 1. For 5-FU very few studies have reported an analytical method for determining concentrations in brain; however, when a single dose of 50 mg/kg of 5-FU was administered *in vivo*, the analytical method was not sensitive enough to quantify 5-FU concentrations over time in brain tissue (Jin *et al*., 2005; Pisano *et al*., 2005). In contrast, we applied our analytical method in describing the PK profile of a single bolus dose of 75 mg/kg 5-FU for 24 h in brain. Chromatograms of LOQ in plasma for both 5-FU and MTX are shown in Figs 2 and 3.

Extraction efficiency and matrix effect

Extraction efficiencies as well as matrix effect for 5-FU and MTX in plasma, brain and urine are reported in Table 3. In all matrices, 5-FU exhibited high extraction efficiency (~90%), while MTX had moderate extraction efficiency (~40%). Matrix effects observed for MTX were moderate in all three matrices (~30%), while for 5-FU matrix effects were high (~70%).

Ion suppression as a result of matrix effect is a common drawback of MS and MS^n detection methods. The mechanisms for such matrix effects are not fully understood; however, ion suppression is most commonly attributed to the presence of endogenous compounds, surface tension, pH, polymers from plastic tubes used for sample preparation, ionization source, basicity, high concentration of analyte or elution of analyte of interest in the solvent front (Jessome and Volmer, 2006; King *et al*., 2000). The presence of ion suppression as a result of matrix effect may be detrimental to the analytical method in terms of accuracy, precision and even detection of analyte (Bakhtiar and Majumdar, 2007; Cullum *et al*., 2004; Jessome and Volmer, 2006). Although the analytical method described for 5-FU exhibited significant matrix effect (Table 3), it did not affect the analytical capability of this method as shown in the method validation report (Table 2). Also, this method did not hinder the detection of analytes, as we could detect drug levels LOQ in brain for 24 h.

Freeze–thaw stability

Both 5-FU and MTX were tested for stability in plasma and brain over two freeze–thaw cycles as described under Experimental. Relative errors for 5-FU and MTX for freeze thaw cycles were each ~20%, indicating acceptable stability for subsequent study conditions. Results are shown in Table 4.

Application to PK study

We have successfully applied our validated bio-analytical method to a PK study determining plasma and brain *C*–*t* profiles and f_e for 5-FU and MTX, when administered as a single i.v.

bolus dose. Plasma and brain *C–t* profiles of animals receiving single i.v. bolus doses of 75 mg/kg 5-FU and 32 mg/kg MTX are shown in Fig. 4. PK parameters obtained by noncompartmental analysis of plasma and brain *C–t* data are shown in Table 5. For 5-FU, *C–t* profile was detectable in the brain for up to 24 h, whereas in plasma drug levels fell below detectable limits of the machine after 45 min. 5-FU showed a monophasic decline over a period of 45 min, whereas the *C–t* profile in the brain was found to be biphasic with a T_{max} of 5 min, indicating no lag time for 5-FU in brain. Plasma $t_{1/2}$ for 5-FU was found to be 5.2 ± 0.9 min, which is similar to the values reported previously (Yi *et al*., 2010). Plasma CL_s and V_{ss} values reported for 5-FU as shown in Table 5 in our study are similar to values reported previously in mice (Jin *et al*., 2005), rats (Jarugula *et al*., 1997; Pinedo and Peters, 1988), dogs (Kuan *et al*., 1998) and humans (Diasio and Harris, 1989). In brain 5-FU has a k_{el} of 0.00035 min^{-1}, which indicates very slow elimination of the drug. Such a prolonged retention for 5-FU has been reported in murine tumors previously, suggesting that the hydrophilic drug may have a tendency to be trapped in more lipophilic tissues (Peters *et al*., 1993).

In plasma MTX has a biphasic decline. Plasma $t_{1/2}$ was 152.5±63.7 min, and is in with accordance values reported previously (Olsen, 1991). CL_s and V_{ss} values reported for MTX as shown in Table 5 in our study are were similar to values reported previously in mice (Wang *et al*., 2011), rats (Fahrig *et al*., 1989; Miglioli *et al*., 1985) and humans (Jolivet *et al*., 1983). Brain *C–t* profile for MTX also showed biphasic decline, with a T_{max} at 5 min, indicating no lag time for MTX detection in brain. In brain, k_{el} for MTX was 0.001 min^{-1}. MTX is also known to exhibit nonlinear kinetics attributed to saturable renal excretory mechanisms and enterohepatic re-cycling (Hendel and Brodthagen, 1984; Hendel and Nyfors, 1984; Olsen, 1991).

PK studies for 5-FU in the brain have not been reported extensively. Characterization of the profile of 5-FU in the brain opens up new avenues for the study of 5-FU-related neurotoxicity with or without other drugs such as MTX. Cytotoxicity synergism between 5-FU and MTX has been reported in the past (Bertino *et al*., 1983; Cadman *et al*., 1981; Fernandes and Bertino, 1980; Herrmann *et al*., 1984; Katzir *et al*., 2000). Given the sensitivity of the analytical method described above for both 5-FU and MTX in plasma and brain, we plan to explore this synergism between 5-FU and MTX by performing a PK study to delineate a possible cause for the reported neurotoxic effects by our group (Foley *et al*., 2008). In our future studies we plan to perform PK studies for 5-FU and MTX at different dose levels administered individually and in combination, via multiple dosing routes. Such a drugdrug interaction (DDI) study will be extremely crucial in providing a novel perspective to explain increased cognitive deficits observed in mice receiving a combination of 5-FU and MTX.

Conclusions

In conclusion, a rapid and sensitive bio-analytical method for the simultaneous determination 5-FU and MTX was developed and validated in mouse plasma, brain and urine. The above bioanalytical method was used for quantitating 5-FU and MTX in plasma

and brain over time for up to 24 h. Also, this method will be used in the future for analyzing possible DDIs between 5-FU and MTX in mice.

