

D.1 Introduction

The laboratory diagnosis of inborn errors of metabolism cannot be completed by analysis of amino acids, organic acids, and acylcarnitines alone. Many defects are situated in other parts of the metabolic chart such as the breakdown of nucleotides and glycoproteins or the biosynthesis of cholesterol (and other steroids) or glycoproteins. Accumulating metabolites or biosynthetic intermediates may be analyzed in plasma or urine by a variety of techniques. The most widely used approaches are described here.

D.2 Purines and Pyrimidines

The purine and pyrimidine bases are the building blocks of DNA and RNA, in which they are incorporated as nucleotides. All purines and pyrimidines are produced by de novo synthesis; there is a continuous turnover. The majority of the purine and pyrimidine bases (and their nucleosides) has a strong UV-absorbance, facilitating their detection by HPLC. Humans produce uric acid as the end product of purine catabolism, its concentration may serve as an indicator of purine metabolism.

Purine and pyrimidine bases are present in plasma and urine; because of their low renal threshold, urine is preferred for analysis. Little use has been found for the analysis of cerebrospinal fluid. As a 24-h-urine sample is often difficult to obtain, the excretion of purine and pyrimidine bases (and nucleosides) is best expressed as mol/mol creatinine. Urine uric acid declines with age from values of 0.5–1.5 mol/mol creatinine in the first year of life to 0.2–0.5 mol/mol creatinine in adulthood [6]. Reference values for the most common purines/pyrimidines are given in Table D.1.

Table D.1. Reference values of purines and pyrimidines in urine. All values in mmol/mol creatinine

Purines		Pyrimidines	
Xanthine	4–48	Uracil	<43
Hypoxanthine	1–36	Uridine	<3
Inosine	<1	Thymine	<6
Guanosine	<1	Thymidine	<1
Adenosine	<1	Orotic acid	<3.3
Adenine	<1	Orotidine	<1
Succinyl-adenosine	<5	Dihydrouracil	<82
		Dihydrothymine	<18
		Pseudo-uridine	8–142
		N-carbamoyl- β -alanine	<53
		N-carbamoyl- β -AIB	<8

Patient selection for purine/pyrimidine screening is based on the occurrence of either neurological symptoms, renal stones, or immune deficiency disease. Symptoms may occur at any age; it is also important to check family members as the extent of the clinical abnormalities is not always clear. The sequential presentation of different symptoms in one subject is highly suspect. Tables giving the variability of the clinical signs are shown in Chap. 23.

The pre-analytical handling of the urine samples requires notice of the low solubility of various purine and pyrimidine bases such as xanthine, uric acid, dihydroxyadenine, and orotic acid. Care should be taken to dissolve all concrements by gently heating and ultrasonic treatment. All analytes are better soluble at a high pH.

Dietary and drug interference is a major threat to the diagnosis of purine/pyrimidine defects, as many foods, beverages, and drugs may contain modified purines. The problem gets worse when drugs that actively interfere with the catabolizing enzymes are involved. Examples are 5-fluorouracil and allopurinol. A shortage of folate will result in interference with the purine biosynthesis; as a consequence 4-amino-5-imidazole carboxamide and its riboside accumulate.

The analysis of purines and pyrimidines has historically always been performed by high-performance liquid chromatography (HPLC) with UV-detection [11]. The specificity of this detection method has improved following the introduction of dual-wavelength and multiple wavelength (diode-array) instruments. Nevertheless, the presence of interfering substances such as methylated purines (cf. coffee, tea, chocolate, soft drinks) and aromatic amino acid metabolites (cf. intestinal malabsorption) seriously hampers the interpretation. Moreover, many drugs such as antibiotics, aromatics (paracetamol, aspirin), methylated purines (theophylline), and pur-

ine/pyrimidine analogs (fluorouracil, allopurinol) will disturb the excretion profiles considerably.

