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Title: A HPLC-SRM-MS Based Method for the Detection and Quantification of Methotrexate in Urine at Doses Used in Clinical Practice for Patients with Rheumatological Disease: A potential measure of adherence

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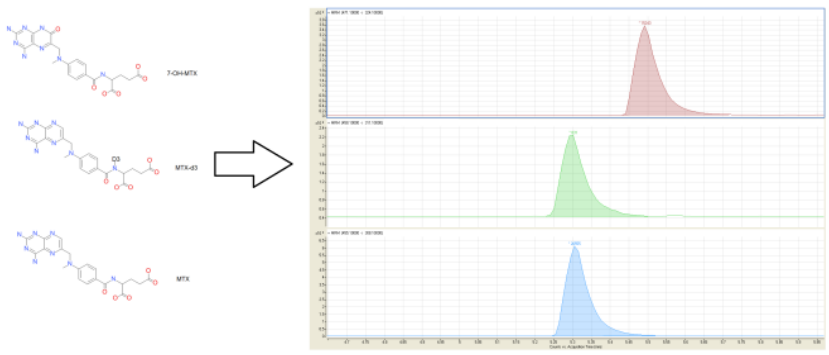
Table of contents entry



Abstract

Rheumatoid Arthritis (RA) is a common autoimmune disease that causes significant disability and reduced life expectancy. The folate antagonist methotrexate (MTX) is first-line therapy for RA when used weekly at low doses (5-25mg). However, the true rate of adherence to MTX is uncertain. This is in part due to the different methods of measurement of adherence employed with no biochemical test currently available to determine adherence to low dose MTX. Common methods of MTX measurement include immunoassays in patients with high dose therapy, but these assays cross-react with MTX metabolites and lack the sensitivity required to measure adherence to low dose MTX. HPLC-SRM-MS (Selected Reaction Monitoring-Mass Spectrometry) has several theoretical advantages over immunoassays with improved specificity, minimal cross-reaction and higher sensitivity. The aim of this study was to develop an assay to measure MTX and its major metabolite 7-OH-MTX in urine as a tool to monitor adherence to low dose MTX in clinic. As a proof of concept, urine samples from 4 participants with RA were measured after directly observed therapy. The assay showed improved sensitivity compared to that reported by immunoassays, with low carryover and high within-run precision. In participant samples, MTX was measurable in the urine for up to 105 hours after administration and 7-OH-MTX was detectable up to 98 hours after administration, suggesting that this assay is suitable for the measurement of adherence to therapy. The assay requires minimal sample preparation and can be adopted by other laboratories with minimal study set up.

Introduction

Rheumatoid arthritis (RA) is a multisystem autoimmune disease that affects up to 1% of the adult population [1]. The disease causes joint inflammation with resultant destruction and disability. 32% of adults with RA are unable to work after only 5 years of disease [2]. The folate antagonist methotrexate (MTX) is the treatment of first choice for most patients with RA [3]. The rate of adherence to MTX in RA ranges from 59% to 107%; the wide range of adherence rates is in part due to the different methods of measurement of adherence employed [4, 5]. Pharmacy records, pill counts and electronic monitoring systems all have their unique advantages and disadvantages and there is currently no gold standard method of measuring adherence to MTX therapy in RA.

MTX is currently used at high doses (1 g/m^2) in acute lymphoblastic leukaemia where it is routine practice to measure MTX levels to guide folinic acid rescue therapy; for the average 10 year old child this is equivalent to a single dose of 877 mg [6]. However, it is used at much lower doses in RA (5-25 mg weekly) where its main mechanism of action may be anti-inflammatory in nature [7]. The commonly used methods of MTX measurement in childhood leukaemia are the fluorescent polarisation immunoassay (FPIA) and enzyme-multiplied immunoassay technique [8]. These immunoassays have the advantage that they are rapid and samples require little sample preparation prior to testing. However, they have low specificity, with cross-reaction with MTX metabolites such as 2,4-diamino-N10-methylpteroic acid (DAMPA), and have a lower limit of quantification (LLOQ) of 50 nM which may be inadequate for measuring adherence to the low dose of MTX in RA [9]. Furthermore, it has been suggested that the major metabolite of MTX, 7-OH-MTX may be a more suitable measurement of adherence due to its longer half-life [10].

