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Liquid chromatography–tandem mass spectrometry method for quantification of thymidine kinase activity in human serum by monitoring the conversion of 3′-deoxy-3′-fluorothymidine to 3′-deoxy-3′-fluorothymidine monophosphate

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ABSTRACT

Thymidine kinase 1 (TK1) is an enzyme involved in DNA synthesis whose activity in serum is indicative of tumor proliferation and the severity of blood malignancies. 3′-deoxy-3′-fluorothymidine (FLT), a specific exogenous substrate for TK1, is phosphorylated by TK1 in the presence of a phosphorylating buffer, therefore the conversion of FLT to 3′-deoxy-3′-fluorothymidine monophosphate (FLT-MP) can be measured to assess serum TK1 activity. Here we describe a liquid chromatography–MS/MS (LC–MS/MS) method for quantification of FLT and FLT-MP from serum using protein precipitation and column switching followed by detection on an Applied Biosystems SCIEX API 4000 QTrap mass spectrometer. The method was linear over the range of 0.5–500 ng/mL for FLT and 2.5–2000 ng/mL for FLT-MP with a mean correlation coefficient of 0.9964 and 0.9935 for FLT and FLT-MP, respectively. The lower limit of quantification was 0.5 ng/mL for FLT and 2.5 ng/mL for FLT-MP. Intra-assay accuracy and inter-assay accuracy was within ±12% for both FLT and FLT-MP. Intra-assay precision was 2.8% to 7.7% for FLT and 3.3% to 5.8% for FLT-MP. Inter-assay precision was 4.6% to 14.9% for FLT and 4.9% to 14.6% for FLT-MP. Serum TK1 activity was measured in serum from hepatocellular carcinoma patients and age-matched controls under standardized conditions. Elevated TK1 activity was detected in 26.3% of hepatocellular carcinoma samples compared to controls. This method provides a robust alternative to radiometric and immunochemical assays of serum TK1 activity.

1. Introduction

Thymidine kinase 1 (TK1, ATP; thymidine 5′-phosphotransferase; EC.2.7.1.21) is a key cellular enzyme in DNA synthesis which catalyzes the one step pyrimidine salvage pathway [1]. Specifically, TK1 catalyzes the transfer of terminal phosphate from ATP to the 5′ hydroxyl group of deoxythymidine (dThd) to produce deoxythymidine monophosphate (dTMP). Increased activity of serum or plasma TK1 has been reported in diseases involving DNA metabolism, e.g., viral infections [2,3], vitamin B_{12} deficiency [3] and in a variety of malignant diseases including acute and chronic leukemia [4], Hodgkin's disease [5], non-Hodgkin's lymphoma [6,7], lung cancer [6] and ovarian cancer [6,8]. TK1 also phosphorylates derivatives of thymidine or deoxyuridine (dUrd). In particular, TK1 can phosphorylate nucleotide analogs with modifications at the 5′-position of the pyrimidine ring, and the 3′-position of the ribose including 5-fluoro-2′-dideoxythymidine and 3′-azido-3′-deoxythymidine (Zidovudine) [9,10]. These nucleotide analogs can therefore be used to monitor the activity of TK1 [3,6,11]. For example, ^{18}F-3′-deoxy-3′-fluorothymidine (^{18}F-FLT), has been used to monitor tumor proliferation via positron emission tomography [12–14]. Phosphorylation of FLT by TK1 leads to cellular trapping and accumulation of FLT-monophosphate (FLT-MP) without incorporation into DNA. Since FLT has low affinity for TK2 in comparison with FLT-MP, it acts as a selective substrate to monitor TK1 activity [10,15].

Serum TK1 activity can reflect tumor burden and proliferation, and is most commonly measured using a commercially available thymidine kinase radioenzymatic assay (TK-REA), which uses ^{125}I-deoxyuridine as a substrate to measure thymidine kinase 1 activity [3]. This assay is time-consuming and requires radioactive waste management. A competitive enzyme-linked immunosorbent assay (ELISA) has also been used for measuring serum TK1 activity for measuring phosphorylation of the selective TK1 substrate 3′-azido-2′-deoxythymidine (AZT) [11]. Alternatively, 5-bromodeoxyuridine (BrdU) incorporation into DNA can be monitored by ELISA [16]. After phosphorylation by TK1,

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bromodeoxyuridine monophosphate (BrdUMP) is processed to bromodeoxyuridine triphosphate (BrdUTP) by yeast enzymes, and the BrdUTP is immobilized by incorporation into an immobilized DNA strand using a recombinant reverse transcriptase. Further, the amount of BrdUTP incorporated in DNA is estimated using an ELISA. This assay is more sensitive than the assay using AZT as a substrate [16]. However, complex sample processing, reagent expense, and selectivity issues associated with ELISA methods are limitations to its application.

