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Pre- and post-dialysis quantitative dosage of thymidine in urine and plasma of a MNGIE patient by using HPLC-ESI-MS/MS

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Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive disorder characterized by severe gastrointestinal dysmotility, cachexia, ptosis, ophthalmoparesis, peripheral neuropathy and leukoencephalopathy. The disease is due to a thymidine phosphorylase defect. This enzyme catalyses the phosphorolysis of thymidine to thymine and deoxyribose 1-phosphate. For this reason, increased levels of thymidine in plasma and urine are found in MNGIE patients. Haemodialysis can reduce circulating plasma thymidine levels and can be beneficial in some MNGIE patients. We developed a fast analytical method based on HPLC-ESI-MS/MS capable of identifying pyrimidine nucleotides (thymine, cytosine, uracil) and nucleosides (thymidine, citidine, uridine) in plasma and urine after direct dilution of the samples without pre-treatment.

In the patient studied, we observed a significant reduction of plasmatic and urinary thymidine levels during and after dialysis. However, we noted a progressive reduction of the initial thymidine level after some dialytic trials. This method will be useful not only for thymidine level follow-up during dialysis in MNGIE patients but also for the improvement of the diagnosis or diagnostic suspect in other pyrimidine defects such as dihydropyrimidine dehydrogenase deficiency, dihydropyrimidinase deficiency and ureidopropionase deficiency. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: MNGIE; thymidine; pyrimidine; dialysis; tandem mass spectrometry

INTRODUCTION

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive disorder defined clinically by severe gastrointestinal dysmotility, cachexia, ptosis, ophthalmoparesis, peripheral neuropathy and leukoencephalopathy.[1] Skeletal muscle biopsies of patients have revealed abnormalities of mtDNA and mitochondrial respiratory chain enzymes.[2] The disease is caused by the thymidine phosphorylase defect. This enzyme catalyses phosphorolysis of thymidine to thymine and deoxyribose 1-phosphate.[3] For this reason, MNGIE patients show increased levels of thymidine in plasma and urine. The gene has been cloned and mapped by Nishino *et al*. in 1999.[4] Some authors hypothesized that mitochondrial deoxyribonucleotide triphosphate (dNTP) pool imbalance is likely responsible for mutagenesis in mitochondrial DNA.[5]

Hemodialysis can reduce circulating plasma thymidine levels and can be beneficial in some MNGIE patients.[6]

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In recent years several methods for thymidine quantitation have been developed using HPLC-UV or HPLC-MS/MS.[6–8] We have developed a fast analytical method based on HPLC-ESI-MS/MS, capable of detecting a spectrum of some pyrimidine metabolites (including thymidine) in plasma and urine. This method can be useful for the accurate determination of the dosage of thymidine during dialytic treatments in MNGIE patients.

EXPERIMENTAL

Materials and method

Chemical standard cytosine, cytidine, uracil, uridine, thymine and thymidine were purchased from Sigma Aldrich (St Louis, USA). Labelled standard Me-^{13}C-thymidine, uracil-1,3-$^{15}N_2$, thymine-$^{2}H_4$ and uridine-ribose-1-^{13}C were purchased from Cambridge Isotopes Laboratories (Andover, MA, USA); cytosine-2,4-$^{13}C_2$,-$^{15}N_3$, cytidine-$^{15}N_3$ were purchased from Aldrich (St Louis, USA).

Stock solutions (1–3 mM) were made in H_2O + ammonium acetate 50 mM.

All solvents were HPLC grade (Sigma Aldrich, St Louis, USA).

An Applied Biosystems-Sciex (Toronto, Canada) API 4000 triple-quadrupole mass spectrometer equipped with a TurboIonSpray (TIS) source was employed in this study. The quantitation experiments were performed using a series PE 200 micropump (Perkin Elmer, USA) coupled to a PE well-plate autosampler, both fully controlled by the API 4000 data system (Analyst 1.4.1 software).

The TIS source was operated in positive ion mode at 350 °C. Collision-activated dissociation (CAD) MS/MS was performed in the LINAC Q2 collision cell, operating with nitrogen at 10 mTorr pressure as the collision gas.

The declustering potentials (DP) for all standards (chemicals and labelled) were in the range of 30–80 V, the collision energies (CE) in the range of 12–27 V and the collision exit potential (CXP) in the range of 5–8 V (laboratory frame). MS and MS/MS spectra were collected in continuous flow mode by connecting the infusion pump directly to the TIS source. A standard solution of 5 ng/µl of each chemical and labelled standards in H_2O + Ammonium Acetate 50 mM was infused at 5 µl/min. The HPLC method had a gradient starting from 100% H_2O + ammonium acetate 50 mM (solvent A) up to 100% MeOH + solvent A 50 : 50 (solvent B).