Ideally, a test for MTX adherence would involve measurement of MTX/7-OH-MTX in urine, as this would undoubtedly have the advantage of being more acceptable to patients since it allows for regular monitoring without the need for invasive blood sampling. HPLC methods have been

developed for MTX detection which use fluorometric detection [11-14] , but this is subject to interference by folates, thus reducing specificity and sensitivity [15]. The development of a method for the detection of these compounds using HPLC-SRM-MS (Selected Reaction Monitoring-Mass Spectrometry), specifically has the advantage of improved specificity with minimal cross-reaction and improved LLOQ [9]. Methods utilising HPLC-MS to measure MTX have been developed but they are either designed either for use in plasma [16], or measure to MTX alone [17, 18] or have an LLOQ too high for the measurement of adherence [19-21]. The aim of this study was to develop an assay to simultaneously measure MTX and 7-OH-MTX levels in urine, at a sufficient LLOQ which could be used to monitor adherence in RA.

Materials and Methods

Reagents and chemicals

7-OH-MTX and deuterium-labelled MTX (MTX-d_3) were purchased from Toronto Research Chemicals (Ontario, Canada). Their structures can be seen in Figure 1. MTX-d_3 was used as the internal standard. MTX, LC-MS grade acetonitrile (ACN), water, methanol and formic acid were purchased from Sigma-Aldrich (Dorset, United Kingdom).

Preparation of standards and samples

Stock solutions were prepared in water at concentrations of 0.1 nM, 1 nM, 10 nM, 100 nM and 1000 nM for MTX/7-OH-MTX and 500 nM for MTX-d_3 and stored at -80°C. A working solution was prepared freshly for each batch of samples. Human urine, used for both spiking of samples and to prepare calibration curves was obtained from pooled samples (n=3) donated from patients with RA participating in the Rheumatoid Arthritis Medication Study (RAMS), a multi-centre observational study in the UK, who were MTX naïve at baseline (REC reference: 08/H1008/25).

Frozen urine was thawed at room temperature and vortex mixed. Samples were prepared in 1.5ml safe-lock tubes (Eppendorf®). Samples were spiked with MTX, 7-OH-MTX and MTX-d_3 to make a final concentration of 50 nM MTX-d_3 and the required concentration of MTX/7-OH-MTX. Protein precipitation was performed by the addition of 200 µl ACN to 50 µl sample, and subsequently vortex mixed at room temperature for 10 minutes. Samples were then centrifuged at 10,000 g for 10 minutes. The supernatant was removed and dried in a concentrator (Eppendorf concentrator plus) for 3 hours at room temperature and reconstituted in 50 µl water prior to LC-MS/MS analysis.

Chromatographic conditions

An autosampler auto-injector (Agilent 1200 series Autosampler with Thermostat, Cheadle, UK) was used to inject 5µl of sample. Chromatographic separation was performed using a Thermo Scientific™ Hypersil GOLD™ HPLC column with a particle size 1.9 µm, 100 mm length and 2.1 mm diameter maintained at 25 °C on an Agilent 1200 series HPLC on-line to the MS. The mobile phase consisted of ACN with 0.1% formic acid as the organic component (B) and water with 0.1% formic acid as the aqueous phase (A). The system was maintained in 8% buffer B at a flow rate of 0.3 mL/min. The gradient elution timetable is shown in Table 1.

Mass-Spectrometry Detection

Analyses were performed on an Agilent® 6460 triple quadrupole mass spectrometer equipped with an electrospray ionisation (JetStream source) operated in positive ion mode and MTX/7-OH-MTX

were detected using the following selected reaction monitoring (SRM) transitions: 455.1>308.1 for MTX, 471.1>324.1 for 7-OH-MTX, and 458.1>311.1 for MTX-d_3. The mass spectrometer settings were optimised as follows: cone voltage 1500 V, capillary voltage 4000 V, collision energy 25 eV, source temperature 350 °C, desolvation temperature 350 °C with a nitrogen gas flow of 10 L/min and cone gas flow 11 L/min. Nitrogen was used as collision gas at a pressure of 45 psi. Quantitation was calculated using the peak-area ratio of the analyte to internal standard using Agilent MassHunter Workstation Software Quantitative analysis B.04.00.

Method Validation

Validation of the assay was tested in several domains as adapted from European Medicines Agency guidelines [22]. Specifically, we determined the lower limit of quantification (LLOQ), carryover, accuracy, linearity, precision, recovery and stability of the assay.