The HPLC separation using reversed-phase columns is fairly critical, no matter whether classical phosphate buffers or volatile acetate buffers are used [6]. Even columns from the same manufacturer may give different results and rigorous control of the column temperature is a necessity. Coupling of the LC purine/pyrimidine separation to a mass spectrometer has recently been achieved [8]. Tandem electrospray MS using a variety of stable isotope-labelled internal standards and specific parent/daughter conversion for each analyte were used, enabling an analysis time of less than 10 minutes. Another advantage of this approach is the possibility of analyzing UV-negative substances such as the distal metabolites of the pyrimidine breakdown pathway, including the N-carbamoyl amino acids. Proton magnetic resonance spectroscopy is another elegant, potentially powerful technique (see Chap. F).

Thin-layer chromatography is no longer in use on a large scale, but may be helpful in isolated cases such as adenylosuccinase deficiency [3]. The diazo reagent not only stains with succinyl aminoimidazole carboxamide riboside (SAICAR), but also with xanthine and guanine.

D.3 Glycoprotein Constituents (Carbohydrates, Alditols, Oligosaccharides, Mucopolysaccharides, Sialic Acid)

Carbohydrates are very important metabolic intermediates; not only are they the prime fuels for delivering energy, but they also constitute the glycan moieties of the glycoproteins, which have many important functions. There are innumerable enzyme steps involved in the carbohydrate interconversion as well as in the biosynthesis and breakdown of glycoproteins. Defects in this area will result in the accumulation of abnormal glycoproteins, mucopolysaccharides, oligosaccharides, sugars, sugar phosphates or sugar alcohols. Selective screening for these defects will involve the analysis of urine and plasma, with a follow-up by investigating intracellular content/enzyme activities.

There is no general clinical chemical parameter available for this group of disorders with exception of the urine test for reducing substances. This will give a positive result (together with a negative glucose!) when fructose or galactose accumulate.

■ Biosynthesis of Glycoproteins (Congenital Disorders of Glycosylation)

The CDG syndromes are genetic defects in the assembly, attachment, and processing of N-linked glycans. These processes take place in the cytosol, the endoplasmatic reticulum, and the Golgi apparatus. As a result of these defects, glycoproteins are formed with a normal protein backbone, but abnormal sugar chains attached to it. As the sugar chains have a fixed composition with the charged N-acetylneuraminic acid as terminal sugar, changes of the number of sugar chains will result in differently charged glycoproteins having different mobilities in electrophoretic systems. Because of the abundance of the glycoprotein transferrin in serum, the iso-electric focusing (IEF) of serum transferrin is considered to be the diagnostic method of choice for the CDG syndromes [2].

Serum is the preferred material for transferrin IEF, as plasma may give blurred bands in isolated cases. Transferrin may accommodate as many as eight sugar chains, with four chains (called tetrasialotransferrin) as the most abundant form [4].

The IEF pattern may be inspected visually and most patients will be picked up by an increase of asialo- and disialotransferrin (CDG type 1 pattern). Some patients may display a so-called CDG type 2 pattern, with additional increase of monosialo- and trisialotransferrin. A better evaluation stems from densitometric scanning of the IEF gels. The reference values for the various transferrins are shown in Table D.2 (G. de Jong, manuscript in preparation).

It should be noted that there are several patients with CDG type 1 syndrome having a virtually normal transferrin IEF pattern.

Patient selection should not be too limited. Any patient with a variety of neurological symptoms, mental retardation, dysmorphic signs, hepatic insufficiency or fibrosis, protein losing enteropathy, failure to thrive, hypoto-

Table D.2. Reference values^a of sialotransferrins in serum of children aged 0–6 years (N=20). Data are expressed as percentage of total

Number of sialic acid residues	Mean	Range
0	0.5	0.0–1.0
1	0.5	0.0–1.1
2	3.5	1.8–5.2
3	9.1	5.5–12.7
4	51.8	45.4–58.2
5	24.8	22.8–26.8
6	8.6	5.0–12.2
7	1.1	0.3–1.8
8	0.3	0.0–1.4

Table D.3. Sialotransferrin pattern

Artefactual changes
– Alcoholism
– Galactosemia (untreated)
– Fructose intolerance (untreated)
– Hemolytic uremic syndrome (bacterial infections)
– Hepatoblastoma
– Organic solvent intoxication
– Polymorphism of the protein structure

^a Dr. G. de Jong, Erasmus University Rotterdam

nia or convulsions may be a candidate for selective screening. No precautions concerning the timing of the sample or the condition of the patient have to be taken. Several secondary changes of the transferrin IEF pattern have been recognized. These are summarized in Table D.3.

