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Short communication

Simultaneous determination of methotrexate and its polyglutamate metabolites in Caco-2 cells by liquid chromatography–tandem mass spectrometry

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ABSTRACT

In normal and malignant human cells, the folate antagonist methotrexate (MTX) is converted to a series of polyglutamates ($MTXGlu_n$, $n = 2–5$) which play a role in its therapeutic efficacy. Here we report an assay to determine MTX and $MTXGlu_n$ in Caco-2 cells exposed to MTX. After a simple protein precipitation step, cell homogenates (2×10^6 cells) were analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) using aminopterin as internal standard. Separation was by reversed phase HPLC on a C8 column using gradient elution with 0.1% formic acid and acetonitrile. Detection was by electrospray ionization in the positive ion mode followed by multiple reaction monitoring of the transitions of the $[M+H]^+$ ions of MTX and $MTXGlu_n$ to their common product ion at m/z 308.2 and of aminopterin at m/z 441.3 → 294.2. Calibration curves for all analytes were linear in the range 2–250 nM ($r^2 > 0.996$). Intra- and inter-day precisions (as coefficient of variation) were 3.4–15.1% and 4.3–18.4%, respectively with corresponding accuracies (as relative error) of −3.6 to +6.6% and −5.5 to +7.5%, respectively. Recoveries were in the range 60 ± 4 to 108 ± 13%. It was found that MTX undergoes only limited polyglutamation in Caco-2 cells exposed to MTX over 24 h.

1. Introduction

Methotrexate (MTX) is a folate antagonist that binds to the active site of dihydrofolate reductase leading to DNA damage and cell death [1]. At high doses (up to 3 g/m^2 body surface) MTX is widely used as chemotherapy for various neoplastic diseases such as acute leukemia, osteogenic sarcoma and breast carcinoma [2]. At low doses (<25 mg/m^2) it is used as an immunosuppressive agent to treat rheumatoid arthritis (RA) and inflammatory bowel disease (IBD) [2]. In both normal and malignant human cells, MTX is converted by folylpolyglutamate synthase to a series of polyglutamates ($MTXGlu_n$, $n = 2–5$, Fig. 1). These are retained intracellularly longer than MTX and play an important role in its therapeutic action [3]. Recently, MTX and $MTXGlu_n$ levels in red blood cells have been evaluated as a means of monitoring response to treatment in patients taking MTX for RA [4] and IBD [5].

MTX and $MTXGlu_n$ in biological specimens have been determined using a number of analytical techniques (Table 1) [6–8]. Although Caco-2 cell monolayers have been used in several studies of the transport [9,10] and cytotoxicity [11] of MTX, polyglutamate formation and degradation of MTX in Caco-2 cells has not been fully elucidated. Because the use of radiolabelled MTX is of limited application and because other methods for the determination of MTX and $MTXGlu_n$ such as HPLC with photochemically induced fluorescence lack the sensitivity required to determine the anticipated picomolar concentrations of $MTXGlu_n$ [6], we have developed a rapid and sensitive method based on liquid chromatography–tandem mass spectrometry (LC–MS/MS).

2. Materials and methods

2.1. Chemicals and reagents

Aminopterin (AMP) for use as internal standard (IS) and ammonium salts of 4-amino-10-methylpteroylglutamic acid (MTX), 4-amino-10-methylpteroyldiglutamic acid ($MTXGlu_2$), 4-amino-10-methylpteroyltriglutamic acid ($MTXGlu_3$), 4-amino-10-methylpteroyltetraglutamic acid ($MTXGlu_4$) and 4-amino-10-methylpteroylpentaglutamic acid ($MTXGlu_5$) were purchased from Schircks Laboratories (Jona, Switzerland). Acetonitrile, formic acid and 35% ammonia were AnalaR® grade purchased from BDH Chemicals Ltd (Poole, United Kingdom). Hank's balanced salt solution (HBSS), N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES) and glucose were purchased from Sigma–Aldrich New Zealand Ltd. (Auckland, New Zealand). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), nonessential amino acids, penicillin and streptomycin were purchased from Invitrogen New Zealand Ltd. (Auckland, New Zealand). Caco-2