Measurement of Adherence

As a proof of concept experiment to explore the ability of the assay to measure adherence, 4 participants with RA taking oral MTX once weekly with folic acid (5 mg) were recruited (REC: 13/NW/0653). Following informed consent, participants were admitted to the Wellcome Trust Clinical Research Facility, Manchester, United Kingdom, prior to MTX administration. Directly observed MTX administration was undertaken and serial urine samples collected on 2 subsequent days within 7 days of MTX administration at variable time points. A mid-stream urine sample (60ml) was collected into chemical free sterile pots. Samples were centrifuged for 15 minutes at 1,000 g at 4 °C. The samples were aliquoted to remove the sediment, aliquotted into 5 mL samples and stored at -80 °C prior to testing for MTX and 7-OH-MTX levels as described above. Samples were analysed in triplicate.

Results

Lower Limit of Quantification and Carryover

A signal five times greater than the noise observed was considered to be the LLOQ. 50 µL of urine was spiked with MTX/7-OH-MTX to achieve final concentrations of 0.05 nM, 0.075 nM, 0.1 nM, 0.25 nM, 0.5 nM, 0.75 nM, 1 nM, 2.5 nM, 5 nM and 10 nM. MTX-d_3 remained constant at 50 nM. Samples were prepared as described previously in triplicate. Blank samples of pooled urine were injected during each experimental run to ensure that there was no detectable contamination. Carryover was calculated as the percentage of analytes residual signal present in a blank sample following an injection of 1000 nM MTX/7-OH-MTX.

The LLOQ for MTX and 7-OH-MTX was determined to be 2.5 nM and 10 nM respectively as those concentrations gave a signal to noise ratio (SNR) of ≥ 5. A typical chromatogram following injection of MTX-naïve urine is shown in Figure 2. Carryover of sample following injection of a 1000 nM MTX/7-OH-MTX with 50 nM internal standard was < 1%. The concentrations obtained for the blank samples did not reach the LLOQ.

Accuracy, Linearity and Precision

Accuracy and linearity was tested in independent triplicate samples. 50 µL of urine was spiked with MTX/7-OH-MTX to achieve final concentrations of 5 nM, 10 nM, 50 nM, 100 nM, 500 nM and

1000 nM, whilst the concentration of MTX-d_3 remained constant at 50 nM. Samples were prepared as described previously. Accuracy of the assay was calculated by expressing the experimentally obtained result as a percentage of the expected concentration. Linearity was assessed using linear least-squared regression analysis.

Precision (within-run) was calculated by repeated measurements (n=5) of the same urine sample following the sample preparation protocol and at a range of concentrations of MTX/7-OH-MTX; 2.5 nM, 10nM, 50 nM, 500 nM and 1000 nM. Precision was expressed as CV%.

The results of accuracy testing are presented in Table 2. The mean accuracy of MTX and 7-OH-MTX ranged from 89 to 110% and 90 to 108% respectively with all CV% recorded as less than 12%. Linearity was calculated from the data presented in Table 2 for MTX and 7-OH-MTX and is shown in Figure 3a and 3b. The r^2 for MTX and 7-OH-MTX was 0.9995 and 0.9986 respectively. Notably there was no appreciable loss of accuracy or linearity as the assay approached the LLOQ. Precision testing revealed an intraday CV% for MTX and 7-OH-MTX of < 6% at all concentrations tested.

Recovery

Recovery was performed by comparing the measured concentration of MTX/7-OH-MTX in samples that were spiked with known analyte concentrations and then protein precipitated to samples that were protein precipitated and then spiked immediately prior to LC-MS analysis. The following concentrations were prepared in triplicate of MTX/7-OH-MTX: 5 nM, 10 nM, 25 nM, 50 nM, 100 nM, 250 nM, 500 nM, 750 nM and 1000 nM. The internal standard MTX-d_3 remained at 50 nM in all samples. The extraction recovery percentage was calculated by dividing the response obtained from the samples which were spiked prior to protein precipitation, to that obtained when analyte was spiked into deproteinated urine directly prior to analysis.

The mean extraction recovery for MTX and 7-OH-MTX ranged from 104% to 126% (mean 118%) and 67% to 122% (mean 86%) respectively across all concentrations as shown in Table 3.

Stability

Stability of the analyte in urine was assessed by spiking urine samples with MTX/7-OH-MTX at 5 nM, 10 nM, 50 nM and 1000 nM from stock solutions in triplicate. To avoid the effect of freeze-thawing-freezing, samples were aliquoted according to their intended time of testing. Samples were subsequently stored at -80°C or room temperature in dark conditions. Samples were processed as described in the above, spiked with the internal standard MTX-d_3 (50nM) and precipitated with ACN at the following time points: 3, 72 and 168 hours. Fresh calibration curves were prepared daily in urine. Analyte stability was calculated as a percentage of loss of analyte compared to the day 0 sample.