The column was a Luna C18, 5 µm, 150 mm × 3 mm (Phenomenex, USA) and the flow rate was 400 µl/min.

The injection loop was 40 µl and the injection volume was 3 µl.

Table 1. Declustering potentials, collision energies, collision exit potentials and transitions of each compound in positive ESI mode

Compound	DP (V)	CE (V)	CXP (V)	Q1 (Th)	Q3 (Th)
Cytosine	51	27	8	112.1	95.0
Cytosine-2,4-$^{13}C_2$,-$^{15}N_3$	51	27	8	117.1	100.0
Cytidine	35	23	8	244.2	112.1
Cytidine-$^{15}N_3$	35	23	8	247.2	115.1
Uracil	80	24	5	113.1	70.0
Uracil-1,3-$^{15}N_2$	80	24	5	115.1	71.0
Uridine	30	15	6	245.2	113.1
Uridine-ribose-1-^{13}C	30	15	6	246.2	113.1
Thymidine	30	12	8	243.2	127.1
Me-^{13}C-thymidine	30	12	8	244.2	128.1
Thymine	80	24	6	131.1	114.1
Thymine-$^{2}H_4$	80	24	6	127.1	110.1

The acquired data were processed using the Analyst 1.4.1 proprietary software including the 'Explore' option for chromatographic and spectral interpretation. For quantitative data, MS/MS spectra were collected operating in multiple reaction monitoring (MRM) mode for all compounds. MRM

