ERNDIM ADVISORY DOCUMENT OF THE QUANTITATIVE ANALYSIS OF PURINES AND PYRIMIDINES.

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This document provides a guideline for the analysis of purines and pyrimidines in body fluids for diagnostic purposes. First, defects in purine and pyrimidine metabolism are reviewed along with critical metabolites, second a general method for the quantitative analysis of purines and pyrimidines using reversed-phase HPLC with UV-detection is described and third the ERNDIM quantitative purine and pyrimidine scheme is presented.

1. Introduction to purines and pyrimidines.

Purines and pyrimidines are essential compounds that are encountered in several forms. One can distinguish bases, nucleosides and nucleotides (figure 1). The former two are present in body fluids, while the latter group of compounds is only present intracellularly.

Purines and pyrimidines are the precursors of DNA and RNA, are pivotal for the regulation of the cell cycle, store and transport energy, are precursors to numerous cofactors (coenzymes) and are carriers of components of cell membranes and carbohydrates.

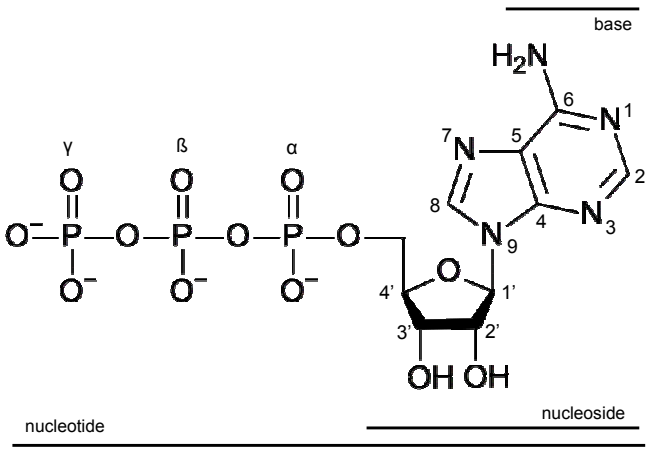

Figure 1 Structure of a ribonucleotide

A ribonucleotide is made up of a ribose ring in which the carbon atoms are numbered 1' to 5' (figure 1). In case of a deoxyribonucleotide, the 2' hydroxyl group is reduced to a hydrogen atom (figure 2). At the 1' position a nitrogenous cyclic base is covalently bound. At the 5' position of the ribose, an inorganic mono-, di, or triphosphate ester is bound. The phosphate ions are designated α, β and γ, the α

phosphate group being the one adjacent to the ribose. A nucleotide without the 5' phosphate ester is called a nucleoside. There are two families of nitrogenous bases, the purines and the pyrimidines. The most abundant purine bases are adenine and guanine, their respective (deoxy)ribonucleosides being (deoxy)adenosine and (deoxy)guanosine. Both DNA and RNA contain adenine and guanine. The pyrimidine bases are cytosine, uracil and thymine. The corresponding ribonucleosides of cytosine and uracil are cytidine and uridine, respectively. Both cytidine and uridine are incorporated into RNA. The pyrimidine ribonucleotides incorporated into DNA are deoxycytidine and thymidine, the corresponding deoxyribonucleoside of thymine.

