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Review

Diagnosis and monitoring of inborn errors of metabolism using urease-pretreatment of urine, isotope dilution, and gas chromatography–mass spectrometry

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Abstract

To diagnose inborn errors of metabolism, it would be desirable to simultaneously analyze and quantify organic acids, purines, pyrimidines, amino acids, sugars, polyols, and other compounds using a single-step fractionation; unfortunately, no such method currently exists. The present article will be concerned primarily with a practical yet comprehensive diagnostic procedure of inborn errors of metabolism (IEM). This procedure involves the use of urine or eluates from urine on filter paper, stable isotope dilution, and gas chromatography–mass spectrometry (GC–MS). This procedure not only offers reliable and quantitative evidence for diagnosing, understanding and monitoring the diseases, but also provides evidence for the diagnosis of new kinds of IEM. In this review, the differential diagnosis for hyperammonemia are described; deficiencies of ornithine carbamoyl transferase, argininosuccinate synthase (citrullinemia), argininosuccinate lyase and arginase, lysinuric protein intolerance, hyperammonemia–hyperornithinemia–homocitrullinemia syndrome, and citrullinemia type II. The diagnosis of IEM of purine and pyrimidine such as deficiencies of hypoxanthine–guanine phosphoribosyl transferase, adenine phosphoribosyl transferase, dihydropyrimidine dehydrogenase, dihydropyrimidinase and β-ureidopropionase are described. During the pilot study for newborn screening, we found neonates with diseases at a rate of 1 per 1400 including propionic acidemia, methylmalonic acidemia, orotic aciduria, β-ureidopropionase deficiency, lactic aciduria and neuroblastoma. A rapid and reliable prenatal diagnosis for propionic acidemia is also described.

Keywords: Reviews; Inborn errors of metabolism; Urease pretreatment; Neonatal screening; 5-Fluorouracil

Contents

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1. Introduction

Human urine contains numerous organic acids and other chemical groups of compounds at a variety of concentrations. In the urine of a patient with a deficiency of an enzyme or its cofactor, the substrate of the enzyme reaction and/or the metabolites formed secondarily via side-paths owing to the accumulation of the substrate, increase markedly. In some cases, instead of the substrate or the secondary metabolites, a substrate precursor increases, owing to the de-repression of end-product inhibition. Therefore, human urine can provide evidence for the diagnosis of inborn errors of metabolism (IEMs). Since the discovery of isovaleric acidemia in 1966 [1], many IEMs that are classified as organic acidemias, in which organic acids accumulate in the urine, have been discovered using gas chromatography–mass spectrometry (GC–MS). GC–MS, due to its high chromatographic performance and highly sensitive and specific identification and quantification, is indispensable for chemical diagnoses of organic acidemias. In GC–MS, urinary organic acids are extracted with ethyl ether and/or ethyl acetate under acidic conditions with or without adding sodium chloride, and are then dehydrated with sodium sulfate and evaporated to dryness; the residues are derivatized to increase their volatility and therefore their suitability for GC–MS analyses [2,3]. Trimethylsilylation has been used by most laboratories for this purpose.

Several polar acids are important for diagnosis: i.e., orotate, the most important target for the screening of hyperammonemia or orotic aciduria, methylcitrate, the key target for propionic acidemia, and glycerol-3-phosphate, the target for fructose-1, 6-bisphosphatase deficiency. To measure these polar acids quantitatively, they can be extracted with DEAE–Sephadex [4], but the procedure takes several hours, and inorganic acids such as phosphate are also recovered well. It has not been possible to simultaneously analyze and quantify organic acids, purines, pyrimidines, amino acids, sugars, polyols, and other compounds using a single-step fractionation procedure. Shoemaker and Elliott reported that urinary organic compounds can be analyzed simultaneously after excessive urea in urine is degraded with urease and removed [5]. A highly modified and simplified procedure has been developed by Matsumoto and Kuhara [6,7], as a device for multiple sample analysis that has a potential use in neonatal screening. Our attempts to extract as much metabolic information as possible without fractionation, and even to use our procedure for mass screening, have been aided by the remarkable advances in GC–MS instrumentation and computer software during the last two decades.

This review will be concerned with our practical yet comprehensive diagnostic procedure for the chemical diagnosis of and screening for a variety of IEMs and for monitoring patients with IEMs.

2. Assay procedures

2.1. Chemicals

Urease Type C-3, methylcitrate, thymine, 5,6-dihydrothymine, 5,6-dihydrouracil, 5-fluorouracil and uracil are used. The following stable isotope-labeled compounds were used as internal standards; [$^{2}H_{3}$]creatinine, [$^{15}N_{2}$]uracil, [$^{2}H_{3}$]methionine, [^{13}C],

[$^{15}N_2$]adenine, [^{13}C], [$^{15}N_2$]guanine, [$^{15}N_2$]orotate, [$^{15}N_2$]urate, [$^{2}H_8$]homocystine, [$^{2}H_4$]cystine, [$^{2}H_5$]glycine, [$^{2}H_3$]leucine, [$^{2}H_4$]lysine, [$^{2}H_5$]phenylalanine, [$^{2}H_4$]tyrosine, [$^{2}H_3$]methylmalonate and [$^{2}H_3$]methylcitrate. The purity of stable isotope-containing compounds used as internal standards should be higher than 99%, except for uracil (98%), adenine (98%), and guanine (98%), as judged by the lack of additional peaks on GC–MS. The isotope enrichment of the stable isotopes should be also more than 99%.

2.2. Preparation of urine on filter paper and other samples

Sample preparation of liquid urine samples or eluates from urine on filter paper was based on the method of Matsumoto and Kuhara, which has been previously described [6,7]. A volume of 100 μl of urine was used. The urine was pretreated with urease at 37°C for 10 min to decompose and remove excess urea. For quantification, the urine was spiked with a fixed amount of several standards. Twenty-five nanomoles each of 2,2-dimethylsuccinate and 2-hydroxyundecanoate, the internal standards, and heptadecanoate, an external standard, were added to the urine. The urine was also spiked with stable isotope-labeled internal standards: creatinine, 100 nmol; uracil, orotate, adenine, guanine, and methylmalonate, 4 nmol; methylcitrate, 5 nmol; methionine, homocystine, leucine, phenylalanine, tyrosine, and cystine, 10 nmol; urate, 25 nmol; glycine and lysine, 50 nmol.

2.3. Creatinine determination

As previously described by Shoemaker et al. [5], and confirmed by us [6,7], creatine is almost completely converted to creatinine during the urease pretreatment procedure. Thus, the value obtained by GC–MS is of endogenous creatinine plus creatine, and it is this value that we use as the standard (total creatinine). We also determined the amount of creatinine and creatine separately by an enzymatic method, using an autoanalyzer, Beckman CX5, and evaluated the urinary metabolite levels obtained by both methods. Trimethylsilylation of creatinine gives two peaks, tri-TMS (major) and di-TMS derivative (minor), and their ratio is not constant. Therefore we did not use d_3-creatinine as an internal standard to quantify all the metabolites, but only to quantify endogenous creatine plus creatinine [6–8].

2.4. GC–MS measurement

Aliquots (0.5 or 1 μl) of derivatized extracts were injected into a bench-top GC–MS apparatus using an automatic injection mode with a split ratio of 1:30 (1:10–1:50). A Hewlett-Packard GC–MSD (HP6890/MSD5973) was used for the GC–MS measurement. Separation was carried out using a fused-silica DB-5 capillary column (30 m×0.25 mm I.D. with a 0.25-μm film thickness, J&W, Folsom, CA). The programming for the oven temperature, and the temperatures of the injection port and the transfer-line were the same as described [6–8]. Electron impact mass spectra were obtained by repetitive scanning at the scan rate of 2.5 cycles/s from m/z 50 to m/z 650. All other conditions for GC–MS measurement were the same as described previously [6,7].

3. Differential chemical diagnosis of hyperammonemia

There are more than 27 etiologies that give rise to hyperammonemia. Primary hyperammonemia is caused by any of six urea cycle enzyme defects [9] and two membrane transport system defects. The diseases caused by the transport system defects are known as hyperornithinemia–hyperammonemia–homocitrullinuria (HHH) syndrome (MIN 258870) [10] and lysinuric protein intolerance (LPI, MIN 247900) [11]. Secondary hyperammonemia is caused by several organic acidemias and other IEMs that cause hepatic dysfunction, such as tyrosinemia or Wilson's disease. In the eight conditions that give rise to primary hyperammonemia, the levels of uracil and/or orotate in urine are not altered in deficiencies of carbamoylphosphate synthetase and *N*-acetylglutamate synthetase, which are involved in the urea cycle [12]. In the remaining six conditions, the utilization of carbamoylphosphate is impaired and it accumulates in the mitochondria. The accumulation of

mitochondrial carbamoylphosphate causes an increase in the de novo synthesis of pyrimidine in the cytosol, resulting in an increase in uracil and/or orotate. Thus orotate and uracil are the useful indicators for screening and diagnosis of these primary hyperammonemias.

Ornithine transcarbamylase (EC 2.1.3.3) is a hepatic mitochondrial protein that catalyzes the formation of citrulline from ornithine and carbamoylphosphate. An ornithine transcarbamylase deficiency (MIN 311240) does not necessarily show an increase in specific amino acids except for pyroglutamate, glutamine, alanine, or proline. Argininosuccinate synthase (EC 6.3.4.5), the rate-limiting enzyme in the urea cycle, is located in the hepatic cytosol. It catalyzes the formation of argininosuccinate from citrulline and asparagine. A deficiency of argininosuccinate synthase causes citrullinemia (MIN 215700). Argininosuccinate lyase (EC 4.3.2.1) catalyzes the formation of arginine. A deficiency of this enzyme (MIN 207900) causes argininosuccinic acidemia. Arginase (EC 3.5.3.1), a cytosolic enzyme, releases urea and ornithine from arginine. An arginase deficiency (MIN 207800) causes hyperargininemia. In HHH syndrome, ornithine and homocitrulline increase, and in LPI, lysine increases in the urine but not in the serum. Therefore, to prevent the misdiagnosis of LPI, examination of the urine is critical. For patients with orotic aciduria and/or uraciluria, the levels of citrulline, arginine, and homocitrulline are further examined using a conventional amino acid autoanalyzer, or soft ionization MS, such as FAB- or ESI- (not GC–MS), and such patients are differentially diagnosed for ornithine carbamoyltransferase deficiency, citrullinemia, argininosuccinic aciduria, arginase deficiency, LPI, or HHH syndrome.