The results of stability testing are presented in Table 4 and 5. Room temperature samples were not extended to 168 hours due to significant loss of 7-OH-MTX at 72 hours. However, storage of samples at -80°C for up to one week showed no appreciable losses of MTX or 7-OH-MTX.

Measurement of Adherence

To demonstrate that this assay is a useful tool for determining adherence, urine samples taken from patients who had been directly observed taking MTX were analysed to study the levels of MTX and 7-OH-MTX and to determine the length of time duration for which a signal from MTX could be

observed. The data in Table 6 demonstrates that it is possible to detect MTX in urine using this assay for at least 4 days following drug administration, with a maximum in this small cohort of 4 days 10 hours post-therapy. Possibly due to the higher LLOQ, levels of 7-OH-MTX were undetectable in 2/4 patients at this time point - the latest time at which 7-OH-MTX could be detected in all four patient samples was 46 hours. This suggests that MTX, and not 7-OH-MTX is likely to be the more sensitive marker for adherence using LC-MS assays in this setting.

Discussion

In the establishment of an assay to measure adherence, LLOQ and lower limit of detection (LLOD) is of vital importance to ensure that adherence is measured with high sensitivity. We have developed a novel HPLC-SRM-MS assay for the measurement of MTX and 7-OH-MTX in urine. The assay exhibited within-run precision, LLOQ and carryover within the guidelines established by the European Medicines Agency for a validated bioanalytical method [22]. Carryover of a blank sample following injection of 1000 nM MTX/7-OH-MTX was less than 1%. As required by the European Medicines Agency, the analyte signal of the LLOQ sample is at least 5 times the signal of a blank sample. Six calibration concentrations were used for MTX and 5 for 7-OH-MTX with a mean accuracy for these samples ±15% as per EMA guidelines. Stability testing of MTX and 7-OH-MTX demonstrated that MTX and 7-OH-MTX are stable at room temperature up to 24 hours and at -80 °C up to 168 hours, demonstrating that urine samples must be frozen within 24 hours. This data is key to the design of optimal sample collection, transport and storage protocols for future use as an assay for therapeutic adherence.

Whilst other methods have been developed for the detection of MTX in urine this proposed method has several distinct advantages [10-14, 17, 18, 21, 23]. Firstly, we have developed an assay that measures both MTX and 7-OH-MTX concurrently, which due to its longer half-life may be a more robust biomarker of adherence, while further samples and modelling of data is required to explore the ability of 7-OH-MTX to measure adherence [10], our initial data suggests that MTX may provide a more long-lived signal for determination of adherence. In part, this may be due to the less sensitive detection of 7-OH-MTX in this assay, rather than a reflection on the relative half-life of MTX and its metabolite. On this basis, we would recommend that measurement of both MTX and 7-OH-MTX simultaneously is advantageous. Analysis of both the drug and its metabolite also allows more accurate PK/PD modelling, and potentially prediction of response. Secondly, there is limited sample preparation required and that required is simple and straightforward compared to other assays [10, 11, 13, 17, 18, 24]. Therefore this assay could be easily adopted by other laboratories without the need for liquid or solid phase extraction. When compared specifically with immunoassays such as FPIA assays or HPLC based assays, our method is able to measure both MTX and 7-OH-MTX concurrently and has a lower LLOQ and may therefore be more suitable to monitor adherence in diseases such as rheumatoid arthritis, where low dose intermittent treatment schedules are used [12, 21, 23].

There are limitations of the assay which need to be recognised. The assay has been developed to measure adherence and therefore we required an assay with a low LLOQ. The assay has therefore not been validated for concentrations higher than 1000 nM and samples with measured concentrations above the upper limit of quantification (ULOQ) need to be diluted and re-analysed. Improvement in the LLOQ of the current assay could theoretically be achieved through the inclusion of solid phase extraction, but this would increase the associated cost and subsequent preparation time. For its adoption as a clinical tool, the assay needs to be fully validated in a clinical setting to

determine the timing of sampling which could measure adherence to oral MTX and the influence of concomitant medication. Pharmacokinetic modelling will be required to fully evaluate the ability of the assay to measure adherence 7 days after MTX administration.

Conclusions

In summary, we have developed a novel HPLC-SRM-MS assay for the measurement of MTX and 7-OH-MTX in human urine. The method requires limited sample preparation and has a lower LLOQ compared to currently available immunoassays. The assay may be a suitable test for adherence for MTX in patients with RA.

