SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Metabolomic studies in healthy and pathological conditions

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1. Introduction

1.1. Metabolomic analyses of endogenous molecules

Metabolomic analysis is a comprehensive study of metabolic processes in biological systems, investigating the concentration and dynamics of endogenous molecules such as lipids, amino acids and carbohydrates. Endogenous molecules are substances naturally produced in the body and play an important role in the biochemical processes of living systems. Metabolomics research involves the identification and quantification of these molecules and inferring molecular interactions and biological responses to specific metabolites. For this reason, studying endogenous molecules in the body, such as nitrogen-containing organic molecules, is crucial for the functional interpretation of the homeostasis of living organisms.

1.1.1. Structure and physiological functions of amino acids

Amino acids are the building blocks of proteins, of which 20 α-amino acids and the imino acid proline are used in the synthesis of proteins in the human body. As their names imply, these compounds are carboxylic acids in which an amino group is attached to the α-carbon atom. Each amino acid differs in the side chains attached to the α-carbon atom and, except glycine, contains at least one chirality center. Proteinogenic amino acids are L-amino acids, with only small amounts of D-amino acids typically found in humans. Amino acids can be classified by different features, one common grouping is based on the chemical characteristics of the side chain.

The side chain, which can range from a single hydrogen atom, as in the case of glycine, to complex aromatic rings, as in the case of tryptophan, gives each amino acid a unique chemical and physical property. The diversity of these side chains gives the special chemical behavior and functions of each amino acid.

Another grouping is based on the essentiality of amino acids. By definition, those amino acids that cannot be synthesized by the body and must be obtained from the diet are called essential amino acids. These include phenylalanine, valine, tryptophan, threonine, isoleucine, methionine, histidine, leucine and lysine. By contrast, non-essential amino acids can be synthesized in the body from other molecules and do not need to be taken up by the diet. In addition, there are amino acids that are normally non-essential but may become essential in certain situations, these are called conditionally essential amino acids.

In addition to their role in protein synthesis, amino acids can also regulate metabolic pathways. Certain amino acids have been identified as potential biomarkers for various

pathological conditions. Decreased serum tryptophan levels have been observed in individuals with depression and other mental disorders, while alterations in the levels of several amino acids have been detected in patients with lung, stomach, colon, breast and prostate cancer. Although the level of different amino acid shown to be indicative for different tumor types, glutamine is a common essential amino acid for cell division in all tumor lesions. In addition to mental disorders and tumor lesions, amino acids are also involved in the pathomechanism of diabetes. Insulin inhibits the breakdown of proteins while increasing the uptake of amino acids into the cells. In addition, lysine, aspartate, threonine, methionine and alanine are potential biomarkers for type 2 diabetes.

1.1.2 Structure and physiological function of biogenic amines

Biogenic amines belong to the group of nitrogen-containing organic compounds. Most of the biogenic amines can be produced by various cell types, while some biogenic amines, such as serotonin, can only be produced by certain dedicated cell types, such as neurons.

Biogenic amines have diverse physiological functions. Histamine plays an important role in cell proliferation and differentiation, regeneration and wound healing, vascular permeabilisation, neurotransmission, as well as in inflammatory responses. Tryptamine, tyramine, and 2-phenethylamine are neuromodulators and vascular regulators involved in neurovascular functions, while cadaverine and putrescine are important molecules in cell division. In addition to their physiological role, biogenic amines are also involved in the pathomechanisms of many diseases such as hypertension, schizophrenia, and cancer. A study in a Japanese population showed a negative correlation between the level of ethylamine and the risk for developing type 2 diabetes. The role of methylamine has been observed in central nervous system disorders, and it has been shown that in pregnancy toxemia, methylamine concentrations remain higher for a longer period after delivery than in normal pregnancy. Ethanolamine is present in all cells of the human body as a building block of phospholipids and is also found in free form in body fluids. As a component of phosphatidylethanolamine, ethanolamine plays a role in the progression of neurodegenerative disorders such as Parkinson's disease, tumor development and in ferroptosis. Serotonin, an important neurotransmitter in the central nervous system, plays a key role in the development of depression and has also been linked to obesity and diabetes.

1.1.3 Analysis of amino acids and biogenic amines

Amino acids and biogenic amines can be analysed by a variety of biochemical and analytical methods, such as gas chromatography (GC), liquid chromatography (LC), nuclear magnetic resonance (NMR) and mass spectrometry (MS). Highly sensitive LC-MS systems can be used to analyze molecules present in low concentrations in complex biological samples. However, LC and LC-MS analysis often require derivatization of the target compounds to improve the limits of detection and quantification. Derivatization is a chemical reaction between a molecule of interest and a reagent that changes the chemical and physical properties of the molecule. By the modification of the target analytes, derivatization improves detection, separation efficiency and volatility, and stabilizes the analytes, allowing better chromatographic analysis. Several reagents can be used to derivatize amino acids and biogenic amines, such as dansyl chloride (DnsCl), phthalaldehyde (OPA), 9-fluorenyl- methyl-chloroformate- chloride (FMOC-Cl) or AccQ-Tag. In many cases, mass spectrometry can be used as a complement to chromatographic analyses to increase the selectivity and sensitivity of analyses and to confirm the identity of components identified by ultraviolet (UV) or fluorescence detection.

1.1.3.1 The AccQ-Tag derivatization technique

The AccQ-Tag derivatization technique (Waters) uses 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (ACQ) to convert primary and secondary amines to highly stable fluorescent derivatives. In the first step of the reaction, the primary and secondary amines are reacted with the ACQ reagent. The second step is a slower reaction, where the remaining reagent reacts with water to form 6-aminoquinoline (AMQ), N-hydroxysuccinimide, and CO_2 by-products. In the final step of the derivatization, the main by-product, AMQ, and the residual reagent interact to form a highly stable bis-aminoquinolineurea. These by-products do not affect the identification or quantification of the amino acids, but can serve as a qualitative control in the chromatographic analysis to demonstrate the success and efficiency of the derivatisation reaction. The derivatized amino acids can be separated by liquid chromatography, and fluorescent derivatives can be detected at 260 nm.

1.2 Metabolomic analyses of xenobiotics

Xenobiotics are substances that are not naturally part of the metabolism of living organisms but can enter into the body. Examples of xenobiotics are drugs, chemicals, industrial pollutants, pesticides or other environmental toxins. Metabolomic analyses provide a way to study the effects of these substances on biological systems, providing information on how these xenobiotics alter the metabolic processes of living organisms. Xenobiotics trigger complex and dynamic processes in the organism, such as biotransformation reactions, in which the organism tries to inactivate and/or remove the compounds. During these processes, the substances are transformed into new metabolites that act on biological systems in various ways.