Quantification of nucleotide analogs can be performed using high performance liquid chromatography (HPLC) with ultraviolet or fluorescence detection [17,18]. In the last decade, mass spectrometric (MS) detection has increasingly been used for quantification of nucleoside and nucleotide analogs [19,20]. Mass spectrometry allows extremely specific and sensitive quantification of nucleotide analogs from biological matrices including serum. Thus, LC–MS/MS can be used as a tool to measure the conversion of FLT to FLT-MP in serum.

In our previous work, a method was developed to quantify the intracellular conversion of FLT to FLT-MP in cell lysates using LC–MS/MS [21]. Separation of the analytes was achieved using C18 column and detection using Applied Biosystems SCIEX API 4000 QTrap mass spectrometer. This method was able to monitor proliferating cell TK1 activity in as few as 500 cells per well in LNCaP prostate cancer cells. In the present report, we describe the development and analytical validation of a suitable non-isotopic, non-immunologically based assay for quantitative monitoring of FLT to FLT-MP in human serum. This method uses a labeled isotope internal standard, and column trapping to improve assay ruggedness.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade acetonitrile and methanol were purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA). 3′-Deoxy-3′-fluorothymidine was purchased from Sigma–Aldrich (St. Louis, MO, USA). 3′-Deoxy-3′-fluorothymidine-5′-monophosphate disodium salt (FLT-MP), 3′-deoxy-3′-fluorothymidine-d3 (FLT-d3) and 3′-deoxy-3′-fluorothymidine-5′-monophosphate-d3 (FLT-MP-d3) were purchased from Toronto Research Chemicals Inc. (TRC, Toronto, ON, Canada). Citric acid, formic acid, sodium acetate, adenosine 5′-triphosphate (ATP), uridine 5′-monophosphate (UMP), magnesium chloride ($MgCl_2$) and dithioerythritol (DTE) were obtained from Sigma–Aldrich (St. Louis, MO, USA). High purity water was obtained in-house using a NANOpure Diamond Life Science ultrapure water System from Barnstead International (Dubuque, IA, USA). Nitrogen was obtained from a Parker Balston Tri Gas Generator LCMS-5000 (Haverhill, MA, USA). Microcentrifuge tubes (1.5 mL) and disposable glass centrifuge (10 mL) were purchased from VWR International (Westchester, PA, USA). Human serum was obtained from Biochemed Services, Inc. (Winchester, VA, USA).

2.2. Instruments and HPLC conditions

High performance liquid chromatography (HPLC) separations were performed using the following equipment: Shimadzu system controller SCL-10A VP, pumps LC-10AD VP, solvent degasser DGU14A (Shimadzu, Kyoto, Japan) and a Waters Acquity UPLC® system (Waters Corporation, Milford, USA). Phenomenex Security Guard column (Gemini C18, 4 mm × 2.0 mm, 5 μm) from Phenomenex (Torrance, CA, USA) was used as the loading column and an Aquasil C18 column (100 mm × 2.1 mm I.D., 5 μm) from Thermo Scientific (Waltham, MA, USA) was used as the analytical column. The analytical column was maintained at 40 °C.

Sample loading was achieved using Waters Acquity UPLC® system. Pumps A and B delivered loading mobile phase A (0.1% formic acid) and loading mobile phase B (0.1% formic acid in acetonitrile), respectively. Sample loading was carried out with 100% loading mobile phase A maintained at a flow rate of 0.300 mL/min. Following an initial loading time of 1.5 min, the diverter valve was switched to position B and the elution initiated. A 10-port Cheminert switching valve and a microelectric actuator obtained from Valco Instruments Co. Inc. (Houston, TX, USA) was used to divert flow to the analytical column in position B. After a running time of 5 min, the diverter valve position was switched back to allow flushing of the loading column. The flushing of the loading column was carried out at 0.400 mL/min with 50% loading mobile phase B. After flushing for 1 min, the loading mobile phase composition was changed back to 100% loading mobile phase A pumped at a flow rate of 0.300 mL/min. Elution was carried out under gradient conditions using two Shimadzu pumps which were operated with a Shimadzu system controller. The elution mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The flow rate was set to 0.300 mL/min. Gradient conditions were as follows: 0.0–2.0 min, isocratic 5% B; 2.00–4.0 min, linear from 5% to 75% B; 4.1–7 min, isocratic 5% B. The total running time was 7 min and the injection volume was 25 μL. The column switching LC–MS/MS design is shown in Fig. 1.

