Chapter 3

Quantitative measurement of thiopurine metabolites in biological fluids using ultra-performance liquid chromatography-tandem mass spectometry

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submitted

Abstract

Thiopurines are used as anti-inflammatory drugs in a broad spectrum of diseases, therapy monitoring is of importance because of the clinical consequences of high intracellular concentrations of thiopurine nucleotides. The measurement of thiopurine degradation products is not routinely performed in thiopurine therapy, although this can give important information on drug efficacy.

Sophisticated techniques like UPLC-tandem mass spectrometry are excellent tools to measure metabolites in biological fluids. Using stable isotope dilution techniques a method was developed for the quantitative determination of thiopurine metabolites in urine and plasma of patients treated with thiopurines.

All compounds showed excellent linearity and recovery, both in urine and plasma. Stability of 6-TU, 6-MP and 6-TG in urine was insufficient, concentrations decreased >50% over a 3 week period; the methylated compounds 6-MMP and 6-MTG were stable. In plasma all compounds were stable over a period of 3 months. Intra assay variation was within 5%, both in urine and plasma. Inter assay variations for the thiopurines in urine were unsatisfactory for the above mentioned decay over time, for the methylated compounds the inter assay variation varied from 22% for low concentrations (0.3 µmol/l) to 4% for the control sample with concentrations of 3 µmol/l. Plasma inter assay varied between 4 and 15%.

In control samples thiopurine metabolites were not detectable. In urine and plasma from patients treated with thiopurine medication the method was used to quantify the thiopurine metabolites.

Introduction

Thiopurines are a class of drugs used for the treatment of patients suffering from a wide variety of diseases. Today they are the standard steroid sparing therapy in (systemic) inflammatory diseases like Crohn's disease, colitis ulcerosa and sarcoidosis[1,2]. Furthermore they are used as therapeutics in lymphatic proliferative diseases and, although to a lesser extent nowadays, to avoid rejection in organ transplantation[3] .
The metabolism of thiopurines is a complex network of activation and degradation. Genetic factors influence how thiopurines are handled: metabolites accumulate or are converted into less efficient or more toxic compounds[4,5]. It is apparent that measurement of the different (classes of) compounds is important to fully understand the pathway and to detect disturbances in thiopurine handling[6]. In a clinical setting, measurement of thiopurine derivatives has two objectives. The first one is the detection and quantification of the active compounds, the thiopurine nucleotides; the second objective is the quantification of the thiopurine metabolites, either to detect increased degradation or to ascertain therapy compliance.
Measurement of thiopurine nucleotides is mainly part of the therapeutic drug monitoring (TDM) protocol[7]. The goal is to monitor the levels of the active, i.e. toxic, thionucleotides in order to avoid undesired adverse drug reactions (ADR). These reactions can be either the formation of an excess of active metabolites or non-responsiveness to therapy. The majority of methods used for the measurement of intracellular thionucleotides are HPLC based methods. In these procedures the phosphate groups are removed from the thionucleotides, either by acid hydrolysis or enzymatically, and the remaining thionucleosides and thiobases are separated and quantified by HPLC[8-10]. The general drawback of these methods is that they measure the 'total' amount of thioguanine or mercaptopurine and their methylated forms. To be able to determine the true intracellular (methyl)thionucleotide content a more sophisticated separation method is needed, using either ion exchange chromatography or ion-pair reversed phase LC separation[11]. The long separation times and the very low concentrations of the individual intracellular (methyl)thionucleotides makes these methods not suitable for routine TDM purposes.
The measurement of the degradation products of thiopurines has not become common practice, although the measurement of these compounds can give important information on the efficacy of therapy and pharmacokinetic properties[12,13]. Recently a revised HPLC method for the determination of 6-mercaptopurine and other thiopurine metabolites in plasma and erythrocytes was published[14].

We describe the development and validation of a ultra-performance liquid chromatography tandem mass spectrometry method for the determination of thiopurine end-products in biological fluids, requiring minimum amounts of sample and less sample preparation.

