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Chemical diagnosis of Lesch–Nyhan syndrome using gas chromatography-mass spectrometry detection

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Abstract

Lesch–Nyhan syndrome (LNS) is caused by a severe deficiency of hypoxanthine-guanine phosphoribosyltransferase (HPRT) and clinically characterized by self-injurious behavior and nephrolithiasis; the latter is treatable with allopurinol, an inhibitor of xanthine oxidase which converts xanthine and hypoxanthine into uric acid. In the HPRT gene, more than 200 different mutations are known, and de novo mutation occurs at a high rate. Thus, there is a great need to develop a highly specific method to detect patients with HPRT dysfunction by quantifying the metabolites related to this enzyme. A simplified urease pretreatment of urine, gas chromatography–mass spectrometry, and stable isotope dilution method, developed for cutting-edge metabonomics, was further applied to quantify hypoxanthine, xanthine, urate, guanine and adenine in 100 μl or less urine or eluate from filter-paper-urine strips by additional use of stable isotope labeled guanine and adenine as the internal standards. In this procedure, the recoveries were above 93% and linearities (r^2=0.9947–1.000) and CV values (below 7%) of the indicators were satisfactory. In four patients with proven LNS, hypoxanthine was elevated to 8.4–9.0 SD above the normal mean, xanthine to 4–6 SD above the normal mean, guanine to 1.9–3.7 SD, and adenine was decreased. Because of the allopurinol treatment for all the four patients, their level of urate was not elevated, orotate increased, and uracil was unchanged as compared with the control value. It was concluded that even in the presence of treatment with allopurinol, patients with LNS can be chemically diagnosed by this procedure. Abnormality in the levels of hypoxanthine and xanthine was quite prominent and *n*, the number of standard deviations above the normal mean, combined for the two, was above 12.9.

Keywords: Lesch–Nyhan syndrome; Hypoxanthine-guanine phosphoribosyltransferase

1. Introduction

Hypoxanthine-guanine phosphoribosyltransferase (E.C.2.4.2.8, HPRT) in the purine salvage pathway catalyzes the conversion of hypoxanthine and guanine to inosine monophosphate (IMP) and guanosine monophosphate (GMP), respectively (Fig. 1). Lesch–Nyhan syndrome (MIM 308000, LNS), originally described in 1964 [2], is caused by a severe deficiency of HPRT enzyme activity [3].

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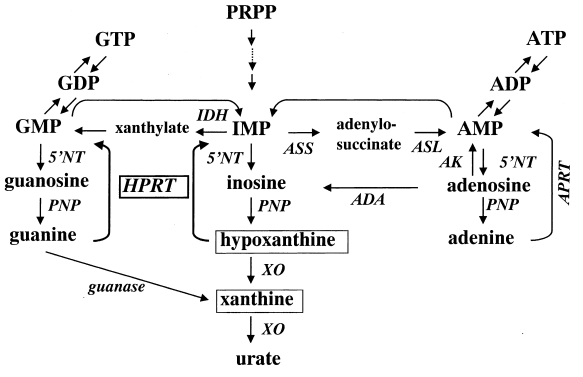

Fig. 1. The role of HPRT in purine metabolism. In LNS, guanine and hypoxanthine are not reutilized to purine nucleotides. The increase of both purine causes overproduction of hypoxanthine, xanthine and urate. 5′NT, 5′-nucleotidase; ADA, adenosine deaminase; AK, adenosine kinase; APRT, adenine phosphoribosyltransferase; ASS/ASL, adenylosuccinate-synthase/lyase; HPRT, hypoxanthine-guanine phosphoribosyltransferase; IDH, IMP dehydrogenase; PNP, purine nucleoside phosphorylase; PRPP, phosphoribosylpyrophosphate; XO, xanthine oxidase.