2.3. Mass spectrometry conditions

Mass spectra were obtained using an Applied Biosystems SCIEX API 4000 QTrap mass spectrometer operated in positive electrospray ionization mode (ESI). Tuning and optimization of the mass spectrometer parameters were performed for the analytes and internal standard (IS, see below) by direct infusion of a 1 μg/mL standard solution at a flow rate of 12 μL/min. The multiple reaction monitoring (MRM) transitions, declustering potential (DP) entrance potential, collision energy (CE) and collision cell exit potential (CXP) for all the compounds are listed in Table 1. The mass spectrometric parameters were as follows: ion source temperature (TEM = 450 °C), ion transfer voltage (IS = 5500 V), collision gas (CAD = high), curtain gas (CUR = 20), ion source gas 1 (GS1 = 55) and ion source gas 2 (GS2 = 45). The units for gases are arbitrary. The data were acquired with Analyst software, Version 1.5.

2.4. Preparation of stock solutions, standards and quality control samples

Stock solutions were prepared by dissolving FLT and FLT-MP in methanol to yield 12.5 and 50 μg/mL solutions, respectively. Intermediate stock solutions were prepared by further diluting the stock solution in methanol to prepare spiked serum samples. Internal standard stock solutions were prepared by dissolving FLT-d3 and FLT-MP-d3 in methanol to yield a concentration of 500 μg/mL. A working IS solution (final concentration of 200 ng/mL) was subsequently prepared by diluting the stock solutions of each compound in methanol. Stock solutions, QC solutions and IS solutions were stored at −20 °C.

2.5. Preparation of calibration standards and quality control samples in human serum

Calibration standards and quality control samples were freshly prepared daily by spiking working solutions and quality control solutions in pooled human serum. The concentration range of the calibration standards was FLT (0.500–500 ng/mL) and FLT-MP (2.5–2000 ng/mL). The quality control samples (lower limit of

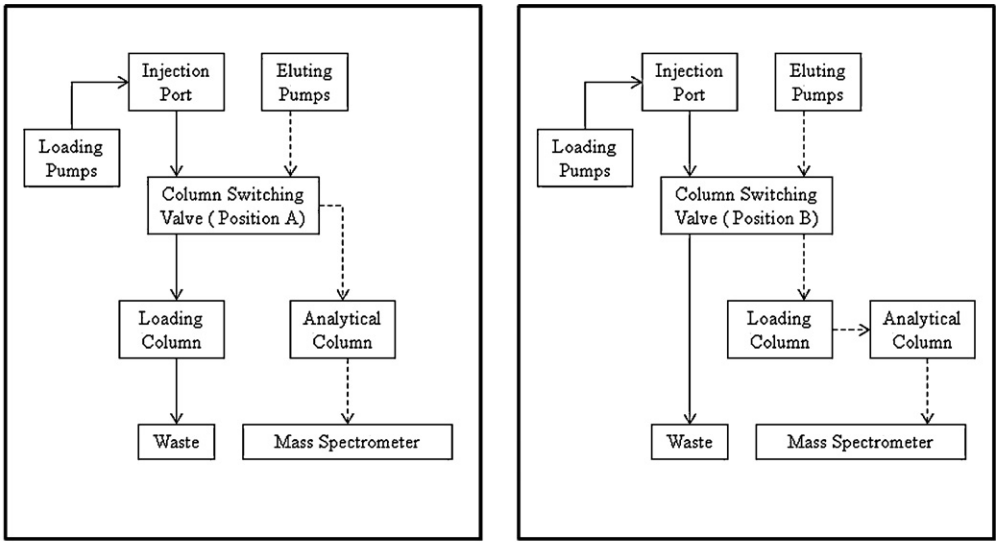

Fig. 1. Column switching LC–MS/MS design.

quantification (LLOQ), low quality control (LQC), medium quality control (MQC), high quality control (HQC) and dilution quality control) had the following concentrations: FLT (0.50, 2.00, 40, 375 and 2000 ng/mL) and FLT-MP (2.50, 6.00, 60, 1800 and 5000 ng/mL).

2.6. Preparation of phosphorylation buffer and reconstitution solution

Phosphorylation buffer was prepared fresh prior to sample analysis. The buffer comprised 0.1 M sodium acetate, 7.8 mM ATP, 2 mM UMP, 12 mM DTE and 30 mM $MgCl_2$ in HPLC grade water. Reconstitution solution was prepared by dissolving 19.2 mg of citric acid and 37.2 mg of EDTA in 100 mL HPLC grade water to obtain a mixture of 1 mM Citric acid and 0.5 mM EDTA. This was used for reconstituting the samples prior to injection on to LC–MS/MS system.