Metabolomic analyses of xenobiotics are of great importance in toxicological research as they help to assess the safety and efficacy of industrial and pharmaceutical products. Furthermore, analyzing the biotransformation profile of individual compounds can also help in personalized medicine, as genetic differences between people can affect the metabolism of xenobiotics, so that treatment can be optimized.

Metabolomics also has a key role in therapeutic drug monitoring (TDM), as it allows a detailed and comprehensive study of the effects, metabolism and excretion of drugs in the body. The aim of TDM is to ensure the effective yet safe use of a drug by monitoring the concentration of drugs and their metabolites in blood plasma or other biological samples and determining the optimal dose. This concerns in particular medicinal products with a narrow therapeutic range and toxic side effects, as well as therapeutic products whose biotransformation may result in the formation of inactive or toxic metabolites.

1.2.1. Autoimmune hepatitis and its treatment

Autoimmune hepatitis (AIH) is a rare chronic inflammatory liver disease that primarily affects women, but can occur in any sex and at any age. AIH is characterized by elevated levels of serum immunoglobulin G and circulating autoantibodies, increased activity of transaminases and liver inflammation. To prevent the development of recurrent liver diseases such as cirrhosis, acute hepatitis and end-stage liver disease, the treatment of AIH requires lifelong therapy in most cases. In most cases, treatment starts with steroid monotherapy followed by maintenance treatment with azathioprine (AZA). Although long-term steroid therapy can cause several side effects, such as diabetes, hypertension and weight gain, the incidence of these can be reduced by the combined use of AZA and steroids.

1.2.2 Metabolism of azathioprine

The metabolism of AZA is complex and involves several enzymatic pathways that result in active, inactive and potentially toxic metabolites.

Approximately 88% of AZA is converted to 6-mercaptopurine (6-MP) and methylnitro-thioimidazole in red blood cells (RBCs) via glutathione-S-transferase (GST) after absorption from the gastrointestinal tract. 6-MP can be further metabolized via several pathways. In a reaction catalyzed by the enzyme thiopurine-methyltransferase (TPMT), it can be methylated to 6-methylmercaptopurine riboside (6MMPr), a potentially hepatotoxic compound. It can be also converted to inactive 6-thiouronic acid (6-TA) by xanthine oxidase (XO); or to 6-thiosine-monophosphate (6-TIMP) by hypoxanthine-guanine phosphoribosyl-transferase (HGPRT). TPMT is also able to catalyze the S-methylation of 6-TIMP to give 6-methylthioinosine monophosphate (6-MeTIMP). Both 6-TIMP and 6-MeTIMP inhibit the phosphoribosyl pyrophosphate aminotransferase enzyme, thereby reducing the rate of de novo purine biosynthesis. 6-TIMP is then converted to 6-thioguanine nucleotides (6-TGN) in a two-step reaction. In the first step, it is converted to 6-thioxanthosine monophosphate (6-TXMP) in a reaction catalyzed by inosine-5-monophosphate dehydrogenase (IMPDH), which is further converted to 6-TGN by guanidine-5-monophosphate synthetize (GMPS). 6-TGNs are considered to be the major therapeutic metabolites of AZA. The 6-TGNs are converted in a series of phosphorylation steps to 6-thioguanine monophosphate (6-TGMP), then to 6-thioguanine diphosphate (6-TGDP) and finally to 6-thioguanine triphosphate (6-TGTP). Although 6-TGNs are considered to be the main therapeutic metabolites, their high levels can cause life-threatening myelosuppression.

1.2.3 Therapeutic use of azathioprine

In addition to the treatment of AIH, AZA and its metabolites are also applied in the treatment of chronic inflammatory diseases, childhood acute lymphoblastic leukemia (ALL) and inflammatory bowel disease (IBD). AZA was originally developed for the treatment of childhood leukemia, but due to the recognition of its antiproliferative effect on T cells, it has been successfully used in the treatment of autoimmune diseases and to prevent rejection after transplantation. The exact molecular mechanism of the immunosuppressive effect of thiopurines is not fully understood yet, but it is known that they may interfere with DNA replication by antagonizing endogenous purines. Thiopurine drugs are effective in preventing disease relapse but are not suitable for inducing remission. In combination with biological drugs, such as anti-tumor necrosis factor (TNF) agents, they can have a beneficial effect by

improving clinical remission and mucosal healing. However, patients who develop antibodies against an anti-TNF agent are significantly more likely to develop antibodies during subsequent anti-TNF therapy. In such cases, the use of TDM in combination anti-TNF and thiopurine therapy may help to prevent immunogenicity in the second anti-TNF treatment. The use of TDM in anti-TNF and thiopurine combination therapy should include not only monitoring of the level of the biological agent, but also control of 6-TG levels and optimization of thiopurine concentrations to maximize biological effects. Optimizing thiopurine levels by dose modification or pharmacological manipulation may be more cost-effective than increasing the dose of the biological agent.

AZA requires a 2-4 weeks treatment to reach its stable concentration, which is longer than other immunosuppressant treatments. Laboratory monitoring of AZA treatment includes a complete blood count and liver function tests, with particular attention to the signs of hepatitis and leukopenia. Laboratory monitoring is recommended every two weeks during the first two months of treatment and every three months thereafter. The British Gastroenterological Society has recently made a recommendation that thiopurine metabolites should also be monitored during AZA treatment of IBD to prevent toxicity and dosing errors. Most of the available data are from studies involving patients with IBD and specific recommendations for AZA in the treatment of autoimmune AIH have not yet been developed. Thiopurine metabolites can be monitored by analyzing the concentrations of 6-TGN and 6-MMP in erythrocytes, and the dose of AZA can then be adjusted to maintain 6-TGN and 6-MMP concentrations within the normal therapeutic range. This is particularly important in patients who do not show an adequate response to treatment or who accumulate toxic metabolites. However, monitoring of thiopurine metabolites is only an additional analysis to standard laboratory analyses.

1.3 Liquid chromatography-mass spectrometry

During the analysis of biological samples such as blood or other body fluids, it is very important to reduce the complexity of the samples to obtain as much information as possible from one sample. One of the most widely used forms of separation techniques is liquid chromatography, including high-performance liquid chromatography (HPLC) and ultra-high-performance liquid chromatography (UHPLC). Due to its high resolution, good reproducibility, wide range of separation interaction types and compatibility with mass spectrometry analysis, liquid chromatography is widely used in various omics research, such as proteomics and metabolomics. In case of chromatography, the separation of the compounds of interest is based on their physical and chemical properties, whereas in the mass spectrometer, the components

eluting from the chromatographic column are separated based on the mass/charge (m/z) ratio after ionization. For the analysis of complex biological samples, the advantages of both separation methods can be exploited. Proper chromatographic separation can reduce matrix effects, ion suppression, and provide the possibility to separate and analyze isobaric compounds.