In our urease-pretreatment procedure, lysine can be quantified and ornithine can be measured semiquantitatively, using d_4-lysine as the internal standard, in addition to orotate and uracil [8]. Table 1 shows the evaluation of abnormalities in the levels of uracil, orotate, lysine, and ornithine in patients with hyperammonemia. In one case, a female infant with ornithine carbamoyltransferase deficiency showed a marked increase in urinary uracil and orotate, and even after protein restriction both metabolites were still elevated. In other patients with ornithine carbamoyltransferase deficiency, both uracil and orotate were detected in urine in large amounts, but only uracil was detected in the sera, and at lower levels than in urine [8]. Therefore, analysis of urine is far more sensitive than analysis of serum. Sass and Skladal also mentioned that the very high renal clearance of orotate, in addition to the fact that urinary values integrate changes over time, probably renders orotate measurement more relevant in the urine than in the plasma of ASD patients [13]. The extraction efficiency of orotate was 31% in the reported solvent extraction method (ethyl acetate twice with sodium chloride under acidic conditions) [14], but was 83% in the simplified urease pretreatment procedure [15,16]. When quality control was performed at six institutions in

Table 1
Differential chemical diagnosis for seven cases with hyperammonemia by simplified diagnostic procedure

Cases	Abnormality: n in mean+nSD ($\mathrm{Log}_{10}x$)				FAB–MS or other method
	Uracil	Orotate	Lys	Orn	
OTC:					
Before treatment	5.9	16.6	4.0	–	
Protein-free diet	4.0	12.0	−0.8	–	
Citrullinemia	7.7	16.1	3.5	–	Cit ↑↑
Argininosuccinic aciduria	4.4	6.1	−1.3	–	Argininosuccinate ↑↑
Hyperargininemia	5.0	12.7	5.4	–	Arginine ↑↑
LPI[a]	10.6	9.6	7.5	–	
HHH syndrome[b]	3.6	7.0	−1.8	5	Homocitrulline ↑
(CPS deficiency)	−0.7	1.4	2.4	–	

[a] Lysinuric protein intolerance.
[b] Hyperornithinemia–hyperammonemia–homocitrullinuria syndrome.

Japan with a urine specimen containing orotate at a level of 6 SD above the mean, the researchers who used the conventional solvent extraction method failed to detect the increase in orotate. Those who used the simplified procedure with urease pretreatment, however, did detect it. This procedure, therefore, is very suitable for monitoring and evaluating the effects of dietary restriction or of medications, which include oral sodium phenylbutyrate, arginine supplements or both, or intravenous sodium benzoate/sodium phenylacetate plus arginine, depending on the specific enzyme deficiency and the conditions, e.g., acute hyperammonemic crises. Such available therapies often result in dramatic improvement in the conditions of patients with hyperammonemia. However, the limitations of the available therapies have also been reported, from a perspective of 20 years of experience [17]. Recently, liver transplantation in IEMs becomes available. The urinary levels of orotate and uracil normalized in the female patient with ornithine transcarbamylase deficiency in Table 1, after she received a living related liver transplantation (not shown). We think that our highly quantitative orotate determination is a reliable monitoring method for the evaluation of liver transplantation for patients with the above six primary hyperammonemias. In Fig. 1, urinary metabolic profile of a patient with ASS deficiency is shown. We also expect it to be useful for evaluating experiments in animal models of these diseases as well, especially the developing new therapies, gene therapy, etc.

4. Screening of adult-onset type II citrullinemia

Citrullinemia has been classified into three types according to the enzyme abnormality and into two forms according to the pathogenesis. The classical form includes neonatal/infantile onset types I and III (CTRN1; OMIM 215700) [9]. In these cases, the

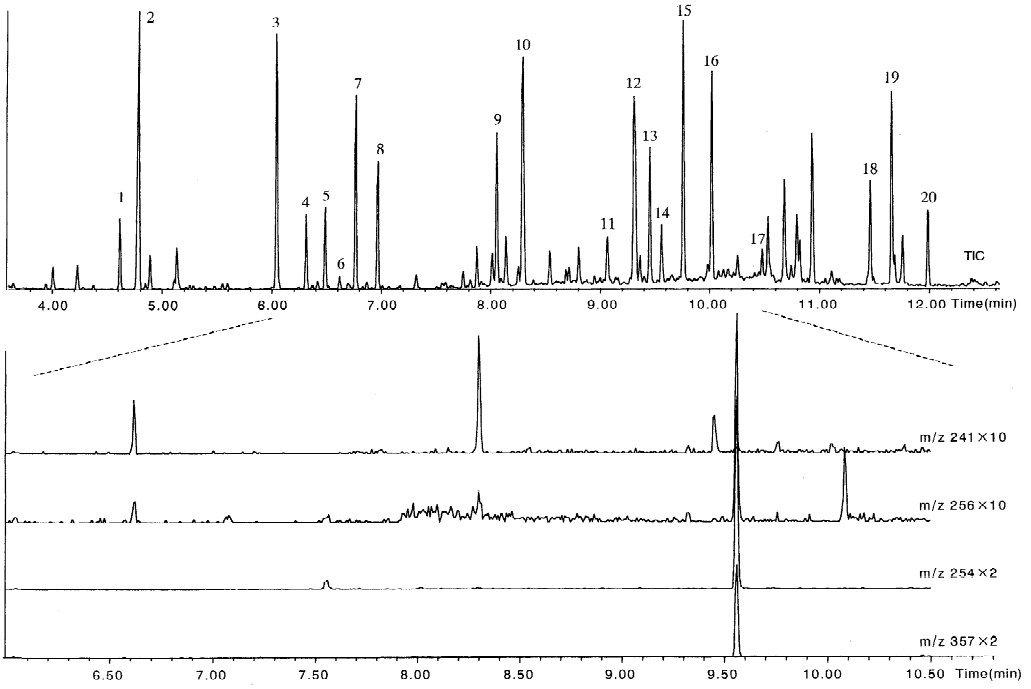


Fig. 1. TIC and mass chromatograms of TMS derivatives of urinary metabolites from a neonate with ASS deficiency. Peak identifications are: (1) alanine-2; (2) glycine-2; (3) phosphate-2; (4) proline-2; (5) 2,2-dimethylsuccinate-2 (I.S.$_1$); (6) uracil-2; (7) serine-3; (8) threonine-3; (9) 5-oxoproline-2; (10) creatinine-3; (11) asparagine-3; (12) lysine-3; (13) 2-hydroxyundecanoate-2 (I.S.$_2$); (14) orotate-3; (15) glutamine-3; (16) citrate-4; (17) glucose-5 (1); (18) *myo*-inositol-5; (19) *myo*-inositol-6; (20) heptadecanoate-1 (I.S.$_3$). Ions monitored for orotate and uracil were m/z 254 and 357, and m/z 241 and 256, respectively.

enzyme defect is found in all tissues in which the ASS gene on chromosome 9q34 is expressed. The other form is adult-onset, type II citrullinemia (CTLN2, OMIM 603471) with liver-specific ASS deficiency [18]. CTLN2 is characterized by decreased ASS enzyme activity with normal kinetic properties in the liver, but normal ASS levels in other tissues. CTLN2 has been mostly found in Japanese patients [19], and is clinically characterized by the sudden appearance of consciousness disturbance, restlessness, and abnormal behavior as well as a high serum citrulline concentration and hyperammonemia. Since most conservative treatments are not effective, the prognosis is very poor. However, liver transplants have recently been performed in several patients, and have resulted in the elimination of metabolic abnormalities and the improvement of clinical symptoms [20,21]. Recently, it was found that this disease is caused by a deficiency of the citrin protein encoded by the LSC25A13 gene on chromosome 7q21.3 [22], and it was later shown that citrin is a calcium-stimulated aspartate/glutamate transporter in mitochondria [23]. ASS is the rate-limiting enzyme in the urea cycle. For this enzyme, which is located in hepatic cytosol, to metabolize citrulline and thereby detoxify ammonia, asparagine is required in the cytosol as a substrate.