Patients with LNS exhibit a clinical syndrome characterized by hyperuricemia, hyperuricosuria, choreoathetosis, mental retardation, self-injurious behavior, and nephrolithiasis [1,4]. In addition, patients with partial HPRT deficiency lack the full clinical manifestations; these variants of LNS are known as neurological variants or hyperuricemic variants [4,5]. HPRT deficiency is an X-linked recessive inherited disorder; 218 different HPRT mutations have been summarized by Jinnah et al. [6], and about 30% of the patients are caused by a de novo mutation. It is essential to develop a simple yet specific method to detect individuals with not only LNS but also partial HPRT deficiency by analyzing abnormal concentrations of metabolites in body fluids. Allopurinol inhibits the oxidation of xanthine and hypoxanthine into uric acid [7]. The method is also invaluable for the monitoring of treatment with allopurinol in order to develop an optimal regimen for the prevention of urolithiasis.

Several methods to screen for disorders of purine metabolism have been reported, using HPLC [8], two-dimensional TLC [9], gas chromatography–mass spectrometry (GC–MS) [10,11], capillary electrophoresis (CE) [12], or NMR [13]. Recently, methods using electrospray tandem mass spectrometry (ESI-MS–MS) [14] or HPLC–ESI-MS–MS [15] have also been developed for more rapid and specific screening of purine disorders. To date, however, these methods have been less quantitative, lacked sensitivity or specificity, or needed further analysis to establish a chemical diagnosis of HPRT deficiency. A simple diagnostic procedure, which involves stable isotope dilution, simplified urease treatment of urine, and GC–MS, extended the chemical diagnostic capacity to new categories of metabolic disorders [16–18]. In the present study, we established a procedure for the chemical diagnosis of LNS by quantifying purines, uracil, and orotate in filter-paper-urine specimens.

2. Experimental

2.1. Chemicals

8-^{13}C (92%), 6-amino-^{15}N (78%), 1-^{15}N (22%), 9-^{15}N (98%)-adenine (i.e. ^{13}C, $^{15}N_2$ [p+3: 96.7% on average]-adenine), 8-^{13}C (98%), 7,9-^{15}N (98%)-guanine, and 1,3-$^{15}N_2$ (98%+)-urate were purchased from Cambridge Isotope Laboratory (Andover, MA, USA). Xanthine and allopurinol were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), urate from Sigma (St. Louis, MO, USA), hypoxanthine from Wako Pure Chemical Industries (Tokyo, Japan), guanine from Kohjin Co., Ltd. (Tokyo, Japan), and adenine from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Creatinine-methyl-$^{2}H_3$ (d_3-creatinine), 1,3-$^{15}N_2$-uracil, 1,3-$^{15}N_2$-orotate, uracil, orotate, and urease type C-3 were purchased as described previously [18,19]. The purity of stable isotope-incorporated compounds as well as other chemicals was confirmed by the lack of additional peaks on GC–MS.

2.2. Subjects and urine samples

Samples from four LNS patients from the University of California San Diego (CA, USA) were examined for the analysis of urinary metabolites. All the patients were receiving treatment with allopurinol. The urine specimens used for the present study were obtained at the ages of 3 years and 7 months (case 1), 19 years (case 2), 21 years (case 3),

and 19 years (case 4), respectively. The four specimens were sent by airmail as urine-soaked filter-paper strips from the University of California San Diego to Kanazawa Medical University, where they were used for the present study after elution with distilled water.

2.3. Sample preparation and GC–MS analysis

The procedures for sample preparation and the GC–MS measurement conditions were the same as previously described [16,17]. Extract (10–100 μl), obtained after elution from urine-soaked filter paper with distilled water, was pretreated with urease. To permit accurate quantification, 20, 20, 25, 4, 4, and 100 nmol of stable isotope-labeled adenine, guanine, urate, uracil, orotate, and creatinine, respectively, were spiked into 0.1 ml of urine as internal standards (IS). To obtain healthy control values, the amounts of labeled adenine and guanine were reduced to 4 nmol. The labeled adenine was used as the IS for the quantification of adenine, hypoxanthine, and allopurinol, and the labeled guanine was the IS for guanine, xanthine, and oxypurinol. In our procedure, creatine is almost completely converted to creatinine [18]. Measurement of creatinine using d_3-creatinine and an auto-analyzer (Beckman CX5), and the evaluation of urinary metabolite levels were described previously [18]. In this paper, the urinary metabolite levels were evaluated on the basis of creatinine but not creatinine plus creatine.

