ELSEVIER

Available online at www.sciencedirect.com
SCIENCE DIRECT®

Journal of Chromatography B, 792 (2003) 89–97

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Monitoring method for pre- and post-liver transplantation in patients with primary hyperoxaluria type I

Yoshito Inoue[a,*], Hiroaki Masuyama[b], Hiromichi Ikawa[b], Hiroshi Mitsubuchi[c], Tomiko Kuhara[a]

[a]Division of Human Genetics, Medical Research Institute, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 9200293, Japan

[b]Department of Pediatric Surgery, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 9200293, Japan

[c]Department of Pediatrics, Kumamoto University School of Medicine, 1-1-1 Honjou, Kumamoto City, Kumamoto 8600811, Japan

Received 8 November 2002; received in revised form 6 February 2003; accepted 17 February 2003

Abstract

Differential diagnosis of primary hyperoxaluria type I (PH1) may become difficult once end-stage renal failure and anuria have occurred. Here we describe a rapid and sensitive method to simultaneously quantify glycolate and oxalate in plasma using a stable isotope dilution and gas chromatography–mass spectrometry. The results are provided within 2 h. The linearity of the method was validated up to 200 μmol/l of these compounds and the inter-assay precision for glycolate and oxalate was 2.4 and 2.6%, respectively ($n=5$), when the control plasma was spiked with 50 μmol/l of glycolate and oxalate. For healthy subjects, 1.0 ml of plasma was required and 0.1 ml for the PH1 patients. Using this method, plasma levels in non-PH1 patients under hemodialysis and in healthy subjects were determined. This method proved to be useful when used for differential diagnosis of PH1 and for monitoring the plasma levels of glycolate and oxalate in two PH1 patients before and after dialysis and liver transplantation. Plasma glycolate in these patients was dramatically decreased after liver transplantation, but plasma oxalate decreased more slowly due to remobilization of the calcium oxalate stores deposited throughout the body.
© 2003 Elsevier Science B.V. All rights reserved.

Keywords: Primary hyperoxaluria type I; Liver transplantation; Glycolate; Oxalate

1. Introduction

Primary hyperoxaluria type I (PH1) is an autosomal recessive metabolic disorder caused by a deficiency of the liver-specific peroxisomal enzyme alanine:glyoxylate aminotransferase (AGT) [1]. The accumulation of glyoxylate results in elevated levels of glycolate and oxalate. Although the key metabolite is glycolate rather than oxalate, these two metabolites in urine or blood are thus indicators for the chemical diagnosis of PH1. Hepatic overproduction of oxalate leads to its deposition in various organ systems, and calcium oxalate deposits in the kidney lead to nephrocalcinosis and progressive renal failure. Once end-stage renal failure (ESRF) and anuria have occurred, chemical diagnosis of PH1 may become difficult. Under these conditions, blood

*Corresponding author. Tel.: +81-76-286-2211x3602; fax: +81-76-286-2312.

E-mail address: yosh@kanazawa-med.ac.jp (Y. Inoue).

1570-0232/03/$ – see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S1570-0232(03)00278-2

levels of glycolate and oxalate need to be measured for chemical diagnosis.

Although various procedures to measure glycolate and oxalate have been proposed, each method has some disadvantages [2–11]. The determination of oxalate in plasma requires complex sample manipulation to prevent spontaneous oxalate generation [2,8,9,11]. Radioisotope-labeled oxalate dilution methods require the handling of radioactive materials [8]. Radioactive material is also required to test the amount of recovery in the ion chromatographic method [2,4]. In most previous reports, plasma glycolate and oxalate have been measured using separate preparations and/or measurement techniques [4,6,10,11]. In this study, we have established a stable isotope dilution and selected ion monitoring mode (SIM) gas chromatography–mass spectrometry (GC–MS) method to measure plasma glycolate and oxalate simultaneously. We also applied this method to monitor plasma glycolate and oxalate in PH1 patients, hemodialysis (HD) patients, and healthy subjects.

The combined liver (LTx)-kidney (KTx) transplant is thought to be the most suitable treatment for PH1 in Europe [12,13] and the United States [14]. However, it is very difficult to perform a simultaneous LTx/KTx in Japan for various reasons, including organ availability, cultural attitudes about organ donation, and politics. Recently in Japan a sequential LTx/KTx was attempted for two patients with PH1. If the LTx is followed by KTx while the oxalate level is still high, the kidney graft may fail. Monitoring not only glycolate but also oxalate may be thus important in the decision as to when to perform the KTx. We therefore monitored these cases with PH1 before and after living-related-liver-transplantation (LRLT).

2. Materials and methods

2.1. Subjects

Patient 1 with PH1 was a 30-year-old man who had developed ESRF. He had no symptoms until hematuria occurred at the age of 23. When he was 26 years old, he was diagnosed with renal failure and uremia, and HD was started. Joint pain occurred when he was 29 years old, and bone marrow puncture revealed hyperoxaluria. Despite having HD four times a week, renal function deteriorated and neuropathy and severe osteodystrophy developed. When he was 30 years old, he was diagnosed with PH1 when increased plasma glycolate and oxalate were detected by GC–MS at Kanazawa Medical University. He was admitted to receive an LRLT in the same university hospital, and his plasma levels of the indicators were monitored before and after the LTx.