Acknowledgments

The work was partially supported by award number R01CA129092 from the National Institutes of Health to E.A.W.

Abbreviations used

5-FU	5-fluorouracil
AUC	area under curve
AUMC	area under the first moment curve
CS	calibration standards
EE	extraction efficiency
f_e	fraction of drug eliminated unchanged in urine
ME	matrix effect
MRT	mean residence time
MTX	methotrexate
NCA	non compartmental analysis
PK	pharmacokinetics

References

Aboleneen H, Simpson J, Backes D. Determination of methotrexate in serum by high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl. 1996; 681:317–322.

Ahles TA, Saykin AJ. Breast cancer chemotherapy-related cognitive dysfunction. Clin Breast Cancer. 2002; 3(suppl 3):S84–90. [PubMed: 12533268]

Ahles TA, Saykin AJ. Candidate mechanisms for chemotherapy-induced cognitive changes. Nat Rev Cancer. 2007; 7:192–201. [PubMed: 17318212]

Bakhtiar R, Majumdar TK. Tracking problems and possible solutions in the quantitative determination of small molecule drugs and metabolites in biological fluids using liquid chromatography–mass spectrometry. J Pharmacol Toxicol Methods. 2007; 55:262–278. [PubMed: 17174577]

Batey MA, Wright JG, Azzabi A, Newell DR, Lind MJ, Calvert AH, Boddy AV. Population pharmacokinetics of adjuvant cyclophosphamide, methotrexate and 5-fluorouracil (CMF). Eur J Cancer. 2002; 38:1081–1089. [PubMed: 12008196]

Bertino JR, Mini E, Fernandes DJ. Sequential methotrexate and 5-fluorouracil: mechanisms of synergy. Semin Oncol. 1983; 10:2–5. [PubMed: 6867754]

Blakeley JO, Olson J, Grossman SA, He X, Weingart J, Supko JG. New Approaches to Brain Tumor Therapy (NABTT) Consortium. Effect of blood brain barrier permeability in recurrent high grade gliomas on the intratumoral pharmacokinetics of methotrexate: a microdialysis study. J Neurooncol. 2009; 91:51–58. [PubMed: 18787762]

Bourke RS, West CR, Chheda G, Tower DB. Kinetics of entry and distribution of 5-fluorouracil in cerebrospinal fluid and brain following intravenous injection in a primate. Cancer Res. 1973; 33:1735–1746. [PubMed: 4198466]

Büchel B, Rhyn P, Schürch S, Bühr C, Amstutz UR, Largiadèr C. LC-MS/MS method for simultaneous analysis of uracil, 5,6-dihydrouracil, 5-fluorouracil and 5-fluoro-5,6-dihydrouracil in

human plasma for therapeutic drug monitoring and toxicity prediction in cancer patients. Biomedical Chromatography. 2013; 27:7–16. [PubMed: 22454320]

Cadman E, Heimer R, Benz C. The influence of methotrexate pretreatment on 5-fluorouracil metabolism in L1210 cells. J Biol Chem. 1981; 256:1695–1704. [PubMed: 6161926]

Casale F, Canaparo R, Muntoni E, Serpe L, Zara GP, Della Pepa C, Berno E, Costa M, Eandi M. Simultaneous HPLC determination of 5-fluorouracil and its metabolites in plasma of cancer patients. Biomed Chromatogr. 2002; 16:446–52. [PubMed: 12378555]

Chu D, Gu J, Liu W, Paul Fawcett J, Dong Q. Sensitive liquid chromatographic assay for the simultaneous determination of 5-fluorouracil and its prodrug, tegafur, in beagle dog plasma. J Chromatogr B Analyt Technol Biomed Life Sci. 2003; 795:377–382.

Ciccolini J, Mercier C, Blachon MF, Favre R, Durand A, Lacarelle B. A simple and rapid high-performance liquid chromatographic (HPLC) method for 5-fluorouracil (5-FU) assay in plasma and possible detection of patients with impaired dihydropyrimidine dehydrogenase (DPD) activity. J Clin Pharm Ther. 2004; 29:307–315. [PubMed: 15271097]

Coe RA, Earl RA, Johnson TC, Lee JW. Determination of 5-fluorouracil in human plasma by a simple and sensitive reversed-phase HPLC method. J Pharm Biomed Anal. 1996; 14:1733–1741. [PubMed: 8887721]

Cullum, N.; Meng, CK.; Zavitsanos, P. Effect of Sample Matrix on Suppression of Ionization in Water Samples Using LC-ESI-MS. Agilent Technologies; Santa Clara, CA: 2004. p. 1-10.

de Bruijn EA, Driessen O, Leeflang P, van Strijen E, van den Bosch N, Hermans J. Interactions of methotrexate and cyclophosphamide with the pharmacokinetics of 5-fluorouracil in an animal model. Cancer Treat Rep. 1987; 71:1267–1269. [PubMed: 3690535]

den Boer E, Heil SG, van Zelst BD, Meesters RJ, Koch BC, Te Winkel ML, van den Heuvel-Eibrink MM, Luider TM, de Jonge R. A U-HPLC-ESI-MS/ MS-based stable isotope dilution method for the detection and quantitation of methotrexate in plasma. Ther Drug Monit. 2012; 34:432–439. [PubMed: 22689188]

Diasio RB, Harris BE. Clinical pharmacology of 5-fluorouracil. Clin Pharmacokinet. 1989; 16:215–237. [PubMed: 2656050]

Dietrich J, Han R, Yang Y, Mayer-Pröschel M, Noble M. CNS progenitor cells and oligodendrocytes are targets of chemotherapeutic agents in vitro and in vivo. J Biol. 2006; 5:22. [PubMed: 17125495]