2.7. Sample preparation

A 50 μL aliquot of serum (samples, calibration and quality control samples) was added to a 1.5 mL microcentrifuge tube containing 50 μL of phosphorylation buffer solution. 10 μL of freshly prepared FLT (10 μg/mL), in phosphate buffered saline (PBS), was transferred to the microcentrifuge tubes containing serum samples, whereas 10 μL of PBS alone was transferred to the microcentrifuge tubes containing calibration standards and quality controls. The samples were incubated for 2 h at 37 °C in Labline Orbit Shaker Bath model 3540 (Melrose Park, IL, USA) at 125 rpm. The reaction was terminated by addition of 300 μL of methanol to each tube. The calibration standards and quality control samples were not incubated; instead the reaction was terminated immediately by addition of 300 μL of methanol to each tube. To each tube, 50 μL of internal standard was added and the tube was vortex mixed for 2 min and centrifuged for 10 min at 4 °C/10,000 rpm using an Eppendorf centrifuge model 5805 (Hamburg, Germany). Approximately, 400 μL of supernatant was transferred into a 10 mL disposable glass centrifuge tube and evaporated to dryness under nitrogen gas at 50 °C for ~15 min. The samples were reconstituted with 50 μL of a reconstitution solution. The reconstituted samples were transferred to a 1 mL 96-well plate and analyzed by LC–MS/MS.

2.8. Matrix effects

In order to evaluate matrix effects, a post-column infusion study was conducted. A 100 ng/mL solution (FLT and FLT-MP) in methanol was prepared and continuously infused at 20 μL/min post-HPLC column directly into the mass spectrometer using a "tee" connection. After stabilization of the baseline, a blank sample was injected and the profiles were investigated for suppression or enhancement at the retention time of FLT and FLT-MP.

2.9. Validation procedures

Validation of the assay was performed according to the FDA guidelines for Bioanalytical Method Validation [22]. Validations runs containing duplicate calibration standards, blank samples, and blank sample spiked with internal standard and replicates of QC samples were run on three separate days.

2.9.1. Selectivity

Human serum samples from six different sources were analyzed in duplicate along with spiked LLOQ serum calibration standards to evaluate selectivity of the analytical method. Each individual lot was extracted according to the sample preparation procedure for calibration standards and quality control samples given above.

Table 1
Mass spectrometer parameters and approximate retention times (Tr).

Analyte	~t_r (min)	Multiple reaction monitoring transitions (parent ion → product ion)	DP (V)	EP (V)	CE (V)	CXP (V)
FLT	3.95	245.1 → 127.1	40	10	20	10
FLT-d3	3.95	248.0 → 130.2	40	10	20	10
FLT-MP	3.53	325.3 → 81.2	50	10	28	10
FLT-MP-d3	3.53	328.1 → 81.2	50	10	28	10

Table 2
Inter-assay and Intra-assay precision and accuracy for FLT and FLT-MP.

	Nominal concentration (ng/mL)			
	2.00	40.0	375	2000
Observed FLT concentration (ng/mL)				
Inter-assay mean ± SD	2.0 ± 0.19	40 ± 5.9	377 ± 39	1830 ± 84
Inter-assay precision (%RSD)	9.4	14.9	10.4	4.6
Inter-assay accuracy (%DFN)	−0.2	−1.1	0.4	−8.5
Intra-assay mean ± SD (n = 6)	2.1 ± 0.16	45 ± 2.6	378 ± 14	1797 ± 50
Intra-assay precision (%RSD)	7.7	5.9	3.6	2.8
Intra-assay accuracy (%DFN)	3.5	11.6	0.9	−10.2
	Nominal concentration (ng/mL)			
	6.00	60.0	1800	5000
Observed FLT-MP concentration (ng/mL)				
Inter-assay mean ± SD	6.6 ± 0.96	65 ± 5.7	1939 ± 95	5159 ± 342
Inter-assay precision (%RSD)	14.6	8.8	4.9	6.6
Inter-assay accuracy (%DFN)	9.4	7.8	7.7	3.2
Intra-assay mean ± SD (n = 6)	6.7 ± 0.30	66 ± 2.5	1958 ± 64	5275 ± 304
Intra-assay precision (%RSD)	4.4	3.7	3.3	5.8
Intra-assay accuracy (%DFN)	11.8	10.4	8.8	5.5

SD, standard deviation; %DFN, percent deviation from nominal value; %RSD, percent relative standard deviation.
Note: Samples were diluted 10-fold for the FLT (2000 ng/mL) and FLT-MP (5000 ng/mL) controls.

Selectivity requirements were that any peak area co-eluting at the retention time of analytes (FLT/FLT-MP) must be less than 20% of the peak area of the average of LLOQ samples for all six lots of blank serum samples. Additionally, any peak area co-eluting at the retention time of internal standards (FLT-d3 and FLT-MP-d3) must be less than 5% of the average peak area of the internal standard concentration for all six lots of blank serum samples.