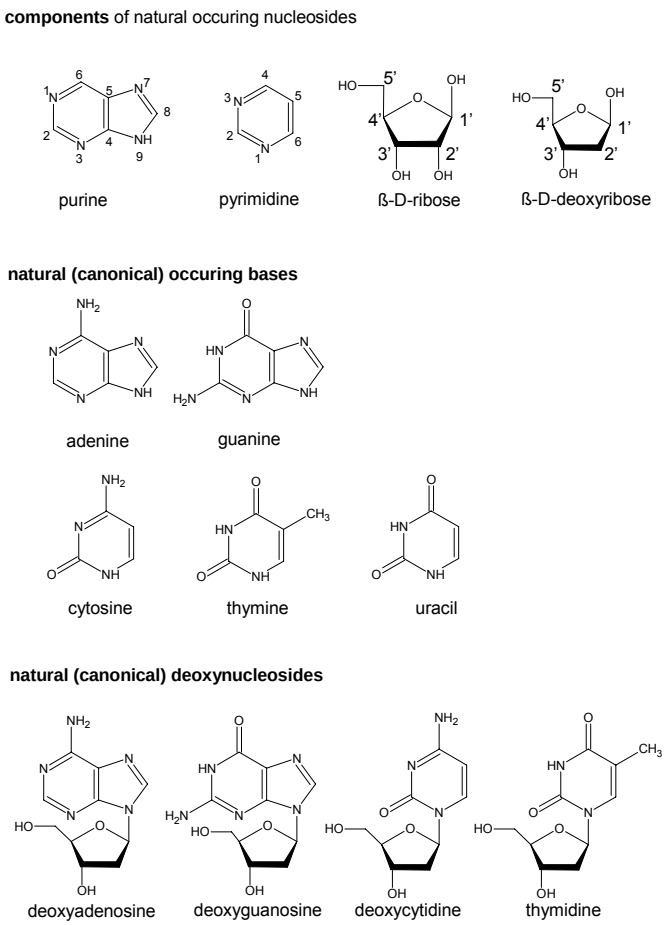

Figure 2 Chemical structures of purines, pyrimidines and their structural parts

Purine metabolism

The precursor of all nucleotides is phosphoribosylpyrophosphate (PRPP). PRPP is synthesised from ribose-5-phosphate and ATP by PRPP synthetase. The central purine nucleotide IMP is synthesised via a series of ten reactions (figure 3). IMP can be phosphorylated further to ITP by nucleoside monophosphate kinase (NMPK) and nucleoside diphosphate kinase (NDPK), respectively. ITP is, however, actively hydrolysed back to IMP by ITPase. From the branch-point nucleotide IMP, the adenine nucleotides are synthesised from the intermediate succinyl-AMP, resulting in AMP. AMP is subsequently phosphorylated to ADP and ATP by AMP kinase and NDPK, respectively. The other purine nucleotides, the guanine nucleotides, are synthesised via xanthine monophosphate (XMP), formed from IMP by IMP dehydrogenase (IMPDH). GMP is subsequently synthesised from XMP by GMP synthetase. GDP and GTP are synthesised from GMP by subsequent phosphorylation by GMP kinase and NDPK. IMPDH is the rate-limiting enzyme in the synthesis of the guanosine nucleotides. The purine deoxyribonucleotides are synthesised by reduction of ADP and GDP by ribonucleotide reductase.

The purine salvage pathway of the (deoxy)nucleosides is analogous to the pyrimidine salvage pathway and both share nucleoside monophosphate kinase (NMPK) and nucleoside diphosphate kinase NDPK. The nucleoside kinases, however, are different. Adenosine is phophorylated by adenosine kinase (ADK) and dAdo is phophorylated by ADK, dCK and deoxyguanosine kinase (dGK), which also catalyses the phophorylation of dGuo. To date, no existence of a human guanosine, inosine or xanthosine kinase has been reported. In addition to salvage of the (deoxy)nucleosides, the purine bases adenine, hypoxanthine and guanosine are salvaged to their respective nucleotides AMP, IMP and GMP by the phosphorybosyltransferases APRT and HGPRT, respectively. This is in contrast to the pyrimidine bases, which can only be converted to their corresponding nucleosides and, subsequently, to their nucleotides.

Purines are degraded by the deaminases adenosine deaminase (ADA) and guanosine deaminase and purine nucleoside phosphorylase to hypoxanthine and xanthine. The last two compounds are oxidised and yield the endpoint of purine metabolism in man, uric acid. In many other species, the poorly soluble uric acid is further oxidised by uricase to form allantoin, which has a much greater solubility.