Materials and methods

Patients

Urine, plasma and erythrocytes were obtained from patients on thiopurine therapy in the frame of TDM. Samples were used according to the “Code for proper use of human tissue” as formulated by the Dutch Federation of Medical Scientific Societies.

Chemicals

Thiopurine bases and nucleosides were obtained from Sigma (Zwijndrecht, the Netherlands) or from Jena Bioscience (Jena, Germany). 6-thiouric acid (6-TU), $^{13}C_1$-6-thiouric acid ($^{13}C_1$-6-TU) and $^{13}C_1$-6-mercaptopurine ($^{13}C_1$-6-MP) were synthesised by SyMO-Chem (Eindhoven, the Netherlands). 6-Methyl-D_3-mercaptopurine (6-D_3-MMP) was obtained from C/D/N Isotopes Inc (Pointe-Claire, Quebec, Canada). Tris(2-carboxyethyl)phosphine (TCEP) was obtained from Sigma (Zwijndrecht, The Netherlands). LCMS-grade acetonitril, methanol and water were purchased from VWR (Amsterdam, the Netherlands). All other chemicals were of the highest quality and purchased from Sigma (Zwijndrecht, the Netherlands).

Sample preparation

To 50 µl sample, either urine, plasma or a mixture of standards in water, an equal volume of a mixture of stable isotope labelled internal standards (concentrations ~ 3 µM) was added. Reduction of protein bound thiopurines was achieved using 100 µl 25 mM TCEP in 50 mM ammoniumformiate (pH 6.3) as the reducing agent. After the addition of 300 µl ice cold acetonitrile / 0.1% Formic acid / 0.025% TFA (v/v/v) the mixture was vigorously vortexed. After centrifugation 400 µl supernatant was evaporated to dryness under nitrogen at room temperature. The residue was dissolved in 150 µl 0.2 M ammoniumformiate pH 4.0 and 2 µl was injected into the UPLC using full loop injection.

Ultra performance liquid chromatography

Separation of the compounds of interest was achieved using an Acquity ULPC system equipped with an Acquity HSS T3 column, 100 * 2.1 mm, particle size 1.8 µM (Waters, Milford, MA). Mobile phases were as solvent A 0.01 M Ammoniumformiate (pH 4.00) and as solvent B methanol / 0.01 M Ammoniumformiate (pH 4.0) (60/40, v/v). A gradient program (see Table 3.1) was used to achieve sufficient retention of the compounds of interest. Run to run time was 18 minutes.

Table 3.1 Gradient program used for separation of thiopurine metabolites.

Time (min)	Flow (ml/min)	%A	%B
Initial	0.45	98	2
2	0.45	98	2
6	0.45	60	40
7	0.45	0	100
8	0.45	0	100
10	0.45	98	2

Tandem mass spectrometry conditions

Mass spectrometry experiments were performed using a Micromass Quattro Premier XE Tandem Mass Spectrometer (Waters, Milford, MA). The mass spectrometer was used in the multiple reaction mode (MRM), both in electrospray ionisation (ESI) positive and negative mode. Desolvatation temperature was 450°C, source temperature 130°C. Capillary voltage setting was 0.5 kV, cone and collision settings for the different compounds are shown in Table 3.2. Nitrogen gas was used both as desolvatation and cone gas, flows were 800 l/h and 50 l/h, respectively. Argon was used as collision gas for MRM and daughter ion scans (flow 0.15 ml/min, pressure $2.8 * 10^{-3}$ bar). Settings of the mass spectrometer for detection of the compounds of interest and internal standards used are shown in Table 3.2. Resolution parameters for the compounds of interest (LM1HM1) and internal standards (LM2HM2) were set to 12 in both positive and negative mode.

Table 3.2 Mass spectrometer settings for detection and quantification of thiopurine metabolites.