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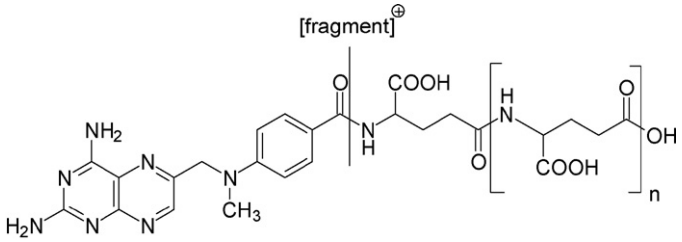

Fig. 1. Structure of MTX ($n=0$) and $MTXGlu_{2-5}$. $[Fragment]^+$ shows the cleavage point giving rise to the product ion from MTX and $MTXGlu_{2-5}$ monitored at m/z 308.2.

cells were obtained from the American Type Culture Collection (Rockville, MD, USA).

2.2. LC–MS/MS

Tandem mass spectrometry was carried out on an API 3200™ triple quadrupole mass spectrometer (MDS-Sciex, Concord, ON, Canada) equipped with an electrospray ionization (ESI) source operated in the positive ion mode. Full scan and product-ion mass spectra and compound-dependent parameters were determined by infusing 1 μM solutions of each compound in 1:1 acetonitrile:water at a flow rate of 10 μL/min. MS source-dependent parameters optimized by flow injection of each analyte were as follows: curtain gas, collision gas, nebulizer gas and auxiliary gas (all nitrogen) 10, 12, 60 and 50, respectively; ionspray voltage 5.5 kV; ion source temperature 400 °C. The most abundant precursor and product ions in the mass spectrum of each analyte were used to select transitions for MRM of MTX, $MTXGlu_n$ and IS at a dwell time of 100 ms per channel. Analyst software version 1.4.2 was used for data manipulation.

The HPLC system (Agilent 1200 series) consisted of a binary pump, autosampler, temperature controller and vacuum degasser (Agilent, USA). Separation was achieved on a C8 column (5 μm, 3.9 mm × 150 mm, SymmetryShield RP8**,** Waters) maintained at 40 °C proceeded by a C8 guard column. Gradient elution utilized 0.1% formic acid as solvent A and acetonitrile as solvent B at a flow rate of 0.5 mL/min. The gradient was as follows: 0–7.0 min, 10–90% B; 7.0–9.0 min, 90% B; 9.0–10 min, 90–10% B followed by 5 min equilibration with 10% B before injection of the next sample. An in-line motorized six-port divert valve was used to divert the eluent flow to waste for the first 3 min.

2.3. Caco-2 cell study

Caco-2 cells were cultured in complete DMEM with 10% FBS, 1% nonessential amino acids, 100 units/mL penicillin and 0.1 mg/mL streptomycin under an atmosphere of 5% CO_2/95% air at 37 °C and 95% relative humidity. Caco-2 cells at passage 30–40 were counted by a hemacytometer and their viability checked by 0.4% Trypan blue staining before being seeded into six-well culture dishes (9.6 cm^2, Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) at a density of 4×10^6 cells per well. After 24 h, the plated cells were rinsed twice and preincubated with HBSS buffer containing 25 mM HEPES and 25 mM D-glucose at pH 7.4 for 10 min at 37 °C. Cells were then incubated with 2 mL HBSS buffer containing 5 μM MTX. After 3, 6 and 24 h, MTX solution was aspirated, cells washed twice with ice cold phosphate buffered saline to remove extracellular MTX and then lysed by adding 400 μL 5% ammonia. An aliquot of cell lysate (200 μL) was then homogenized on ice for 20 s in 2 s pulses at 30% intensity using a Vibra Cell sonicator (model CV26) and stored at −20 °C until analysis.

2.4. Sample preparation

To the thawed cell homogenate, 20 μL IS solution (1 μM in 1:1 acetonitrile:water) and 900 μL ice-cold acetonitrile were added. After vortexing for 20 s, the mixture was centrifuged at 12,000 × g for 10 min and the supernatant (1000 μL) evaporated to dryness in a Speedvac (Savant Instruments Inc., Farmingdale, NY, USA) at room temperature. The residue was reconstituted in 1:1 acetonitrile:water (70 μL) and injected (30 μL) into the LC–MS/MS system. The concentrations of MTX and $MTXGlu_n$ were normalized to the number of cells.