Dietrich J, Monje M, Wefel J, Meyers C. Clinical patterns and biological correlates of cognitive dysfunction associated with cancer therapy. Oncologist. 2008; 13:1285–1295. [PubMed: 19019972]

Fahrig L, Brasch H, Iven H. Pharmacokinetics of methotrexate (MTX) and 7-hydroxymethotrexate (7-OH-MTX) in rats and evidence for the metabolism of MTX to 7-OH-MTX. Cancer Chemother Pharmacol. 1989; 23:156–160. [PubMed: 2924373]

Fardell JE, Vardy J, Logge W, Johnston I. Single high dose treatment with methotrexate causes long-lasting cognitive dysfunction in laboratory rodents. Pharmacol Biochem Behav. 2010; 97:333–339. [PubMed: 20828582]

Fernandes DJ, Bertino JR. 5-Fluorouracil-methotrexate synergy: enhancement of 5-fluorodeoxyridylate binding to thymidylate synthase by dihydropteroylpolyglutamates. Proc Natl Acad Sci USA. 1980; 77:5663–5667. [PubMed: 6160578]

Foley JJ, Raffa RB, Walker EA. Effects of chemotherapeutic agents 5-fluorouracil and methotrexate alone and combined in a mouse model of learning and memory. Psychopharmacology (Berlin). 2008; 199:527–538. [PubMed: 18463849]

Guo P, Wang X, Liu L, Belinsky MG, Kruh GD, Gallo JM. Determination of methotrexate and its major metabolite 7-hydroxymethotrexate in mouse plasma and brain tissue by liquid chromatography–tandem mass spectrometry. J Pharm Biomed Anal. 2007; 43:1789–1795. [PubMed: 17289326]

Han R, Yang YM, Dietrich J, Luebke A, Mayer-Pröschel M, Noble M. Systemic 5-fluorouracil treatment causes a syndrome of delayed myelin destruction in the central nervous system. J Biol. 2008; 7:12. [PubMed: 18430259]

Hao X, Cheng G, Zou M, Sun J, Cui F. Organ distribution of 5-fluorouracil loaded gelatine microspheres in mice. Pharmazie. 2004; 59:709–712. [PubMed: 15497754]

Heggie GD, Sommadossi JP, Cross DS, Huster WJ, Diasio RB. Clinical pharmacokinetics of 5-fluorouracil and its metabolites in plasma, urine, and bile. Cancer Res. 1987; 47:2203–2206. [PubMed: 3829006]

Hendel J, Brodthagen H. Entero-hepatic cycling of methotrexate estimated by use of the D-isomer as a reference marker. Eur J Clin Pharmacol. 1984; 26:103–107. [PubMed: 6714281]

Hendel J, Nyfors A. Nonlinear renal elimination kinetics of methotrexate due to saturation of renal tubular reabsorption. Eur J Clin Pharmacol. 1984; 26:121–124. [PubMed: 6714284]

Herrmann R, Manegold C, Schroeder M, Tigges FJ, Bartsch H, Jungi F, Fritze D. Sequential methotrexate and 5-FU in breast cancer resistant to the conventional application of these drugs. Cancer Treat Rep. 1984; 68:1279–1281. [PubMed: 6525599]

Jarugula VR, Boudinot FD. High-performance liquid chromatographic determination of 5-fluorouracil and its prodrugs, tegafur and 4-deoxy-5-fluorouracil, in rat plasma. J Chromatogr B Biomed Sci Appl. 1996; 677:199–203.

Jarugula VR, Lam SS, Boudinot FD. Nonlinear pharmacokinetics of 5-fluorouracil in rats. J Pharm Sci. 1997; 86:756–758. [PubMed: 9188061]

Jessome LL, Volmer DA. Ion suppression: a major concern in mass spectrometry. LCGC North America. 2006; 24:8.

Jin Y, Li J, Rong LF, Lü XW, Huang Y, Xu SY. Pharmacokinetics and tissue distribution of 5-fluorouracil encapsulated by galactosylceramide liposomes in mice. Acta Pharmacol Sin. 2005; 26:250–256. [PubMed: 15663907]

Jolivet J, Cowan KH, Curt GA, Clendeninn NJ, Chabner BA. The pharmacology and clinical use of methotrexate. N Engl J Med. 1983; 309:1094–1104. [PubMed: 6353235]

Joulia JM, Pinguet F, Grosse PY, Astre C, Bressolle F. Determination of 5-fluorouracil and its main metabolites in plasma by high-performance liquid chromatography: application to a pharmacokinetic study. J Chromatogr B Biomed Sci Appl. 1997; 692:427–435. [PubMed: 9188833]

Katzir I, Shani J, Wolf W, Chatterjee-Parti S, Berman E. Enhancement of 5-fluorouracil anabolism by methotrexate and trimetrexate in two rat solid tumor models, Walker 256 carcinosarcoma and Novikoff hepatoma, as evaluated by 19F-magnetic resonance spectroscopy. Cancer Invest. 2000; 18:20–27. [PubMed: 10701363]

King R, Bonfiglio R, Fernandez-Metzler C, Miller-Stein C, Olah T. Mechanistic investigation of ionization suppression in electrospray ionization. J Am Soc Mass Spectrom. 2000; 11:942–950. [PubMed: 11073257]

Kong AN, Jusko WJ. Definitions and applications of mean transit and residence times in reference to the two-compartment mammillary plasma clearance model. J Pharm Sci. 1988; 77:157–165. [PubMed: 3361431]

Kosovec JE, Egorin MJ, Gjurich S, Beumer JH. Quantitation of 5-fluorouracil (5-FU) in human plasma by liquid chromatography/electrospray ionization tandem mass spectrometry. Rapid Commun Mass Spectrom. 2008; 22:224–230. [PubMed: 18085512]

Kuan HY, Smith DE, Ensminger WD, Knol JA, DeRemer SJ, Yang Z, Stetson PL. Regional pharmacokinetics of 5-fluorouracil in dogs: role of the liver, gastrointestinal tract, and lungs. Cancer Res. 1998; 58:1688–1694. [PubMed: 9563484]

Li KM, Rivory LP, Clarke SJ. Rapid quantitation of plasma 2′-deoxyuridine by high-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry and its application to pharmacodynamic studies in cancer patients. J Chromatogr B Analyt Technol Biomed Life Sci. 2005; 820:121–130.