Compound	mode	parent m/z	daughter m/z	Cone V	collision V	CV kV	DT sec
6-thio-urinezuur	neg	182.9	140.1	35	15	0.25	0.1
$^{13}C_1$-6-thiourinezuur	neg	184.1	141	35	15	0.25	0.1
6-thioguanine	pos	167.8	150.7	40	19	3.5	0.005
6-methyl-thioguanine	pos	181.8	133.8	40	20	3.5	0.005
6-mercaptopurine	pos	152.7	118.9	40	20	3.5	0.005
$^{13}C_1$-6-mercaptopurine	pos	153.7	119.9	40	20	3.5	0.005
6-methyl-mercaptopurine	pos	166.8	151.8	40	22	3.5	0.005
6-D_3-methyl-mercaptopurine	pos	169.8	151.6	30	25	3.5	0.005

Cone: Cone voltage; collision: Collision cell voltage ; CV : capillary voltage ; DT : dwell time ; mode : detection mode

Linearity and recovery

All compounds were added to water and three different urine and plasma samples to establish linearity in the expected range of 0-5 µM. For quantification stable isotope labelled internal standards were used, for 6-TG and 6-MTG we used $^{13}C_1$-6-MP and 6-D_3-MMP respectively as internal standards. Recovery of the compounds of interest was deduced from the slope of the calibration curves. The limit of detection (LOD) was determined in water at a signal to noise ratio (S/N) of 3, for the limit of quantification the lower limits were determined in urine and plasma samples at S/N of 10.

Intra- and inter-assay variability

The intra-assay variation was determined by 10 consecutive analysis of spiked plasma and urine samples, samples were spiked with low (0.3 µmol/l), medium (1.2 µmol/l) and high (3.0 µmol/l) concentrations of thiopurine metabolites. The inter-assay variation was determined by measuring the spiked urine and plasma samples on 10 consecutive days.

Results

Chromatography

Baseline separation for the compounds of interest was achieved within 8 minutes, using the gradient conditions listed in Table 3.1. In Figure 3.1 a chromatogram of a standard solution of thiopurine metabolites is shown.

Linearity

Concentrations of the thiopurine metabolites were linear up to 10 µM, either in water, plasma and urine (R^2>0.99). Recoveries were 100 ± 5% for the compounds measured using this method. In Figure 3.2 the addition curves for 6-TU in urine and plasma are shown. The curves for the other compounds were comparable (data not shown).

Precision/accuracy

Intra- and inter-assay variations for the plasma samples at the different concentrations were between 2.8 and 4.8 % for all compounds for the low concentrations and between 1.8 and 3.7 % for the high concentrations. Intra-assay variation in urine was comparable to plasma. In urine only the methylated thiopurines were stable, and showed a reasonable inter-assay variation. Inter-assay variations for thiopurines could not be established. In Table 3.2 the intra- and inter-assay variations for 6-TU, 6-MP and 6-MMP in plasma and urine are shown.