2.5. Assay validation

Stock solutions containing MTX and $MTXGlu_n$ ($n=2$–5) (all at 10 μM) and an IS solution (10 μM) were prepared in 0.1 M sodium hydroxide and stored at −80 °C until required. Quantitation was based on determination of peak areas of each analyte normalized to the IS signal. Calibration curves were prepared using spiked standards containing 0, 2, 5, 10, 25, 50, 100 and 250 nM of each analyte in cell homogenates. Linearity was assessed by unweighted linear least-squares regression of calibration curves determined in quadruplicate.

Accuracy as relative error (RE%) and precision as coefficient of variation (CV%) were determined by analysis of low, medium, and high quality control (QC) samples (4, 40 and 200 nM, respectively) prepared by spiking cell homogenates. Intra-day and inter-day precision and accuracy were determined by assay of five replicates on 1 day and assay of duplicate on five different days, respectively. The lower limit of quantitation (LLOQ) for each analyte was determined as the lowest concentration giving a CV ≤ 20% for intra-day precision.

-1 Total recovery and matrix effects were assessed using replicates ($n=5$) of QC samples. Total recovery of MTX and $MTXGlu_n$ was evaluated by comparing QC samples with standard solutions in 5% ammonia. Matrix effects were investigated by comparing post-extraction spiked blank samples with standard solutions in 5% ammonia. Stability of MTX and $MTXGlu_n$ was tested by analyzing QC samples under the following conditions: Room temperature for 4 h; −20 °C for 30 days; after three freeze–thaw cycles; and in autosampler vials at 4 °C for 24 h (post-preparative).

Table 1
Comparison of the assay with previous analytical methods for MTX and $MTXGlu_n$ in biological samples. *Abbreviations*: ACN, acetonitrile; EA-iP, ethyl acetate–isopropanol; extr, extraction; MeOH, methanol; PCA, perchloric acid; prec, precipitation.

Matrix	Preparation	Internal standard	Column type	Detection method	Run time (min)	LLOQ	Reference
Cell homogenates	prec-MeOH	NA	ODS Ultrasphere	Ultraviolet and radioactivity in HPLC eluates	80	0.01 pmol/6 × 10^6 cells	[6]
Human whole blood	prec-PCA, extr EA-iP	Protriptyline	NA	Capillary zone electrophoresis fused-silica capillary	14	1 μM	[8]
Human erythrocytes	prec-PCA	NA	Terra MS C18	Post-column photochemically induced fluorescence detection	20	5 nM	[7]
Cell homogenates	prec-ACN	Aminopterin	SymmetryShield C8	Tandem mass spectrometry	15	2 nM	This study

Table 2
Transitions and source-dependent parameters for multiple reaction monitoring of MTX, $MTXGlu_n$ and aminopterin (IS).

Compound	Precursor ion (m/z)	Product ion (m/z)	Declustering potential (V)	Entrance potential (V)	Collision cell entrance potential (V)	Collision energy (eV)	Collision cell exit potential (V)	Retention time (min)
MTX	455.2	308.2	41	7.5	20	35	4	5.6
$MTX\text{-}Glu_2$	584.1	308.2	51	7.5	24	35	4	5.7
$MTX\text{-}Glu_3$	713.1	308.2	61	8.5	32	47	6	5.9
$MTX\text{-}Glu_4$	842.1	308.2	76	10	34	53	6	6.0
$MTX\text{-}Glu_5$	971.1	308.2	91	11	40	65	6	6.1
IS	441.3	294.2	41	5.5	20	27	10	4.0

3. Results and discussion

3.1. Method development

MRM transitions and source-dependent parameters optimized to give the maximum intensity and sensitivity for each analyte are listed in Table 2. MRM chromatograms revealed a peak from an unidentified compound (with the same molecular weight as MTX) at 6.9 min (Fig. 2) which does not interfere with the analysis of $MTXGlu_n$ in cell homogenates.