2.9.2. Linearity and LLOQ

Eleven calibration standards were extracted in duplicate and analyzed in 3 independent runs. Calibration curves were fitted using linear regression of the ratio of the peak area response of the analyte and the internal standard versus concentration. A weighting factor of $1/x^2$ was used for both the analytes (FLT and FLT-MP). The acceptance criteria followed FDA guidelines for bioanalytical method validation [22].

2.9.3. Accuracy and precision

Accuracy and precision were determined from QC samples (LQC, MQC, HQC, dilution QC) in three independent runs. A criterion of ±15% of the nominal concentration was used to assess accuracy and precision was expressed as %RSD, which should not exceed ±15%. Intra-assay precision and accuracy were determined from 6 replicates of each QC sample on a single assay. Inter-assay precision and accuracy were determined by analyzing three different validation runs.

2.9.4. Recovery and carryover

Analyte recovery of the extraction procedure was determined by comparing peak areas. Blank serum was spiked with FLT and FLT-MP at two levels (LQC and HQC). These samples were compared to samples spiked after extraction with the same final concentrations of FLT and FLT-MP to compensate for variations in instrument response. The criterion for acceptance in this recovery experiment was that recovery was consistent over the two QC levels. Carry over was assessed by injecting LQC immediately after each of the highest calibration standards in an analytical run. The acceptance criterion for this experiment was that the LQC must be accurate to within 15% of the nominal concentrations.

2.9.5. Stability studies

Post-preparative stability was assessed using blank serum samples spiked with FLT and FLT-MP at two levels LQC and HQC. These quality control samples were processed and maintained at 5 °C in the autosampler. After 48 h, these samples were analyzed against a freshly spiked calibration standard curve. A criterion of ±20% of the nominal concentration was used to assess for 48 h post-preparative stability. Additionally, the intermediate stability of samples, i.e., supernatant, was assessed. Spiked serum LQC and HQC samples of FLT and FLT-MP were processed as described above. The supernatant (400 μL), obtained after protein precipitation and vortex mixing, was transferred to a 10 mL disposable glass centrifuge tube, then stored at 4 °C. After 48 h, the supernatant was processed and analyzed against a freshly spiked calibration standard curve. A criterion of ±20% of the nominal concentration was used for intermediate stability.

2.9.6. Precision of the FLT phosphorylation reaction

In order to assess the precision of the phosphorylation procedure, a serum sample having high TK1 activity (serum H) was

Table 3
Precision of the FLT phosphorylation reaction.

	Enzyme level-1	Enzyme level-2	Enzyme level-3	Enzyme level-4	Enzyme level-5	Enzyme level-6
TK1 activity units[a]	1.00	0.50	0.20	0.10	0.04	0.00
Average concentration of FLT-MP (ng/mL)[b]	965.33	599.80	245.80	131.83	58.05	5.09
SD	34.915	60.726	32.980	14.662	4.972	1.273
%CV	3.62	10.12	13.42	11.12	8.56	25.03

[a] The serum sample with high TK1 activity (serum H) was defined arbitrarily to have an activity equivalent to 1 while the serum sample having low TK1 activity (serum L) was defined as an activity equivalent to 0. Activity units for the six levels were calculated based on the ratio of the activity of serum H to serum L.
[b] The FLT-MP concentrations were generated under standardized conditions of 2 h incubation at 37 °C.