This disease, although it is termed adult-onset type II citrullinemia, is now known to present in early infancy with an intrahepatic cholestatic syndrome characterized by hepatic steatosis, elevated liver enzymes, and a unique serum amino acid pattern [24–26]. Some of these infants present with hypermethioninemia, hyperphenylalaninemia, or hypergalactosemia during the neonatal period, and these conditions can be detected by neonatal mass screening [25,26]. Thus, neonatal screening for hyperamino acidemia and galactosemia provides an important

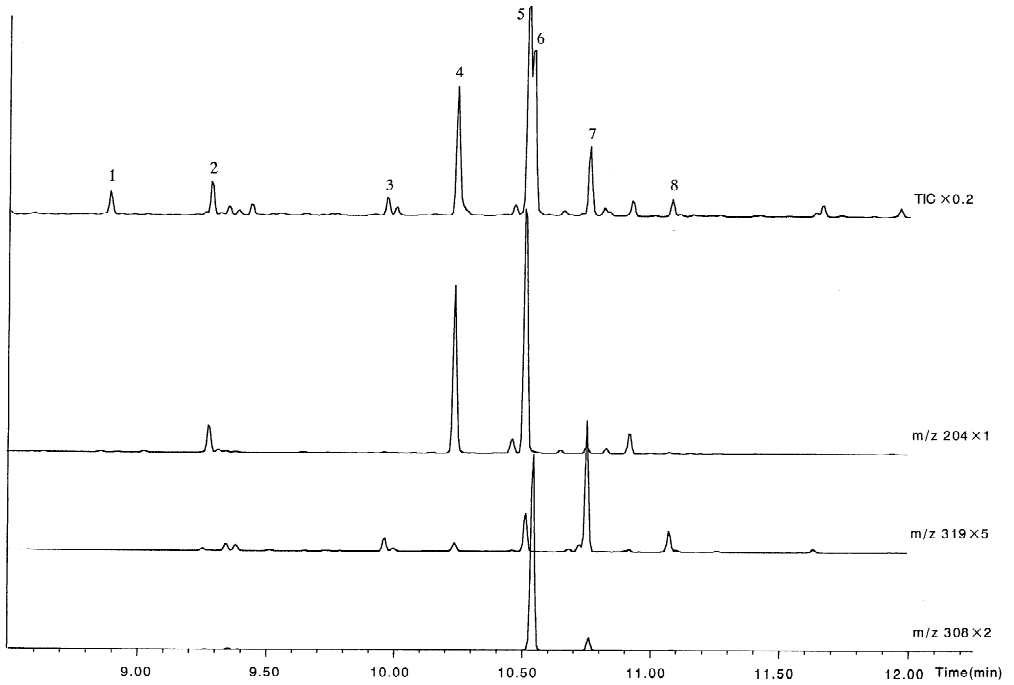


Fig. 2. Partly shown TIC and mass chromatograms of TMS derivatives of urinary metabolites from a patient with citrullinemia type II. Age at sampling time was 2 years when the presence of combined tyrosinuria and galactosuria were still noticed. Peak identifications are: (1) *p*-hydroxyphenylacetate-2; (2) lysine-3; (3) fructose-5(2); (4) galactose-5(1); (5) galactose-5(2); (6) *p*-hydroxyphenyllactate-3; (7) galactitol-6; (8) galactonate-6. Ions monitored for galactose-5(1) and (2), galactitol-6 and galactonate-6, and *p*-hydroxyphenyllactate-3 were at m/z 204, m/z 319 and m/z 308, respectively. Significant increase of phenyllactate and *N*-acetyltyrosine were also found (not shown in this figure).

clue for diagnosing CTLN2 in early infancy. In addition to citrulline and the above indicators, tyrosine, threonine lysine, and arginine are elevated in early infancy. In adults, citrulline is almost the only amino acid that is elevated in serum.

Millington et al. reported a rapid detection of citrullinuria and argininosuccinic aciduria by a procedure for recognizing elevated citrulline or argininosuccinate in urine. This method is based on a combination of fast atom bombardment mass spectrometry with tandem mass spectrometry [27]. Currently, elevated citrulline in blood is recognized by electrospray tandem mass spectrometry [28]. These methods are very sensitive and specific. Neonatal screening by electrospray tandem mass spectrometry on a blood spot can be used to screen for CTLN2. In our simplified urease-pretreatment procedure, methionine, phenylalanine, lysine and tyrosine in the urine were quantified by stable isotope dilution, and galactose, galactitol, galactonate, threonine, proline, pyroglutamate, phenyllactate (derived from phenylalanine), and *p*-hydroxyphenyllactate, *p*-hydroxyphenylpyruvate, and *N*-acetyltyrosine (derived from tyrosine), can be semi-quantified (Figs. 2 and 3).

The occurrence of such a variety of transient metabolic abnormalities led us to believe that CTRN 2 could be screened for by our procedure efficiently and successfully, although no screening test will be sufficient to catch these transient phenomena in all patients at a given point in time. For example, a female infant who had been discovered to have hyperphenylalaninemia by routine neonatal screening (Guthrie test) was examined for the evaluation of the hyperphenylalaninemia at the age of 26 days [29]. In her urine, not only galactose but also several other metabolites were elevated, as shown in Fig. 2. Several months later, her clinical abnormality disappeared and her abnormal urinary metabolite profile also normalized (not shown). In another infant, a male who was shown to have hypermethioninemia

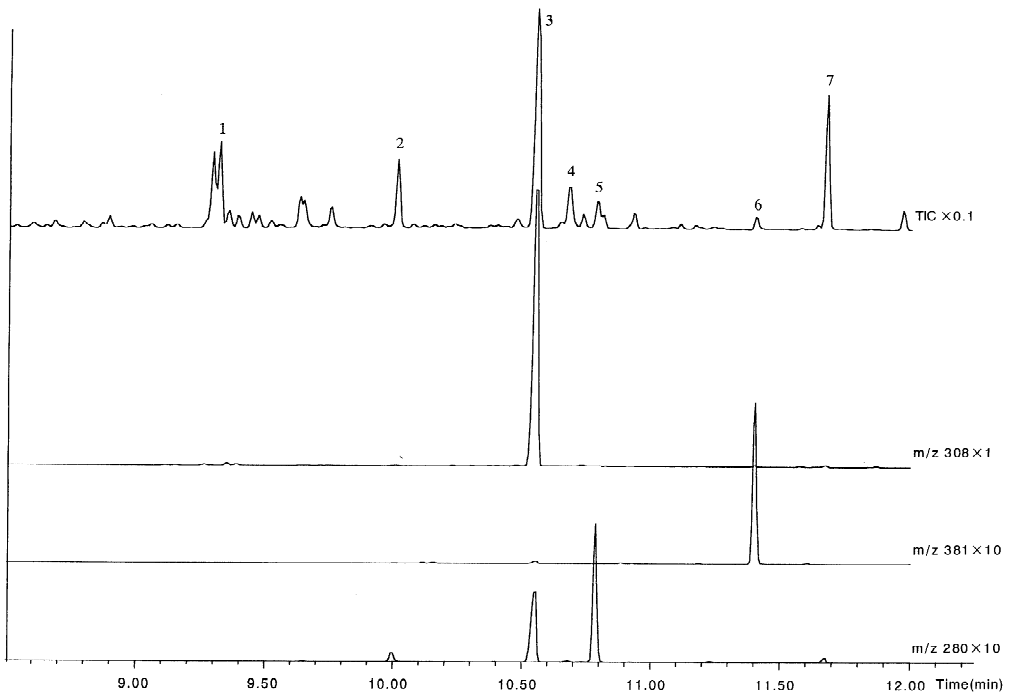


Fig. 3. Partly shown TIC and mass chromatograms of TMS derivatives of urinary metabolites from a patient with citrullinemia type II. Age at sampling time was 3 months when the presence of tyrosinuria was found. Peak identifications are: (1) lysine-3; (2) citrate-4; (3) *p*-hydroxyphenyllactate-3; (4) histidine-3; (5) tyrosine-3; (6) *p*-hydroxyphenylpyruvate-3; (7) urate-4. Ions monitored for *p*-hydroxyphenyllactate-3, *p*-hydroxyphenylpyruvate-3 and tyrosine-3 were at *m*/*z* 308, *m*/*z* 381 and *m*/*z* 280, respectively. Significant increase of methionine and *N*-acetyltyrosine were also found (not shown in this figure).

by the neonatal Guthrie test and later was proven to have CTRN2, a similar but slightly different metabolic profile was obtained using our urease pretreatment method. His metabolic profile during the time he had clinical symptoms is shown in Fig. 3. After 2 months, following clinical normalization, normalization of the urinary metabolite pattern was confirmed (not shown).

5. Diagnosis of inborn errors of purine metabolism

Inherited enzyme defects in the de novo synthesis of purines and pyrimidines or in their salvage and catabolism can cause alterations in cellular nucleotide patterns and the accumulation of normal or abnormal purines, pyrimidines, and their degradation products in body fluids. Twenty-four disorders have now been recognized. These defects manifest clinically with a broad spectrum of symptoms, including severe neurological abnormalities, fatal immunodeficiency, anemia, or urolithiasis. Thus, screening and chemical diagnosis for these defects should not be focused too much on selected groups of patients.

Inborn errors of purine and pyrimidine metabolism still present a diagnostic problem [30]. To date, several methods have been reported including HPLC [31], thin-layer chromatography [32], capillary electrophoresis [33], GC–MS [34,35], and nuclear magnetic resonance [36] for this purpose. These methods, however, target only a portion of the index metabolites in urine, and either could not be used readily for quantitation, lacked specificity or sensitivity, or required further analysis for a differential diagnosis [37]. Very recently, HPLC–ESI-MS–MS methods have been developed [38,39]. Here, we describe a rapid and specific procedure for chemical diagnoses of Lesch–Nyhan syndrome and adenine phosphoribosyl transferase deficiency using GC–MS.

5.1. Lesch–Nyhan syndrome

Hypoxanthine–guanine phosphoribosyltransferase (HPRT EC 2.4.2.8) catalyzes the conversion of hypoxanthine and guanine to inosine monophosphate (IMP) and guanosine monophosphate (GMP), respectively, in the salvage pathway for purine nucleotide synthesis. Lesch–Nyhan syndrome (LNS, MIM 30800) is caused by a severe or complete deficiency of HPRT [40]. Patients with LNS exhibit hyperuricemia, nephrolithiasis, and self-injurious behavior, but are frequently identified only after they present with gout or acute renal failure. This most often occurs in children or adolescents who have been institutionalized for cerebral palsy of unknown cause. Purine analogues such as azathiopurine, the standard immunosuppressive agent used in renal transplantation, is first converted to 6-mercaptopurine, a first-order cancer therapeutic agent but also an analogue of hypoxanthine. 6-Mercaptopurine is activated by HPRT to 6-mercaptopurine ribosephosphate, which has no effect on patients with LNS [41].

LNS is an X-linked (q2.6–2.7) recessive disorder, but 30% of cases are caused by de novo mutations [40,42]. More than 270 different mutations have been reported. Genotype-phenotype correlations have provided no indication that specific disease features are associated with specific mutation locations [43]. Although mutational analysis does not provide precise information for predicting disease severity, it continues to provide a valuable tool for genetic counseling in terms of confirmation of diagnoses, identifying potential carriers, and prenatal diagnosis [43].