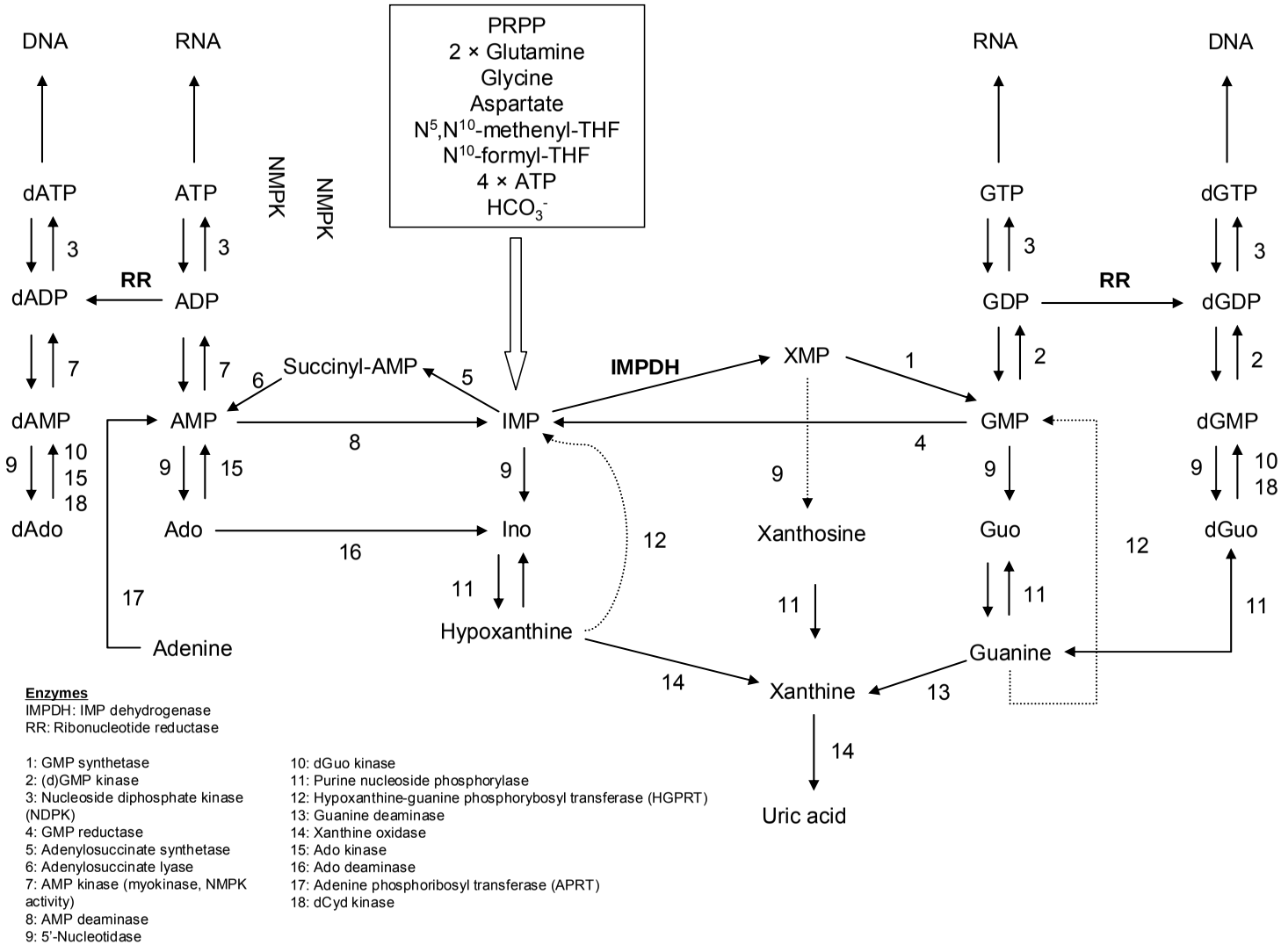

Figure 3 Purine metabolism

Pyrimidine metabolism

The first three steps in the *de novo* synthesis of the pyrimidines are catalysed by CAD, a trifunctional enzyme cluster that contains carbamylphosphate synthetase, aspartate carbamyltransferase and dihydro-orotase activities (figure 4). Dihydro-orotate is reduced to yield orotate by dihydro-orotate dehydrogenase. UMP is subsequently synthesised from orotate by the bifunctional enzyme UMP synthetase, which contains the orotate phosphoribosyl transferase and orotidine-5'-phosphate decarboxylase activities. Subsequent phosphorylation of UMP by nucleoside monophosphate kinase (NMPK) and nucleoside diphosphate kinase (NDPK), respectively, yields UTP. CTP synthetase (CTPs) catalyses the conversion of UTP into CTP. CDP is a substrate for ribonucleotide reductase (RR), which catalyses the reduction of all ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates (dNDP's). dNDP's are subsequently phosphorylated to dNTP's by NDPK and incorporated into DNA, with the exception of dUTP. Although UDP is a substrate for RR, dUTP is only erroneously incorporated into DNA and directly excised by uracil-DNA glycosylase. Both dUDP and dUTP are a source of dUMP, from which TMP is synthesised by thymidylate synthase (TS). TMP is the precursor of TTP, which is ultimately incorporated into the DNA.

Alternatively, pyrimidine nucleotides are synthesised by salvage of uridine and cytidine. In pyrimidine metabolism, salvage is the phosphorylation of a nucleoside by a nucleoside kinase. Uridine and cytidine are phosphorylated by uridine/cytidine kinase (UK), deoxycytidine is phosphorylated by deoxycytidine kinase (dCK) and thymidine and deoxyuridine are phosphorylated by thymidine kinase (TK). The reverse reaction is catalysed by enzymes with 5'-nucleotidase activity.

The pyrimidines are degraded via a common pathway. First, cytidine and deoxycytidine are converted into uridine and deoxyuridine, respectively, by (deoxy)cytidine deaminase. Thymidine phosphorylase catabolises (deoxy)uridine and thymidine into uracil and thymine, respectively. Uracil and thymine are degraded in three steps to ß-alanine and ß-aminoisobutyrate, respectively, by subsequently dihydropyrimidine dehydrogenase (DPD), dihydropyrimidase (DHP) and ß-ureidopropionase. The amino groups of the pyrimidine degradation end products are removed by transamination to yield malonyl semialdehyde and methylmalonyl

semialdehyde, respectively, which are converted into malonyl CoA and methylmalonyl CoA. Pyrimidine degradation enters the citric acid cycle (TCA cycle) via conversion of malonyl CoA and methylmalonyl CoA into propionyl CoA and succinyl CoA respectively.

Inborn errors of metabolism

As is evident from figures 3 and 4, purine and pyrimidine metabolism is an intricate network of biochemical reactions catalysed by a myriad of enzymes. In theory, any genetically determined aberration in the activity or function of any of the enzymes in purine and pyrimidine metabolism can result in an inborn error of metabolism. However, an inborn error does not necessarily cause overt disease. At present 39 disorders in purine and pyrimidine metabolism have been described. These include inborn errors of metabolism, pharmacogenetic risk factors and oncological changes. Inborn errors of purine and pyrimidine metabolism are associated with a great diversity of clinical symptoms. Therefore, the diagnosis of these defects can be challenging. As a guideline, the clinical signs and symptoms requiring analysis of (urinary) purines and pyrimidines are listed in table 1. In general, a symptom from this list in isolation does not necessarily imply the need for analysis of purines and pyrimidines. Analysis of purines and pyrimidines is considered based on the whole clinical presentation and differential diagnosis. The defects that are encountered in the field of biochemical genetics are listed in tables 2 and 3. For all disorders listed in tables 1 and 2 confirmatory testing is available by means of enzyme activity measurements, molecular diagnostics or both.