2.4. GC–MS measurement

Aliquots (0.5 or 1 μl) of derivatized extracts were injected into a bench-top GC–MS apparatus, Hewlett-Packard GC–MSD (HP6890/MSD5973), using an automatic injection mode with a split ratio of 1:30 (1:10−1:50) [17].

2.5. Standard curves, intra-assay variability, and statistical analysis

Quantification was performed using the relative peak area of the target ions between each compound and its IS. The ions used for targets were m/z 265 and 280 for hypoxanthine and allopurinol, m/z 353 and 368 for xanthine and oxypurinol (metabolite of allopurinol), m/z 352 and 367 for guanine, m/z 264 and 279 for adenine, and m/z 441 and 456 for urate, corresponding to $[M]^+$ and $[M–CH_3]^+$. The target ions of creatinine, uracil, orotate and their ISs were the same as previously described [18]. As shown in Fig. 2, allopurinol was distinguished from hypoxanthine by its retention time and mass spectrum, oxypurinol was similarly distinguished from xanthine. The retention time of oxypurinol-3TMS was 10.15 min and that of xanthine-3TMS was 11.23 min. In both mass spectra, prominent ion peaks of $[M]^+$ and $[M–CH_3]^+$ were observed at m/z 368 and m/z 353, respectively. In the latter, however, an additional intense ion peak of $[M–H]^+$ at m/z 367. Similarly, the retention time of allopurinol-2TMS was 8.73 min and that of hypoxanthine-2TMS 10.00 min. In the latter mass spectrum, the prominent ion peaks of $[M]^+$ and $[M–CH_3]^+$ at m/z 280 and m/z 265 were observed but in the former an intense ion peak of $[M–H]^+$ at m/z 279 was additionally observed.

To quantify metabolites in the urine samples, calibration curves were obtained from mass chromatograms. Various amounts of authentic compounds except oxypurinol were added to 100-μl urine specimens spiked with fixed amounts of IS. These mixtures were processed as described above and analyzed by GC–MS. A correction was also made for the quantitation of adenine, guanine, and urate (as for uracil, orotate and creatinine), because endogenous compounds (due to natural abundance) and labeled compounds (due to the presence of unlabeled ones) both contribute significantly to the other ions. The intra-assay variability of the GC–MS measurement was obtained by single sample preparation ($n=1$) and repeated GC–MS analysis ($n=10$, but $n=6$ for urate) of sample that was prepared from urine (each purine was added in an amount that was half of its IS). Additionally, the concentration of oxypurinol in urine was estimated from the concentrations of the labeled guanine and xanthine and corrected using the ratio of the total ion and the ion used for targets: Σ(%). Except for allopurinol and oxypurinol, the urinary concentrations of the five purines and two pyrimidines in healthy controls determined by the same procedure were not normally distributed. Accordingly, the data were $\log_{10}$-transformed before statistical analysis, and the transformed data were