To detect and identify the first case in a family as early as possible, it is critical to develop a sensitive yet specific method to identify patients with LNS. An accurate quantitation of hypoxanthine and xanthine adapting the stable isotope dilution and urease pretreatment procedure has enabled fast and accurate chemical diagnosis of LNS [44]. Fig. 4 shows the metabolic profile of a patient with LNS under treatment with allopurinol which is used as it inhibits xanthine oxidase. The urine specimens, kindly supplied by Dr. W.L. Nyhan, Professor UCSD, were sent via air mail from USA as dried urine on filter paper, and only 0.1 ml of the eluate from the paper was used for analysis. The most prominent abnormality was in the hypoxanthine levels. The next most severe abnormality was in the xanthine levels. Guanine increased only slightly. On the other hand, adenine decreased probably due to the elevated activity of

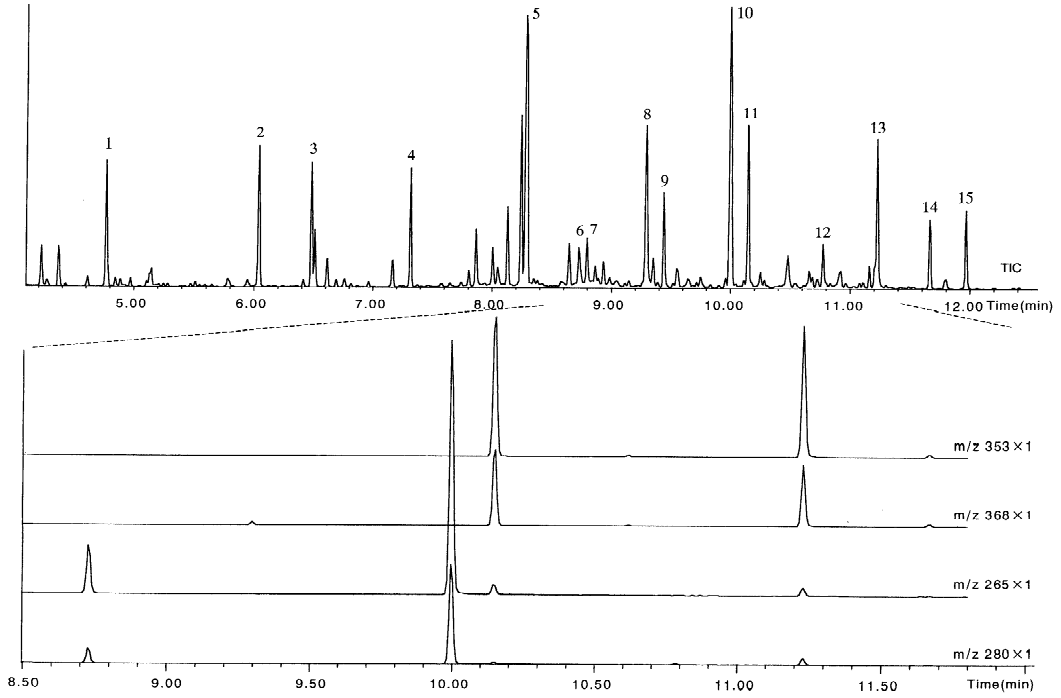


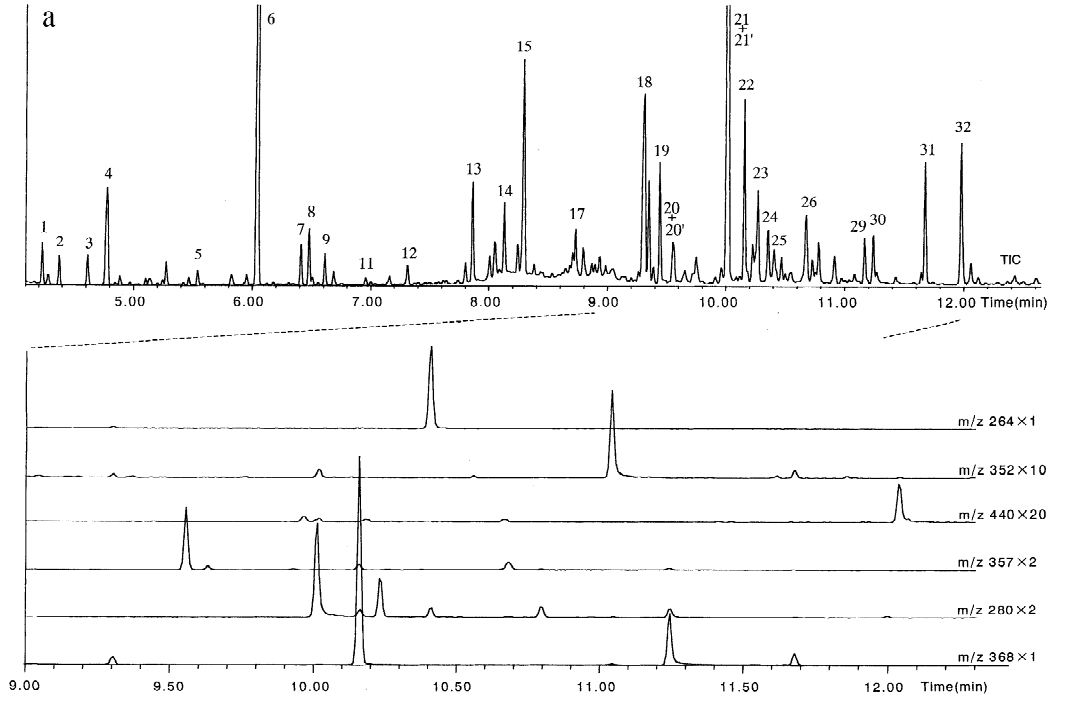
Fig. 4. TIC and mass chromatograms of TMS derivatives of urinary metabolites from a patient with Lesch–Nyhan syndrome under allopurinol treatment. Peak identifications are: (1) glycine-2; (2) phosphate-3; (3) 2, 2-dimethylsuccinate-2 (I.S.$_1$); (4) 2-deoxytetronate-3; (5) creatinine-3; (6) allopurinol-2; (7) phenylalanine-2; (8) lysine-3; (9) 2-hydroxyundecanoate-2 (I.S.$_2$); (10) hypoxanthine-2; (11) hydroxyallopurinol-3; (12) tyrosine-3; (13) xanthine-3; (14) urate-4; (15) heptadecanoate-1 (I.S.$_3$). Ions monitored for hypoxanthine and xanthine were m/z 265, 280 and m/z 353, 368, respectively.

APRT in LNS [45]. Orotate also increased because of the treatment with allopurinol, which also inhibits orotidine monophosphate decarboxylase [46].

5.2. Adenine phosphoribosyltransferase deficiency

Adenine phosphoribosyltransferase (APRT, EC 2.4.2.7) catalyzes the synthesis of adenine monophosphate (AMP) from adenine and 5-phosphoribosyl-1-pyrophosphate (pp-ribose-p) in the salvage pathway for purine nucleotide synthesis. APRT deficiency (MIM 1026000) blocks the use of adenine, which is oxidized by xanthine oxidase via 8-hydroxyadenine to 2,8-dihydroxyadenine. As 2,8-dihydroxyadenine is extremely insoluble, its accumulation in the kidney leads to the formation of urinary stones [47]. In many cases it has taken several years before the exact nature of the stones was recognized, following the initial presentation with urolithiasis [48–50].

The clearest indicator of this disease is an accumulation of adenine and its oxidation products, 8-hydroxyadenine and 2, 8-dihydroxyadenine. We examined urinary metabolites of patients with APRT deficiency using urease pretreatment, stable isotope dilution with labeled adenine, and GC–MS [51]. Only 100 μl of urine were used for the sample preparation, and it was analyzed as described in Section 2. Adenine increased markedly, and 8-hydroxyadenine and 2,8-dihydroxyadenine were clearly detected (Fig. 5a). The level of urinary adenine was markedly elevated. The younger brother of this patient was also tested, and his adenine level was also high, and 8-hydroxyadenine and 2,8-dihydroxyadenine were clearly detected (Fig. 5b). As the elder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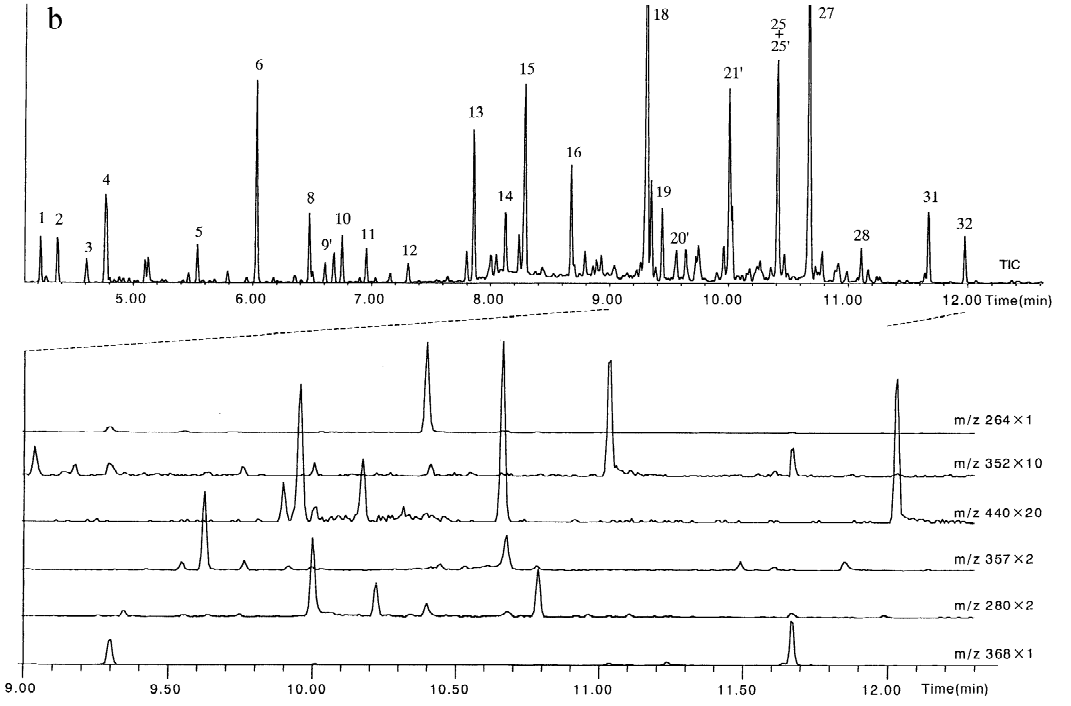
m/z 280×2
m/z 368×1
Time(min)