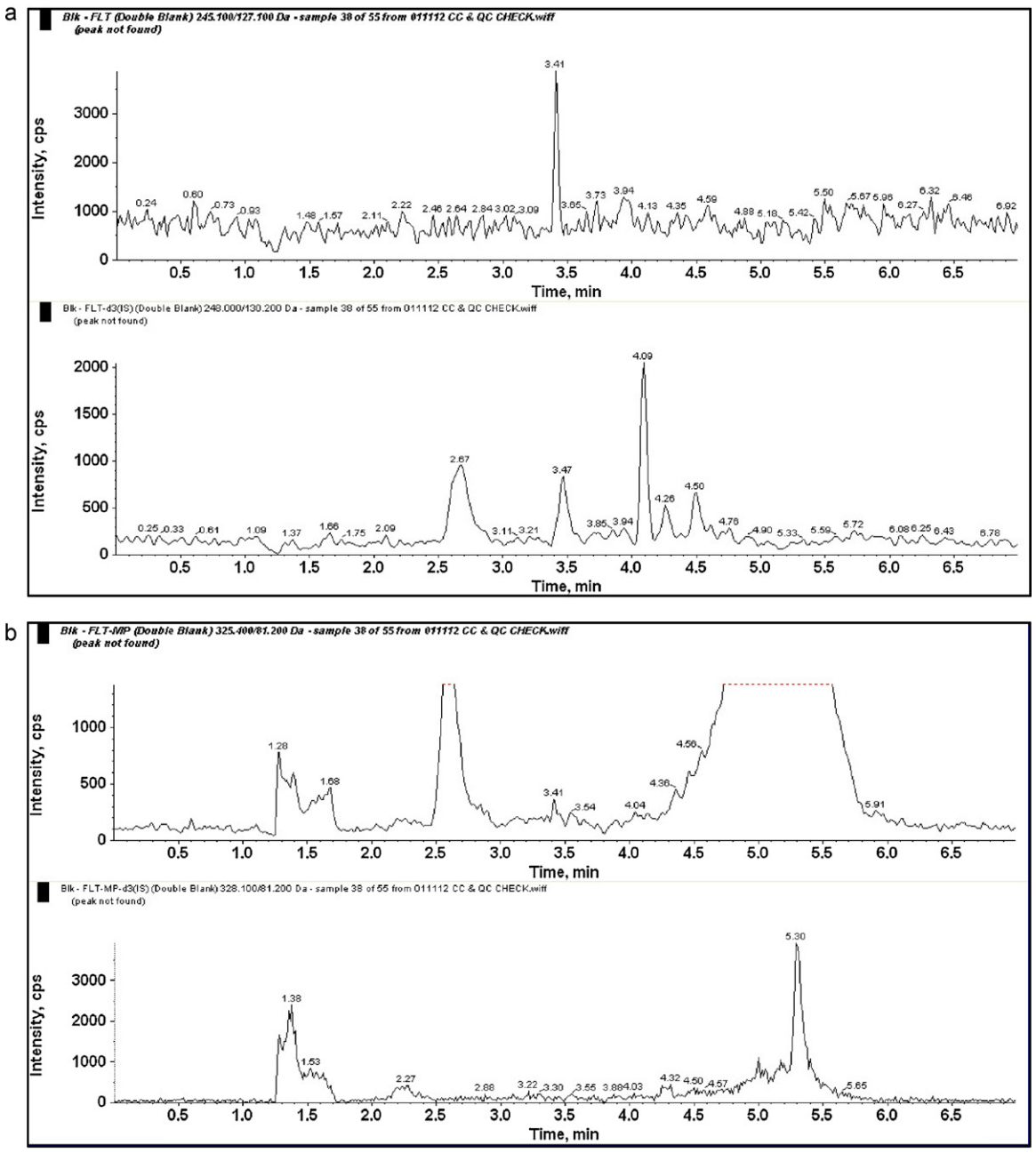

Fig. 2. (a) Representative chromatogram of blank (FLT and FLT-d3). (b) Representative chromatogram of blank (FLT-MP and FLT-MP-d3).

diluted with a serum sample having low TK1 activity (serum L) to obtain six different control concentrations as follows: (1) undiluted serum H, (2) serum H diluted 2 times with serum L, (3) serum H diluted 5 times with serum L, (4) serum H diluted 10 times with serum L, (5) serum H diluted 25 times with serum L and (6) undiluted serum L. Six replicates were analyzed and assay precision was expressed as %RSD for each control concentration. The serum sample with high TK1 activity (serum H) was defined arbitrarily to have an activity equivalent to 1 while the serum sample having low TK1 activity (serum L) was defined as an activity equivalent to 0. Activity units for the six levels were calculated based on the ratio of the activity of serum H to serum L. These activity units were used to correlate TK1 with the amount of FLT-MP generated at the end of 2 h incubation at 37 °C.

2.10. Application of the validated method

The validated method was used for determination of TK1 activity in 19 serum samples obtained from hepatocellular carcinoma patients and 40 healthy, age-matched controls (20 male and 20 female; Bioreclamation, Inc., Westbury, NY, USA).

3. Results and discussion

This serum TK1 activity assay monitors the phosphorylation of FLT, a thymidine analog and selective TK1 substrate, to its metabolite FLT-MP. The reaction is performed at 37 °C in a 0.1 M sodium acetate phosphorylation buffer supplemented with