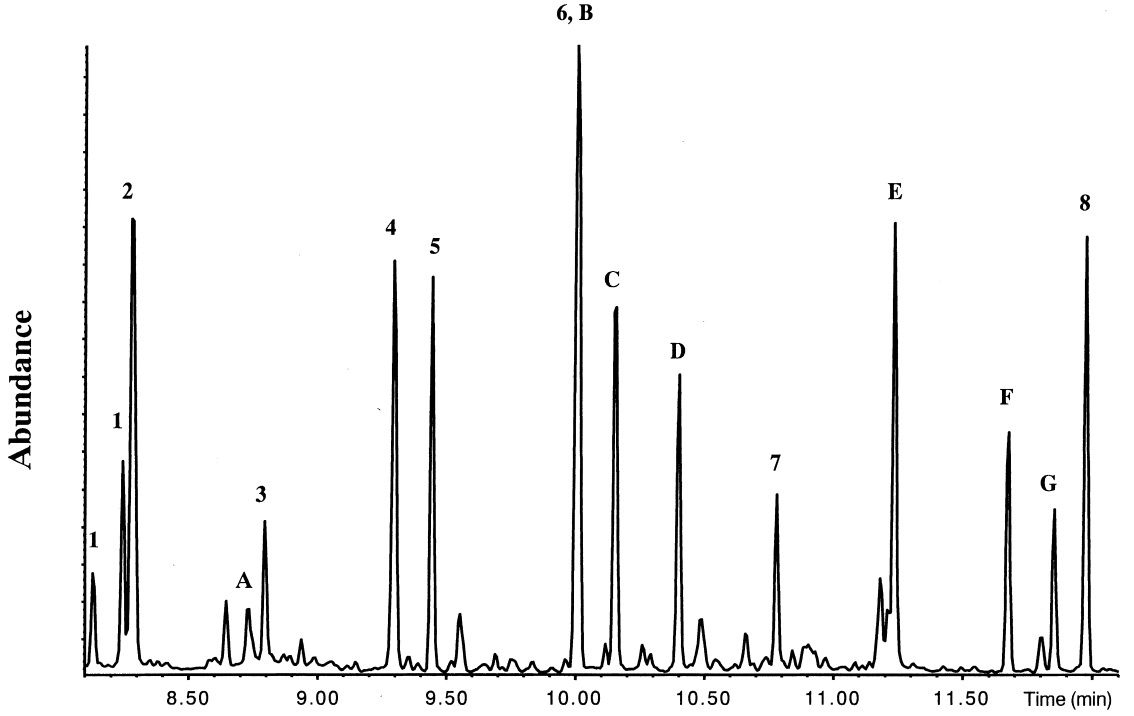

Fig. 2. TIC chromatograms of trimethylsilyl derivatives of metabolites from the urine of a patient with LNS (case 1) under allopurinol treatment. The eluate from the filter-paper-urine strip was treated with urease, deprotenized by ethanol, evaporated to dryness and trimethylsilylated. Hewlett-Packard GC–MSD (HP 6890/MSD5973) was used. The chromatographic separation is 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 and W, Folsom, CA USA). The oven temperature was programmed to increase at the rate of 17 °C/min from 60 °C to 320 °C, with a final holding for 10 min [18]. Peaks are: (1) tetronate; (2) d_0- and d_3-creatinine; (A) allopurinol; (3) d_0- and d_5-phenylalanine; (4) d_0- and d_4-lysine; (5) 2-hydroxyundecanoate (IS_1); (B) hypoxanthine; (6) citrate; (C) oxypurinol; (D) $^{13}C_0$, $^{15}N_0$- and $^{13}C_1$, $^{15}N_2$-adenine; (7) d_0- and d_4-tyrosine; (E) xanthine; (F) $^{15}N_0$- and $^{15}N_2$-urate; (G) $^{13}C_0$, $^{15}N_0$- and $^{13}C_1$, $^{15}N_2$-guanine; (8) heptadecanoate (IS_2).

used for evaluating the abnormalities of the patients with LNS, as previously described [20].

3. Results and discussion

The HPRT deficiency results in the accumulation of hypoxanthine and guanine. Hypoxanthine is oxidized to xanthine, and xanthine to urate, by xanthine oxidase. As guanine is metabolized to xanthine by guanase, the important urinary indicators for HPRT deficiency are hypoxanthine, xanthine and urate. Allopurinol is very similar to hypoxanthine in chemical structure, and inhibits xanthine oxidase. The inhibition of the overproduction of urate by allopurinol treatment greatly reduces the risk of nephrolithiasis and gout [1,5], although the treatment for the neurobehavioral features of LNS are limited. Under allopurinol treatment, orotate increases, because the drug also inhibits orotate monophosphate decarboxylase. It is desirable to detect LNS patients before transplantation, because azathioprine, a standard immunotherapeutic agent, has no effect on patients with LNS [21].