b
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m/z 352×10
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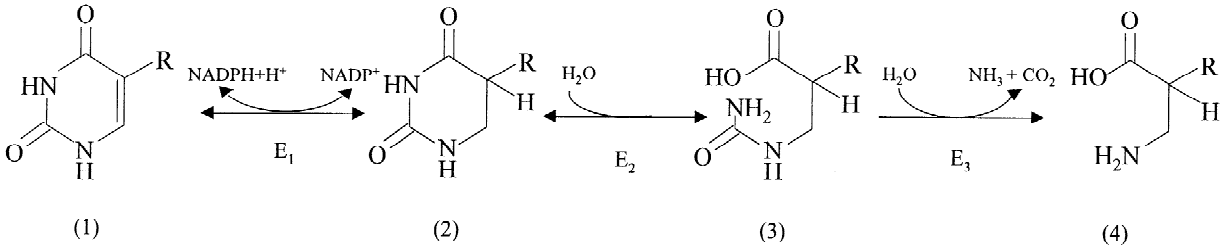

Fig. 6. The degradative pathway of pyrimidines. E1, dihydropyrimidine dehydrogenase; E2, dihydropyrimidinase; E3, β-ureidopropionase [*R*(−)3-amino-2-methylpropionate-pyruvate aminotransferase (D-3-aminoisobutyrate-pyruvate aminotransferase), β-alanine-pyruvate aminotransferase, 4-aminobutyrate aminotransferase]. R=H: uracil (1), 5, 6-dihydrouracil (2), β-ureidopropionate (3), β-alanine (4). R=CH_3: thymine (1), 5, 6-dihydrothymine (2), β-ureidoisobutyrate (3), β-aminoisobutyrate (4). R=F: 5-fluorouracil (1), 5,6-dihydro-5-fluorouracil (2), α-fluoro-β-ureidopropionate (3), α-fluoro-β-alanine (4).

sister was treated with the xanthine oxidase inhibitor, the level of adenine was markedly higher than in the younger.

6. Deficiencies of pyrimidine degradation

Pyrimidines are degraded in four steps in humans. The degradation is catalyzed by dihydropyrimidine dehydrogenase (5,6-dihydropyrimidine: $NADP^+$ oxidoreductase; DHPDH, EC 1.3.1.2), dihydropyrimidinase (5,6-dihydropyrimidine amidohydrolase; DHP, EC 3.5.2.2), β-ureidopropionase (UP, EC 3.5.1.6), and three aminotransferases (Fig. 6). Many cases with DHPDH deficiency (MIM 274270) or DHP deficiency (MIM 222748) are reported [52]. In both deficiencies, asymptomatic infants and adults have been reported and the clinical abnormalities in those with symptoms are variable and nonspecific [37,53].

No treatment for these enzyme defects has been described, but withdrawal of pyrimidine analogues from cancer chemotherapy regimens is proven critical for DHPDH deficiency [54,55]. The catabolic route shown in Fig. 6 plays a significant role for the degradation of pyrimidine analogues in vivo. DHPDH was shown to be responsible for the breakdown of widely used antineoplastic agent 5-fluorouracil (5FU), because more than 89.7% of the administered 5FU was excreted as unchanged into urine in DHPDH deficiency [56]. In healthy human, however, more than 87% is metabolized to 5-fluoro-β-alanine by this pathway [57]. Although the side-effects of 5FU for patients with DHP deficiency have not been reported, the same recommendation was also suggested for patients with this second enzyme deficiency on the bases of the results of a uracil loading test in DHP deficiency [53]. Therefore, finding out whether a cancer patient has pyrimidine metabolism deficiencies beforehand could prevent this devastating side effect.

DHPDH deficiency, DHP deficiency and UP deficiency are characterized by the markedly increased concentrations of uracil and thymine [58], dihydrouracil and dihydrothymine accompanied with

Fig. 5. TIC and mass chromatograms of trimethylsilyl derivatives of metabolites from urine of patients with adenine phosphoribosyl transferase deficiency; elder sister under allopurinol treatment (a) and younger brother (b). Peak identifications are: (1) lactate-2; (2) glycolate-2; (3) alanine-2; (4) glycine-2; (5) β-aminoisobutyrate-2; (6) phosphate-3; (7) succinate-2; (8) 2,2-dimethylsuccinate-2 (I.S.$_1$); (9) $^{15}N_2$-uracil-2 (I.S.); (9′) 4-deoxytetronate-3; (10) serine-3; (11) threonine-3; (12) 2-deoxytetronate-3; (13) erythritol-4; (14) tetronate-4(1); (15) creatinine-3; (16) ornithine-3; (17) allopurinol-2; (18) lysine-3; (19) 2-hydroxyundecanoate-2 (I.S.$_2$); (20) orotate-3; (20′) *cis*-aconitate-3; (21) hypoxanthine-2; (21′) citrate-4; (22) oxypurinol-3; (23) hippurate-1; (24) unknown; (25) adenine-2; (25′) glucose-5(1); (26) histidine-3; (27) lysine-4; (28) gluconate-6; (29) unknown; (30) xanthine-3; (31) urate-4; (32) *n*-heptadecanoate-1 (I.S.$_3$). Ions used for mass chromatography of adenine-2, 8-hydroxyadenine-3 and 2,8-dihydroxyadenine-4 are *m*/*z* 264, 352 and 440, respectively. Those of orotate, hypoxanthine and xanthine are *m*/*z* 357, 280 and 368, respectively. Note that creatinine concentration is 1.8 times larger in the upper sample than the lower one.

moderately increased uracil and thymine [59], and β-ureidopropionate (βUP) and β-ureidoisobutyrate (βUIB) in urine [60], respectively. Although several methods to screen for disorders of pyrimidine metabolism have been reported [32,61–63], these methods have been either time-consuming or have lacked specificity or sensitivity. Recently, a rapid and specific screening method was described that involves the use of urine and HPLC–ESI-MS–MS [38], where uracil, thymine, 5-hydroxymethyluracil, and orotate were targeted, but not dihydrothymine, dihydrouracil, creatinine or amino acids. Very recently, a method for screening of all the three pyrimidine degradation disorders was developed by which thymine, uracil, dihydrothymine, dihydrouracil, βUP and βUIB are simultaneously targeted but not orotate nor creatinine [39].

We have developed a GC–MS procedure for diagnosing these three pyrimidine degradation defects, in which the levels of pyrimidines, dihydropyrimidines, β-ureides, creatinine and orotate are determined by the simplified urease pretreatment and stable isotope dilution [16,64,65]. Recovery and C.V. were satisfactory, and the values of healthy controls were determined [16].

To prove the efficacy of our procedure, we have examined artificial urine specimens that simulated DHPDH deficiency and DHP deficiency [16]. We also investigated how to output the degree of quantitative abnormality of the indicators using the urine specimens taken from three patients with proven DHPDH deficiency and those from six patients with proven DHP deficiency [64]. Dr. A.H. van Gennip, Professor, University of Amsterdam, and Dr. Y. Wada, Professor, Nagoya City University Medical School, kindly supplied these samples. The levels of the indicators, thymine, uracil, dihydrothymine and dihydrouracil in urine from healthy controls are not normally distributed with a frequency distribution. Thus for statistical analysis, the data were $\log_{10}$-transformed. In DHPD and DHP homozygotes, the abnormality n in mean plus nSD of target metabolites was above 5.9 for the lower target, above 8 for the higher one, and 8 still on average. The abnormality was n was 12 for thymine and 5.9 for uracil and 5-hydroxymethyluracil was distinctly detected in three cases with proven DHPD deficiency. The value was 8.4–12 for dihydrothymine and 7.2–11 for dihydrouracil in six cases with proven DHP deficiency. Thus our output of abnormality n of the indicators in the urease-pretreatment procedure, has sensitive diagnostic value, although we could not detect any difference between DHP carriers and control. Although there was no relationship between the gene mutation and clinical severity [66], our data showed the close relationship between mutation and the magnitude of abnormality of the metabolites level [64].

UP deficiency (MIN 210100) was originally described in 1998 [67] with the use of NMR spectroscopy [68] in a 22-month-old female who developed the dystonia, scoliosis and microcephaly during the neonatal period. Later, the enzyme deficiency was proven [69]. This disease is characterized by an increase in βUP and βUIB in the urine [68,69,74]. These very polar compounds are not recovered by the conventional organic solvent extraction methods [70].

During the pilot study of neonatal screening and diagnosis (see below), we detected the second, but asymptomatic case, of this disease [65]. As shown in Fig. 7, the highly polar ureides are recovered and more popular GC–MS techniques can lead to the diagnosis of UP deficiency because of the involvement of the urease-pretreatment. In the urine of the newborn, βUP and βUIB were markedly increased and 5,6-dihydrouracil and 5,6-dihydrothymine were moderately increased. Uracil and thymine were within the normal range. Therefore, UP deficiency can clearly be diagnosed differentially from a first or second enzyme deficiency. This specific as well as sensitive procedure to screen and diagnose UP deficiency will stimulate the understanding of the clinical heterogeneity of this disorder and prevent possible side effects of cancer chemotherapy with pyrimidine analogues.