Licea-Perez H, Wang S, Bowen C. Development of a sensitive and selective LC-MS/MS method for the determination of alpha-fluoro-beta-alanine, 5-fluorouracil and capecitabine in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci. 2009; 877:1040–1046.

Liu HY, Ding L, Yu Y, Chu Y, Zhu H. Comparison of three derivatization reagents for the simultaneous determination of highly hydrophilic pyrimidine antitumor agents in human plasma by LC-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci. 2012; 893–894:49–56.

Liu K, Zhong D, Zou H, Chen X. Determination of tegafur, 5-fluorouracil, gimeracil and oxonic acid in human plasma using liquid chromatography–tandem mass spectrometry. J Pharm Biomed Anal. 2010; 52:550–556. [PubMed: 20138454]

Lobo ED, Balthasar JP. Pharmacokinetic–pharmacodynamic modeling of methotrexate-induced toxicity in mice. J Pharm Sci. 2003; 92:1654–1664. [PubMed: 12884252]

Loos WJ, de Bruijn P, van Zuylen L, Verweij J, Nooter K, Stoter G, et al. Determination of 5-fluorouracil in microvolumes of human plasma by solvent extraction and high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl. 1999; 735:293–297. [PubMed: 10670744]

Miglioli PA, Businaro V, Manoni F, Berti T. Tissue distribution of methotrexate in rats. A comparison between intravenous injection as bolus or drip infusion. Drugs under experimental and clinical research. 1985; 11:275–279. [PubMed: 3836130]

Moore MJ, Erlichman C, Thiessen JJ, Bunting PS, Hardy R, Kerr I, Soldin S. Variability in the pharmacokinetics of cyclophosphamide, methotrexate and 5-fluorouracil in women receiving adjuvant treatment for breast cancer. Cancer Chemother Pharmacol. 1994; 33:472–476. [PubMed: 8137456]

Olsen EA. The pharmacology of methotrexate. J Am Acad Dermatol. 1991; 25:306–318. [PubMed: 1918470]

Peer CJ, McManus TJ, Hurwitz HI, Petros WP. Development and utilization of a combined LC-UV and LC-MS/MS method for the simultaneous analysis of tegafur and 5-fluorouracil in human plasma to support a phase I clinical study of oral UFT®/leucovorin. J Chromatogr B Analyt Technol Biomed Life Sci. 2012; 898:32–7.

Peters GJ, Lankelma J, Kok RM, Noordhuis P, van Groeningen CJ, van der Wilt CL, et al. Prolonged retention of high concentrations of 5-fluorouracil in human and murine tumors as compared with plasma. Cancer Chemother Pharmacol. 1993; 31:269–276. [PubMed: 8422689]

Pinedo HM, Peters GF. Fluorouracil: biochemistry and pharmacology. J Clin Oncol. 1988; 6:1653–1664. [PubMed: 3049954]

Pisano R, Breda M, Grassi S, James CA. Hydrophilic interaction liquid chromatography–APCI–mass spectrometry determination of 5-fluorouracil in plasma and tissues. J Pharm Biomed Anal. 2005; 38:738–45. [PubMed: 15967302]

Remaud G, Boisdron-Celle M, Morel A, Gamelin A. Sensitive MS/MS-liquid chromatography assay for simultaneous determination of tegafur, 5-fluorouracil and 5-fluorodihydrouracil in plasma. J Chromatogr B Analyt Technol Biomed Life Sci. 2005; 824:153–160.

Sabatini L, Barbieri A, Tosi M, Violante FS. A new high-performance liquid chromatographic/electrospray ionization tandem mass spectrometric method for the simultaneous determination of cyclophosphamide, methotrexate and 5-fluorouracil as markers of surface contamination for occupational exposure monitoring. J Mass Spectrom. 2005; 40:669–674. [PubMed: 15739158]

Sartori T, Murakami Seigi F, Cruz Pinheiro A, de Campos Machado A. Development and validation of a fast RP-HPLC method for determination of methotrexate entrapment efficiency in polymeric nanocapsules. J Chromatogr Sci. 2008; 46:505–509. [PubMed: 18647471]

Shah VP, Midha KK, Dighe S, McGilveray IJ, Skelly JP, Yacobi A, Layloff T, Viswanathan CT, Cook CE, McDowall RD. Analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies. Conference report. Eur J Drug Metab Pharmacokinet. 1991; 16:249–255. [PubMed: 1823867]

Sparreboom A, Loos WJ, Nooter K, Stoter G, Verweij J. Liquid chromatographic analysis and preliminary pharmacokinetics of methotrexate in cancer patients co-treated with docetaxel. J Chromatogr B Biomed Sci Appl. 1999; 735:111–119. [PubMed: 10630896]

van Kuilenburg AB, van Lenthe H, Maring JG, van Gennip AH. Determination of 5-fluorouracil in plasma with HPLC–tandem mass spectrometry. Nucleosides Nucleotides Nucleic Acids. 2006; 25:1257–1260. [PubMed: 17065102]

Wang K, Nano M, Mulligan T, Bush ED, Edom RW. Derivatization of 5-fluorouracil with 4-bromomethyl-7-methoxycoumarin for determination by liquid chromatography–mass spectrometry. J Am Soc Mass Spectrom. 1998; 9:970–976. [PubMed: 9725015]