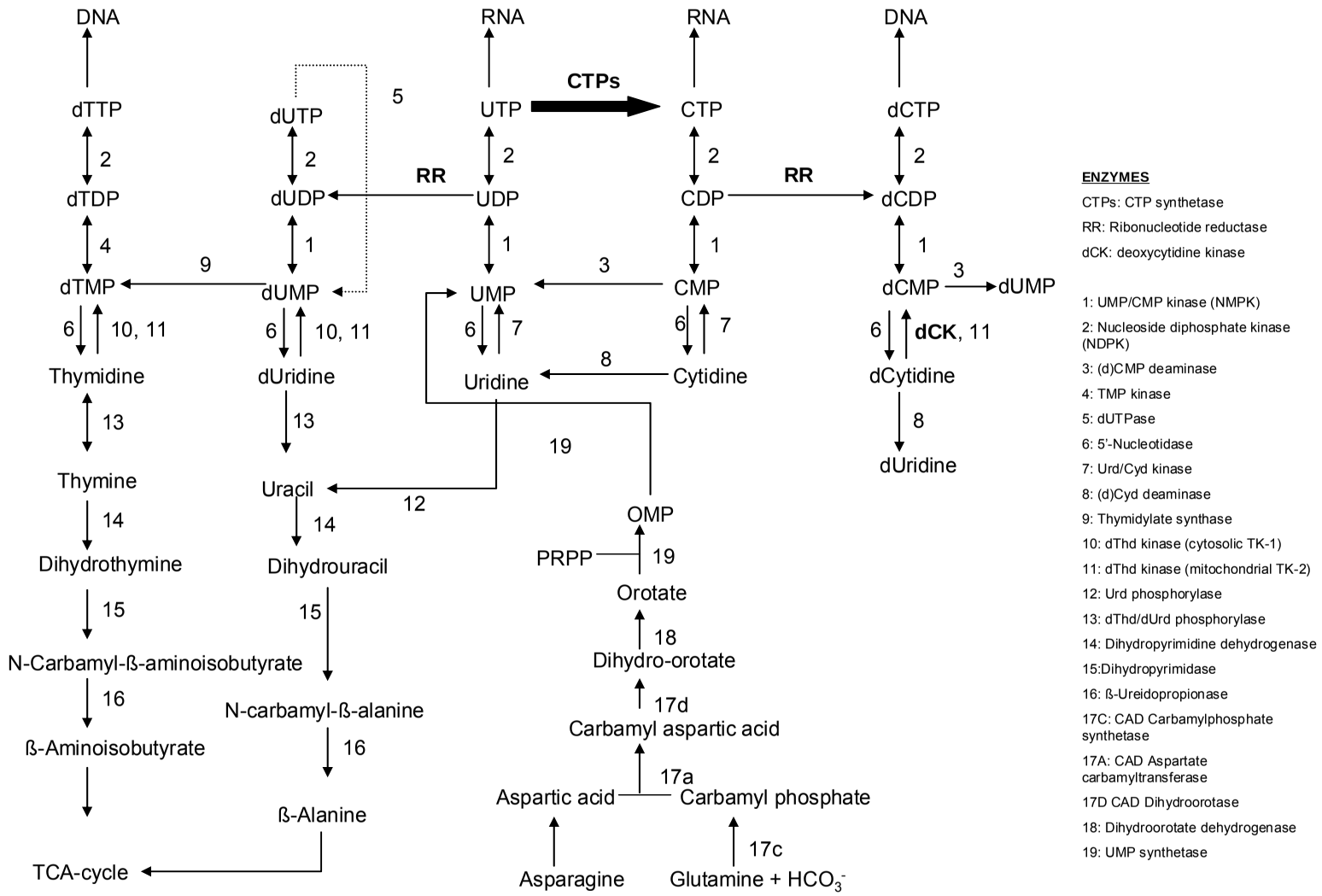

Figure 4 Pyrimidine metabolism

Table 1: Symptoms that may point to the need of purine and pyrimidine analysis
Anaemia (megaloblastic, haemolytic)
Arthritis
Autism
Automutilation
Cachexia, feeding difficulties
Cerebral palsy
Developmental delay
Dysmorphic features
Encephalopathy
Epilepsy, seizures, fitting
Exercise intolerance
Gout
Haematuria
Hepatomegaly
Hyperactivity, short attention span
Hyperuricaemia
Hypo-/hypertonia
Immunodeficiency
Impaired hearing, deafness
Lactic acidosis
Lens dislocation
Lymphopaenia
Microcephaly
Mitochondrial DNA-depletion
Muscle weakness
Psychomotor retardation
Nephropathy
Nephro/urolithiasis
Optic atrophy
Renal failure (acute and chronic)
Scoliosis
Severe combined immunodeficiency
Spastic diplegia
Splenomegaly
T-cell immunodeficiency
Tetraparesis