The mass spectrometer produces gas-phase ions from neutral particles and separates the ions based on their mass-to-charge (m/z) ratio. Each mass spectrometer contains different ion sources and mass analyzers that have different physical/chemical properties for ionization and ion separation.

1.3.1 Ionization techniques

Various ionization techniques are used to ionize the samples and convert them to gas phase. The three most commonly used ionization techniques in LC-coupled setups are Electrospray Ionization (ESI), Atmospheric Pressure Chemical Ionization (APCI) and Atmospheric Pressure Photoionization (APPI). The applied ionization technique depends mainly on the size and polarity of the analyzed components. These ionization techniques belong to the group of so-called soft ionization techniques, which means that the molecules are not fragmented during ionization. In ESI, the sample eluting from the chromatography column is passed through a capillary with high voltage, and after evaporation of the solvent by a temperature gradient and drying gas, the ionized target molecules are introduced into the mass spectrometer where the ions are separated based on their m/z ratio.

1.3.2. Mass analyzers

The role of the analyzer is the separation of the ions arriving from the ion source according to their m/z ratio. The separation can be done using an electric current or a magnetic field. The analyzers can be classified according to their principle of operation and their resolution, therefore, low, medium and high resolution mass analyzers can be classified. The most common analyzers are the Quadrupole Analyzer (Q), Time of Flight (TOF), Ion Trap (IT), Ion Cyclotron Resonance (ICR), Orbitrap (OT) and Ion Mobility Cell (IM).

1.3.3 Tandem mass spectrometry

In addition to the simple mass spectrometers, tandem mass spectrometers are very often applied. Tandem mass spectrometers contain several analyzers and can be used to fragment the target molecules, thus, important structural information can be obtained from tandem mass

spectra (MS/MS). Tandem mass spectrometers can contain multiple copies of the same analyzer, such as three quadrupoles, or they can be so-called hybrid instruments, which include different type of analyzers, such as the QTRAP hybrid tandem mass spectrometer (Sciex), which includes quadrupole and ion trap analyzers. A special feature of the QTRAP system is that depending on the configuration, the third quadrupole can also operate in linear ion trap mode. Due to the hybrid design, the instrument can be used for different types of analysis, such as fragment ion analysis, precursor ion analysis, neutral loss analysis or Selected/Multiple Reaction Monitoring (SRM, MRM).

When operating in SRM mode, the instrument allows only one predefined precursor ion to enter the collision cell, only one predefined fragment ion will pass through the third quadrupole to enter into the detector. Thus, the mass spectrometer registers a signal only in the presence of the selected precursor ion and the corresponding selected fragment ion. The analysis of the m/z values associated with the precursor ion-fragment ion pair, called SRM transitions, ensures the selectivity and specificity of the method. The area under the curve (AUC) is proportional to the amount of compound entering the mass spectrometer, making the method an excellent choice for quantitative studies. In the semi-quantitative mode, the SRM method is well suited for relative quantification, but absolute quantification is also possible with appropriate settings. For both relative and absolute quantification, it is necessary to use stable isotope-labelled (SIL) reference molecules, which used as internal standards during the analyses. In SRM analysis, the mass spectrometer is capable of the simultaneous analysis of hundreds of transitions allowing the analysis of multiple target molecules from the same sample. The sensitivity of SRM methods can be further improved by monitoring the target molecules within a given retention time window in a so-called "scheduled" SRM way, which allows the sensitive analysis of hundreds of components with relatively short chromatographic separations.

2. Objectives

The aim of my research was to develop and apply targeted mass spectrometry-based analytical methods to address questions in metabolomics. The specific objectives of the study are as follows:

1. Development of a UHPLC-SRM method for the quantitative analysis of amino acids and biogenic amines.
2. Validation of the developed method and its application to the analysis of complex biological samples.
3. Development of a UHPLC-SRM method for the detection and quantification of azathioprine metabolites.
4. Validation of the developed method and its application to the analysis of clinical samples.

3. Materials and methods

3.1 Materials

The amino acid and biogenic amine standards were obtained from Waters Ltd. (alanine, arginine, aspartic acid, glycine, glutamic acid, histamine, cysteine, lysine, serine, proline, threonine, tyrosine, methionine, valine, leucine, isoleucine and phenylalanine) and Sigma-Aldrich Ltd. (asparagine, glutamine, taurine, histamine, ethanolamine, methylamine, ethylamine, citrulline, ornithine, putrescine, cadaverine, tyramine, tryptamine, 2-phenethylamine). Stabile isotope labelled tryptophan was purchased from Cambridge Isotope Laboratories. The AccQ-Tag Ultra derivatization kit and AccQ-Tag Ultra A and B eluents were supplied by Waters Ltd, while LC grade methanol and water and the 3kDa Nanosept columns were purchased from VWR Ltd.

The LC grade acetonitrile, methanol and water used for the analysis of azathioprine (AZA) metabolites were purchased from VWR Ltd, while the HPLC grade trifluoroacetic acid, 6-thioguanine (6-TG), 5-bromouracil and DL-dithiothreitol (DTT) were purchased from Sigma-Aldrich. 6-Methylmercaptopurine (6-MMP) and stable isotope-labelled 6-thioguanine-$^{13}C^{15}N$ (isotopic purity 98 atom % ^{13}C, 98 atom % ^{15}N) and 6-methylmercaptopurine-D_3 (isotopic purity 98 atom % D_3) were purchased from Toronto Research Chemicals Inc.

3.2. Sample collection

Amino acids and biogenic amines were determined in serum and tear samples. Serum and tear samples were collected from 5 healthy donors. The sample collection was performed according to the principles of the Helsinki Declaration and ethical approval was obtained from the University of Debrecen Ethics Committee (DEOEC RKEB/IKEB 4701A-2016). Tear samples were collected using a glass capillary (VWR Ltd.) without stimulation and local anesthesia. Tear samples were collected from both eyes of each donor.

Blood samples from AIH patients treated with AZA-containing Imuran (Aspen Pharma Trading Ltd.) were provided by the members of the Department of Gastroenterology, Faculty of General Medicine, University of Debrecen. The samples were collected according to the Helsinki Declaration, and ethical approval was obtained from the University of Debrecen Ethics Committee (12759-6/2019/ECIG).