Wang Z, Zhou Q, Kruh GD, Gallo JM. Dose-dependent disposition of methotrexate in Abcc2 and Abcc3 gene knockout murine models. Drug Metab Dispos. 2011; 39:2155–2161. [PubMed: 21841039]

Yi H, Cho HJ, Cho SM, Lee DG, Abd El-Aty A, Yoon SJ, Bae GW, Nho K, Kim B, Lee CH, Kim JS, Bartlett MG, Shin HC. Pharmacokinetic properties and antitumor efficacy of the 5-fluorouracil loaded PEG-hydrogel. BioMed Central Cancer. 2010; 10:211. [PubMed: 20482808]

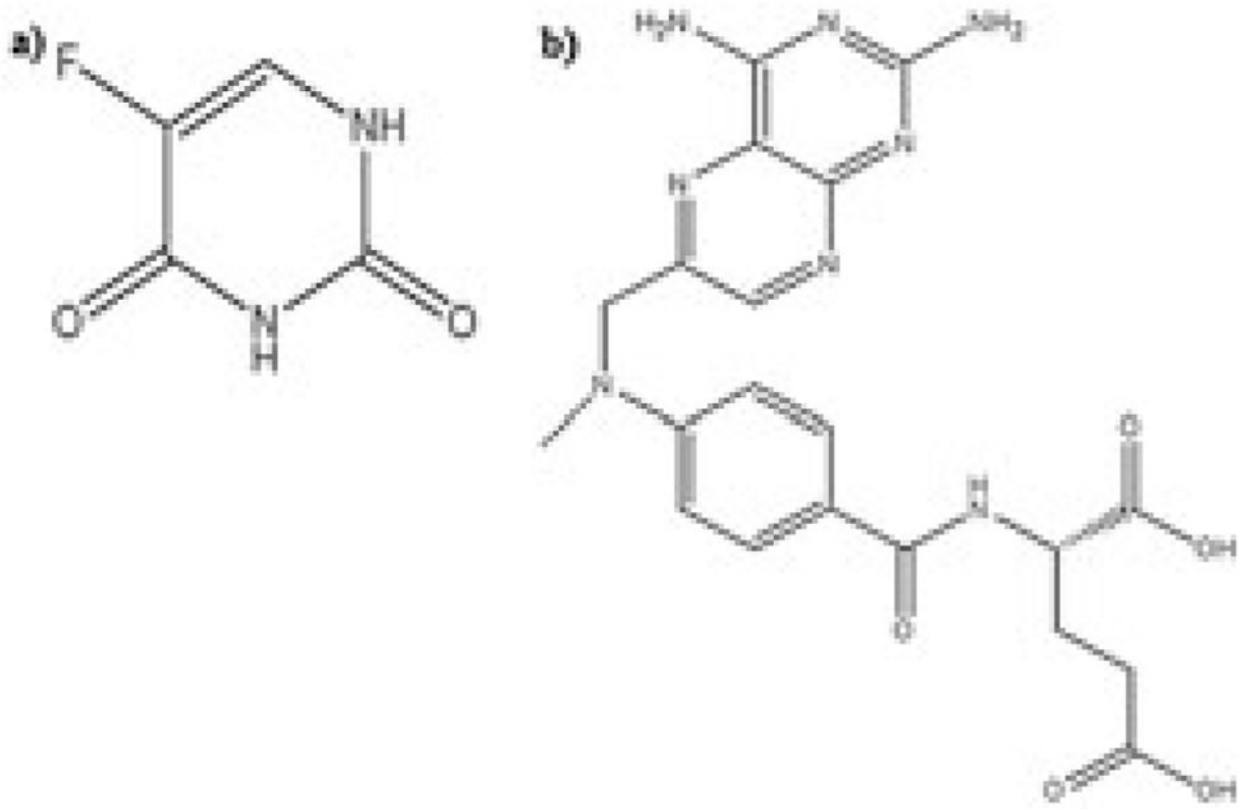

Figure 1.
Chemical structures for (a) 5-fluorouracil (5-FU), molecular weight-130.08 Da; (b) methotrexate (MTX), molecular weight 454.44 Da.