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Time after injection (min)	A (%)	B (%)
0	92	8
1	92	8
4	50	50
5.5	50	50
5.6	5	95
8	5	95
8.21	92	8
11	92	8

Table 1. Gradient elution schedule.

Expected MTX/7-OH-MTX concentration (nM)	Mean MTX measured concentration (nM) (SD)	CV (%)	Mean accuracy (%) (SD)	Mean 7-OH-MTX measured concentration (nM) (SD)	CV (%)	Mean accuracy (%) (SD)
5	4.56 (0.54)	11.93	91.22 (10.88)	< LLOQ		
10	9.74 (0.17)	1.70	97.40 (1.65)	10.79 (0.69)	6.36	107.90 (6.86)
50	44.31 (1.37)	3.10	88.62 (2.74)	51.41 (0.92)	1.78	102.82 (1.83)
100	87.58 (3.08)	3.51	87.58 (3.08)	107.28 (2.48)	2.32	107.28 (2.48)
500	549.48 (8.06)	1.47	109.90 (1.611)	485.19 (3.40)	0.70	97.04 (0.68)
1000	1058.69 (34.11)	3.22	105.87 (3.41)	902.19 (27.75)	3.08	90.22 (2.77)

Table 2. Results of accuracy testing for MTX/7-OH-MTX samples in urine (n=3)

Expected Concentration (nM)	Mean MTX Extraction recovery (%)	Mean 7-OH-MTX Extraction recovery (%)
5	121.42	< LLOQ
10	122.55	< LLOQ
25	118.64	121.97
50	126	93.26
100	103.96	72.01
250	116.89	93.13
500	118.49	67.08
750	103.64	80.32
1000	129.74	76.91

Table 3. Mean extraction recovery for MTX and 7-OH-MTX (n=3)

Time point (h)	3		72	
Expected concentration (nM)	Mean MTX loss (%) (SD)	Mean 7-OH-MTX loss (%) (SD)	Mean MTX loss (%)	Mean 7-OH-MTX loss (%)
5	11.96 (21.40)	< LLOQ	30.53 (13.33)	< LLOQ
10	-11.63 (5.36)	-14.05 (14.50)	-3.63 (21.06)	9.34 (9.06)
50	-13.59 (6.63)	-14.40 (4.35)	-9.30 (6.59)	5.99 (4.36)
500	-5.87 (8.54)	-20.47 (14.44)	25.84 (7.97)	28.02 (7.36)
1000	5.48 (21.85)	6.98 (24.66)	20.35 (1.90)	33.13 (14.58)
Mean loss for this time point (%) (SD)	-2.73 (11.07)	-10.49 (12.01)	12.76	19.12 (13.46)

Table 4. MTX and 7-OH-MTX stability testing results for samples stored at room temperature demonstrating significant loss of MTX at 72 hours (n=3)

Time point (h)	3		72		168	
Expected concentration (nM)	Mean MTX loss (%)	Mean 7-OH-MTX loss (%)	Mean MTX loss (%)	Mean 7-OH-MTX loss (%)	Mean MTX loss (%)	Mean 7-OH-MTX loss (%)
5	-21.00 (10.74)	< LLOQ	0.13 (11.25)	< LLOQ	-4.05 (8.00)	< LLOQ
10	-14.62 (4.46)	-22.35 (8.45)	0.96 (6.79)	0.30 (11.31)	-3.51 (3.61)	-2.42 (5.90)
50	-25.86 (4.37)	-28.97 (5.32)	-7.40 (6.84)	-7.82 (2.19)	-15.57 (5.76)	-13.46 (3.12)
500	-33.45 (3.00)	-32.05 (5.39)	-18.91 (6.63)	-11.04 (3.69)	-18.35 (5.86)	-9.89 (3.45)
1000	-6.11 (1.26)	-6.49 (2.89)	-12.61 (2.14)	1.22 (2.75)	-23.07 (6.06)	-15.70 (4.61)
Mean loss for this time point (%)	-20.21 (10.46)	-22.47 (11.39)	-7.57 (8.46)	-4.34 (6.04)	-12.91 (8.76)	-10.37 (5.81)

Table 5. MTX and 7-OH-MTX stability testing for samples stored at -80 °C demonstrating no significant loss of 7-OH-MTX at 168 hours (n=3)

Participant	MTX dose (mg)	Hours after MTX	MTX (nM)	SD (nM)	CV (%)	7-OH-MTX (nM)	SD (nM)	CV (%)
1	15	97.5	8.39	0.89	10.58	15.99	2.05	12.79
2	20	91.8	80.35	1.88	2.34	31.50	0.49	1.55
3	10	105.6	7.12	0.33	4.69	< LLOQ		
4	12.5	94.5	4.52	0.24	5.40	< LLOQ		

Table 6. Patient results of MTX and 7-OH-MTX levels following directly observed therapy of MTX (n=3).