During method development, comparison of a C18 column (ZORBAX SR-CN, 4.6 mm i.d. × 150 mm) with the C8 column showed that the latter gave better peak shape and sensitivity. In addition, the order of retention of $MTXGlu_n$ on the C8 column was observed to be directly proportional to the number of glutamate residues (Table 2, Fig. 2B) and the total run time was less than in previous assays (Table 1). In the cell homogenization step, 5% ammonia was used to prevent conversion of $MTXGlu_n$ to MTX by endogenous polyglutamate hydrolase [12]. Perchloric acid (10%), formic acid (10%) and heat treatment (100 °C for 10 min) were also tested as homogenizing fluids to deactivate the enzyme but 5% ammonia produced the best peak shapes with satisfactory recoveries.

3.2. Assay validation

All standard curves for MTX and $MTXGlu_n$ in Caco-2 cell homogenates were linear in the range 2–250 nM with $r > 0.996$ ($n = 4$). Intra- and inter-day precisions were 3.4–15.1% and 4.3–18.4%, respectively with corresponding accuracies of −3.6 to +6.6% and −5.5 to +7.1%, respectively (Table 3). The LLOQ for MTX and $MTXGlu_n$ corresponded to the lowest concentration (2 nM) on the standard curves indicating that the assay is more sensitive than previously reported methods (Table 1). The total recoveries of MTX and $MTXGlu_n$ (Table 3) were independent of concentration but in genreal increased as the number of glutamate residues increased. This may be related to the change in protein binding of analytes consequent on their changing lipophilicities (log *P* values for MTX and $MTXGlu_{2-5}$ −1.97, −4.43, −5.41, −5.90 and −6.32, respectively, as calculated by ALOGPS 2.1 program). With regard to matrix effects, all concentrations were between 80% and 113% of the nominal values (Table 3) suggesting there were no significant matrix effects for MTX and $MTXGlu_n$ and that no co-eluting substance influenced the ionization of the analytes. The results from stability tests (Table 4) show a deviation of ±15% between the measured and nominal concentrations indicating good stability of MTX and $MTXGlu_n$ under all the conditions examined.

3.3. Method application

The assay was applied to the determination of MTX and $MTXGlu_n$ in Caco-2 cells exposed to 5 μM MTX. The time course of MTX uptake and $MTXGlu_n$ formation is shown in Fig. 3. The level of MTX remained relatively constant over time and was much higher than that of $MTXGlu_n$ at all time points. $MTXGlu_2$ and $MTXGlu_3$ were present after 3 h incubation whereas $MTXGlu_4$ was present only after 24 h and $MTXGlu_5$ was not detected at any time point. These results suggest polyglutamation of MTX occurs to only a limited extent in Caco-2 cells in contrast to the situation in Nalm6 cells (a human pre-B cell leukemia cell line) where incubation with

Table 3
Precision, accuracy and recovery for MTX and $MTXGlu_n$ in Caco-2 cell homogenates.

Analyte	Nominal Concentration (nM)	Intra-day ($n = 5$)			Inter-day ($n = 10$)			Recovery ($n = 5$)	Matrix effect ($n = 5$)
		Precision		Accuracy	Precision		Accuracy	Mean ± SD (%)	Mean ± SD (%)
		Mean measured concentration (nM)	CV (%)	Mean RE (%)	Mean measured concentration (nM)	CV (%)	Mean RE (%)		
MTX	4	4.13	8.60	3.20	4.29	12.9	−3.95	60 ± 4	97 ± 5
	40	38.6	7.51	−3.60	38.8	12.1	2.00	73 ± 10	93 ± 5
	200	194	6.63	−3.10	198	8.34	−1.00	79 ± 11	102 ± 4
$MTXGlu_2$	4	4.17	14.6	4.30	3.96	18.4	−4.40	73 ± 3	95 ± 8
	40	40.2	11.7	0.60	40.6	13.3	3.95	83 ± 5	96 ± 4
	200	195	6.80	−2.6	197	9.01	−2.70	77 ± 9	99 ± 3
$MTXGlu_3$	4	4.04	9.43	1.05	4.17	9.51	0.85	98 ± 10	101 ± 9
	40	42.3	6.80	5.80	42.8	8.81	4.65	88 ± 9	102 ± 4
	200	193	2.84	−3.40	202	6.70	3.00	73 ± 9	100 ± 3
$MTXGlu_4$	4	3.90	11.8	−2.60	3.94	10.8	7.05	71 ± 9	88 ± 12
	40	41.4	10.4	3.60	39.7	15.5	−5.45	71 ± 7	97 ± 5
	200	208	3.40	4.20	217	4.29	12.1	78 ± 11	98 ± 3
$MTXGlu_5$	4	3.90	15.1	−2.40	4.04	16.0	6.25	101 ± 19	101 ± 10
	40	41.0	2.92	2.45	40.3	8.24	0.10	95 ± 12	101 ± 5
	200	213	3.94	6.60	208	5.58	−3.10	108 ± 13	100 ± 5