It is likely that more secondary changes will be found. Amino acid changes in the protein part of transferrin may change its IEF behaviour and result in unusual patterns with no clinical significance.

■ Glycoprotein/Glycosaminoglycan Breakdown

Lysosomal storage disorders give rise to intra-lysosomal accumulation of macromolecules, substrates for the failing enzyme reaction. In the case of sialic acid storage disease, a transport defect, there is only accumulation of the low-molecular weight sialic acid. Three selective screening methods are available to date, i.e. urine analysis of either mucopolysaccharides, oligosaccharides, or sialic acid. Patient selection will be based on a typical sequence of symptoms which may include progressive mental retardation, dysmorphic signs (gargoylism), skeletal abnormalities, hepato-/splenomegaly, corneal clouding, hyperactive behaviour, hearing loss, and dysproportionate growth retardation. As the lysosomal breakdown process is continuous, no precautions regarding diet or urine timing have to be taken.

The analysis of urinary mucopolysaccharides is best performed by a colorimetric test using dimethylmethylene blue (DMB) as described by de Jong et al. [5]. Reference values for different age groups, in use in the author's laboratory, are shown in Table D.4.

Table D.4. Reference values for urine mucopolysaccharide excretion, measured with the dimethyl methylene blue (DMB) test

Age group	MPS ($\mu\text{g}/\text{mmol creatinine}$)
0–6 months	10–50
6 months–2 years	8–20
2–10 years	5–15
>10 years	1–8

It is recommended to try and collect one's own reference population in view of the slight variations in color yield of the reagent.

Do not expect excessive MPS excretion in patients with mucopolysaccharidoses; even a 25% increase over the upper normal level may be diagnostic. Serious artefacts may occur by the use of urine collection bags which are made to stick to the body with adhesives.

Every clearly abnormal DMB test should be followed up by a second analysis using electrophoresis of the MPS fractions [7]. This separation is useful for guiding the subsequent enzyme analyses (Table D.5).

Artefacts of MPS electrophoresis may be the same as for the DMB test, i.e. adhesives of urine collection bags. There is a large group of disorders – mostly affecting connective tissue metabolism – that have been associated with increased MPS excretion. Amongst these are the Lowe syndrome, osteopetrosis, rheumatoid arthritis, pycnodysostosis.

Oligosaccharides (2–10 sugar moieties) and sialyloligosaccharides (together with free sialic acid) can easily be separated by thin-layer chromatography (TLC). Roughly their migration is inversely related with the number of sugar moieties in the molecule. Some of the oligosaccharides have a sialic acid as the terminal sugar. This gives them a positive charge and causes them to be retained on an anion-exchange column, a procedure that is helpful for the clean-up of urine samples. Staining of the TLC plates can be done with a resorcinol reagent or an orcinol reagent [10]. Quantitative assays exist only for sialic acid (both bound and free) and may involve chromatographic techniques or spectrophotometric measurements [16]. The free sialic acid excretion (mmol/mol creatinine) is inversely related with age. Some patients with sialic acid storage disease may have variable sialic acid excretion, occasionally in the normal range.

The oligosaccharide excretion profiles are disease-specific [10]. There are, however, a number of conditions interfering with the potential pathological profiles. These are listed in Table D.6.