3.3. Preparation of standards and quality control (QC) samples

The stock solution of the amino acids and biogenic amines was prepared in a 2.5 mmol/L concentration (each molecule had 2.5 mmol/L concentration) and stored at -20°C. Quality control (QC) samples were prepared at three different concentrations (2.5 µmol/L, 7.5 µmol/L and 15 µmol/L) in three different matrices (MilliQ water, serum and tear), which were subsequently used for validation experiments.

Standard solutions of the AZA metabolites (6-TG and 6-MMP) were prepared in methanol-2M NaOH (3:7) for 6-TG and 6-TG_SIL, and in pure methanol for 6-MMP and 6-MMP_SIL in a 1 mg/ml final concentration. The individual standards were stored at -20 °C. For the preparation of calibration curves, methanol-water (3:7) solvent was used for serial dilution of individual standards. QC samples were prepared in serum obtained from healthy volunteers, with final concentrations of each analyte of 5 ng/ml, 20 ng/ml, 625 ng/ml and 1250 ng/ml, which were used for subsequent validation experiments.

3.4. AccQ-Tag derivatization

Derivatization of standards and biological samples for amino acid and biogenic amine analysis was performed using the AccQ-Tag Ultra derivatization kit (Waters) according to the manufacturer's instructions. Briefly, 10 µl of sample was mixed with 70 µl of borate buffer (pH=8.8) and 20 µl of AccQ-Tag Ultra derivatization reagent. The sample was incubated for 1 min at room temperature followed by a 10 min incubation at 55 °C until the reactions discussed in section 1.1.3.1 were carried out.

3.5. Chromatographic and mass spectrometric parameters

LC-MS analyses were performed on a Waters ACQUITY H-Class UHPLC liquid chromatography system (Waters) coupled with a 5500 QTRAP (Sciex) mass spectrometer. The UHPLC instrument was controlled by the Empower 3 software (Waters) and the mass spectrometer was controlled with the Analyst 1.6.3 software (Sciex).

3.5.1. Analysis of amino acids and biogenic amines

Chromatographic separation of the AccQ-Tag derivatized samples was performed on an AccQ-Tag Ultra C18 column (1.7 µm, 2.1 x 100 mm, Waters) equipped with an Acquity pre-column (0.2 µm, 2.1 mm, Waters). A 11 min long gradient elution method was used the flow with 0.65 ml/min rate and the column temperature was 54 °C. For chromatographic separation, four different solvent mixtures were used, solvent A was 100% AccQ-Tag Ultra A eluent,

solvent B was 10% AccQ-Tag Ultra B eluent diluted with LC pure water, solvent C was 100% LC pure water and solvent D was 100% AccQ-Tag Ultra B eluent. UV spectra were detected with a PDA detector at 260 nm wavelength with a data acquisition rate of 10 points per second.

The eluent from the chromatography column was ionized using Electrospray ionization (ESI) with a 5000 V current. SRM spectra were recorded in positive ion mode. Ion source gas 1 was 30 psi, ion source gas 2 was 50 psi, drying gas was 30 psi, ion source temperature was 500 °C, cycle time was 0.5 s, and the number of cycles was 1319. The recorded UV spectra were evaluated using Empower 3 software, while the SRM spectra were evaluated using Skyline software (version 23.1.0.455).

3.5.2. Analysis of AZA metabolites

Liquid chromatographic separation of 6-TG and 6-MMP molecules was performed on an AccQ-tag Ultra C18 column (1.7 μm; 2.1 × 100 mm, Waters) equipped with an Acquity pre-column (0.2 μm, 2.1 mm, Waters). The total analysis time, including equilibration time, was 4 min. The flow rate used for separation was 0.40 ml/min and the column temperature was 40 °C. Solvent A was 0.02 mol/l of ammonium formate dissolved in water containing 0.3% (v/v) formic acid (pH = 3.00) and solvent B was 100% acetonitrile.

SRM-based targeted MS analysis was performed by electrospray ionization in positive ion mode with 5500 V current. The ion source temperature was set to 500 °C, ion source gas 1 was set to 30 psi, ion source gas 2 was set to 50 psi, and sheet gas was set to 30 psi. The applied decluster potential was 100 eV and a collision energy of 40 eV was used for fragmentation. The stable isotope-labelled compounds 6-thioguanine-13C15N (6-TG_SIL) and 6-methylmercaptopurine-D3 (6-MMP_SIL) were used as internal standards. SRM spectra were analyzed using Skyline software (version 23.1.0.455).

3.6. Validation of the developed LC-MS methods

3.6.1 Amino acids and biogenic amines

The developed method was validated according to the FDA guidelines based on the work of Galba et al. and Grey et al. The validation included linearity, accuracy, intra-day and inter-day variability, matrix effect, recovery, limit of detection (LOD), limit of quantification (LOQ) and stability tests.

Linearity was investigated over the concentration range of 0.25-30 μmol/L in all three matrices (MilliQ water, serum and tear). For each of the three tested matrices, three parallel

analyses were performed and the limit of detection (LOD) and limit of quantification (LOQ) were determined using the equations of the calibration curves.

The three QC samples of different concentrations were prepared in each of the three matrices and each sample was analyzed five times and the accuracy, intra-day variability, matrix effect, recovery and stability were determined. To calculate the inter-day variability, the results of three consecutive days (5 measurements per day of each concentration and matrix) were used. The recovery was calculated by comparing the measured values with the theoretical values.

The autosampler stability was tested with the incubation of the QC samples in the autosampler for 12 h at 4°C before analysis. The freeze–thaw stability of the samples was tested by the analysis of the QC samples after three freeze–thaw cycles at −70 °C before and after AccQ-Tag derivatization. The stability was calculated by the comparison of the concentrations after the freeze–thaw cycles with the concentrations of freshly prepared QC samples.

3.6.2. AZA metabolites

The developed LC–MS method was validated according to the European Medicines Agency (EMA) ICH guideline M10 on bioanalytical method validation and study sample analysis, based on the previous work of Yu et al. and Miao et al. The linearity, selectivity, specificity, accuracy and precision, intra- and inter-day variability, carry-over, recovery, matrix effect, lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) were determined.

The calibration standards were prepared in the same biological matrix as the clinical samples subsequently tested. The calibration ranges was tested in a 2.5-5000 ng/ml range according to the EMA guidelines. According to the criteria, the accuracy for LLOQ should be within ±20% of the nominal concentration, while for all other calibration points it should be within ±15% and at least 75% of the calibration points should meet the criteria.

The selectivity was determined by the analysis of matrix samples without the analytes of interest and/or their SIL counterparts.