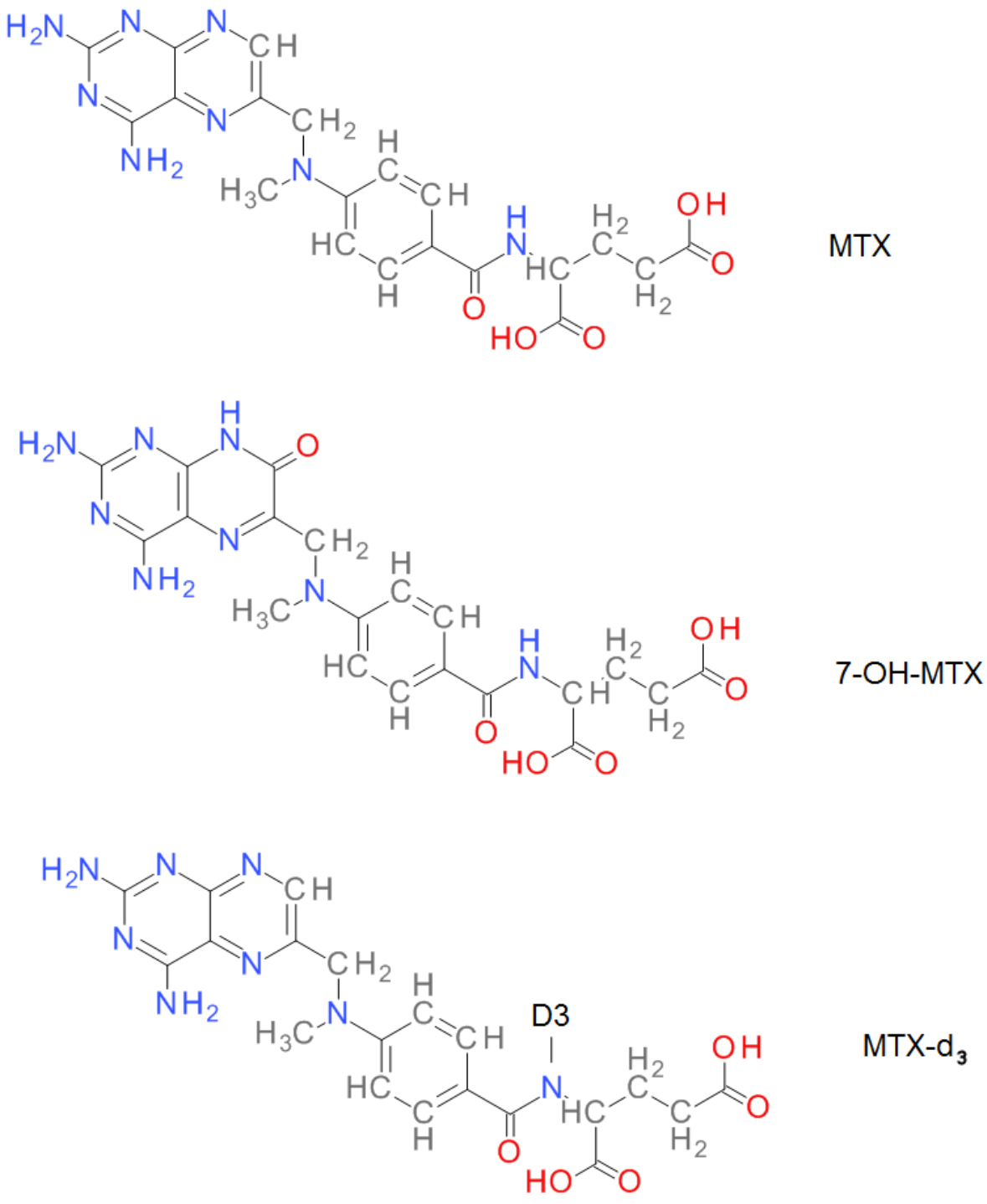

Figure 1. Chemical structures for MTX, 7-OH-MTX and MTX-d_3.