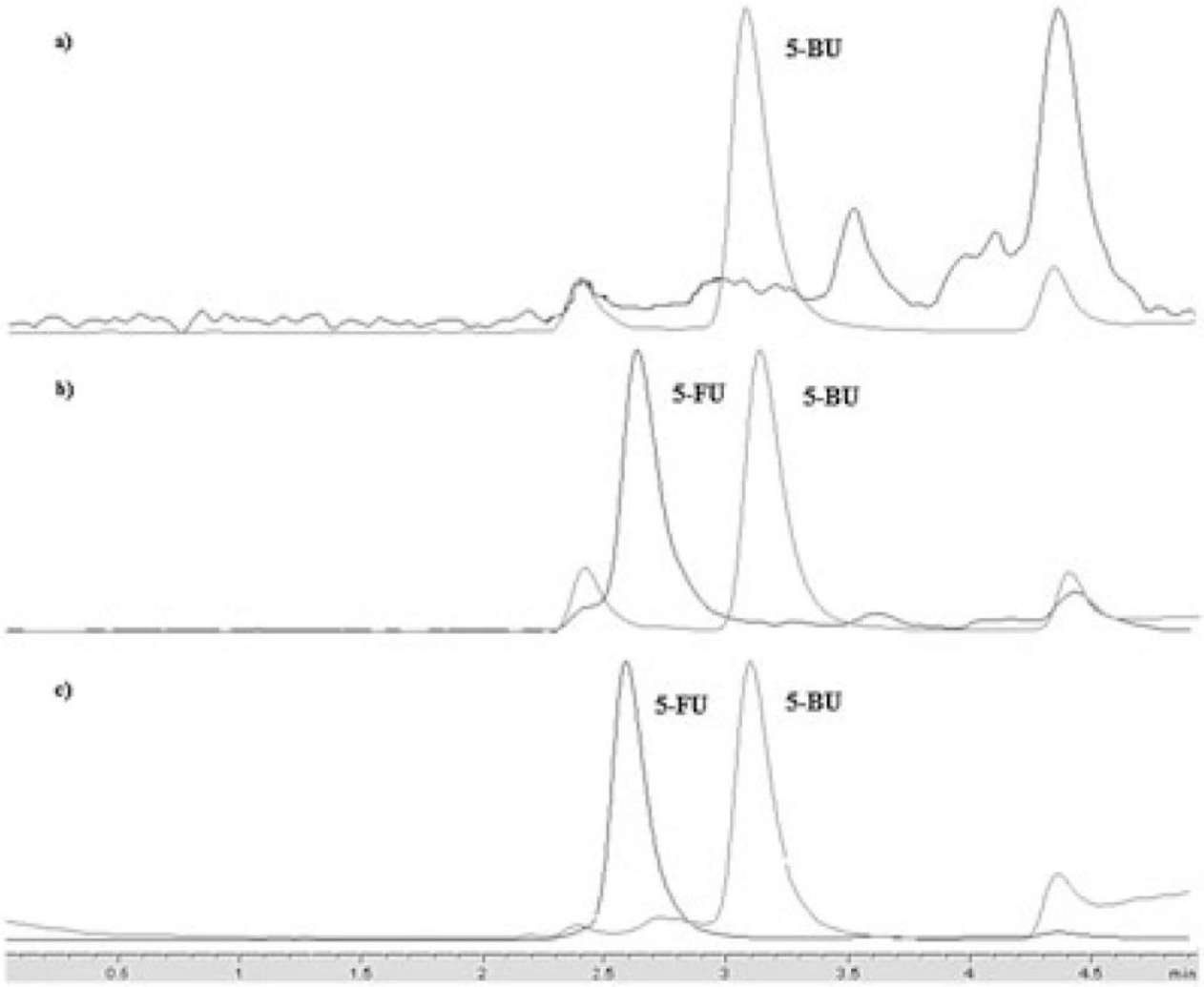

Figure 2.

Representative chromatograms for 5-FU and internal standard 5-bromouracil (5-BU) in (a) blank plasma (only 5-BU is present); (b) plasma spiked with LOQ concentration; and (c) *in vivo* collected sample for 5-FU.

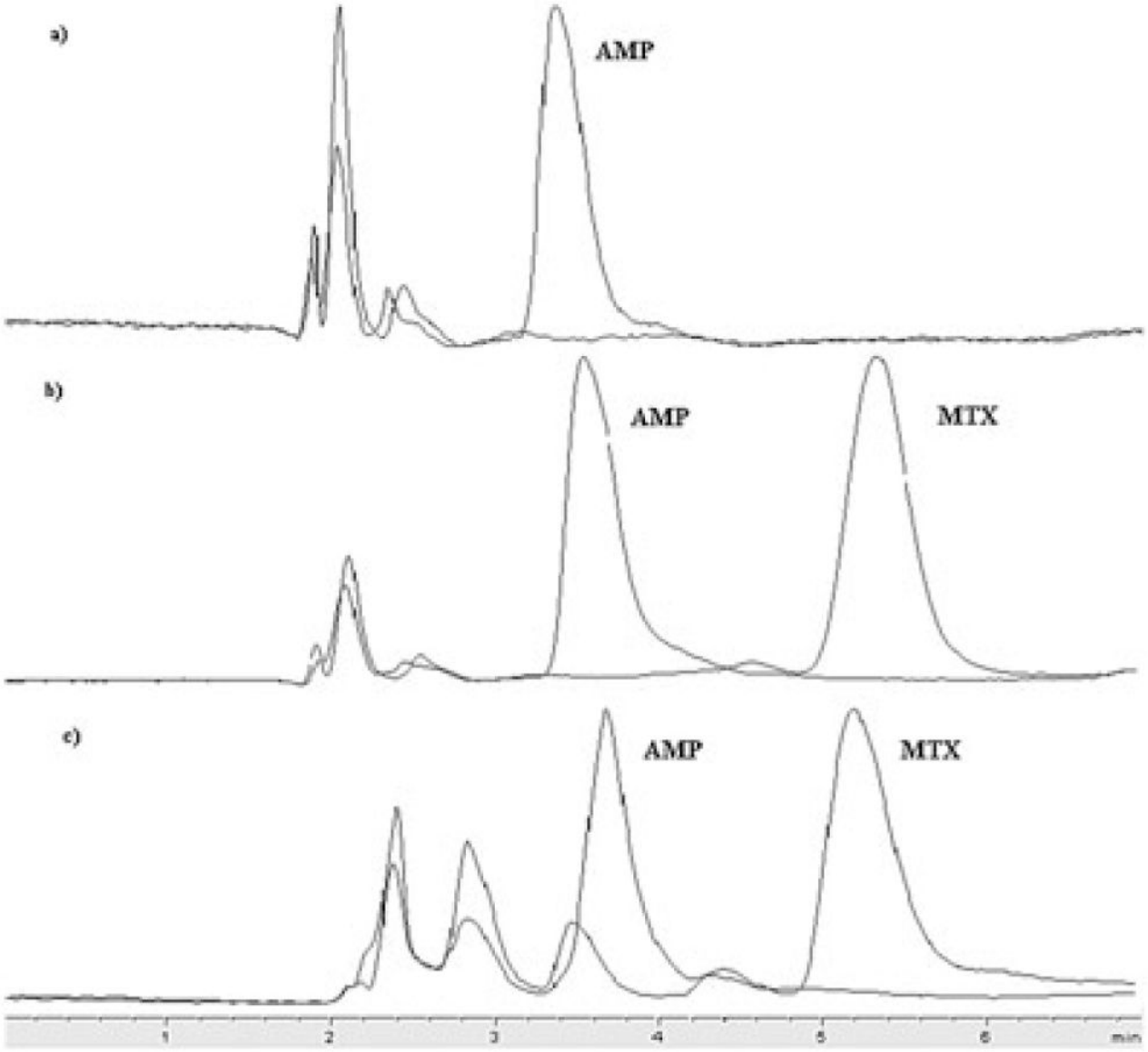

Figure 3.