Specificity was tested by the injection of 5-bromouracil, a purine nucleotide analogue of the molecules of interest. Based on the EMA criteria, 5-bromouracil should not interfere with the analysis of the target molecules and their SIL counterparts.

The accuracy and precision were determined by analysing QC samples in 5, 20, 625 and 1250 ng/mL concentrations. Intra- and inter-day variability was determined by the analysis of five replicates of the QC samples in the same day (intraday) and for 2 days.

The carry-over of the system was tested by the analysis of blank samples after the ULOQ of the calibration curves.

The matrix effect was examined by the analysis of three replicates of low concentration (20 ng/mL) and high concentration (1250 ng/mL) QCs from 6 different batches. According to the EMA criterion, the precision and coefficient of variation should not exceed 15%.

The freeze-thaw stability was investigated by analysis of QC samples after three freeze-thaw cycles at -70 °C. According to the EMA criteria, low and high concentration QC samples (20 ng/ml and 1250 ng/ml, respectively) should be treated in the same way as the clinical samples to be tested. The QC samples should be kept frozen for at least 12 hours between freeze-thaw cycles and stability studies should be performed using freshly prepared calibration standards and QCs as reference. The long-term stability of the analytes was investigated in a matrix stored at -20 ◦C for 10 weeks.

The stability of stock solutions and working solutions of 6-TG and 6-MMP and internal standards was determined using the lowest and highest concentrations under the storage conditions used for the analysis of the test samples. We also determined the stability of the processed samples, including the time to completion of the analysis, as well as the stability of the autosampler and the reproducibility of repeated analyses.

3.7 Analysis of amino acids and biogenic amines in serum and tear samples

From the collected tear samples, 3-3 µl volumes were diluted to 50 µl with MilliQ water and centrifuged on a Nanosept3K filter (VWR Ltd.) to separate the components of interest from proteins and higher molecular weight compounds. The centrifugation was performed for 2x10 min with 16000 g at 4°C, then the filtrate was dried in a vacuum concentrator (Thermo Scientific). The dried samples were dissolved in 80 µl borate buffer, the AccQ-Tag derivatization reaction described in section 3.4 was performed.

100µl of the serum samples were filtered through a Nanosept3K filter and centrifuged 2x10 min at 16000 g at 4°C for 2 x 10 min to prepare the tear samples. AccQ-Tag derivatization was performed from 10 µl filtrate.

Tear samples were analyzed without dilution, while serum samples were analyzed both without dilution and with 10-fold dilution. The injection volume was 1µl in each case, and the concentration of amino acids and biogenic amines were calculated for the original sample volume.

3.8 Analysis of AZA metabolites in blood samples from patients with AIH

Blood samples collected in EDTA-treated tubes were lysed with two volumes of UPLC grade water for 1 min. Then, 100 μL of SIL mixture (0.2 μg/ml for each compound) and 150 μL of DTT (dissolved in water with 0.1 mol/L concentration) were added to 200 μL of lysate, and proteins were precipitated by mixing the samples with 40 μL of 100% trifluoroacetic acid for 1 min. Samples were centrifuged at 16100 g for 15 min at room temperature, then 2 × 200 μL supernatant was pipetted into a new tube and incubated at 100 °C for 45 min, then centrifuged again at 16100 g for 15 min. Then 200 μL supernatant was pipetted into a new tube and the previous centrifugation step was repeated. A volume of 100 μL of the clean supernatant was pipetted into the sample vials, of which 5 μL was injected to the UPLC system. The 6-TGNs were hydrolyzed to 6-TG by heating under acidic conditions, and the concentration of 6-TGN was determined by analyzing the level of 6-TG. The concentrations of 6-TG and 6-MMP were expressed as ng/ml and the concentrations of 6-TGN and 6-MMPr were calculated by normalization to red blood cell count.

4. Results

In our work, we performed targeted mass spectrometry analysis of amino acids and biogenic amines endogenously present in the human body, as well as the metabolites of the xenobiotic azathioprine, including method development, validation and application of the methods on biological samples.

4.1 UHPLC-MS method development for amino acids and biogenic amines

Since quantitative changes of amino acids and biogenic amines may indicate pathological changes in the human body, a rapid UHPLC-MS method for their analysis was developed. The AccQ-Tag derivatization technique, detailed in section 1.1.3.1, was applied on the 33 target molecules and UHPLC-MS analyses were performed.

4.1.1 Determination of mass spectrometry parameters

Since the derivatization reaction used during the sample preparation results in a mass difference on the target molecules, we determined the m/z values of the precursor ions before the mass spectrometry analysis. In the case of histidine, ornithine, citrulline and some biogenic amines, more than one free amino group is present in the molecule, so the possibility of multiple derivatization was investigated.

Based on the results of the mass spectrometry analyses, single derivatization was observed in case of citrulline, histidine, histamine and tryptamine, while double derivatization was identified in case of cadaverine, ornithine and putrescine. In the case of serotonin, both single and double derivatized forms were identified, but the double derivatized form had much lower intensity than the single form. Since the ratio between the singly and double derivatized forms was constant, the values for the double derivatized form were used to generate the method file. After performing fragmentation analysis of the derivatized molecules, an SRM method file was generated for the analysis of the selected ion transitions.

4.1.2 UHPLC-UV-MS method development

The method was developed on an Acquity H-Class UHPLC system coupled to a 5500 QTRAP tandem mass spectrometer. The chromatographic elution profile was optimized, and an 11 min gradient was optimized for the separation of the 33 target amino acids and biogenic amines. The derivatized molecules were detected at 260 nm wavelength using a photodiode array (PDA) detector and in SRM mode with the 5500 QTRAP mass spectrometer. Using the

optimized chromatographic parameters, the separation of derivatized molecules was successful except for tryptamine and 2-phenethylamine, but their individual analysis could be performed by the SRM method due to the mass difference.

4.1.3 Validation of the UHPLC-UV-MS method

The developed method was validated according to FDA guidelines, based on the work of Galba et al. and Grey et al. The calibration range was tested in three different matrices, MilliQ water, serum and tear at concentrations of 0.25-30 μmol/L, which were used to determine the limit of detection (LOD) and the limit of quantitation (LOQ) as well.

The limit of detection (LOD) for the UV data were the following: 0.108-2.023 μmol/l in MilliQ water, 0.097-2.304 μmol/l in serum matrix and 0.124-1.372 μmol/l in tear matrix. For the limits of quantification, higher concentrations were observed: 0.361-6.743 μmol/l in MilliQ water, 0.325-7.679 μmol/l in serum matrix and 0.412-5.575 μmol/l in tear matrix. The linear calibration range was between 1 and 20 μmol/l in MilliQ water matrix for all analytes, in serum and tear matrix, this range is modified to 2,5-25 μmol/l and to 1-15 μmol/l, respectively.