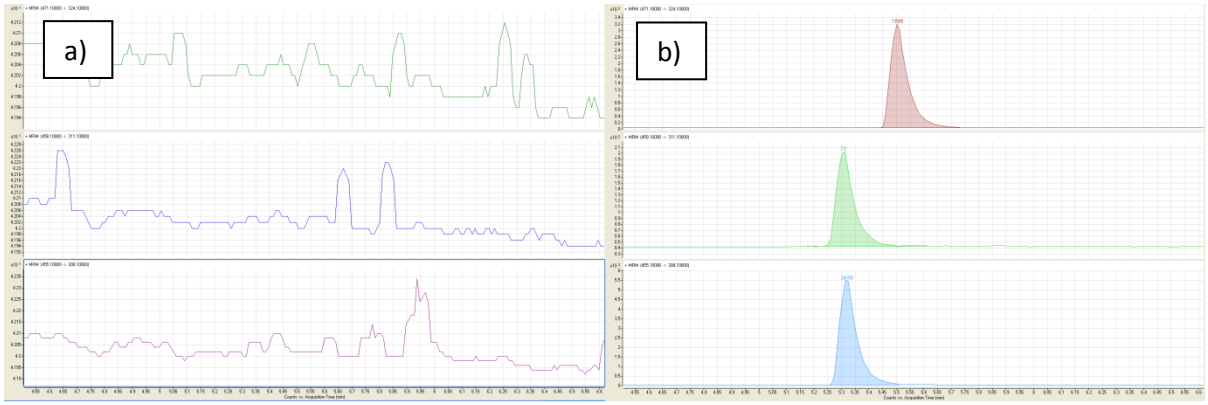

Figure 2. a) Typical chromatograms obtained from a 5uL injection of blank urine showing no detectable signals for 7-OH-MTX (top), MTX-d_3 (middle) or MTX (bottom) and b) from a 5uL injection of urine collected from a patient 23 hours after MTX administration.