Here, we established a specific procedure for the chemical diagnosis of LNS by quantifying urinary target metabolites using urease pretreatment, isotope dilution, and GC–MS. This procedure was validated. For the five purines, the correlation coefficients (r^2) of the calibration curves and the recoveries are shown in Table 1. In the range 0−40 nmol/vial, but 0−50 nmol/vial for urate, of each purine added to the control urines (i.e., 1−401 μ*M*, but 16−660 μ*M* for urate), the linearities of the calibration curves

Table 1
Correlation coefficients of calibration curves and recoveries of urinary purines

Compound	Internal standard	Target ion	r^2	Recovery (%)
Adenine	$^{13}C_1$, $^{15}N_2$-Adenine	$[M-15]^+$	0.9999	99
		$[M]^+$	0.9997	100
Hypoxanthine	$^{13}C_1$, $^{15}N_2$-Adenine	$[M-15]^+$	0.9994	94
		$[M]^+$	0.9991	97
Guanine	$^{13}C_1$, $^{15}N_2$-Guanine	$[M-15]^+$	1.0000	100
		$[M]^+$	0.9998	98
Xanthine	$^{13}C_1$, $^{15}N_2$-Guanine	$[M-15]^+$	0.9959	96
		$[M]^+$	0.9947	96
Urate	$^{15}N_2$-Urate	$[M-15]^+$	0.9999	94
		$[M]^+$	0.9996	93

were good (r^2=0.9947−1.000). Their recoveries were also good (93–100%), although the recovery from the dried filter-paper-urine was slightly decreased by 5% for hypoxanthine and 12% for xanthine as compared with urine specimens. The urine samples from the patients were prepared so that the ratio of each purine to its IS ranged from 0.1 to 10, and then their quantitation showed certain values (data not shown). When the ratio of each purine to its IS was 0.50–0.56 (but 1.64 for urate), the CV values (%) were small especially for adenine, guanine and urate, because these were quantified by using their best internal standards. In intra-day assay, the values were 1.3, 1.8 and 1.4% for adenine, guanine and urate, respectively. Those were 2.1 and 4.5% for hypoxanthine and xanthine, respectively. The CV values in inter-day assay were 7.0, 5.0 and 2.8% for hypoxanthine, xanthine and urate. They were comparable to those obtained using HPLC–ESI-MS–MS or superior, for recovery (i.e., hypoxanthine 64% vs 94–97%; xanthine 79% vs 96%) [15].

Using this procedure, we analyzed four urine samples from four patients with LNS who were treated with allopurinol. Total ion current (TIC) and mass chromatograms of the metabolites in the urine of a patient with LNS (case 1) are shown in Figs. 2 and 3. The dominant peaks of hypoxanthine, xanthine, and oxypurinol, derived from allopurinol, were detected in all cases. The urinary concentrations of five purines and two pyrimidines are shown in Table 2. The concentrations of hypoxanthine were greater than 387 mmol/mol creatinine, and the concentrations of xanthine were greater than 77 mmol/mol creatinine in patients with LNS. The concentrations of urate were lower than 421 mmol/mol creatinine and of orotate were greater than 5 mmol/mol creatinine as a result of the allopurinol treatment.

We expressed the value of the deviation from control levels of the indicators as the abnormality *n*, the number of standard deviations above the normal mean. The abnormality *n* for each indicator is shown in Table 3. Compared with healthy controls, the abnormalities of hypoxanthine levels in all LNS patients were extreme, ranging from mean plus 8.4 SD to mean plus 9.0 SD. The level of xanthine was mean plus 4.0 to 6.1 SD. Guanine was increased only moderately. It is known that the activity of adenine phosphoribosyltransferase (APRT), the other purine salvage enzyme, is increased in HPRT deficiency [22]. The levels of adenine were lower in all cases of LNS as compared with healthy controls, ranging from the mean −2.8 SD to the mean −1.2 SD. These data suggest that the increase in APRT activity is reflected in the elevation of the urinary levels of adenine and that the targeting of adenine is valid in our quantitative assay procedure. Urate was below the mean or slightly above the mean, whereas orotate increased significantly. As uracil was not increased, the increase in orotate can be interpreted as being due to the use of allopurinol but not due to an increase in carbamoylphosphate. The usefulness of this novel metabonomics for the differential diagnosis of six primary hyperammonemia as well as the secondary hyperammonemia has been briefly discussed [23].