The limits of detection determined from the results of the SRM analysis were the following: 0.021-1.042 μmol/l in MilliQ water, 0.025-0.858 μmol/l in serum, and 0.038-1.574 μmol/l in tears. In case of the limits of quantification, higher concentrations were also observed: 0.071-3.472 μmol/l in MilliQ water, 0.083-2.86 μmol/l in serum, and 0.127-5.247 μmol/l in tears. A linear calibration range of 0.25-1 μmol/l was observed for all tested analytes. in case of biogenic amines, the linear correlation between area under the curve and concentration was observed at the low concentration range, whereas for amino acids, a wider range of 0.25-20 μmol/l was observed.

The comparison of the results obtained by UHPLC-UV and SRM analyses showed that while individual analysis of tryptamine and 2-phenethylamine could be only performed by SRM analysis, the calculated linear dynamic ranges were wider for UHPLC-UV analysis compared to SRM. Based on the data, we decided to use UHPLC-UV-based analysis for the quantification of derivatized amino acids and biogenic amines and to further validate the developed method. SRM analysis was used to confirm the chromatographic peaks and for the qualitative and quantitative analysis of tryptamine and 2-phenethylamine.

To validate the method developed, QC samples were generated in MilliQ water, serum and tear matrices containing the analytes of interest at concentrations of 2.5 μmol/l, 7.5 μmol/l and 15 μmol/l. The samples were analysed to determine intra-day and inter-day variability, accuracy and precision. For the 33 derivatized molecules, the precision ranged from 85.66% to

114.41% in MilliQ water, from 90.92% to 114.12% in serum matrix and from 91.39% to 114.80% in tear matrix. Intra-day and inter-day variability and accuracy were found to be below 15% in MilliQ water and tear matrix and below 9% in serum matrix. The recovery calculated from the data obtained from the analysis of QC samples was higher than 85%. Significant interference was observed when the matrix effect was examined. In serum, the matrix effect was over 15% for histidine, glutamine, arginine, histamine, serotonin, tryptophan, tryptamine and 2-phenethylamine, mainly at low concentrations. In tears, matrix effects were over 15% for lysine, tyrosine, valine, cadaverine and tryptophan. The stability of the molecules was investigated under different conditions. Their autosampler stability and freeze-thaw stability were investigated before and after derivatization. Taking the registered data into account, we concluded that samples should not be stored in the autosampler for more than 12 hours and that freeze-thaw cycles should also be avoided for accurate quantification.

4.2 Quantification of the amino acids and biogenic amines in serum and tear samples

The validated method was used to determine the concentration of the selected amino acids and biogenic amines in serum and tear samples collected from healthy volunteers. After sample collection, serum and tear samples were purified on 3 kDa spin columns and subjected to AccQ-tag derivatization. To determine the possible sample loss during sample preparation, the amino acid and biogenic amine standard mixture was analyzed before and after purification. No significant difference was observed between the peak areas of purified and unpurified samples. Serum samples from five healthy volunteers were derivatized, analyzed with the validated method, and the concentration of molecules were calculated for the original sample volume.

All derivatized amino acids and biogenic amines except histamine, tyramine, tryptamine and 2-phenethylamine were identified in the serum samples collected from the 5 healthy volunteers. Ethylamine was detected in serum samples by UHPLC-UV analysis, but the concentration was below the limit of quantification, while methylamine could only be detected by SRM analysis.

The results showed that glutamine and alanine were present in the highest concentrations in serum, while the concentration of serine, glycine, threonine, proline, lysine, valine and leucine were also above 100 μmol/l. The ceoncentrations of the tested biogenic amines were significantly lower compared to the proteinogenic and non-proteinogenic amino acids.

Tear samples were also collected from the same 5 healthy volunteers. During the sample collection, we attempted to collect tears from both eyes, but this was not always possible, so we analyzed a total of 8 tear samples, which were considered as individual samples. After derivatization and analysis, all molecules were identified in the samples except serotonin, putrescine, tryptamine and 2-phenethylamine.

Ethanolamine, ethylamine, methionine, cadaverine, tyramine, leucine and phenylalanine were detectable in the tear samples by UHPLC-UV analysis, but their concentrations were below the limit of quantification. Methylamine could only be detected by SRM analysis.

The validated method allowed the quantification of 17 proteinogenic and 2 non-proteinogenic amino acids and taurine. With respect to the results obtained in the analysis of serum samples, the concentration of biogenic amines in tears was lower compared to amino acids. Our results show that serine has the highest concentration in tears of all the studied biomolecules.

4.3 UHPLC-MS method development for the analysis of azathioprine metabolites

Monitoring the concentrations of AZA metabolites can provide important information for the therapy of patients with AIH, therefore, we developed an SRM-based LC-MS method for the identification and quantification of 6-TG and 6-MMP.

4.3.1. Determination of mass spectrometry parameters

As with amino acid and biogenic amine analysis, the parameters required for the SRM method file were determined using standard molecules for method development. Stable isotope-labelled (SIL) standards were also used for the analyses.

4.3.2 UHPLC-MS method development

The method was developed on an Acquity H-Class UHPLC instrument (Waters) coupled to a 5500QTRAP tandem mass spectrometry (Sciex) system. The chromatographic elution profile was optimized and a rapid 4-min gradient was generated. SIL standards were used as quality control and for accurate quantification. The SRM transitions used were specific for the target analytes and, accordingly, the target molecules and their synthetic counterparts were successfully identified. The observed retention times were 0.73 min for 6-TG and 6-TG_SIL and 1.66 min for 6-MMP and 6-MMP_SIL.

4.3.3 UHPLC-MS method validation

The developed LC-MS method was validated according to the European Medicines Agency (EMA) guidelines, and the selectivity, specificity, carryover, linear range, lower limit of quantitation (LLOQ), upper limit of quantitation (ULOQ), accuracy, precision, recovery, matrix effect and stability of the method were investigated.

To determine the sensitivity and calibration range of the method, the matrix was prepared from blood samples collected from healthy volunteers. The linearity was tested over a concentration range of 2.5-2500 ng/ml for both analytes in the prepared matrix. For the determination of the calibration curve, LLOQ and ULOQ, the linear range was investigated in three independent experiments in two replicates over three days. Using the recorded data, linear regression analysis was performed using logarithmic scales. The analysis yielded equations $y = 0.0073*x + 0.0042$ ($R^2 = 1$) for 6-TG and $y = 0.0087*x - 0.0035$ ($R^2 = 0.9993$) for 6-MMP. LLOQ was found to be 5 ng/ml and ULOQ 1250 ng/ml for both tested analytes.