Dihydrothymine increased to 5SD above the mean in the present case with UP deficiency. This is probably because βUP strongly inhibit the activity of UP [73]. It was reported that the increased urinary βUP level is also observed in patients with ornithine transcarbamylase deficiency [71] and propionic acidemia [72]. Differential diagnosis of hyperammonemias including ornithine transcarbamylase deficiency, can be made because orotate is quantitatively and simultaneously determined as described in this

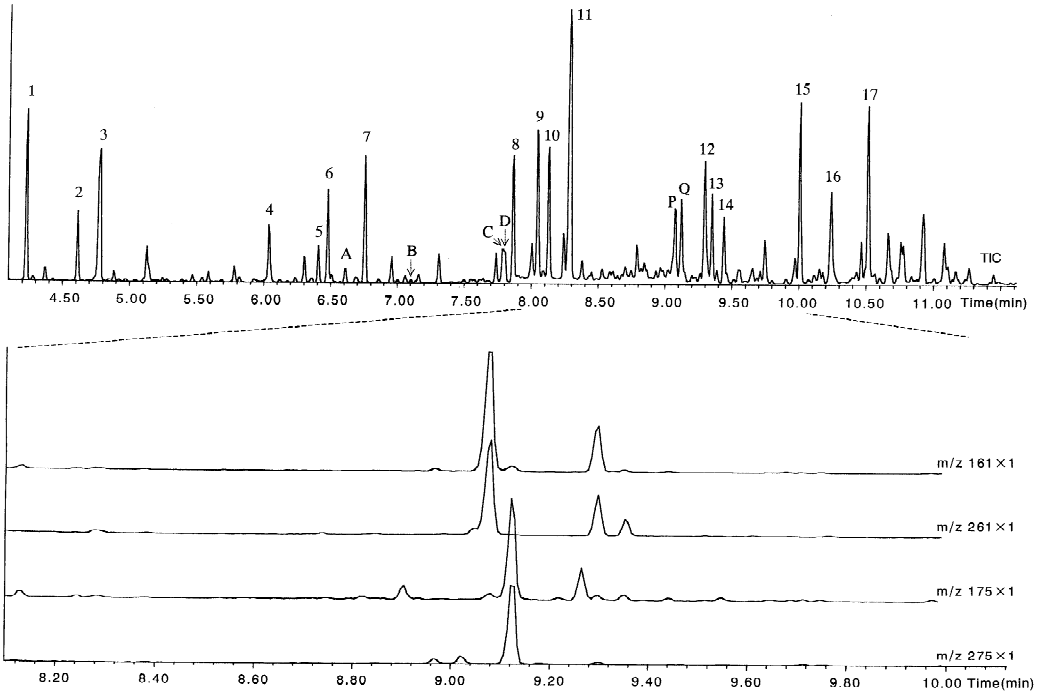

Fig. 7. TIC and mass chromatograms of trimethylsilyl derivatives of metabolites from urine of a neonate with UP deficiency. Peak identifications are: (1) lactate-2; (2) alanine-2; (3) glycine-2; (4) leucine-2; (5) succinate-2; (6) 2,2-dimethylsuccinate-2 (I.S.$_1$); (A) uracil-2; (7) serine-3; (B) thymine-2; (C) 5,6-dihydrothymine; (D) 5,6-dihydrouracil; (8) erythritol-4; (9) 4-hydroxyproline-3; (10) tetronate-4(1); (11) creatinine-3; (P) β-ureidopropionate-2; (Q) β-ureidoisobutyrate-2; (12) d_4-lysine-3; (13) xylitol-5; (14) 2-hydroxyundecanoate-2 (I.S.$_2$); (15) citrate-4; (16) galactose-5(1); (17) galactose-5(2). Ions monitored for β-ureidopropionate-2 and β-ureidoisobutyrate-2 were m/z 161, 261 and m/z 175, 275, respectively.

article. As shown in Fig. 8, a significant increase of βUP was noticed (2.6SD above the mean) in a male patient with ornithine transcarbamylase deficiency. Far more marked increase was, however, demonstrated in orotate (14SD above the mean based on either creatinine or total creatinine). Together with additional information obtainable from the TIC and mass chromatograms, increase of 5-oxoproline, alanine, glutamate, etc., UP deficiency can be denied. In our urease pretreatment procedure, PCCD including asymptomatic one could be detected and identified clearly differentiated from pyrimidine degradative disorders, because the most reliable target methylcitrate is highly recovered and simultaneously analyzed [74].

Defects in the three steps of pyrimidine degradation can now be screened for and diagnosed. No further analysis is required for a differential diagnosis because sensitive, specific, and simultaneous quantification of thymine, uracil, dihydrothymine, dihydrouracil, βUP, βUIB, orotate, methylcitrate and creatinine can be made by using stable isotope dilution, urease pretreatment and GC–MS.

7. Pilot study for newborn screening of 22 target diseases

GC–MS techniques have become indispensable for the diagnosis of IEMs since the 1970s, especially for organic acidurias, and high-risk patients are examined by selected institutions in various countries [2,3,75]. As metabolic abnormalities exist prior to the onset of the disease in most IEMs, chemical diagnosis can be made for presymptomatic neonates by detecting abnormal metabolites or metabolites that are abnormally increased in blood, urine, or other body fluids. Most IEMs result in severe

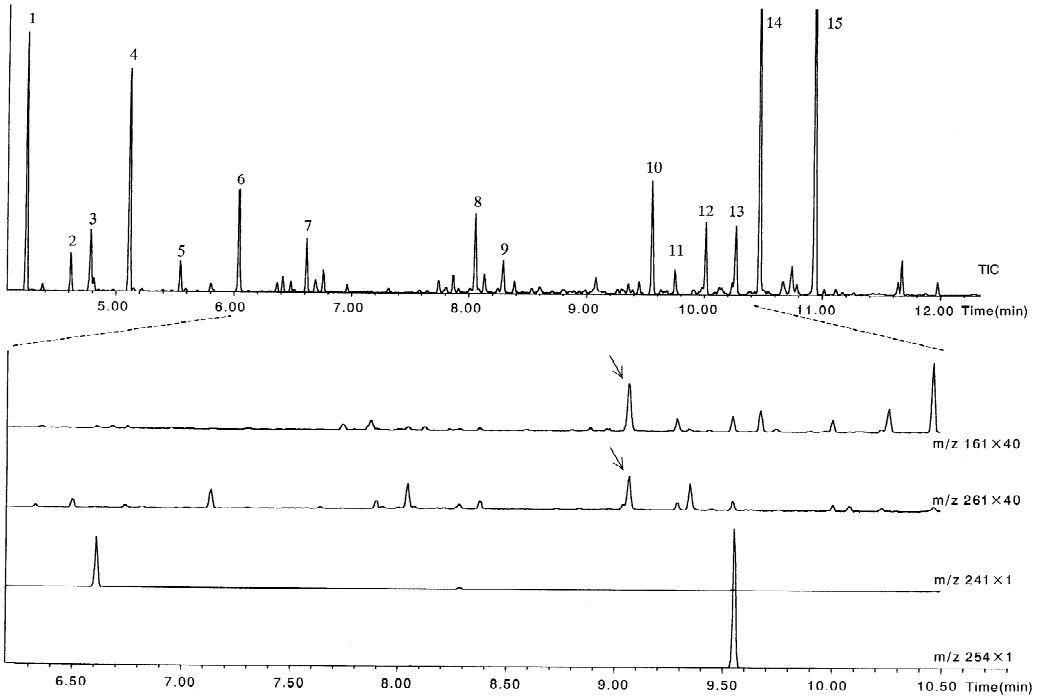

Fig. 8. TIC and mass chromatograms of trimethylsilyl derivatives of metabolites in the eluate from the filter paper urine of 2-year-old boy with ornithine transcarbamylase deficiency during an episode. Peak identifications are: (1) lactate-2; (2) alanine-2; (3) glycine-2; (4) 3-hydroxybutyrate-2; (5) β-aminoisobutyrate-2; (6) phosphate-3; (7) uracil-2; (8) 5-oxoproline-2; (9) creatinine-3; (10) orotate-3; (11) glutamine-3; (12) citrate-4; (13) hippurate-1; (14) glucose-5(1); (15) glucose-5(2). Orotate and uracil are markedly increased. Other metabolites associated with this disease, alanine, 5-oxoproline and glutamine, are also increased as shown in this TIC.

pathological consequences such as mental retardation, sudden infant death, or other irreversible conditions. Early treatment can prevent or significantly reduce the severity of the consequences, but delayed treatment is much less beneficial. Therefore, early diagnosis is critically important [76]. Hence, practical, sufficiently specific, and cost-effective neonatal screening programs (e.g., the Guthrie test) [77] are currently conducted for six IEMs using blood spots on filter paper in Japan. GC–MS is also used for secondary screening or scrutiny of positive cases detected by current neonatal screening [75].

Chamberlin and Sweeley [78] reported in 1987 that urine on filter paper was generally more useful than blood spots on filter paper. Indeed, most laboratories using GC–MS techniques for diagnosis have used and still use urine or urine on filter paper rather than blood, blood spots on filter paper, or serum. In our urine analysis using GC–MS, methylmalonate is the target for diagnosing methylmalonic acidemia, and methylcitrate is the target for diagnosing PCCD, as both methylmalonate and methylcitrate are highly cleared by the kidney. In patients with PCCD, methylcitrate and 3-hydroxypropionate are detected in large amounts in urine, but only 3-hydroxypropionate was detected in serum, and in lower amounts [8,79]. In neonates with benign methylmalonic aciduria, there is a marked increase in methylmalonate in the urine and no such increase in the serum [79]. For mass screening, however, only a limited number of studies have used urine. Tuchman et al. in 1990 adapted GC–MS for use in a mass screening program for neuroblastoma in 3-week-old infants in Quebec, using organic solvent extraction of urine from filter paper under acidic conditions [80], which was further extended to a screening

program for 20 or more different metabolic conditions in 1991 [81]. Recently, a GC–MS–MS method for screening urine specimens for 10 organic acidurias was described. Fourteen markers were quantified after solid-phase extraction, oximation/trimethylsilylation, and a short (10 min) GC–MS–MS measurement time [82]. Because these authors used a solid-phase extraction method, polar organic acids, such as methylcitrate, the most sensitive and reliable indicator for propionic acidemia, were not targeted.