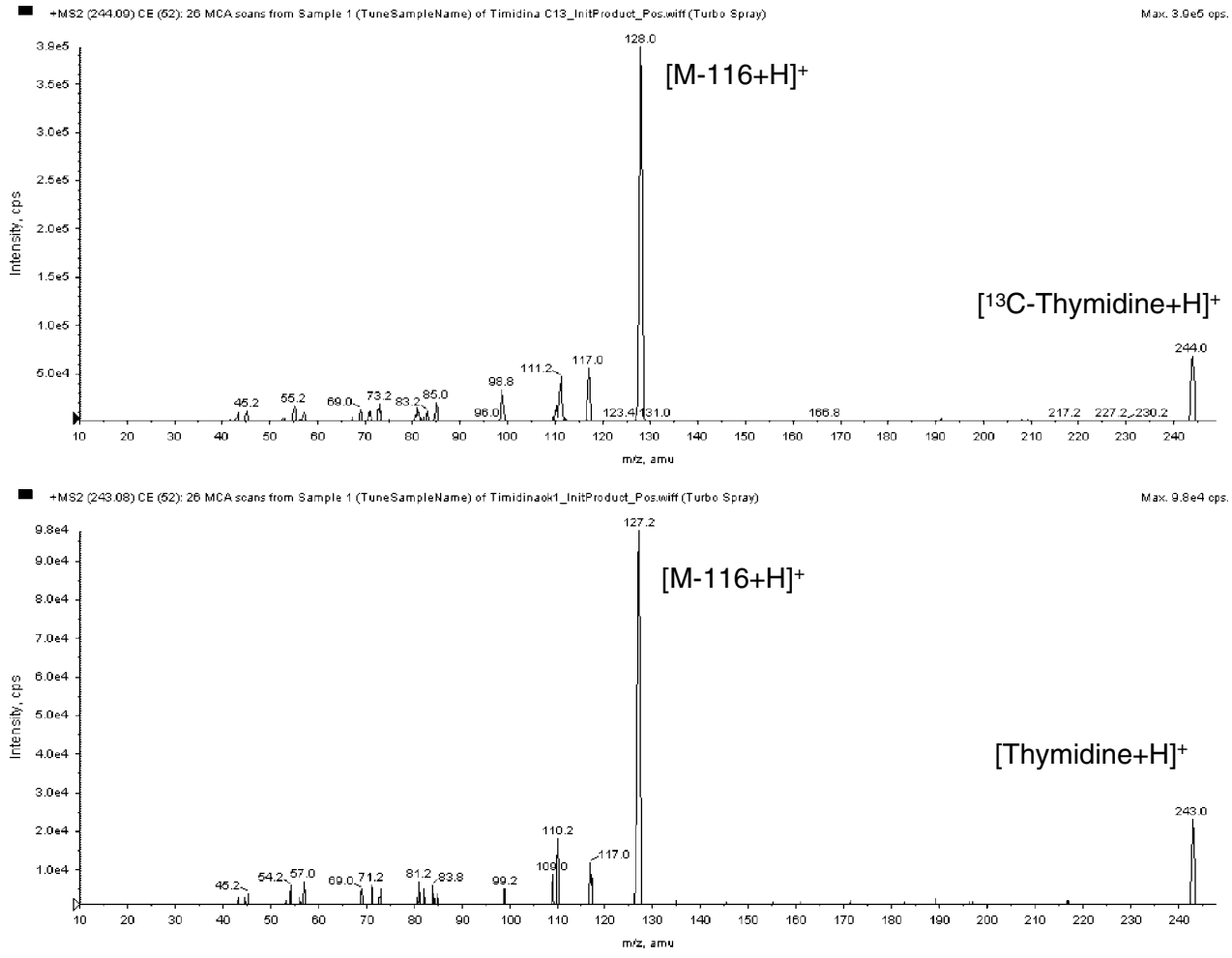

Figure 1. Product ion scan of thymidine and ^{13}C-thymidine.

transitions, DP, CE and CXP for all pyrimidines are reported in Table 1.

The product ion scan spectra of thymidine and ^{13}C-thymidine are reported in Fig. 1.

Samples

Dietary and drug interferences are the major threat to the diagnosis of purine/pyrimidine defects, as many foods, beverages and drugs can contain, especially, purines. We collected the urine and plasma of healthy controls who were recommended to avoid, 48 h before the collection, coffee, tea, chocolate, soft drinks, bananas, not-home-made sweets, liquorice, red meat and, when possible, antibiotics, aromatics (paracetamol, aspirin) and purine/pyrimidine analogues such as fluorouracil and allopurinol. The best condition was

Table 2. Intra-day reproducibility of pyrimidines at various concentrations

Unspiked urine (creatinine 3.59 mM)						
	Thymidine	Thymine	Cytidine	Cytosine	Uridine	Uracil
Expected value	1	7.5	12.2	0	1	19
Average	0.95	7.56	12.43	0.00	0.97	18.7
SD	0.12	0.44	0.96	–	0.1	1.07
CV%	12.43	5.82	7.72	–	10.31	5.72
Accuracy	95.00	100.80	101.89	–	97.00	98.42
Spiked urine (creatinine 3.59 mM)						
	Thymidine	Thymine	Cytidine	Cytosine	Uridine	Uracil
Expected value	21.36	27.86	22.56	20.36	31.36	39.36
Average	20.86	28.44	24.01	19.74	32.01	37.56
SD	0.61	1.3	4.3	3.9	0.99	4.02
CV%	2.92	4.57	17.91	19.76	3.09	10.70
Accuracy	97.68	102.08	106.43	96.95	102.07	95.43
Spiked urine (creatinine 2.11 mM)						
	Thymidine	Thymine	Cytidine	Cytosine	Uridine	Uracil
Expected value	10.03	15.44	18.08	10.11	10.96	24.47
Average	9.45	13.95	19.04	8.87	11.13	25.29
SD	0.41	4.6	2.6	1.6	0.32	2.1
CV%	4.33	32.97	13.66	18.04	2.88	8.30
Accuracy	94.2	90.35	105.31	87.73	101.55	103.35
Spiked urine (creatinine 2.11 mM)						
	Thymidine	Thymine	Cytidine	Cytosine	Uridine	Uracil
Expected value	20.03	35.44	28.08	20.11	20.96	34.47
Average	20.23	33.12	27.44	19.88	22.04	35.82
SD	1.08	4.6	1.9	3.1	2.32	3.1
CV%	5.31	13.89	6.92	15.59	10.53	8.65
Accuracy	100.98	93.45	97.72	98.86	105.15	103.92
Spiked urine (creatinine 0.44 mM)						
	Thymidine	Thymine	Cytidine	Cytosine	Uridine	Uracil
Expected value	11	16.94	15.28	10.01	10.13	21.11
Average	11.29	17.12	16.02	9.91	11.01	20.63
SD	0.73	2.7	1.5	1.1	0.57	1.71
CV%	6.46	15.77	9.36	11.10	5.18	8.29
Accuracy	102.61	101.06	104.84	99.00	108.69	97.73
Spiked urine (creatinine 0.44 mM)						
	Thymidine	Thymine	Cytidine	Cytosine	Uridine	Uracil
Expected value	26	31.94	30.28	25.01	25.13	46.11
Average	25.98	30.12	30.02	25.06	24.44	44.86
SD	1.74	4	1.4	3.1	2.57	5.08
CV%	6.69	13.28	4.66	12.37	10.52	11.32
Accuracy	99.92	94.30	99.14	100.20	97.25	97.29

to perform analysis on urine collected for 24 h or, when not possible, on urine randomly collected and normalized to creatinine. Our reference value for thymidine in urine was 0.09–1.34 μmol/mmol creatinine (5–95th percentiles, based on controls = 80). Plasma thymidine was normally not detectable or was present only in traces.