Table D.5. Mucopolysaccharide excretion profile in the various mucopolysaccharidoses

Name	Hurler/Scheie	Hunter	SanFilippo	Morquio	Marotiaux-Lamy	Sly
Number	I	II	III	IV	VI	VII
Chondroitin-4-S	n	n	n	n	n	↑↑
Chondroitin-6-S	n	n	n	(↑)	n	↑↑
Dermatan-S	↑↑↑	↑↑↑	n	n	↑↑↑	↑↑
Heparan-S	↑↑	↑↑↑	↑↑↑	n	n	n
Keratan-S	–	–	–	↑↑↑	–	–

Table D.6. Artefacts of TLC of (sialyl) oligosaccharides

Condition	Consequence
Calcium lactobionate treatment	Fast migrating band (heavy!)
Amikacin treatment	Slow migrating band
Polyglucose feeding	Multiple bands at regular intervals
Lactating woman	Pattern like α -mannosidosis
Breast-fed infant	Pattern like α -mannosidosis
Secretion of Lewis blood group substance	Several bands
Nephrotic syndrome	General increase
Rheumatoid arthritis	Increased sialyllactose
Intestinal malabsorption	Lactose, saccharose increased

Any unusual set of bands should be interpreted with care; in cases of doubt on the identification, application of a second developing system may be helpful [1]. Alternative staining of the TLC plates with the ninhydrin reagent will disclaim aspartylglycosaminuria.

Most patients with glycogen storage disease will excrete small amounts of the oligosaccharide tetraglucose. This is especially prominent in the one lysosomal glycogen storage disorder Pompe disease. The tetrasaccharide moves very close to the trisaccharide which accumulates in glucosidase deficiency, one of the CDG syndromes that is otherwise characterized by a normal transferrin IEF pattern.

The GC/MS analysis of monosaccharides (sugars) and sugar alcohols (alditols) has received little attention until recently. These substances were mainly associated with the diagnosis of fructose intolerance and galactosemia. Especially galactitol has some use, because it will always be produced in excess by galactosemic patients, regardless of the dietary treatment. Other polyols such as arabitrol and ribitol have been associated with defects of the pentose phosphate pathway [13] and will probably become important diagnostic analytes in the near future.

The analysis of sugars and polyols by GC/MS does not require extensive pretreatment of the biological sample: in most cases evaporation, followed by (eth)oximation and trimethylsilylation will be sufficient.

D.4 Markers of Peroxisomal Function (Very Long-Chain Fatty Acids, Phytanic/Pristanic Acid, Bile Acids)

The growing list of disorders of peroxisomal function, divided into peroxisome biogenesis disorders (PBD) and defects of isolated peroxisomal enzymes, requires selective markers for their biochemical detection. One of the functions of the peroxisomes is the β -oxidation of substances that cannot be handled by the mitochondria such as very long-chain (C_{24} – C_{26})

fatty acids, branched-chain acids like phytanic and pristanic acid, and C₂₇ bile acids.

The peroxisomal processes are stable; no major changes will occur during fasting, eating, exercise or other modulating conditions. An exception is phytanic acid, which is exclusively derived from dietary sources. As a consequence newborns do not show accumulation of this substance and dietary measures may result in lowering of its levels.

Virtually all markers of peroxisomal function are lipid-bound or protein-bound; they are present in the plasma, but they are not excreted into the urine. Exceptions are the water-soluble glycine-, taurine-, or glucuronic acid conjugates of bile acids which may be found in the urine. The general clinical chemistry lab will not give consistent clues to the existence of peroxisomal disease as there is no readily accessible end product of peroxisomal substrates.

Plasma cholesterol may be decreased in PBD patients as several steps of the early part of the cholesterol biosynthetic pathway reside within the peroxisome. However, this is not a permanent finding. Each selective screening program for peroxisomal disorder will focus primarily on the quantitative analysis of the substrates themselves.

Selection of patients is a cumbersome task. The most severely affected patients, i.e. those with the neonatal Zellweger syndrome, display characteristic dysmorphic signs including a broad forehead and an enlarged fontanel. They are extremely hypotonic, have liver abnormalities and may have renal cysts. On the other hand there is a whole range of milder presentations. Retinitis pigmentosa and hearing loss may be important clues.