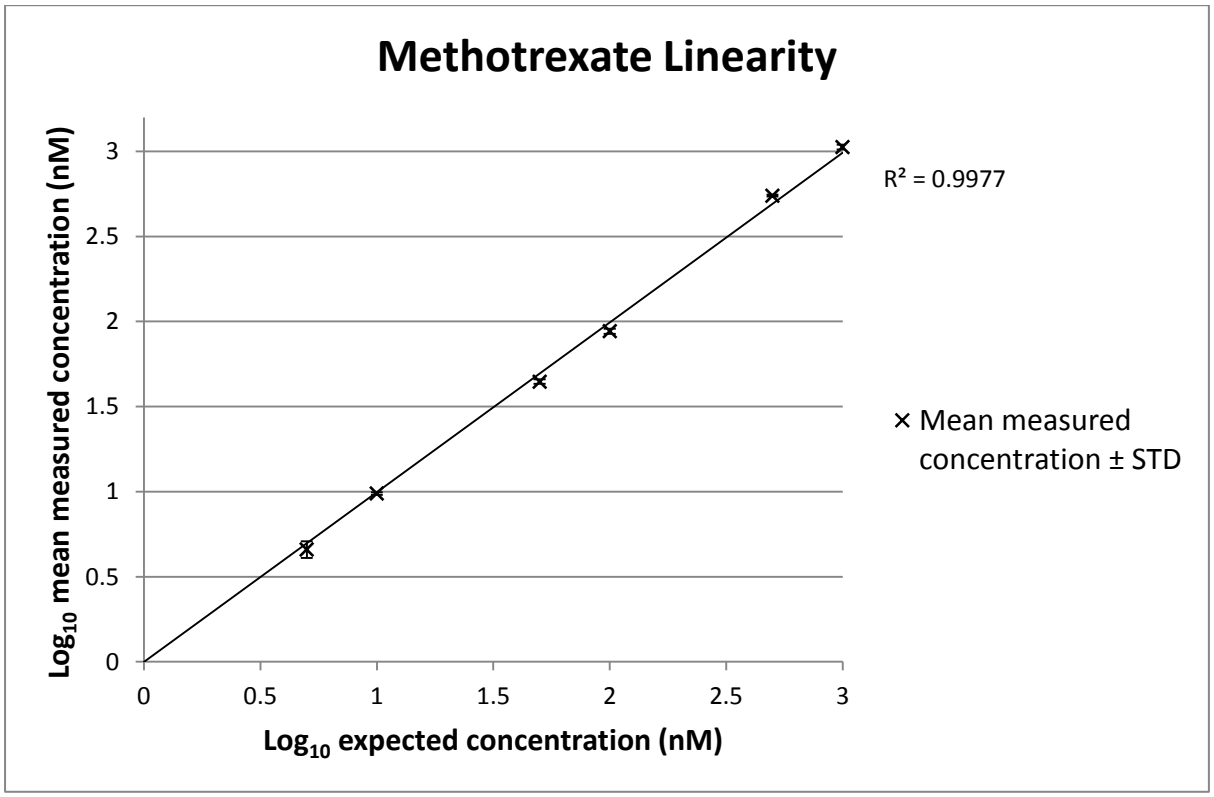

Figure 3a. MTX linearity results. Mean measured concentration ± standard deviation (STD) were plotted against the expected concentration (n=3).

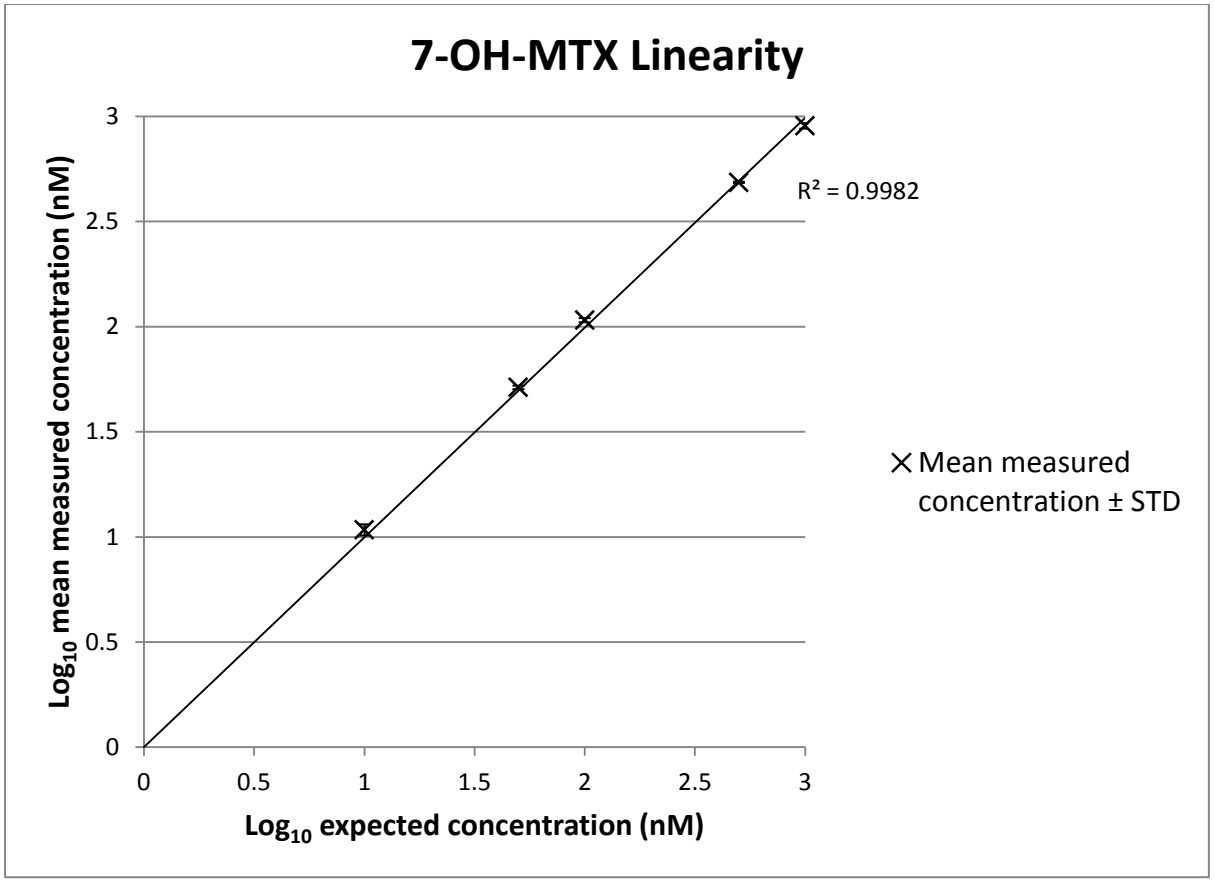

Figure 3b. 7-OH-MTX linearity results. Mean measured concentration ± STD were plotted against the expected concentration (n=3).