The residual HPRT activity in LNS is less than 1.5%; it ranges from 1.5 to 8% in Kelly–Seegmiller syndrome; and it exceeds 8% in gout [1]. In the latter

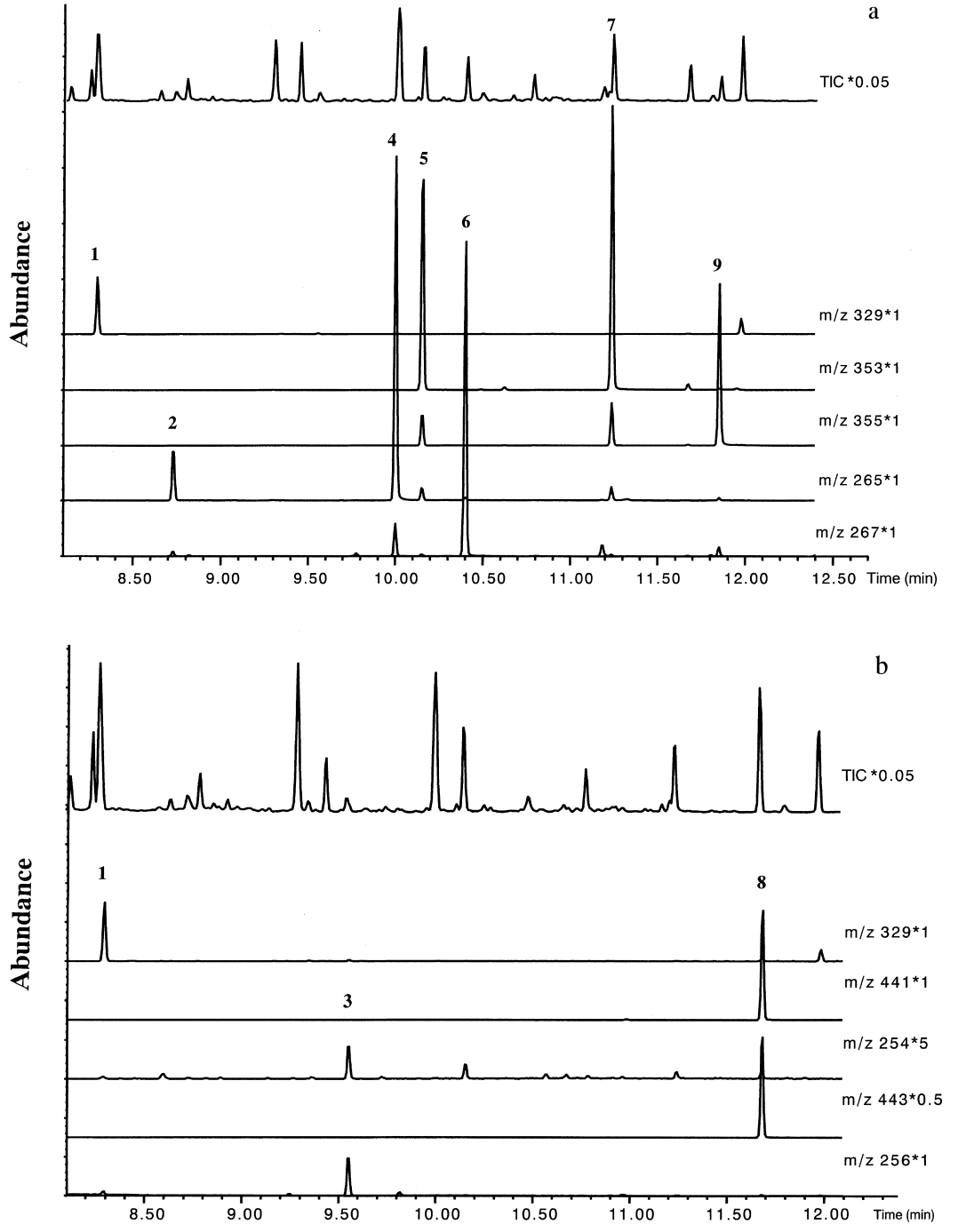

Fig. 3. The mass chromatograms of trimethylsilyl derivatives of metabolites from a patient with LNS (case 1) under allopurinol treatment. The ions targeted for quantification were m/z 329 for creatinine (1), m/z 353 for xanthine (7) and oxypurinol (5), m/z 355 for $^{13}C_1$, $^{15}N_2$-guanine (9), m/z 265 for hypoxanthine (4) and allopurinol (2), m/z 267 for $^{13}C_1$, $^{15}N_2$-adenine (6), m/z 441 for urate (8), m/z 254 for orotate (3), m/z 443 for $^{15}N_2$-urate (8), m/z 256 for $^{15}N_2$-orotate (3). The chromatograms are shown into two parts (a and b).

Table 2
Urinary metabolite levels in four patients with LNS determined in the present procedure[a]

Patients	1	2	3	4	Control (mean±1 SD[b])
Age at sampling	3 years 7 months	19 years	21 years	19 years	>4 years (n=26 or 27)
Hypoxanthine	698	488	387	497	4.01–10.5
Xanthine	1346	77.3	159	403	1.02–5.84
Adenine	1.31	0.405	0.535	0.747	0.814–1.75
Guanine	2.44	1.49	0.996	2.86	0.180–0.578
Uracil	13.3	9.03	32.0	12.5	4.44–15.1
Orotate	21.1	6.52	5.28	8.80	0.542–1.38
Urate	361	138	421	275	78.0–462

[a] The values are expressed as mmol/mol creatinine.
[b] Statistical analysis to obtain the values of mean and SD was carried out after data were $\log_{10}$-transformed as described [20].

two disorders, the clinical presentation is related to the overproduction of urate. Although the present data were obtained from only four patients who were all treated with allopurinol, any case of LNS can be chemically diagnosed by this procedure, which allows the highly sensitive and specific quantitation of the