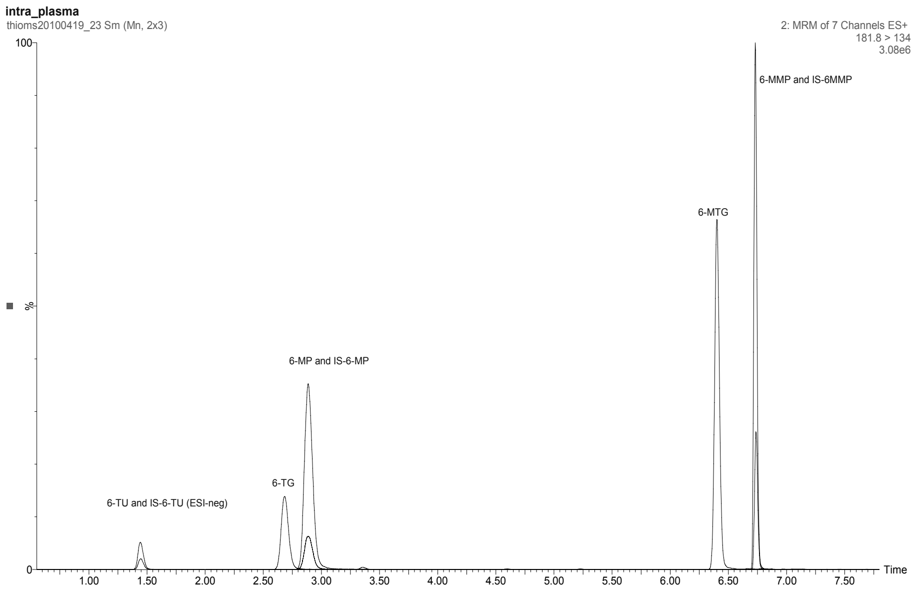

Figure 3.1 LC-MSMS chromatogram of thiourine metabolites. Experimental conditions are described in the methods section.

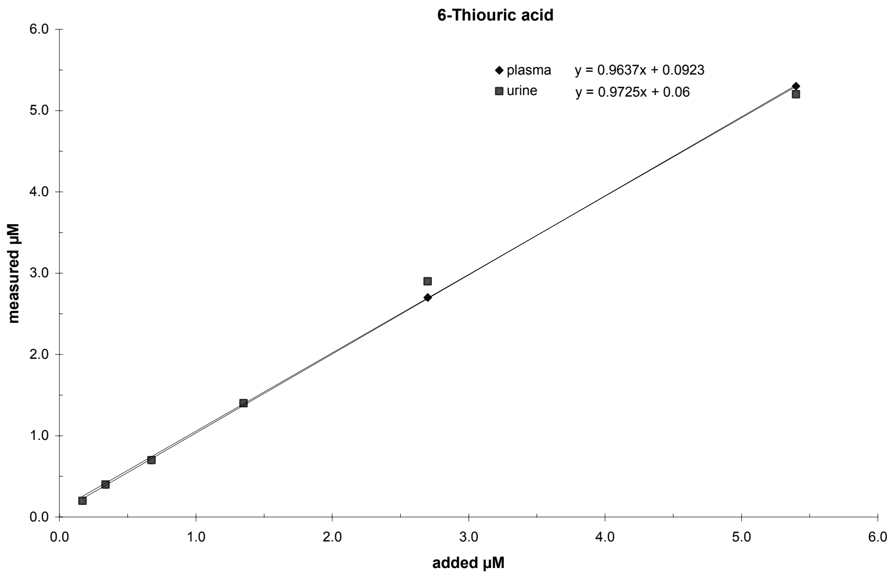

Figure 3.2 Addition curves for 6-TU in urine and plasma.

Table 3.2 Intra- and inter-assay variation (%) for 6-TU , 6-MP and 6-MMP in urine and plasma.

Matrix	6-TU		6-MP		6-MMP	
	Intra	Inter	Intra assay	Inter	Intra	inter
Plasma low	4.8	9.6	2.8	14.9	3.2	5.2
Plasma medium	4.2	8.9	3.6	6.4	2.8	6.9
Plasma High	1.8	5.6	1.9	7.3	3.7	4.7
Urine low	3.6	59	3.5	48	2.9	22
Urine medium	4.1	28	2.7	43	3.3	6.7
Urine High	2.3	51	2.1	n.d.	2.8	3.7

LOD/LOQ

The limit of detection (LOD) of the different compounds was established after dissolving the compounds in water and injection of the solutions. As mentioned earlier, a S/N ratio of 3 was used for the calculation of the LOD. The limit of quantification (LOQ) was established by adding the compounds of interest it plasma and urine samples. LOQ was set at S/N ratio 10. The LOD and LOQ for the different compounds are shown in Table 3.3.

Table 3.3 Limits of detection and quantification of thiopurine metabolites.