Sample preparations

Urine and plasma samples were diluted 1 : 20 with H_2O + ammonium acetate 50 mM, added with labelled standards (conc. 100 μM) and directly injected in the LC/MS/MS system for analysis. Urine and plasma samples were collected and analysed on the same day. If a rapid analysis was not possible, samples were frozen to −20° C immediately to avoid degradation. No addition of preservatives such as chloroform or toluene was made, to avoid electrospray suppression. For this study different urine samples with different creatinine concentrations were spiked with a mixture of chemical standards. Sample dilution rate (20 times) and injection volume (3 μl) were selected in order not to overload the chromatographic column even after a consistent number of sample injections (0.3 μl of original urine was introduced through each single injection). The injection volume was selected after several trials with different injection volumes. The dilution step made with H_2O + ammonium acetate 50 mM and no organic solvent was intended to pack the injected sample plug at the front end of the column.

In order to assess the robustness of the method, several urine and plasma samples (with very different densities and creatinine concentrations) were processed several times. Samples were processed and read in comparison with aqueous standard (results are stated without normalization for the creatinine content). As an example, Table 2 reports the thymidine data obtained for three samples with low (0.44 mmol/l), medium (2.11 mmol/l) and high (3.59 mmol/l) creatinine concentrations. The resulting intra-day reproducibility was below 6.7% for values above 10 μmol/l. In an inter-day reproducibility test, the values were better than 3.6% (Table 3). With the proposed parameters, the estimated thymidine limit of detection (3 × SD) was less than 0.01 μM (similar values for all other pyrimidines). We found the worst limit of detection in the case of uracil of about 0.08 μmol/l.

No deterioration in column efficiency was observed after analyses of 125 urine and 80 plasma samples.

Case report

The proband was a woman born in 1976 who died at the age of 28. Her parents were consanguineous. No other family members, including an older and a younger brother, had any neurological or gastrointestinal abnormalities. The onset of symptoms was in the first months of life with vomiting and diarrhea; when 10 years old, she presented diarrhea, borborygmia and abdominal pain. Gastrointestinal investigations and intestinal biopsy showed no lesion.

At the age of 19 she presented impaired growth: another biopsy showed nonspecific chronic inflammation.

At the age of 20 the patient was examined for unsteady gait: Electromyography (EMG) showed neurogenic changes in muscle, and an axonal, mostly sensory, peripheral neuropathy.

She developed bowel obstruction and went for operative intervention with surgical resection several times.

At the age of 22, the patient was referred to our department for abdominal pain, diarrhoea, diverticula and dilatation of the bowel. ECG, echocardiography, audiometry and neuropsychological examinations were normal, as well as plasma creatine kinase and plasma lactate. The retina was normal. Examination revealed mild ptosis, ophthalmoplegia, areflexia, sensory ataxia and diffuse muscle weakness associated with pronounced cachexia. She was 152 cm tall (<3rd percentile) and weighed 30 kg (<3rd percentile). Gastrointestinal investigations showed chronic pseudo-obstruction due to dysmotility and dilation. Brain MRI demonstrated leukoencephalopathy.

MNGIE was suspected for specific clinical symptoms (intestinal pseudo-obstructions, leukoencephalopathy and peripheral neuropathy) and was diagnosed by markedly raised plasma thymidine and severely reduced thymidine phosphorylase activity in leukocytes.[6] Sequence analysis of the patient's *TP* gene revealed homozygosity for the mutation g3867a.[6] Analysis of muscle mitochondrial enzyme was normal, and muscle histochemical analysis did not show any abnormalities. A tentative therapeutic trial with benzbromarone was started without any clinical improvement.

She underwent three weekly dialysis treatments to reduce thymidine levels. We analysed via our tandem mass spectrometric method, plasma and urine samples collected before and after dialysis. We demonstrated a gradual reduction of thymidine levels during dialytic treatment.

RESULTS AND DISCUSSION

We have developed a new and fast analytical method that allows the quantification of pyrimidine metabolites in biological fluids such as urine and plasma.