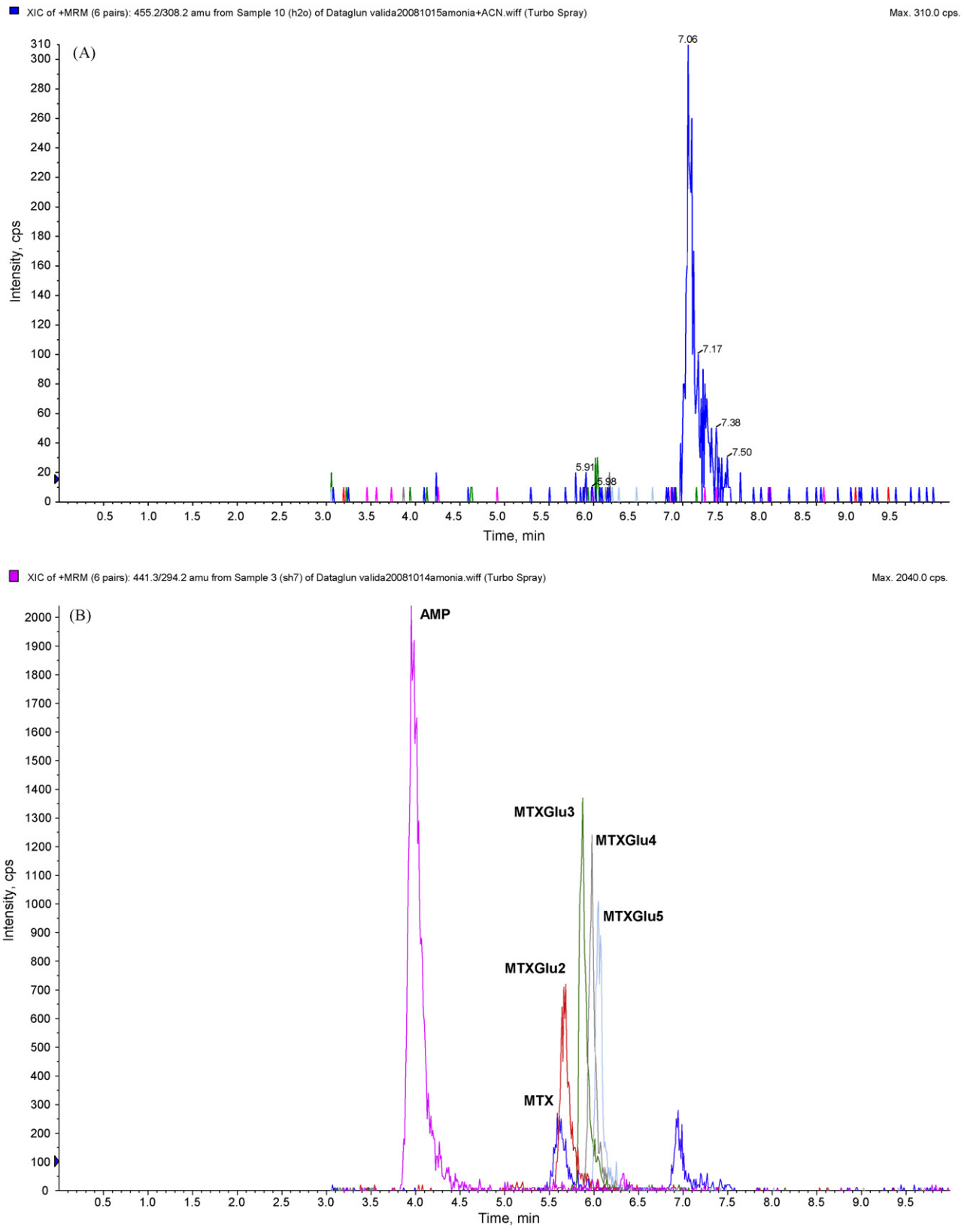

Fig. 2. Representative MRM chromatograms of MTX, MTXGlu$_n$ and AMP(IS) in Caco-2 cell homogenates: (A) Blank cell homogenate; (B) cell homogenate sample at the LLOQ (2 nM).

Table 4
Stability of MTX and MTXGlu$_n$ in Caco-2 cell homogenates.

Storage conditions	Concentration measured (mean ± SD, n = 5)													
	MTX			MTXGlu2			MTXGlu3			MTXGlu4			MTXGlu5	
	4 nM	40 nM	200 nM	4 nM	40 nM	200 nM	4 nM	40 nM	200 nM	4 nM	40 nM	200 nM	4 nM	40 nM	200 nM
At room temperature for 4 h	4.10 ± 0.20	40.8 ± 5.2	194 ± 11	3.95 ± 0.32	41.5 ± 2.6	201 ± 8	3.92 ± 0.37	41.4 ± 2.5	196 ± 7	4.08 ± 0.61	39.0 ± 4.4	198 ± 10	3.87 ± 0.35	41.8 ± 4.5	189 ± 6
At −20 °C for 30 days	4.05 ± 0.15	39.9 ± 4.0	209 ± 12	4.00 ± 0.24	39.7 ± 2.2	199 ± 12	3.98 ± 0.28	39.4 ± 2.7	209 ± 9	3.84 ± 0.42	42.9 ± 3.4	202 ± 8	4.05 ± 0.52	42.3 ± 4.1	202 ± 7
After three freeze–thaw cycles	3.81 ± 0.32	39.4 ± 4.0	190 ± 14	3.90 ± 0.32	38.6 ± 3.4	190 ± 14	4.00 ± 0.32	40.5 ± 3.7	197 ± 8	3.90 ± 0.43	40.4 ± 2.5	218 ± 10	3.75 ± 0.62	39.9 ± 3.8	206 ± 15