Representative chromatograms for MTX and internal standard aminopterin (AMP) in (a) blank plasma (only AMP is present); (b) plasma spiked with LOQ concentration; and (c) *in vivo* collected sample for MTX.

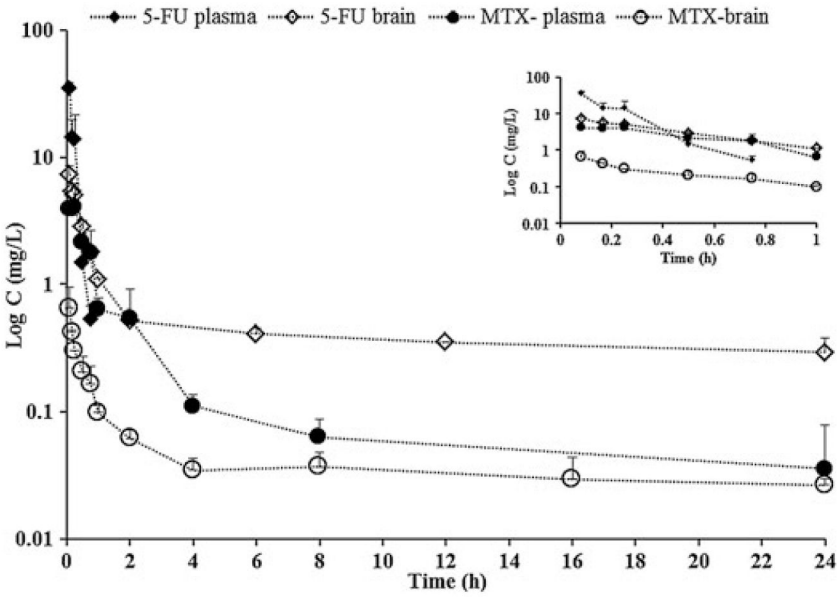

Figure 4.

Concentration–time profiles for 5-FU and MTX in mouse plasma and brain. All values are represented as means ± SD, with $n = 3$. Inset depicts concentration–time profiles for 5-FU and MTX in mouse plasma and brain from 0 to 1 h.

Table 1

Different methods tested for the simultaneous determination of 5-fluorouracil (5-FU) and methotrexate (MTX)

Sample extraction procedures tested [a,b]			
Protein precipitation	**Double protein precipitation**	**Protein precipitation with LLE**	**LLE**
A. Plasma + 3*x*[c] MeOH/3*x* ACN	A. Plasma + ammonium sulfate + MeOH–ACN	A. Plasma + 2*x* perchloric acid + 10*x* EA	A. Plasma + 10*x* dichloromethane
B. Plasma + TCA + GAA	B. Plasma + perchloric acid + MeOH/ACN	B. Plasma + 2*x* ammonium sulfate + 10*x* EA	B. Plasma + 3*x* MeOH–ACN + 10*x* EA
C. Plasma + ammonium sulfate		C. Plasma + 2*x* ammonium sulfate + *n*-propanol- dimethyl ether (8:2)	C. Plasma + 10*x* dichloromethane
D. Plasma + perchloric acid		D. Plasma + 2*x* ammonium sulfate + GAA + 10*x* EA + back extraction with potassium hydroxide (0.5 M)	D. Plasma + 10*x* EA
E. Plasma + 2*x* HCl + 10*x* EA–isopropanol (9:1)		E. Plasma + 3*x* MeOH–ACN + 10*x* EA	
Mobile phases tested (*pH = 4.0*)			
AA (1–20 mM; % v/v AA:MeOH), or AF (1–20mM; % v/v AF:ACN)		40:60	
		50:50	
		60:40	
		70:30	
		90:10	
Reconstitution media tested		50:50 (%v/v) buffer: organic	
		100% organic	
		100% aqueous	
Final method selected based on:		Sample preparation	
Sensitivity (LOQ), short run time, and ease of analysis		Protein precipitation – MTX (A)	
		LLE– 5FU (D)	
		Mobile phase	
		AA (5 mM, pH 4.0)–MeOH, 70:30% v/v	
		Reconstitution media	
		50:50% (v/v) buffer – organic	

Abbreviations: MeOH, methanol; ACN, acetonitrile; TCA, trichloro acetic acid; GAA, glacial acetic acid; LLE, liquid–liquid extraction; EA, ethyl acetate; AA, ammonium acetate; AF, ammonium formate.

[a] Each sample extraction procedure was individually performed with centrifugation speeds 4000, 8000 and 12,000 rpm.

[b] Isocratic elution method was utilized for all combinations.

$^{c}_{x}$, Plasma, brain, urine volume in microliters.