Compound	LOD (nmol/l)	LOQ (nmol/l)	
		Urine	Plasma
6-thiouric acid	30	200	100
6-mercaptopurine	30	200	100
6-thioguanine	30	200	100
6-methylmercaptopurine	10	75	50
6-methylthioguanine	10	75	50

Sample stability

Plasma and urine samples were spiked with 6-TU, 6-MP, 6-TG, 6-MMP and 6-MTG and used as control samples for metabolite sample stability, samples were divided into 500 µl aliquots and stored at -20°C. In plasma all metabolites were stable over a period of 2 months when the samples were stored at -20°C. In urine methylated thiopurines, 6-MMP and 6-MTG were stable, the thiopurine metabolites with a reactive sulfhydryl-group, 6-TU, 6-MP and 6-TG were very unstable. Within 3 weeks the concentrations of 6-MP and 6-TG decreased to 10% of the original concentrations. The decrease of 6-TU was ~50%. Addition of EDTA and acidification of the urine before storage did not prevent the degradation of the sulfhydryl metabolites.

Thiopurine metabolites in patient samples

Urine and plasma samples from patients treated with thiopurine medication were analysed with the above described method. In urine 6-TUA and 6-MP were present in concentrations ranging from 0.2 µM to 30 µM, depending on dose and co-medication. 6-MMP, 6-TG and 6-MTG were only present in trace amounts. In plasma 6-TUA and 6-MP were detectable, with maximum concentrations of 1 µM for 6-TU and 1.5 µM for 6-MP. It must be taken into account that the plasma samples were random samples. The other metabolites were below the limit of detection. In control samples, both urine and plasma, we could not detect thiopurine metabolites.

Discussion

The introduction of liquid chromatography tandem mass spectrometry (LC-MSMS) in clinical laboratory medicine has overcome a lot of the drawbacks of the earlier separation and detection methods[15]. One of the cornerstones of a reliable LC-MSMS method is the availability of stable isotope labelled standards, as was recently shown by our group in a report on the quantitative determination of amino acids in body fluids[16].

HPLC with UV-detection is currently the standard method for the determination of free and total thiopurine metabolites in urine and plasma[14,17]. These methods have their drawbacks and the results must be interpreted carefully[6,18]. Because of the relative low specificity of UV detection, we developed a stable isotope dilution based LC-MSMS method for the quantification of thiopurine bases and nucleosides in urine and plasma. From the results obtained with our method it can be concluded that the thiopurine bases and corresponding nucleosides can easily be detected and quantified in urine and plasma. Recoveries, both in urine and plasma, were within acceptable limits. Limits of quantification, using the conditions described above, were set at 200 nmol/l, both in plasma and urine, although limits of determination of 50 nmol/l for 6-MP were easily attainable. As was expected, thiopurine metabolites were not detectable in samples from patients who did not receive thiopurine therapy. In patients with inflammatory diseases using thiopurines as an anti-inflammatory drug, the metabolites were easily detected. From our results on the stability of the thiopurine bases and nucleosides we concluded that the sulfhydryl compounds were unstable in urine. Sulfhydryl groups are reactive and prone to oxidation and reactions with other reactive groups[19]. A similar phenomenon we have experienced with the measurement of free homocysteine in urine (data not shown). In plasma the sulfhydryl compounds are mainly protein bound to other sulfhydryl compounds in the protein (cysteine-groups) and can be liberated by reduction of the S-S bond by TCEP. Although thiopurines are cleared from the circulation within 3-4 hours after administration, they are detectable in low concentrations in plasma. It is apparent that only a part of the administered 6-MP is

absorbed in the gastrointestinal tract. 80% of the circulating 6-MP is metabolised to 6-TU and only 25% of the dose is effectively absorbed in the intestine and will enter the activation, deactivation or degradation pathways[2]

In conclusion we have developed a simple and fast method for the determination of thiopurine bases and nucleosides in biological fluids. Furthermore this method can be used for monitoring therapy compliance to thiopurine therapy using urinary 6-TU as a biomarker.

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