Table 3. Inter-day (6 days) reproducibility on spiked urine

	Spiked urine (creatinine 0.44 mM)					
	Thymidine	Thymine	Citidine	Cytosine	Uridine	Uracil
Expected value	11	16.94	15.28	10.01	10.13	21.11
Average	11.07	17.33	15.55	10.81	11.91	21.35
SD	0.39	0.7	0.95	1	0.34	1.01
CV%	3.52	4.04	6.11	9.25	2.85	4.73
Accuracy	100.65	102.30	101.77	107.99	117.57	101.14

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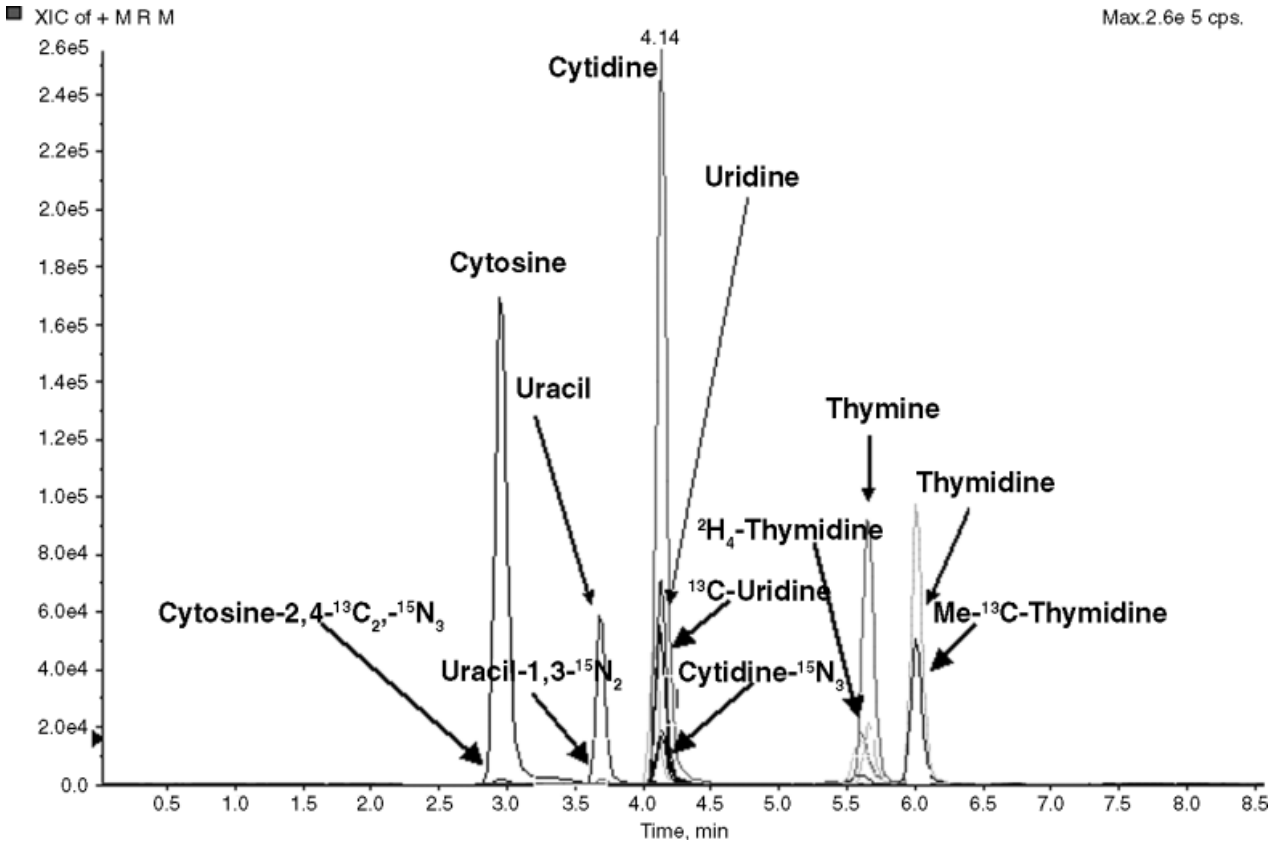

Figure 2. LC-MS/MS pyrimidine standards profile.