Table 2: Inborn errors of purine and pyrimidine metabolism with urinary biomarkers				
Purines			**Index metabolites in urine**	
Disorder	**OMIM**	**Gene**	**Increased excretion**	**Decreased excretion**
Phosphoribosyl pyrophosphate synthetase deficiency	301835 311850	PRPS1	orotic acid	uric acid
Phosphoribosyl pyrophosphate synthetase superactivity	300661 311850	PRPS1	uric acid	
Adenylosuccinate lyase deficiency	103050 608222	ADSL	SAdo, SAICAR	
AICAR transformylase/IMP cyclohydrolase deficiency	608688	ATIC	AICAR	
Hypoxanthine guaninine phosphoribosyl transferase deficiency	300322 300323 30800	HPRT	hypoxanthine, xanthine, uric acid	
Adenine phosphoribosyl transferase deficieny	102600	APRT	2,8-dihydroxyadenine	
Adenosine deaminase deficiency	102700 608958	ADA	(deoxy)adenosine	
Purine nucleoside phosphorylase def.	613179 164050	PNP	(deoxy)inosine, (deoxy)guanosine	
Xanthine dehydrogenase def. Isolated	278300 607633	XDH	hypoxanthine, xanthine	Uric acid
Xanthine dehydrogenase def. combined with aldehyde oxydase deficiency	602841	AOX1	hypoxanthine, xanthine	Uric acid
Xanthine dehydrogenase def. due to Molybdenum cofactor deficiency	252150 603707 603708 603930	MOCS1 MOCS2 GEPH	hypoxanthine, xanthine S-sulfocysteine, sulfite, thiosulfate	Uric acid, cystine

Table 2 continued				
Pyrimidines			**Index metabolites in urine**	
Disorder	**OMIM**	**Gene**	**Increased excretion**	**Decreased excretion**
Orotate phosphoribosyl transferase deficiency (Uridine-5'-monophosphate synthase deficiency)	258900	UMPS	orotic acid	
Thymidine phosphorylase deficiency	603041 131222	TYMP	thymidine, deoxyuridine	
Dihydropyirimidine dehydrogenase deficiency	274270 612779	DPYD	uracil, thymine	
Dihydropyrimidinase deficieny	222748	DPYS	dihydrouracil, dihydrothymime	
Beta-ureidopropionase deficiency	613161 606673	UPB1	N-carbamoyl-ß-alanine N-carbamoyl-ß-amino-isobutyric acid	
ß-alanine-α-ketoglutarate aminotransferase deficiency	237400		ß-alanine	
ß-aminoisobutyrate-pyruvate aminotransferase deficiency	210100		ß-aminoisobutyric acid	
Uridine-5'-monophosphate hydrolase superactivity (pyrimidine specific 5'-nucleotidase superactivity)	266120 606224	UMPH1		uric acid

Table 3: Inborn errors of purine and pyrimidine metabolism without specific urinary biomarkers

Disorder	OMIM	Gene	Biomarker
Adenosine-5'-monophophate deaminase deficiency (Myoadenylate deaminase def.)	102770	AMPD1	Ischemic forearm test: decreased NH_3, increased creatine kinase after exercise
Inosine-5'-triphosphate pyrophosphohydrolase deficiency	147520	ITPA	Intracellular accumulation of inosine-5'-triphosphate (ITP)
Deoxyguanosine kinase deficiency	251880 601456	DGUOK	Hepatocerebral mtDNA depletion, increased plasma lactate
Thymidine kinase 2 deficiency	609560 188250	TK2	Muscular mtDNA depletion, increased plasma lactate
Thiopurine S-methyltransferase deficiency	610460 187680	TPMT	Inability to methylate thiopurines
Uridine-5'-monophosphate hydrolase deficiency (pyrimidine specific 5'-nucleotidase deficiency)	266120 606224	UMPH1	Accumulation of pyrimidine nucleotides in erythrocytes
Mitochondrial Ribonucelotide Reductase subunit 2 deficiency	604712	RRM2B	Hepatocerebral mtDNA depletion, increased plasma lactate

2. Recommendations for the analysis of purines and pyrimidines in biological fluids

The most commonly used method to analyse purines and pyrimidines in biological fluids is reversed phase high performance (pressure) liquid chromatography (HPLC) with UV-Visible detection. The preparation of the samples varies, some protocols require only dilution of the sample while other protocols prescribe solid phase extraction in order to concentrate the compounds of interest prior to analysis. Alternatively, some compounds are also detected by GC-MS in the analysis of organic acids.