The primary diagnosis of peroxisomal disorders is made by the GC analysis of very long-chain fatty acids in plasma. Lipids have to be extracted and the analytes have to be derivatized by transesterification [9]. Historically the methyl ester formation was preferred, but alternative derivatives may be more suited for GC/MS analysis [14].

Not only the plasma concentrations of the very long-chain fatty acids, but also their molar ratios are important diagnostic parameters.

Reference ratios are:

$$C24/C22 < 0.95$$

$$C26/C22 < 0.02$$

Usually both ratios are increased in peroxisomal disorders. Nonperoxisomal increase of very long-chain fatty acids are rare (Table D.7).

Phytanic acid levels in neonates are not reliable, because its dietary intake has been insufficient by definition. In older patients it is important to evaluate the levels of both branched-chain fatty acids; an increase of phytanic acid with a decrease of pristanic acid suggests either Refsum disease or rhizomelic chondrodysplasia punctata. All other peroxisomal defects will show an increase of both analytes.

Table D.7. Abnormal very long-chain fatty acids

Condition	Consequence
Severe neonatal cholestasis	General increase
Hemolysis	General increase
Ketogenic diet	Mainly unsaturated fatty acids

Bile acids are formed from cholesterol by a series of reactions involving one round of peroxisomal β -oxidation. Accordingly patients with general peroxisomal disorders accumulate C_{27} bile acids instead of C_{24} bile acids. These can be measured by GC/MS or by electrospray tandem MS using a simple HPLC prefractionation. Both urine and plasma can be used.

D.5 Sterols

Defects of the biosynthesis of cholesterol were not included in the previous edition of this book, because they were practically 'non-existing'. Since the mid-nineties it has been recognized that patients with the Smith-Lemli-Opitz syndrome lack the final enzyme of cholesterol biosynthesis, i.e. 7-dehydrocholesterol reductase. Consequently the patients have hypocholesterolemia and accumulate 7-dehydrocholesterol and 8-dehydrocholesterol in their plasma [12].

Affected patients had a range of clinical symptoms and dysmorphic signs of which the syndactyly of the 2nd/3rd fingers/toes were the most consistent. The extent of the clinical symptoms was inversely related to the residual enzyme activity. Accordingly mild patients were found later, having normal plasma total cholesterol and only traces of the dehydrocholesterols.

Low cholesterol levels may also be found in other disorders (Table D.8).

Table D.8. Decreased cholesterol levels

Observed in
<ul style="list-style-type: none"> – Neonatal inadequate feeding – Smith-Lemli-Opitz syndrome – Other cholesterol biosynthesis defects – Generalized peroxisomal disorders – Abetalipoproteinemia – Some cases of cerebrotendinous xanthomatosis – Inborn errors of bile acid synthesis – Congenital defects of glycosylation (CDG)

Both nutritional and metabolic effects play a role, although not all inter-relations are well understood at present. There are even patients with low cholesterol levels in whom no underlying cause has been found yet. All patients with hypocholesterolemia should be screened for biosynthesis defects as there have been a number of other conditions identified affecting the biosynthetic pathway [15]. These defects comprise the Conradi-Hünemann syndrome (sterol δ^8 - δ^7 isomerase), CHILD syndrome (sterol C-4 demethylase), desmosterolosis (sterol δ^{24} -reductase), Greenberg skeletal dysplasia (sterol δ^{14} -reductase), and sitosterolemia.

All defects can be picked up by a careful analysis of plasma cholesterol and its precursors by GC-MS. Following the saponification of cholesterol esters, the cholesterol(s) are extracted and derivatized to form trimethylsilyl ethers. The levels of all precursors are very low in controls, less than 1 $\mu\text{mol/l}$. Only few artefactual increases are known such as the increase of plant sterols (sitosterol) following the administration of intralipid, and the accumulation of 7- and 8-dehydrocholesterol following haloperidol drug treatment.

An alternative way of diagnosing these defects is the analysis of sterols in the supernatant of fibroblasts that have been cultured in a dilipidated culture medium. The expression of the defect may be more clear than in plasma. Enzymatic and molecular genetic confirmation is now possible for virtually all defects.

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