indicators. The number of standard deviations above the normal mean for hypoxanthine and xanthine when combined was above 12.9. The combined abnormality n, for three indicators, hypoxanthine, xanthine, and guanine was above 15.1. The combined abnormality n for three indicators, hypoxanthine, xanthine, and urate would exceed 10–12 in patients who were not treated with allopurinol. We believe this procedure, practical yet comprehensive from a metabolic point of view, will also be useful to follow, evaluate the treatment of, and develop more effective strategies for clinical management by direct metabolite analysis. The method would also be of use in the diagnosis of hereditary orotic aciduria, and in the differential diagnosis of patients with hyperammonemia, in whom the excretion of orotic acid serves to distinguish patients with OTC deficiency from those with CPS deficiency or other primary hyperammonemia.

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Table 3
Abnormality n, the number of SD above the mean of controls, in four patients with LNS

Patients	1	2	3	4
Age at sampling	3 years 7 months	19 years	21 years	19 years
Control age group	1–4 years	>4 years	>4 years	>4 years
Hypoxanthine	8.6	8.9	8.4	9.0
Xanthine	6.1	4.0	4.8	5.9
Adenine	−2.1	−2.8	−2.1	−1.2
Guanine	2.1	2.6	1.9	3.7
Uracil	0.30	0.16	2.2	0.69
Orotate	5.7	4.3	3.9	5.0
Urate	0.013	−0.33	0.90	0.43

Statistical analysis was carried out after data were $\log_{10}$-transformed as described [20]. The values were derived from the concentrations shown in Table 2. The values for case 1 were calculated by the data for 1–4 years control (n=50), and those of cases 2–4 were calculated by the data for >4 years control (n=27).

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