At present liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is becoming more and more the first-line analytical tool for the analysis of many compounds and groups of compounds, including purines and pyrimidines. LC-MS/MS allows automation and thus greater sample throughput and a broader spectrum of analytes within one run, e.g. HPLC-MS/MS makes it possible to analyse all purine and pyrimidine metabolites in one run. Contrary to UV-Vis detection, the lack of UV-absorption of some compounds is irrelevant for MS/MS-detection. Unfortunately, this technique requires substantial investments and is therefore not yet available to all laboratories working in the field of biochemical genetics. Therefore, a standard procedure using HPLC with UV-detection is described in this document. For a method using LC-MS/MS, please refer to van Kuilenburg et al. in “Laboratory guide to the methods in biochemical genetics”, Blau et al. eds. pp 725-737.

Analysis of purines and pyrimidines in biological fluids by HPLC with UV-detection.

General aspects

Laboratory:

Fume hood should be available.

Method/instrumentation:

Automated reversed-phase liquid chromatography with UV-Vis detection.
High standard high performance (pressure) liquid chromatograph equipped with at least dual wavelength detection (a Diode Array Detector is preferable) and thermostated column holder and thermostated auto-sampler unit.
Column: Nucleosil C18 column (250 mm × 4.6 mm × 5 µm). Guard-column: standard reversed-phase guard column. Mobile phase: solution A: 350 ultra-pure water: 335 acetonitrile: 400 methanol (v:v:v), solution B: 0.1 mol/l KH_2PO_4 pH 5.0.

Linearity

Approximately 300 – 6000 µmol at λ = 254 nm, depending on the compound.

Sensitivity

The lower detection limit is approximately 1 – 10 µmol, depending on the compound.

Sample volume (absolute minimum):

Urine : 50 µl
Plasma : 200 µl
CSF : 200 µl

Additional apparatus and disposable objects required:

Plastic safe Lock vials
Microcentrifuge
Safe Lock Bio vials 1.5 ml
Waterbath
Autosampler vials
Microcentrifuge tube filters
Vortex-mixer
Micro pipettes
Disposable pipette tips

Sample collection and storage

Urine:

Traditionally 24-hour urine collection or overnight collection is preferred for diagnostic purposes. In practice, many laboratories use urine portions for diagnostic purposes. No preservatives are added to the sample.

The recommended procedure for 24-hour urine collection is a follows: during the collection period the urine aliquots are kept refrigerated (4°C) and after completion the urine is sent to the laboratory in a well-isolated package and stored in the refrigerator for max. one week at 4°C until analysis or stored frozen at -20°C when analysis is carried out after more than one week but within 2 months. For longer period store at -80 °C.

Dipstick tests for nitrite and pH should be carried out directly after receipt of the urine in order to check for bacterial contamination. In addition qualitative tests for glucose, reducing substances, sulphite and ketone bodies should be performed. Analysis should not be performed in severely bacterially contaminated samples (pH>7 and/or nitrite is positive).

Plasma

The analysis of purines and pyrimidine can be performed in plasma obtained from blood heparin coagulated with heparin as well as EDTA. This can be adjusted according to local protocols. In the case of capillary blood, clean and disinfect skin thoroughly before taking the blood sample, to avoid contamination from the skin surface.

Plasma samples should be stored at –20 º C or at –80 ºC, if stored for a prolonged period. Plasma samples should be deproteinized before analysis (see below).

CSF

Please refer to your own hospital protocol for the lumbar punction procedure.

CSF samples should be deproteinized before analysis (see below). CSF samples should be stored at –80 ºC

Sample preparation for analysis

Urine:

Thaw frozen urine sample in a water bath at 37 ºC, and leave at 37 ºC for 30 minutes. Mix thoroughly. Dilute the urine sample five times in ultra pure water (thus 1+4) and mix using a vortex-mixer. Usually 50 µl of the urine sample is used. Filter the sample using a 0.2 µm nylon micro centrifuge filter. Transfer the filtrate to a clean injection vial.

Plasma and CSF:

Quickly thaw the frozen sample in a water bath at 37 ºC. Place the sample vial on ice immediately after thawing. From this point on the sample is kept on ice. Gently mix the sample. To 200 µl of sample 200 µl of 2 mol/l perchloric acid ($HClO_4$) in water is added. Mix using a vortex-mixer. Leave to stand for 10 minutes on ice in order to deproteinize the sample. Centrifuge the sample at 20.000 × *g* at 4 ºC for 10 minutes. Transfer 300 µl of the supernatant to a clean vial. Add 10 µl of 0.5%(w/v) phenol red in water and 100 µl of 4 mol/l KOH + 1mol/l KH_2PO_4 prepared in water. After thorough mixing, the colour should be of a salmon pink quality. If necessary add more of the 4 mol/l KOH + 1 mol/l KH_2PO_4 solution in 10 µl portions to obtain a solution having the colour described. Leave to stand on ice for 15 minutes. Centrifuge the sample at 20.000 × *g* at 4 ºC for 5 minutes. Filter the supernatant using a 0.2 µm nylon micro centrifuge filter. Transfer the filtrate to a clean injection vial.

Control samples:

Pooled and for some compounds enriched samples are prepared using the same procedures as for patient samples and included in each series for diagnostic analysis. Control samples should only be deproteinized together with patient samples.