Six individual matrix samples (without analyte and SIL molecules) were used to determine the selectivity and carry-over of the system. LLOQ analyte responses were 7.60% for 6-TG and 2.31% for 6-MMP, while the SIL responses were lower. In the carry-over assay, the responses for 6-TG and 6-MMP after ULOQ were 10.37% and 3.28%, respectively, while SIL responses were 1.09% and 0.01%, respectively. The specificity was tested using 100 ng/ml 5-bromouracil and the analyte responses were 5.17 % and 1.48 %.

The intra- and inter-day accuracy, precision and recovery were tested. The results showed that accuracy and precision remained within 15% for all concentrations. The matrix effect was tested at low (20 ng/ml) and high (1250 ng/ml) concentrations and the precision, recovery and matrix effect were less than 15% of the nominal concentration.

The freeze-thaw, long-term (10 weeks) and working solution stability was investigated during the storage of the samples at -20°C. Sample stability was analyzed immediately after sample preparation and after six hours storage in the autosampler. Repeated injection reproducibility was tested by five parallel injections of low, medium and high concentration QC samples. For 6-TG, the recovery was within 15%, while for 6-MMP the differences ranged from 25% to 60%, with the best recovery at 20 ng/ml concentration level.

4.4 Determination of AZA metabolite concentrations in blood samples from patients with AIH

To demonstrate the usefulness of the validated method for the analysis of clinical samples, the concentration of 6-TG and 6-MMP in blood samples from patients with AIH was investigated. Patients were treated with different doses of AZA (Imuran). 6-TGN and MMPr concentrations, important for therapeutic efficacy, were calculated by normalizing 6-TG and 6-MMP concentrations to red blood cell count.

The results show that the metabolite concentration determined by the validated method is proportional to the applied therapeutic dose.

5. Discussion

Metabolomics studies play a key role in the analysis of endogenous molecules and xenobiotics, as they can provide a comprehensive picture of the chemical composition of biological systems. Endogenous molecules, such as metabolites, provide information on the state of cells and tissues, while xenobiotics, such as drugs, toxic substances or environmental pollutants, can affect metabolic networks. Metabolomics enables the mapping of molecular interactions, biochemical pathways and physiological responses, which is essential for understanding the mechanism of diseases, biomarker identification, and for drug development and toxicity prediction. LC-MS analysis is a particularly important tool in this field, as it is able to analyze metabolites and xenobiotics sensitively and specifically. The LC-MS technique combines the advantages of chromatography with mass spectrometry, which allow the separation of compounds and the identification and quantification of analytes. This method is particularly useful for understanding metabolic networks, studying the effects and toxicity of drugs, and revealing the mechanisms of disease.

In this research, I aimed to design, validate and apply targeted LC-MS methods on biological samples.

5.1 Amino acid and biogenic amine determination

We have developed an LC-MS method for the analysis of amino acid and biogenic amine content of serum and tear samples that was validated according to FDA guidelines. From the results of the validation experiments, we found that the method has sensitive and has a wide dynamic range for the analysis of complex biological samples. By comparing the results of UV and SRM-based detection, we concluded that UV detection has a wider dynamic range and therefore this detection method was used for the quantification of target molecules. SRM analysis was used to confirm chromatographic peaks and for the qualitative and quantitative analysis of tryptamine and 2-phenethylamine. The variability, accuracy and precision were tested and the results showed a variation of less than ±15% that is in accordance in the FDA criteria. Significant matrix effects were identified in our studies, indicating that the calibration curves should be prepared in the appropriate matrix for accurate quantification. Stability studies suggest that long-term storage of samples and repeated freeze-thaw cycles should be avoided.

Among all of the human body fluids, serum is the most widely used biological sample in medicine, particularly in the diagnosis of diseases and therapeutic monitoring. Serum is rich in proteins and metabolites and can be obtained in a minimally invasive way, making it a widely

used sample for biomarker research. Using the validated method, we analyzed serum samples from five healthy volunteers. We identified all target molecules except histamine, tyramine, tryptamine and 2-phenethylamine and were able to quantify them except ethylamine and methylamine. The results show that concentrations of glutamine and alanine are the highest in serum, which is in agreement with the scientific literature. In addition to the major nitrogen transporters, serine, glycine, threonine, proline, lysine, valine and leucine were found to have concentrations greater than 100 μmol/L. The levels of biogenic amines tested were significantly lower in serum than the proteinogenic and non-proteinogenic amino acids.

Monitoring the changes in the serum amino acid profile has an important role in both biological and medical sciences, as amino acids play an important role in the homeostatic processes of the body and in the pathogenesis of certain diseases. A correlation has been found between serum glycine concentrations and insulin resistance and metabolic syndrome. Elevated levels of branched-chain amino acids such as valine, leucine and isoleucine have been identified in maple syrup disease, while significant decreases have been shown in autism caused by branched-chain ketoacid dehydrogenase kinase deficiency. Besides amino acids, biogenic amines also play an important role in homeostatic and pathological processes. Ethanolamine, as a constituent of plasmalogens, may play a role in the development of neurological problems such as Alzheimer's disease. Polyamines, such as putrescine and cadaverine, have different functions in the human body and play a significant role in pathological conditions such as chronic renal failure or autoimmune thyroid diseases. In addition, cadaverine has also been identified as a regulator of breast cancer development.

Although serum samples are most commonly tested for the diagnosis of various pathological problems, tear also has considerable medical relevance. Changes in the protein content of tears have been associated with several local and systemic pathological changes, thus, tear has an important role in biomarker research. An additional advantage of tear sample analysis is the non-invasive and relatively simple sample collection method. Analysis of tears collected from the same five healthy individuals successfully identified all target molecules except serotonin, tryptamine, putrescine and 2-phenethylamine. Quantitative identification of all the test molecules except ethanolamine, ethylamine, methylamine, methionine, cadaverine, tyramine, leucine and phenylalanine was also possible. Similar to the results obtained from the analysis of serum samples, the concentration of biogenic amines in tears was lower than the amino acids. Furthermore, we have shown that the analyzed amino acids and biogenic amines show different concentrations in tears and serum. Although a valuable source of biomarkers, changes in the metabolite profile of tears have not been studied as thoroughly as in serum.