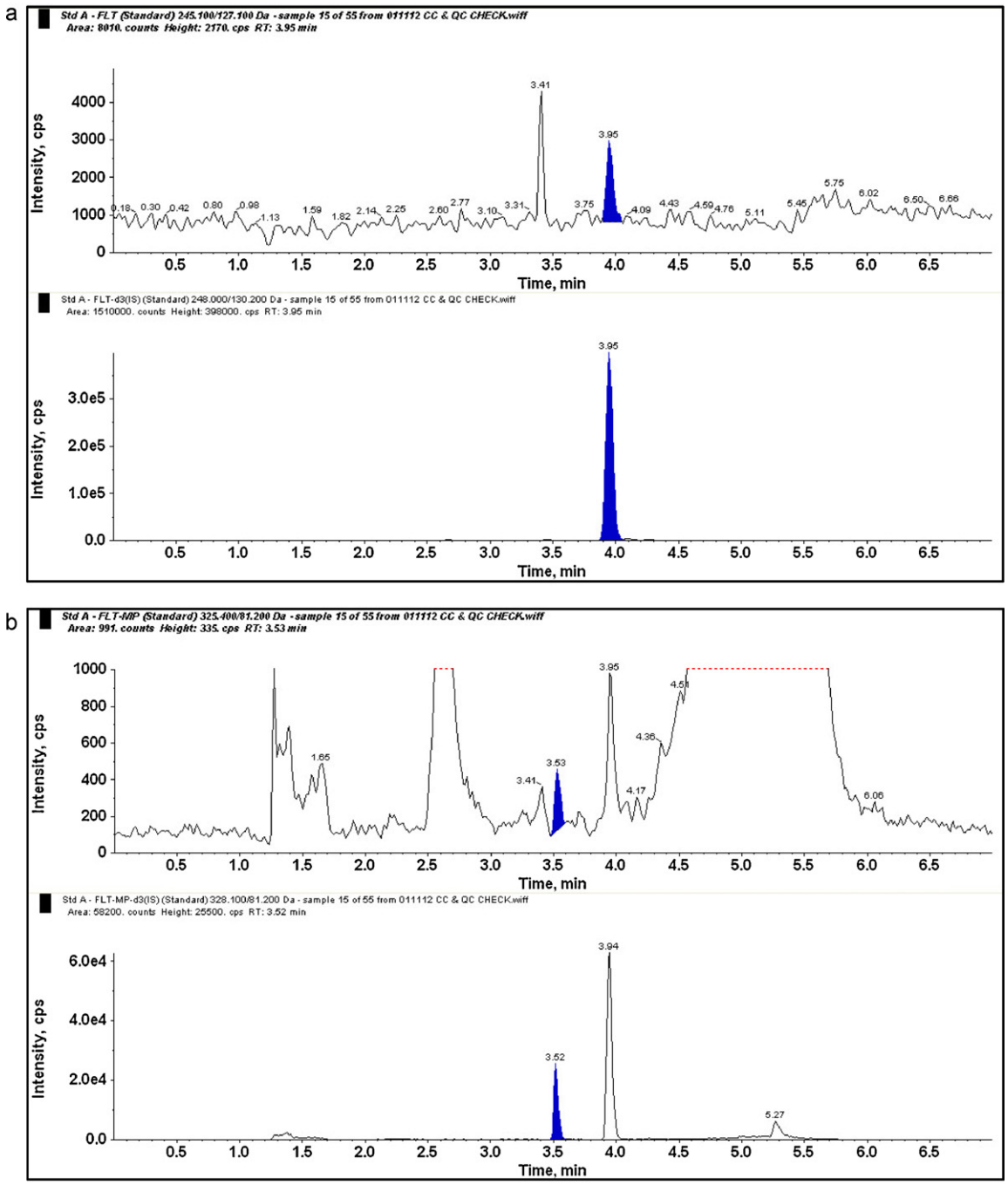

Fig. 3. (a) Representative chromatogram of FLT (LLOQ) and FLT-d3. (b) Representative chromatogram of FLT-MP (LLOQ) and FLT-MP-d3.

enzyme co-factors and stabilizing reagents [11,23]. ATP serves as a phosphate donor in the reaction. The reducing agent DTE liberates TK1 from inhibitory serum protein complexes, and UMP serves as a FLT-MP degradation inhibitor [11]. The FLT phosphorylation FLT phosphorylation reaction is terminated by methanol extraction. Calibration and quality control standards were freshly prepared to avoid analyte degradation in human serum that can occur after extended storage [11]. Since recombinantly produced TK1, and TK1 generated from cell lysates may not be biochemically representative of TK1, serum with high endogenous TK1 activity was diluted with low-activity serum to serve as positive controls [13,14]. Target analytes were extracted using protein precipitation, and chromatographically separated using a column switching strategy. Specifically, samples were loaded onto a C18 Phenomenex security guard column, which retains the analytes while impurities were eluted to waste. After column switching, analytes were separated on an Aquasil C18 analytical column and detected using the QTrap in MRM mode. Without column switching, a build-up of matrix on the column after multiple injections was observed, which resulted in a loss of sensitivity (data not shown).

LC–MS/MS conditions were optimized for the analytes (FLT, FLT-MP, FLT-d3 and FLT-MP-d3), allowing their quantification with a total run time of 7 min. Retention times and mass transitions are shown in Table 1. Representative chromatograms of blank samples for FLT and FLT-MP are shown in Fig. 2 and FLT and FLT-MP at the LLOQ are shown in Fig. 3. Matrix effects were evaluated with

post-column infusion of a 100 ng/mL FLT and FLT-MP solution. No suppression or enhancement was seen at retention time of analyte peaks.