Note that some purines and pyrimidines have a very poor solubility, e.g. 2,8-dihydroyadenine and xanthine, and require sonication and basic conditions to dissolve. Place urine samples in a ultrasonic water bath for 10 minutes after the thawing procedure described above to increase solubility.

Reagents and buffers:

General remark: Always visually inspect buffers and solutions. Discard if contaminated.

2 mol/l Perchloric acid ($HClO_4$). Store at 4 ºC for max. 1 year

0.5 % Phenol-red (w/v). Store at 4 ºC for max. 1 year

2.5 mol/l KOH in ultra pure water. Store at 4 ºC for max. 1 year

4 mol/l KOH, 1 mol/l KH_2PO_4 in ultra pure water. Store at 4 ºC for max. 1 year

Mobile phase solution A: 350 ultra-pure water: 335 acetonitrile: 400 methanol (v:v:v). Store at ambient temperature for max. 1 year.

Mobile phase solution B: 0.1 mol/l KH_2PO_4 pH 5.0. Store at ambient temperature for max. 1 month.

Standards

Internal standards: no internal standards are used in this method.

Calibrators: Aqueous solutions of purines and pyrimidines are used to calibrated each series of analyses. Typical concentrations of the components in the calibrator solution are 150-200 µmol/l.

Analysis

Preparation

Mobile phase solutions are used at ambient temperature. The column is thermostated at 20 ºC. The sample tray of autosampler is kept at 4 ºC. Samples, control samples and calibrators should be ready for analysis.

Analysis

The elution gradient for diagnostics analysis of purines and pyrimidines should achieve separation of the compounds to such a degree that they can be distinguished qualitatively and quantitatively. Since numerous configurations of the equipment mentioned above exist only an example can be given. This example can serve as starting point for optimisation of the method using your own equipment.

In the analytical validation the following is essential:

- Visual inspection of the chromatogram (base line, peak areas, elution pattern)
- Verify calibrators with respect to retention times and peak areas.
- Identification of every single analyte should be made with great care

Interpretation and quantification:

Each purine and pyrimidine base has its own characteristic UV absorbance spectrum which can be used for identification. A library of spectra obtained from pure standards should be obtained. Peaks are identified based on their retention time and by comparison of the recorded absorption spectrum to library of spectra. Single-wavelength UV detection and quantification is usually performed at $\lambda = 254$nm. At this wavelength many substances have an absorbance maximum. Purines also have an absorption maximum at $\lambda = 280$ nm. For practical purposes quantification is often done at one wavelength ($\lambda = 254$nm). 2,8-Dihydroxyadenine is best quantified at $\lambda = 350$nm

Concentrations are calculated by an external-standard method. First, a response factor (Rf, $nmol^{-1}$) is calculated by dividing the peak area of the standard (A_s) by the absolute amount in nmol of the analyte injected on the column (n_s).

$$Rf = \frac{A_s}{n_s}$$

The concentration of the component in the analytical sample (C_i) is calculated by dividing the area of the component of interest in the sample by the response factor, corrected for the injected sample volume.

$$C_i = \frac{f * A_i}{Rf}$$

Representitive system configuration and settings

Shimadzu® High Performance Liquid Chromtograph configuration: LC-10 Adv pump, SIL-10 Adv autosampler, SPD-M10 Avp Diode Array Detector, SL-10Avp System controller. Additional components: Spark Mistral column oven, Alltech (mobile phase) degasser.

Integration software package: Shimadzu Class-VP

Settings chromatograph and gradient:

Flow rate: 1 ml/min
Gradient mode: linear

Time (min)	% Mobile phase solution A	% Mobile phase solution B
0	0	100
8	1	99
25	5	95
30	12	88
35	20	80
40	20	80
50	37	63
60	50	50
65	0	100
90	0	100

Diode Array Detector Settings

Start wavelength: 190 nm
End wavelength: 370 nm
Absorbance: 0-60 mAU
Wave step: 1 nm
Lamp type: deuterium
Sampling frequency: 1.5625 s^{-1}
Run time: 60 min
Acquisition delay: 0 min
Time constant: 0.64 s

Auto sampler Settings:

Injection volume: urine: 25 µl
plasma: 50 µl
CSF: 100 µl
Rinsing volume: 200 µl
Needle stroke: 35 mm
Rinsing speed 35 µl/s
Sampling speed: 15 µl/s
Purge time: 5 min
Rinse mode: After aspiration
Rinse dip time : 2 s

Quality control

Recoveries, detection limits and linearity of all purines and pyrimidines have to be established by analysing urine, plasma and CSF samples before and after enrichment (standard addition method) with reference compounds to define these parameters in the relevant biological matrix. Exact retention times and response factors for all components have to be determined at the wavelength used for quantification. Control urine or plasma samples are analysed in each series of urine, plasma or CSF analyses to control the inter-assay reproducibility

All values obtained for samples are registered and compared with the calculated respectively expected values and in case of deviations of > 2 s.d. the series is repeated.

Instrument performance is checked by observing the baseline and noise level, and the signal to noise ratio.

In general:

Analytical detection depends on:

1. Quality and condition of the analytical column.
2. Response factor in the linear area (is different for each component);
3. Performance of the detector.