Using a simplified diagnostic procedure with urease-pretreatment and GC–MS techniques, a joint pilot study of neonatal screening has been initiated by four institutions in Japan: Kanazawa Medical University, Kurume University Medical School, Shimane Medical University, and Chiba Prefecture Children's Hospital, in 1995 [79]. Twenty-two IEMs have been targeted for this pilot study. After obtaining written informed consent from parents, urine specimens are taken from neonates on days 5–7, when blood is taken for the Guthrie test, and sent for testing as dried urine on filter paper. Early reports from this pilot study have been published [8,79]. At the end of July 2002, a total of 56 600 newborns had been examined at the four institutions. Eighteen diseases were found. Kanazawa Medical University screened 14 200 newborns, and detected 13 cases of metabolic disorders. These were propionic acidemia (one case), lactic acidemia (one case, later diagnosed with an enzyme assay as an α subunit deficiency in pyruvate dehydrogenase), UP deficiency (one case), methylmalonic aciduria (three cases), Hartnup disease (one case), α-aminoadipic aciduria/α-ketoadipic aciduria (three cases), orotic acidemia (one case), transient but combined galactosuria and tyrosinuria (one case), and cystinuria (one case) and neuroblastoma (one case, asymptomatic but required prompt cancer chemotherapy). The incidence of IEMs at Kanazawa Medical University was thus one per 1100. Kurume University (Professor I. Yoshida) analyzed 35 900 samples and detected 26 cases. They were citrullinemia (one case), ornithine carbamoyltransferase deficiency (one case), methylmalonic aciduria (two cases), methylmalonic acidemia accompanied with homocystinuria (one case), propionic acidemia (two cases, but twins), maple syrup urine disease (one case), glyceroluria (two cases), and α-aminoadipic aciduria/α-ketoadipic aciduria (seven cases). This group also detected neuroblastoma (two cases), which is not an IEM. The estimated incidence was thus one per 1380. Shimane Medical University (Professor S. Yamaguchi) examined 4400 samples and found one case of mild phenylketonuria. Chiba Children's Hospital (Dr. M. Takayanagi) analyzed 2100 samples and did not encounter any abnormal cases. Thus, of the 56 600 newborns examined, 40 cases were identified; the incidence was one per 1400 at the four institutions, and one per 1290 at Kanazawa Medical University and Kurume University Medical School alone. This rate of detection (1/1290) is 13 times higher than that obtained for four IEMs (MSUD, PKU, homocystinuria, and galactosemia) identified by the Guthrie test during 21 years in Japan (1/16 400), although the Guthrie test also served to detect several cases with IEMs other than these four.

A method to analyze amino acids and acylcarnitines in blood spots on filter paper by tandem mass spectrometry has been developed by Millington et al. [83], Chace et al. [84], and Rashed et al. [85]. This method has been used recently for neonatal screening; it has been shown to be efficient and the cost is reasonable [86]. This method detected IEMs with an incidence of one per 4300 babies in Pennsylvania and North Carolina [86]. In Japan, Professor Shigematsu, of Fukui Medical University, found an incidence of IEMs of one per 9200 neonates and 6 diseases using blood/tandem mass spectrometry (at 30th annual meeting of Japanese Society for Mass-screening). Although this method permits high-speed analyses, it might be more appropriate to reserve it for screening rather than for chemical diagnosis. Most mass spectroscopists and biochemists believe that GC–MS is unlikely to be economically feasible for large-scale newborn screening. Our procedure is, however, a simple yet comprehensive diagnostic tool for a wide range of metabolic disorders. Methylmalonic aciduria and homocystinuria can also be differentially diagnosed as described [15]. In our procedure, more than 200 compounds are quantified to screen 22 IEMs. Because sufficient informations for a conclusive chemical diagnosis can be obtained in most cases, no further analysis is required. The time required to make a diagnosis and start appropriate treatment for neonates may, therefore, be short

enough to permit its use in large-scale screening of newborns.

8. Neonatal screening and diagnosis of propionic acidemia

Propionic acidemia (PCCD) is a rare but serious disease that is caused by inborn errors of propionyl-CoA metabolism. Propionyl-CoA is a catabolic intermediate derived from several essential amino acids (isoleucine, valine, methionine, threonine), odd-chain fatty acids, and the cholesterol side chain. Propionyl-CoA is normally metabolized to D-methylmalonyl-CoA by biotin-dependent propionyl-CoA carboxylase (propionyl-CoA: carbon-dioxide ligase; PCC, EC 6.4.1.3). D-Methylmalonyl-CoA is racemized to L-methylmalonyl-CoA by L-methylmalonyl-CoA mutase (EC 5.1.99.1) and then isomerized to succinyl-CoA by L-methylmalonyl-CoA mutase (methylmalonyl-CoA CoA-carbonyl-mutase, EC 5.4.99.2). PCCD (McKusick 232000 and 232050, corresponding to mutations affecting genes coding for the α subunit and β subunit, respectively), originally described as ketotic hyperglycinemia [87], is an autosomal recessive IEM in which the activity of PCC is deficient or greatly reduced. Nineteen disease-causing mutations have been identified in the PCC A gene and 28 in the PCC B gene [88]. Methylmalonic aciduria is an autosomal recessive IEM in which the activity of L-methylmalonyl-CoA mutase is deficient or greatly reduced (OMIM 251000). It has four known etiologies, two caused by an apomutase deficiency (mut 0, mut −) and two caused by defective adenosylcobalamine synthesis (cblA and cblB) [89]. Another inborn error in cobalamine metabolism causes combined methylmalonic acidemia and homocystinuria, which was proven detectable by urease-pretreatment procedure.

We identified one asymptomatic male infant with propionic acidemia during a screening program using GC–MS analysis of metabolites in urine dried on filter paper (Fig. 9). There was highly elevated methylcitrate without other elevations, indicating a deficiency of propionyl-CoA carboxylase [74]. This was confirmed by an assay showing low enzymatic activity in lymphocytes and a molecular analysis showing homozygosity for a mutation in the β subunit. Methylcitrate was distinctly elevated at 4.8SD above the normal mean relative to the 2-hydroxyundecanoate internal standard, and a concentration of 14.7SD above the normal mean as calculated by quantitation with a stable isotope-labeled internal standard, and expressing the results per enzyme creatinine. The activity of PCC in lymphocytes was 7% of the control. Gene analysis revealed that a single missense mutation, TAT to TGT, resulting in a Y435C substitution in the β chain was present in a homozygous form. A dietary treatment including carnitine supplementation decreased the patient's MC level and, to date (at 13 months of age), he shows no neurological or somatic abnormalities [74].

Propionylcarnitine is increased in the blood and urine of patients with PCCD or methylmalonic acidemia [89]. Therefore, these diseases are screened by evaluating the level of propionylcarnitine in blood by tandem mass spectrometry [90,91]. Although GC–MS is thought not to be economically feasible for large-scale newborn screening, our study does raise questions about the sensitivity of the more-practical tandem MS newborn screening for the detection of propionic acidemia. In our patient, methylcitrate was highly elevated and clearly diagnostic by the GC–MS of urine at 4 days of age (Fig. 9). The parallel analysis of a dried blood spot at 4 days of age by tandem MS, which was conducted by Professor Y. Shigematsu in Japan, however, showed only borderline elevation of propionylcarnitine. The level of the target was determined to be only 2.1SD above the normal mean, and the ratio of propionylcarnitine to acetylcarnitine, 0.287, was slightly higher than the screening cut-off point (0.21). The ratio of propionylcarnitine to acetylcarnitine in a blood sample taken at 2 months of age, was 0.258. The propionylcarnitine results from the dried blood spot are of great interest because of the growing use of tandem MS to screen for PCCD and methylmalonic acidemia. The ratio of propionylcarnitine to acetylcarnitine for this patient was much higher than the ratio from a healthy control subject (0.053), but the numbers 0.287 and 0.258 are not much higher than the 0.21 cut-off point. Thus, differentiating a patient with this disease from heal-

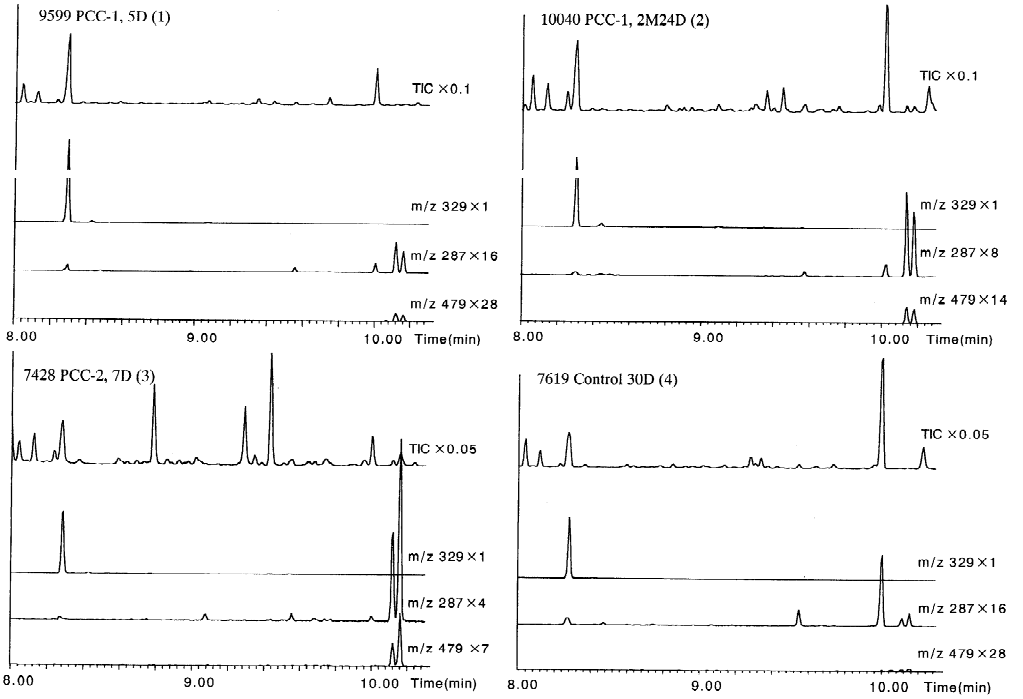

Fig. 9. TIC and mass chromatograms of trimethylsilyl derivatives of metabolites in the eluate from the filter paper urine of a 5-day-old neonate in whom the chemical diagnosis of propionic acidemia was made based on this profile, during the pilot study, and later confirmed by measurement of the PCC activity and mutation analysis [73] (1), from the same patient at 2 months and 24 days of life (2), from the urine of a patient with neonatal onset propionic acidemia on day 7 after birth (3) and from a 30-day-old healthy infant as a control (4). The ions targeted were m/z 329 for creatinine, and m/z 287 and m/z 479 for methylcitrate.