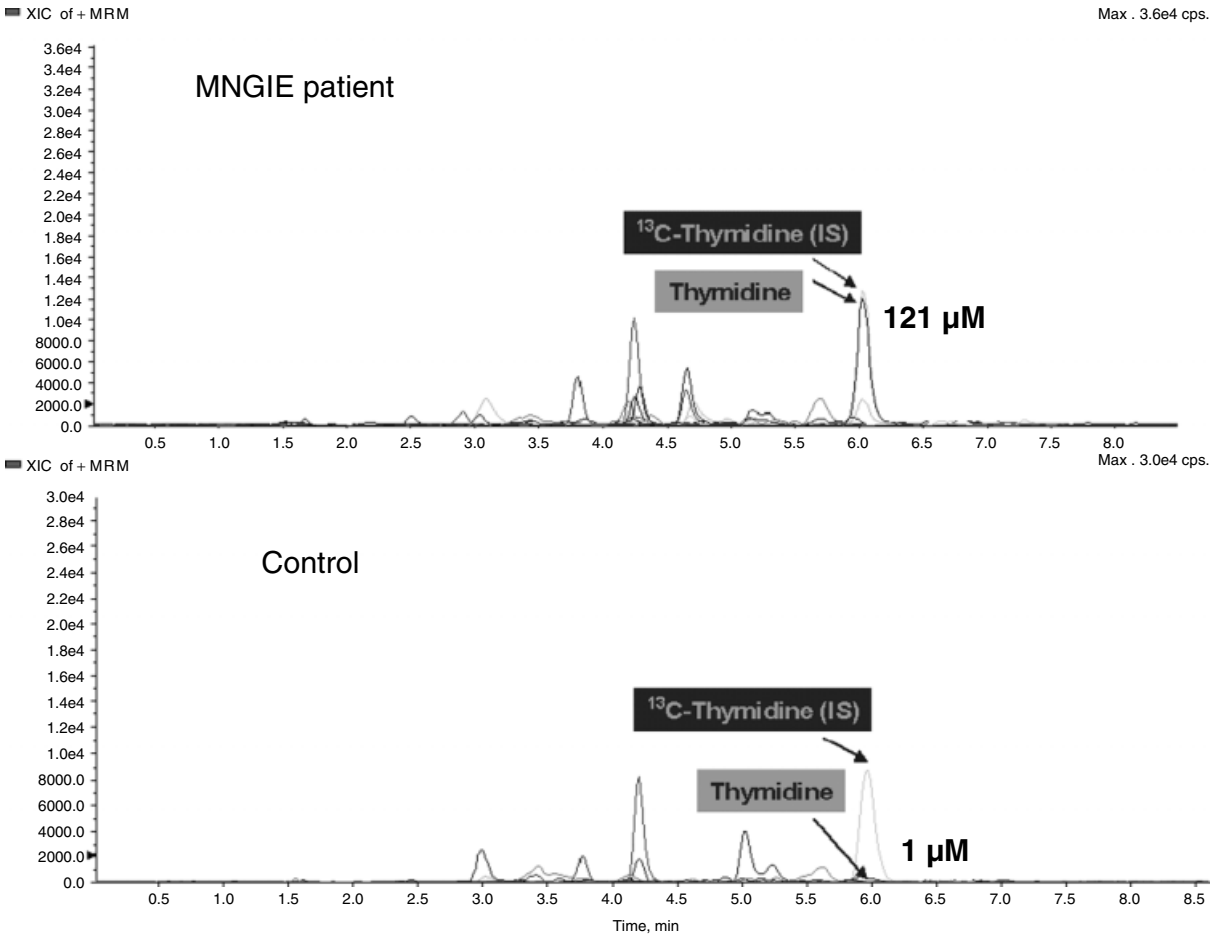

Figure 3. Urine thymidine level: MNGIE patient *versus* control.