Any change or adjustment of the system may cause changes to occur in the linear part of the calibration curve, e.g. new column, new detector lamp.

With every change in the procedure mentioned it is required to check if this influences the analytical performance. Analysis of the pooled control samples and the calibrators will make clear if any adjustments need to be made.

External Quality control

Participation in an external QA scheme like the ERNDIM scheme "Purines and Pyrimidines in Urine" is strongly recommended.

The ERNDIM External Quality control scheme for Purines and Pyrimidines

The ERNDIM (European Research Network for evaluation and improvement of screening, diagnosis and treatment of inherited Disorders of Metabolism) scheme "Purines and Pyrimidines in Urine" is organised by SKZL (Stichting Kwaliteitsbewaking Ziekenhuis Laboratoria) at Nijmegen (NL) on behalf of ERNDIM. This is a purely quantitative scheme dealing with the technical analysis of purines and pyrimidines only. The idea is that good technical performance is essential for diagnostic proficiency. Diagostic proficiency is addressed by ERNDIM in de DPT-schemes.

Aim and scope of the scheme

The aim of the ERNDIM qualitative Purine and Pyrimidine scheme is to monitor the analytical quality of the quantitative assay of purines and pyrimidines in urine in laboratories involved in the screening and diagnosis of patients with inherited metabolic disorders. This allows the participants to test their analytical methods and compare their results with their peers. The ultimate objective of an external quality control scheme is harmonization of the analytical skills of the participants, so that the same results are obtained in one and the same sample, regardless of the laboratory performing the analysis or the method used.

Every year, the scheme organizer and the scientific-advisor provide the participants with information concerning the performance of the participants as a group and each participant individually. The information on the performance of the group as a whole is provided by ERNDIM. In each annual report accuracy, recovery, precision, linearity, interlab CV are discussed.

For each annual series, 8 spiked lyophilised human urine samples are distributed. Each series consists of 4 randomly distributed pairs, allowing determination of the precision of a lab. Linearity over the whole relevant analytical range is another important parameter for analytical quality. This is also addressed in the schemes' design. A second approach to describe accuracy is the percentage recovery of added

analyte. In this approach it is assumed that the amount of the weighed quantity is the target value.

The purine and pyrimidine scheme is a purely quantitative scheme dealing with the performance of the technical analysis of purines and pyrimidines only. Diagnostic proficiency is addressed by ERNDIM in the diagnostic proficiency schemes. However, diagnostic proficiency strongly benefits from good technical performance. ERNDIM achieves to create a comprehensive scheme, bearing in mind that it is not possible to cover all disorders of purine and pyrimidine metabolism. Some disorders lead to aberrant intracellular nucleotide levels only and cannot be detected in body fluids, while other disorders do not have a primary index metabolite. Moreover, not all inborn errors of purine and pyrimidine metabolism that have biomarkers are represented in the scheme. It is, of course, the ultimate goal to include all known measurable relevant compounds in the scheme at a reasonable price. The compounds included in the 2010 series are: Orotic acid, Orotidine, Uracil, Adenine, Thymine, Uridine, Dihydrothymine, Dihydrouracil), Hypoxanthine, Xanthine, Adenosine, Succinyladenosine, 5-amino-4-imidazolecarboxamide riboside (AICAR), 2,8-dihydroxyadenine, Guanosine, Inosine, Deoxy-adenosine, Deoxy-guanosine, Deoxy-inosine, Deoxy-uridine, 5-OH methyluracil, Creatinine, Uric acid and Thymidine. At present succinylamino-imidazole carboxamine riboside (SAICAR) is lacking in the scheme. This is something ERNDIM is working on, but this takes time and may prove not be feasible on short term.

A word on dihydrouracil and dihydrothymine

Dihydrouracil (DHU) is formed from uracil by dihydropyimidine dehydrogenase (DPYD), and is subsequently, degraded to N-carbamoyl-ß-alanine in a reaction catalyzed by dihydropyrimidinase. Thymine is degraded to, subsequently, dihydrothymine and N-carbamoyl-ß-amino-isobutyric acid by the same enzymes. DHU was added to the purine and pyrimidine scheme in 2005 in order to improve the diagnosis of dihydropyrimidinase deficiency, in which there is an accumulation of dihydro-uracil and dihydro-thymine in the body fluids. In the reaction catalyzed by DPYD uracil and thymine lose their conjugated electron configuration and thus their UV-activity. Therefore, the quantification of the dihydropyrimidines is more complicated than the compounds that have UV-absorption and requires different

analytical techniques. The dispersion in the values measured by the participants is extremely large, especially considering the 2005 and 2006 series. Initially, virtually all participants used GC-MS for the quantification of DHU. In the 2007 series, results obtained with LC-MS/MS made their way into the scheme. Still, the overall results were far from perfect. While GC-MS is often off target, the results obtained by liquid chromatography coupled with tandem mass spectrometry are close to the target values and the dispersion is less extreme. It is the scientific advisor's opinion that LC-MS/MS is the superior technique for the quantification of DHU.