thy controls was difficult using the blood tandem MS method because the initial propionylcarnitine value was only 2.1SD above normal, which may well be below the cutoff in many tandem MS screening laboratories. Had tandem been the only screening method used, the diagnosis might not have been made, depending on how the normal cut-off point was defined. Perhaps the GC–MS urine screen might be considered as a secondary screen when the tandem MS dried blood spot screening shows borderline elevations of acylcarnitines that could suggest an organic acid disorder.

PCCD has a wide range of clinical manifestations varying from severe neonatal ketoacidosis with the risk of a major handicap or death to an asymptomatic or mild disease that usually responds well to treatment and has a good long-term outcome [88]. Recent studies have suggested that neurological deterioration occurs in patients even in the absence of ketosis or metabolic acidosis [92–94] and that fatality is not limited to cases with neonatal onset but also include unusual late-onset ones [95]. Even patients with relatively high residual PCC activity (11%) are known to develop serious clinical manifestation later [95,96]. Therefore, PCCD should be considered in all newborn infants with unexplained neurological deterioration even in the absence of ketosis and metabolic acidosis. As neurological abnormalities in newborns are often difficult to notice, a simple, highly sensitive, yet specific screening during the neonatal period seems critical to assure a high quality of life for individuals with PCCD. Although a definitive diagnosis of PCCD among high-risk patients has been accomplished by GC–MS analysis of urine [2,3,75], presymptomatic or neonatal screening and diagnosis of PCCD using urine specimens has not been extensively performed. Methylcitrate is the most reliable indicator for PCCD [97], but also increases in methylmalonic acidemia and multiple carboxylase deficiency. Multiple carboxylase de-

ficiency, for which treatment with biotin is critical, either caused by holocarboxylase synthetase deficiency, biotinidase deficiency, or biotin deficiency, results in the simultaneous deficiency of four human carboxylases: propionyl CoA carboxylase, methylcrotonyl-CoA carboxylase (3-methylcrotonyl-CoA: carbon-dioxide ligase; EC 6. 4. 1. 4), pyruvate carboxylase, and acetyl CoA carboxylase. The differential diagnosis of disorders with elevated methylcitrate depends the presence or absence of methylmalonic acid, 3-hydroxyisovaleric acid, or 3-methylcrotonylglycine, which can also be determined by this GC–MS screening method.

Urease pretreatment without fractionation allows satisfactory recovery of the highly polar methylcitrate and good reproducibility. Thus it permits highly sensitive identification of asymptomatic newborns with PCCD.

9. Prenatal diagnosis of propionic acidemia

The accurate diagnosis of PCCD can be done using GC–MS analysis of urine performed at selected laboratories. Once the index case has been identified in a family, the availability of reliable methods for prenatal diagnosis of potential fetuses becomes an important component of genetic counseling for the family. Methylcitrate is increased in cell-free amniotic fluid when a fetus is affected and is a key indicator for the prenatal diagnosis of PCCD. Since the direct chemical analysis of methylcitrate in cell-free amniotic fluid using the stable isotope dilution GC–MS was introduced by Naylor et al. [98] and Sweetman [99], direct methods have been developed rapidly not only for PCCD [100–103] but also for the other organic acidemias [104]. The direct analysis offers the considerable

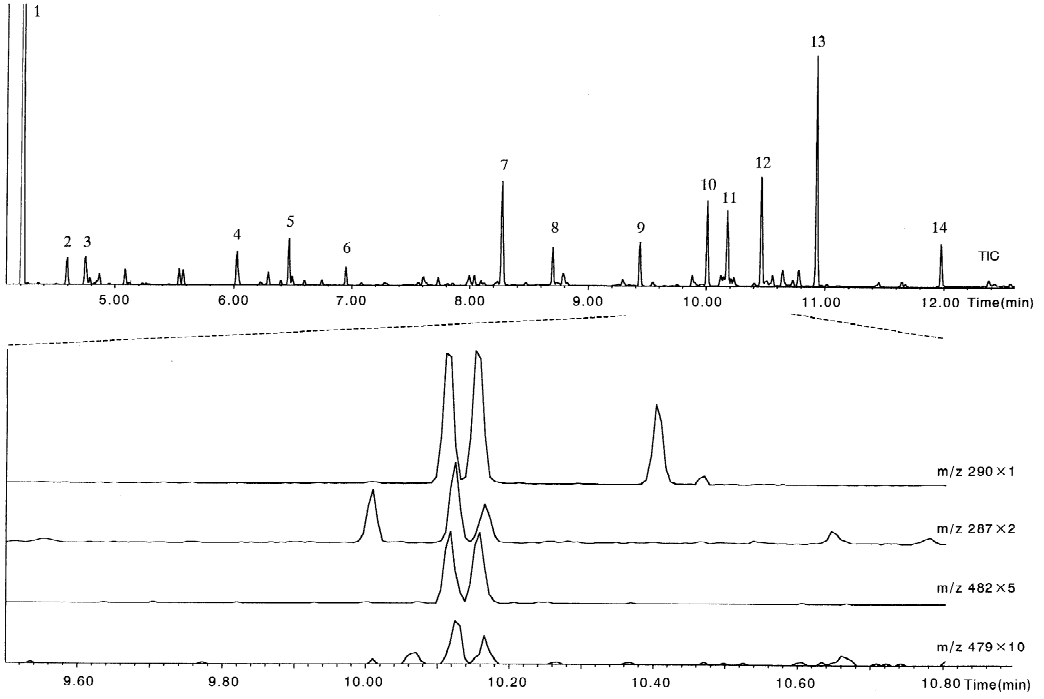

Fig. 10. TIC and mass chromatograms of trimethylsilyl derivatives of metabolites from an amniotic fluid specimen from a pregnant woman with affected fetus. Peak identification are: (1) lactate-2; (2) alanine-2; (3) d_3-glycine-2 (I.S.); (4) d_3-leucine (I.S.) plus phosphate-2; (5) 2,2-dimethylsuccinate-2 (I.S.$_1$); (6) threonine-3; (7) creatinine-d_3; (8) glutamate-3; (9) 2-hydroxyundecanoate-2 (I.S.$_2$); (10) citrate-4; (11) unknown; (12) glucose-5(1); (13) glucose-5(2); (14) heptadecanoate-1 (I.S.$_3$). Ions used for quantitation of methylcitrate and d_3-methylcitrate are [M-CH_3] at m/z 479 and 482, and [M-TMSOH-COOTMS] at m/z 287 and 290, respectively.

advantages of a rapid, repeatable, and reliable diagnosis. On the other hand, methods involving cell culture are time-consuming and potentially unreliable due to the possible contamination of amniotic fluids with maternal cells [105]. The measurement of PCC activity in chorionic villi has also been reported to give false results due to contamination by maternal tissue.

Until now, the organic solvent extraction [100] and solid-phase extraction [98,101–103] methods have been used for the quantitation of methylcitrate in amniotic fluid. Our simplified urease pretreatment procedure was applied to the prenatal diagnosis of PCCD [106]. The levels of methylcitrate in cases where the fetus was not affected were as low as in control cases. However, the concentrations in the amniotic fluid of affected fetuses were 20–30 times higher than in the fluid of unaffected fetus. For the affected cases, 20 μl of amniotic fluid was enough for quantitation with the selected ion monitoring (SIM) mode and 100 μl were enough with the scanning mode (scan range m/z 250 to m/z 500). The recovery of methylcitrate from the amniotic fluid was as high as 91%, and the coefficient of variation was lower than 3% in this procedure [106]. The total ion current (TIC) and mass chromatograms of trimethylsilyl derivatives of the metabolites in an amniotic fluid specimen from an affected fetus are shown (Fig. 10). This new procedure takes 1 h for sample pretreatment, including derivatization, and 15 min for the GC–MS measurement, and provides the final results within 5 min, as total 1.5 h. Thus, our procedure for the prenatal diagnosis of PCCD is rapid, highly sensitive, and more accurate than methods reported previously. This procedure does not require special solvents, columns, or special detection modes in mass spectrometry such as negative chemical ionization and SIM [106].

10. Conclusion

The simplified urease-pretreatment GC–MS diagnostic procedure targets far more than 200 compounds in biological samples, from lactate to homocystine within 15 min. GC allows the most efficient separation and gives chromatographic information of very precise retention times and order of elution of compounds of endogenous as well as exogeneous origin. Mass spectrometry combined with GC enables sensitive detection, accurate identification and reliable quantitation of these compounds. This procedure, technically practical yet comprehensive from the metabolic point of view, will provide valuable tool for screening and chemical diagnosis of more than 100 target diseases. This procedure is also useful to detect abnormal nutritional conditions in patients, such as acquired vitamin deficiency, and to monitor and evaluate patients receiving various treatment, including liver transplantation.

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