In autosampler vials at 4 °C for 24 h	4.06 ± 0.30	39.3 ± 3.5	196 ± 16	4.16 ± 0.35	39.5 ± 2.8	208 ± 10	3.90 ± 0.33	39.9 ± 2.7	200 ± 7	4.11 ± 0.48	38.9 ± 4.2	220 ± 13	3.76 ± 0.51	39.4 ± 2.0	227 ± 11

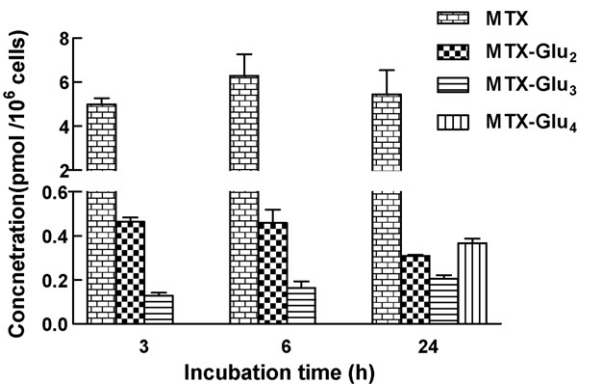

Fig. 3. MTX and MTXGlu$_n$ levels in Caco-2 cells (4×10^6) incubated with 5 μM MTX for 3, 6 and 24 h. Data are mean ± SD for n = 3 wells at each incubation time.

1 μM [^{3}H]-MTX produced high levels of MTXGlu$_{2-5}$ at all times up to 24 h [6].

4. Conclusion

We present for the first time a validated LC–MS/MS method for the determination of MTX and MTXGlu$_n$ in Caco-2 cells. Compared with previously reported assays for MTX and MTXGlu$_n$ in other matixes, the method is more rapid and more sensitive. The method has been successfully applied to a study of MTXGlu$_n$ formation in Caco-2 cells exposed to MTX.

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