Changes in tear metabolite profile have been observed in keratoconus, keratitis and dry eye syndrome, but there is limited information in the literature on the importance of amino acids and biogenic amines in tears. ChenZhuo et al. showed that alanine, aspartate and taurine levels were significantly higher in the tears of patients with dry eye syndrome compared to healthy controls. The work of Nakatsukasa and colleagues showed a decrease in the level of arginine, methionine and taurine, while ornithine, lysine and threonine showed increased level in ocular surface disease.

Although the amount of information available in the scientific literature is limited, the available studies show that the analysis of the amino acid and biogenic amine profile of tears can provide valuable information on the molecular changes that characterize different pathological conditions.

5.2 Determination of azathioprine metabolites

In addition to a method for the analysis of amino acids and biogenic amines, we have developed a targeted mass spectrometry method for the quantitative analysis of azathioprine metabolites that was validated according to the EMA. The results of the validation experiments showed that the method had adequate sensitivity and specificity, and all tested parameters were in accordance with the EMA criteria. For the calibration points, a deviation of less than 20 % of the nominal concentration was observed for the LLOQ value and less than 15 % for all other points. In case of selectivity, specificity and carryover, our results showed less than 20% and 5% difference for analyte and internal standard responses, respectively. In case of accuracy, precision and recovery tests, the differences were found to be less than 15% of the nominal concentration. In the matrix effect assay, we detected much lower interferences compared to the amino acid and biogenic amine assay. Although the three freeze-thaw cycles used in the stability studies did not alter the concentration of the target molecules, the concentration of samples stored at -20 ◦C decreased significantly over the 10 weeks storage. These data suggest that long-term storage of samples should be avoided. Exposure to the autosampler for 6 h did not significantly change the concentrations of analytes, but much larger variations were observed in the reproducibility test of re-injection of samples, especially for low concentrations and 6-MMP. Using the validated method, we examined blood samples from patients treated with different doses of AZA (Imuran) and the concentrations of 6-TG and 6-MMP were determined. The concentration of 6-TGN and 6-MMPr were determined by the normalization of 6-TG and 6-MMP to the red blood cell count. Our data showed that the metabolite

concentration values determined by the validated method were consistent with the applied dose of the drug.

Monitoring the concentration of AZA metabolites during treatment is important to prevent drug toxicity. A correlation between high levels of 6-MMPr and hepatotoxicity has been demonstrated; if 6-MMPr concentrations exceed 5700 pmol/8 × 108 RBC (red blood count), the risk of hepatotoxicity increases threefold. In addition, the analysis of 6-TGN concentration may be useful in case of those patients who do not respond to the therapy despite the optimally adjusted AZA/6-mercaptopurine dose.

Yu et al. recently published a UPLC-UV method for the analysis of 6-TG and 6-MMP in red blood cells. Although their method has proven to be useful for the analysis of AZA metabolites, our developed and validated method uses a targeted mass spectrometry technique and stable isotope-labelled standards, which provides higher precision and selectivity. Although the linear dynamic range of our method is slightly narrower than that of the method developed by Yu et al., the higher sensitivity of MS analysis results in a lower LLOQ value for both analytes. MS-based identification provides high selectivity, which allows the elimination of potential interfering agents. The UHPLC-MS method developed by our group is suitable for the analysis of AZA metabolites of blood samples from patients, even with low metabolite concentrations.

5.3 Limitations of the assays

In the first part of our work, we developed and validated a rapid and sensitive UHPLC-UV method combined with a SRM assay for the simultaneous analysis of 33 AccQ-Tag-derived amino acids and biogenic amines. Although the validated method is well suited for the analysis of test body fluids, sample collection and sample preparation may be challenging. Our results have shown that freeze-thaw cycles should be avoided during sample preparation, and therefore sample storage and use should be performed carefully. Additional limitations may be the individual variability of samples and the low volume of the collected basal tear samples. The rate of tear production varies considerably from individual to individual and in many cases tear collection is not possible. Our method requires 3 µl of tear sample, therefore subjects with low tear production rates cannot be included in the studies. We have shown that the amino acid content of tears shows a high variability not only between individuals but also between the left and right eye. When collecting tear samples, it is preferable to have the same person perform the sample collection to minimize technical variation, but individual differences in tear composition may still be a problem. The method presented here can be further optimized for

the analysis of other medically relevant body fluids, such as saliva and sweat, in addition to the two body fluids under investigation. A further limitation was that the concentration of biogenic amines was mostly below the limit of quantification. While in healthy conditions the concentration of these biogenic amines in body fluids is low, pathological conditions such as diabetes can increase the concentration of several molecules such as putrescine and histamine.

In the second part of our work, we developed and validated a targeted mass spectrometry method for the quantification of two AZA metabolites, 6-TG and 6-MMP. Although the method has proven to be applicabe for the analysis of clinical samples, one limiting factor of our study is that we focused only on these two AZA metabolites, without analyzing other potential metabolites such as 6-thioinosinic acid or potentially toxic derivatives such as 6-thioinosin monophosphate. Another limiting factor is the analytical system, which can only be operated by properly trained persons. However, the selectivity provided by mass spectrometers to distinguish target molecules from potentially interfering substances makes their application necessary, especially in cases where important decisions on therapeutic changes have to be made.

6. Conclusion

In this research, our aim was to develop metabolomic methods for the analysis of human body fluid samples, which are a niche in clinical research. The analysis of amino acids and biogenic amines can provide important information in the medical sciences, but to the best of our knowledge, there is currently no validated analytical method that is suitable for the simultaneous analysis of these molecules. Therefore, we have developed and validated a UHPLC-MS method according to FDA guidelines for the rapid simultaneous analysis of 33 amino acids and biogenic amines from complex biological samples. The validated method was tested on serum and tear samples, demonstrating the reliability and applicability of the method on clinically relevant samples. The results obtained from the analysis of samples collected from healthy volunteers are in agreement with data available in the scientific literature.

The study of Azathioprine metabolites has an important role in the therapeutic drug level setting, which is of paramount importance for treatment efficacy. In our work, we developed and validated a UHPLC-MS method for the analysis of two Azathioprine metabolites, 6-thioguanine and 6-methylmercaptopurine, according to the criteria of the European Medicines Agency. The validated method was tested on blood samples from patients with autoimmune hepatitis under azathioprine treatment. The method developed proved to be suitable for the determination of the concentrations of the two metabolites and the concentration values we determined were also consistent with the dose of the drug. The results suggest that the developed method is suitable for the quantification of the tested molecules in clinical samples and may help to optimize the drug dose during treatment.

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List of publications related to the dissertation

1. **Guba, A.**, Kováts, P., Mezei, Z. A., Papp, M., Csősz, É., Kalló, G.: Analysis of Azathioprine Metabolites in Autoimmune Hepatitis Patient Blood-Method Development and Validation.
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