Table 2

Method validation for 5-FU and MTX in mouse plasma, brain and urine

Matrix		Interday			Intraday		
	Theoretical (ng/mL)	Observed (ng/mL)	CV (%)[a]	RE (%)[b]	Observed (ng/mL)	CV (%)[a]	RE (%)[b]
5-Fluorouracil							
Plasma	15.6	17.2 ± 3.3	19.1	9.3	14.8 ± 1.7	11.5	−5.5
	31.3	33.9 ± 4.5	13.3	8.3	31.8 ± 1.9	5.9	1.8
	125	141.9 ± 21	14.8	13.5	139.7 ± 13.8	9.9	11.8
	500	501.2 ± 74.1	14.8	0.2	493.3 ± 31.6	6.4	−1.3
Brain	15.6	15.4 ± 2.4	15.6	−1.2	14.5 ± 1.5	10.1	−6.8
	31.3	29.5 ± 2.5	8.5	−6.0	29.9 ± 2.3	7.7	−4.2
	125	125.8 ± 14.5	11.5	0.7	128.8 ± 17.9	13.9	2.9
	500	514.7 ± 26.2	5.1	2.9	510.9 ± 26.1	5.1	2.1
Urine	78.1	71.4 ± 11	15.6	−9.4	72.6 ± 1.4	19.1	−4.7
	156.3	143.7 ± 16.5	11.5	−8.7	157.2 ± 23	14.6	0.7
	625	585.9 ± 61.5	10.5	−6.7	568.9 ± 33	5.8	−9.9
	2500	2637.5 ± 308.6	11.7	5.2	2537.3 ± 294.3	11.6	1.5
Methotrexate							
Plasma	15.6	13.9 ± 1.5	11.0	−12.6	14.0 ± 1.6	11.6	−11.9
	31.3	36.7 ± 5.5	15.1	11.5	33.8 ± 4.7	13.8	7.6
	125	124.1 ± 14.5	11.7	−0.8	119.2 ± 14.2	11.9	−4.9
	500	505.2 ± 51.5	10.2	1.0	474.4 ± 39	8.2	−5.4
Brain	15.6	15.4 ± 1.0	6.7	1.9	16.9 ± 2.5	14.6	7.6
	31.3	29.5 ± 2.7	9.4	−10.6	30.1 ± 1.4	4.6	−3.9
	125	123.1 ± 8.0	6.5	−1.5	130.0 ± 7.5	5.8	3.8
	500	512.2 ± 6.1	1.2	2.4	503.3 ± 40.3	8.0	0.7
Urine	78.1	71.6 ± 10.7	14.9	−9.0	78.7 ± 14.2	18.1	0.8
	156.3	167.8 ± 22.7	13.5	6.9	142.7 ± 20	14.0	−9.4
	625	565.3 ± 55.4	9.8	−10.1	629.6 ± 98.2	15.6	0.7
	2500	2513.3 ± 83	3.3	0.51	2446.4 ± 203.1	8.3	−2.1

[a]Precision (CV %) = (standard deviation/mean) × 100.

[b] Bias (RE %) = [(mean observed − theoretical value)/theoretical value] × 100.

Concentrations measured are reported as means ± SD; $n = 5$ for interday and $n = 6$ for intraday.

Table 3

Extraction efficiency and matrix effect for 5-FU and MTX in mouse plasma, brain and urine

Biological matrix	Added (ng/mL)	Extraction efficiency[a] (% mean, *n* = 3)		Matrix effect[b] (% mean; *n* = 3)	
		MTX	5-FU	MTX	5-FU
Plasma	31.3	53.5 ± 15.8	65.4 ± 20.2	42.3 ± 8.1	8.9 ± 10.4
	125	58.1 ± 20.9	53.3 ± 29.0	31.3 ± 7.7	18.6 ± 23.5
	500	36.8 ± 4.9	97.9 ± 20.2	16.5 ± 8.0	78.6 ± 0.9
Brain	31.3	24.1 ± 4.7	114.8 ± 16.9	17.0 ± 6.9	77.2 ± 5.3
	125	43.5 ± 9.2	108.4 ± 13.9	21.0 ± 11.1	45.6 ± 9.1
	500	44.3 ± 4.6	107.4 ± 13.1	20.6 ± 15.0	69.8 ± 1.2
Urine	31.3	31.7 ± 4.6	114.2 ± 1.5	37.0 ± 7.3	75.2 ± 20.2
	125	43.5 ± 11.2	76.1 ± 19.2	21.0 ± 17.0	35.2 ± 5.0
	500	44.2 ± 5.1	83.5 ± 48.2	20.6 ± 10.0	49.5 ± 16.7

All values are represented as means ± SD; *n* = 3.

[a] Extraction efficiency = (set1/set2) × 100.

[b] Matrix effect = 100 − [(set2/set3) × 100]. Sets 1–3 are defined in the Experimental section in the text.

Table 4

Freeze–thaw stability for 5-FU and MTX in mouse plasma and brain samples

Biological matrix		RE%[a] (4 h)		RE% (22 h)	
	Theoretical (ng/mL)	MTX	5-FU	MTX	5-FU
Plasma	31.3	−4.0	−11.2	−2.0	−26.6
	125	−16.2	−25.1	3.0	−5.6
	500	−0.7	−10.5	−6.4	−3.0
Brain	31.3	15.9	−13.1	7.2	21.3
	125	21.2	−8.4	1.5	−7.1
	500	−15.0	−0.3	−3.9	−1.3

[a]Percentage relative error (RE %) = [(mean observed − theoretical value)/theoretical value] × 100.

Table 5

Noncompartmental pharmacokinetic parameters for single i.v. bolus dose of 75 mg/kg 5-FU or i.v. bolus dose of 32 mg/kg MTX

PK parameters (units)	5 FU (75 mg/kg)	MTX (32 mg/kg)
$AUC_{plasma\ (0–\infty)}$ (min mg/L)	631.0 ± 78.9	300.1 ± 65
C_{max} (mg/L)	34.8 ± 4.1	4.3 ± 0.05
T_{max} (min)	5	5
CL_s (L/min/kg)	0.12 ± 0.03	0.11 ± 0.03
$AUC_{brain\ (0–24)}$ (min mg/L)	748.8 ± 122	66.3 ± 12.9
V_{ss} (L/kg)	0.9 ± 03	23 ± 5.1
$t_{1/2}$ (min)	5.2 ± 0.9	152.5 ± 63.7
f_e	0.08 ± 0.01	0.2 ± 0.06