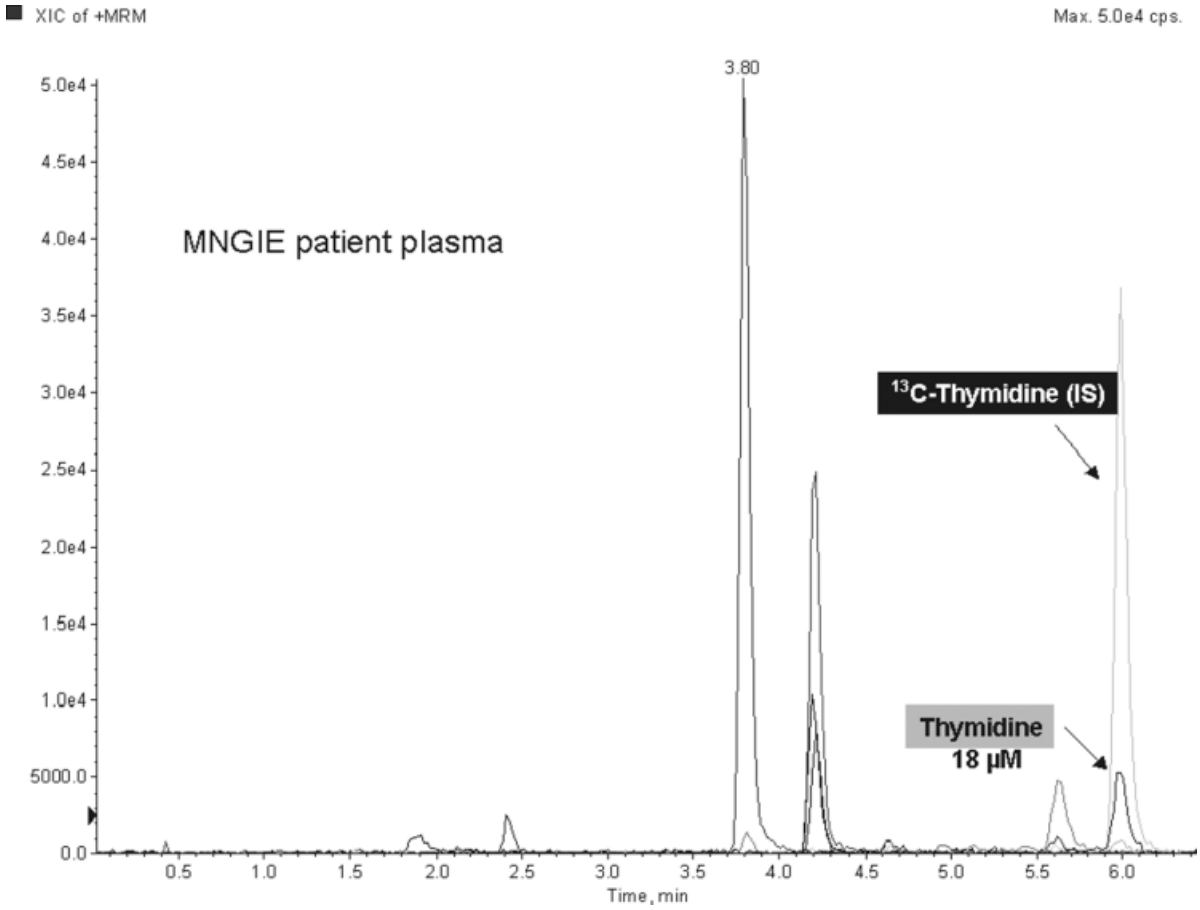

Figure 4. Plasma thymidine level in the MNGIE patient.