3.1. Validation of the analytical method

3.1.1. Selectivity

The peak area at the retention time for all the analytes in the six human serum lots was found to be less than 10% of the respective LLOQ serum calibration standard. Thus, indicating that the method was selective estimating FLT and FLT-MP from human serum samples.

3.1.2. Linearity

The peak area ratio of FLT to FLT-d3 and FLT-MP to FLT-MP-d3 were linear over the range 0.5–500 ng/mL and 2.5–2000 ng/mL, respectively. The calibration curves yielded a mean correlation coefficient of 0.9964 and 0.9935 for FLT and FLT-MP, respectively ($n = 3$). A weighting factor of $1/x^2$ was used for both analytes (FLT and FLT-MP). The percent deviation from nominal (%DFN) for the mean back-calculated values of the calibration standards were between −14.7% and 11.7% for FLT and −4.3% and 7.5% for FLT-MP. Precision of the calibration standards, measured as the percent relative standard deviation for the mean back-calculated values, ranged between 6.0% and 13.4% for FLT and 3.5% and 14.1% for FLT-MP.

3.1.3. Accuracy and precision

The lower limit of quantification (LLOQ) was established at 0.5 ng/mL for FLT with a precision of 8.8%. The LLOQ for FLT-MP was set at 2.5 ng/mL with a precision of 11.5%. Accuracy at the LLOQ was −9.2% and −3.8% for FLT and FLT-MP, respectively. Inter- and intra-assay precision and accuracy for FLT and FLT-MP quality control samples are shown in Table 2.

3.1.4. Extraction recovery and carryover studies

The mean extraction recovery for FLT in human serum, determined at the LQC and HQC levels, was 103.5% and 100.3%, respectively. FLT-MP mean extraction recovery was 57.0% and 56.9% for LQC and HQC, respectively.

All the LQCs were within 15% of the nominal concentrations when injected after a high calibration standard for both analytes (FLT and FLT-MP). Thus, illustrating that carryover is not significant for this method.

3.1.5. Post-preparative stability

For FLT, %DFN for the mean back-calculated values of the quality control standards were between −20.0% and −10.2% for LQC and −10.5% and −5.5% for HQC. For FLT-MP, %DFN for the mean back-calculated values of the quality control standards were between −9.3% and −6.3% for LQC and 1.5% to 2.8% for HQC. Thus, post-preparative stability was found to be within the acceptance criteria.

3.1.6. Intermediate processing stability

For FLT, %DFN for the mean back-calculated values of the quality control standards were between −14.6% and −13.2% for LQC and −2.0% and −3.0% for HQC. For FLT-MP, %DFN for the mean back-calculated values of the quality control standards was between −13.1% and −8.6% for LQC and 19.0% and 9.0% for HQC. Thus, the supernatant obtained during processing was considered stable within specifications when stored at 4 °C for 48 h.

3.1.7. Precision of the FLT phosphorylation reaction

Phosphorylation reaction precision (% RSD) was within 15% at all concentrations except the defined zero level (Table 3). Additionally, the average concentration of FLT-MP in these samples and TK1 activity were found to have a positive linear correlation with a correlation coefficient of 0.9862.

3.2. Application of the method to patient samples

Average FLT-MP generation in serum from healthy male and female volunteers aged 50 or older was 19.1 ± 10.54 ng/mL and 18.7 ± 5.82 ng/mL, respectively, when analyzed using the standardized reaction conditions described here (37 °C, 2 h).

The average FLT-MP concentration in healthy individuals was 18.9 ± 8.46 ng/mL. In contrast, the hepatocellular carcinoma patient serum samples showed a relatively wide variation in TK1 activity. Notably, 5 (26.3%) hepatocellular carcinoma patient serum samples demonstrated concentrations above 44.3 ng/mL (mean + 3 × standard deviation of healthy individuals) indicating high TK1 activity when compared to the matched controls. The varying concentration of FLT-MP in the patient population may have been due to differences in the severity of their disease although this could not be verified since detailed information regarding these samples was not available.

4. Conclusions

Serum TK1 activity is a potentially useful biomarker for monitoring hematological malignancies and solid tumors. A sensitive, non-radiometric LC–MS/MS assay for was developed and validated for monitoring the conversion of FLT to FLT-MP in human serum. Based on established, radiometric TK1 activity assays, this novel method shows good linearity and selectivity in human serum samples. Stability studies demonstrated adequate intermediate processing and post-preparative analyte stability.

The applicability of the method for measuring serum TK1 activity was demonstrated in hepatocellular carcinoma patient serum samples and age-matched control sera. Significantly higher concentrations of FLT-MP were found in 26.3% of the hepatocellular carcinoma patient samples in comparison with controls. This method is proposed as an alternative to ELISA and radio-enzymatic assays for rapid and selective determination of serum TK1 activity.

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