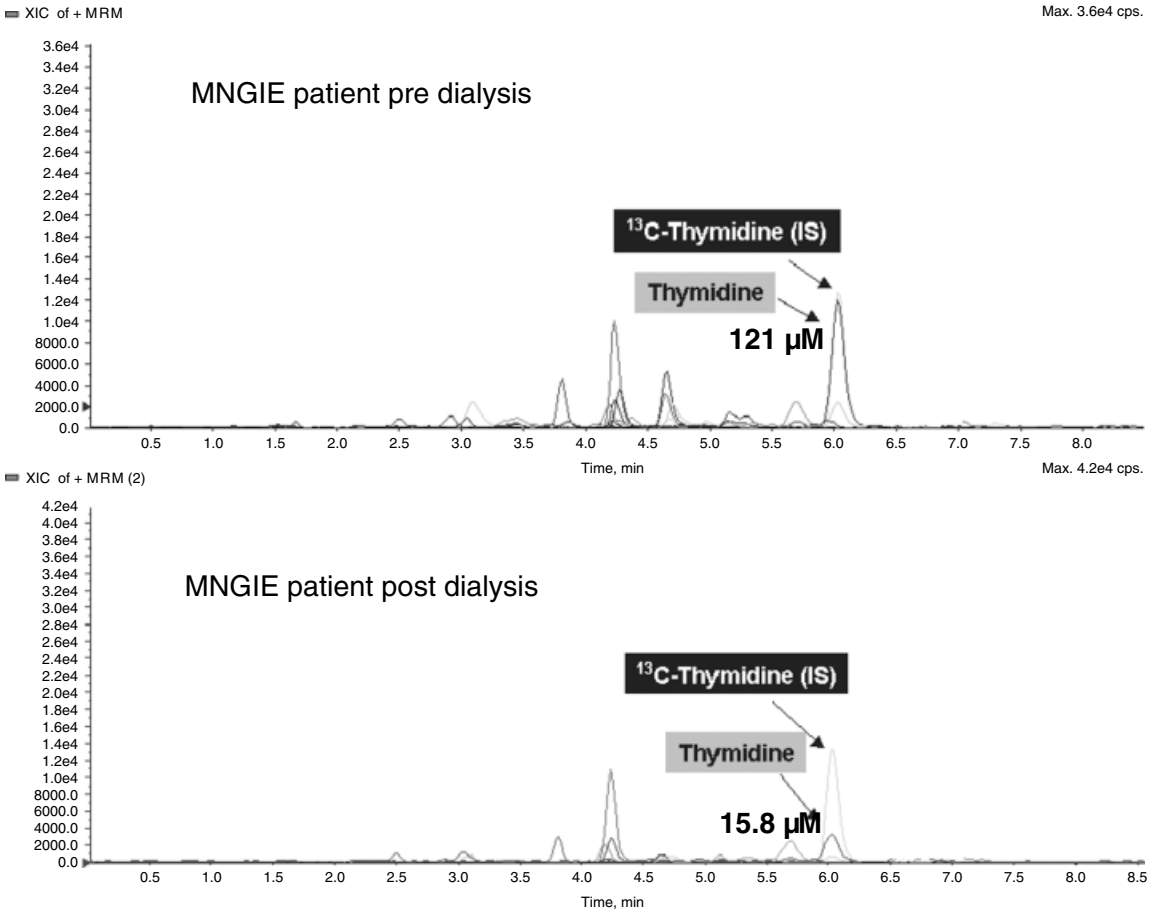

Figure 5. Comparison between pre- and post-dialysis urine thymidine level in the MNGIE patient.

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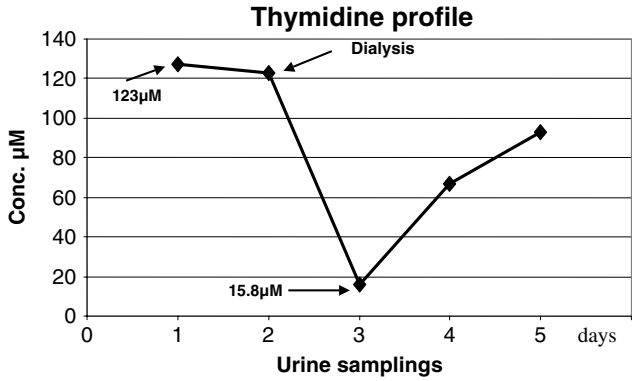

Figure 6. Thymidine level reduction after haemodialysis used as therapeutic approach.

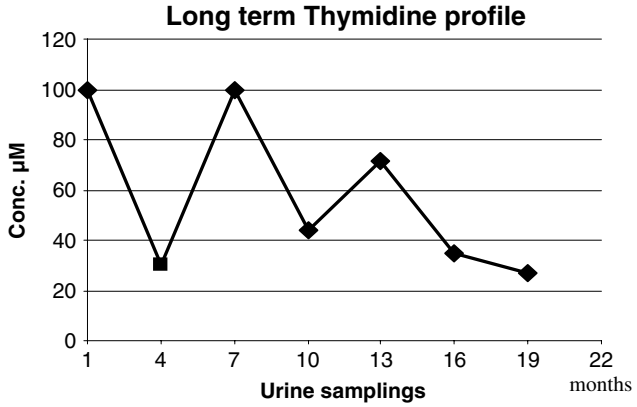

Figure 7. Long-term thymidine profile: the graph shows that repetitive dialysis can reduce the maximum basal level of circulating thymidine in a MNGIE patient.

The proposed method is characterized by a fast and very simple sample preparation. Calibration is made internally with aqueous labelled standards. Instrumental analysis time is less than 17 min. Chromatography combined with the specificity provided by tandem mass spectrometry detection allows a fast and specific procedure for the determination of some pyrimidines. At the moment we are able to identify pyrimidine nucleotides (thymine, cytosine, uracil) and nucleosides (thymidine, citidine, uridine) (Fig. 2). This method allowed the quantification of urine (Fig. 3) and plasma (Fig. 4) thymidine levels in a MNGIE patient before, during and after dialysis used as therapeutic approach (Figs 5, 6).

In the patient studied, we observed a significant reduction of plasmatic and urinary thymidine levels during and after dialysis. However, we noted a progressive reduction of the initial thymidine level after some dialytic trials (Fig. 7).

This method will be useful not only for thymidine level follow-up during dialysis in MNGIE patients but also for improvement of the diagnosis or diagnostic suspect in other pyrimidine defects such as dihydropyrimidine dehydrogenase deficiency, dihydropyrimidinase deficiency and ureidopropionase deficiency.

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