Patient 2 with PH1 was a 15-year-old boy who had ESRF. He was born the third child of healthy consanguineous Japanese parents. When this patient was 2 years old, he and his elder brother, who had progressive bilateral urolithiasis and nephrocalcinosis, were suspected of having hyperoxaluria. A diagnosis of PH1 was established by documenting increased urinary oxalate and glycolate, but not L-glycerate, by GC–MS at Kurume University. When patient 2 was 12 years old, peritoneal dialysis was started. At this time, he was admitted to the Kumamoto University Hospital to receive an LRLT. Since the LTx, his health has been maintained by HD.

Patient 3 with PH1, the elder brother of patient 2, was 19 years old and also had ESRF.

Two patients with oxalosis-unrelated renal failure served as controls for dialysis treatment. Samples from uremic patients were drawn from their arteriovenous fistula prior to and at the end of a dialysis session.

The two living-relative donors were the father of patient 1, and the mother of patient 2.

A total of eight healthy subjects (three males and five females, aged 34.6±10.0 years) were enrolled in the study as healthy controls. Blood samples for glycolate and oxalate determination were taken from the healthy subjects and donors when they were fasting.

2.2. Chemicals

[1,2-$^{13}C_2$]Oxalate ([$^{13}C_2$]OXA, ^{13}C, 99%) was from Cambridge Isotope Laboratories (Andover, MA). [1,2-$^{13}C_2$]Glycolate ([$^{13}C_2$]GLC, ^{13}C, 99%+) was from ISOTEC (Miamisburg, OH). Trimethylchlorosilane (TMCS) was from Tokyo Kasei Kogyo (Tokyo, Japan). Ethyl acetate was from Sigma

Aldrich (Tokyo, Japan). *N*,*O*-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and other reagents were from Wako (Osaka, Japan).

2.3. Preparation and GC–MS analysis

All blood samples were drawn into cooled heparinized tubes and were immediately centrifuged at 4 °C for 10 min at 1800 *g*. Concentrated hydrochloric acid was then added to the cold plasma (40 μl per 1 ml) and the tube was vigorously vortexed. Plasma samples were stored frozen at −20 °C until analysis.

Solvent extraction with ethyl acetate and trimethylsilylation was as described previously [7]. $[^{13}C_2]$OXA and $[^{13}C_2]$GLC, the internal standards (I.S.), were added to 0.1 or 1.0 ml of plasma, to obtain a concentration of 10–50 μ*M*.

An aliquot (2 μl) of the trimethylsilyl (TMS) derivatized sample was injected into a Hewlett-Packard model 6890/5973 gas chromatography–mass selective detector equipped with a fused-silica capillary column (DB-5MS, 0.25 μm×0.25 mm×30 m; J&W Scientific, Folsom, CA, USA) using an automatic injector with a split ratio of 30:1. The column temperature was programmed to increase from 60 to 170 °C at a rate of 17 °C/min. SIM was used for the quantitation of lower levels of glycolate and oxalate (dwell times 80 ms), and mass chromatography for the quantitation of higher levels, m/z 50 to m/z 350, 4.72 scan/s.

2.4. Quantitation

The mass spectra of 2TMS derivatives of glycolate, $[^{13}C_2]$GLC, oxalate, and $[^{13}C_2]$OXA are shown in Fig. 1. Quantitation was performed by SIM. The ions at m/z 190 and m/z 191 were used for oxalate and its I.S., and the ions at m/z 205 and m/z 207 for glycolate and its I.S. A SIM chromatogram of plasma oxalate from a healthy control is shown in Fig. 2. An unknown peak (X) appeared at the retention times of interest on the ion chromatogram of m/z 219. Therefore, the ion pair m/z 190 and m/z 191 was used for the quantitation. A correction was made for the quantitation of glycolate and oxalate, because endogenous (due to natural abundance) and labeled compounds (due to the presence of unlabelled compounds) both contribute significantly to the other ions.

To determine the recovery of glycolate and oxalate extraction and to validate the linearity of the method, $[^{13}C_2]$OXA and $[^{13}C_2]$GLC were added to pooled plasma in quantities sufficient to obtain glycolate and oxalate enrichment varying from 0 to 200 μmol/l, and analyzed.

3. Results

3.1. Accuracy and precision of the method

The qualitative values of both glycolate and oxalate, i.e. the corrected ion ratios, were linear up to 200 μmol/l. The equations for the regression lines for the total glycolate and oxalate found in pooled plasma (y) versus the glycolate and oxalate added to the plasma (x) were $y = 1.035x + 4.002$ for glycolate and $y = 0.999x + 3.821$ for oxalate; the correlation coefficients were 0.9999 and 0.9996, respectively. The extraction recoveries of glycolate and oxalate, when pooled plasma was spiked with 4–200 μmol/l of both, varied from 83 to 107% and 94 to 104%, respectively (average±SD: both 99±4.6%). The intra- and inter-assay precision for the determination of glycolate and oxalate in plasma are shown in Table 1.

3.2. Clinical application

The specimens of PH1 patients taken prior to the LTx were measured by GC–MS using scan mode, but other specimens could not be measured in this way. Mass chromatograms of target ions of glycolate and oxalate extracted from a plasma sample of PH1 patient 1 are shown in Fig. 3. Values from specimens of PH1 patients and non-PH1 uremic patients, and fasting samples from the donors and the healthy subjects are listed in Table 2. The glycolate and oxalate plasma levels of PH1 patients 1 and 2 were measured between the 11th day before and the 33rd day after the LTx, and the 57th day before and the 261st day after the LTx, respectively. Plasma glycolate was enormously increased in the samples from the PH1 patients obtained prior to the LTx, whereas it was only mildly above control values in the other

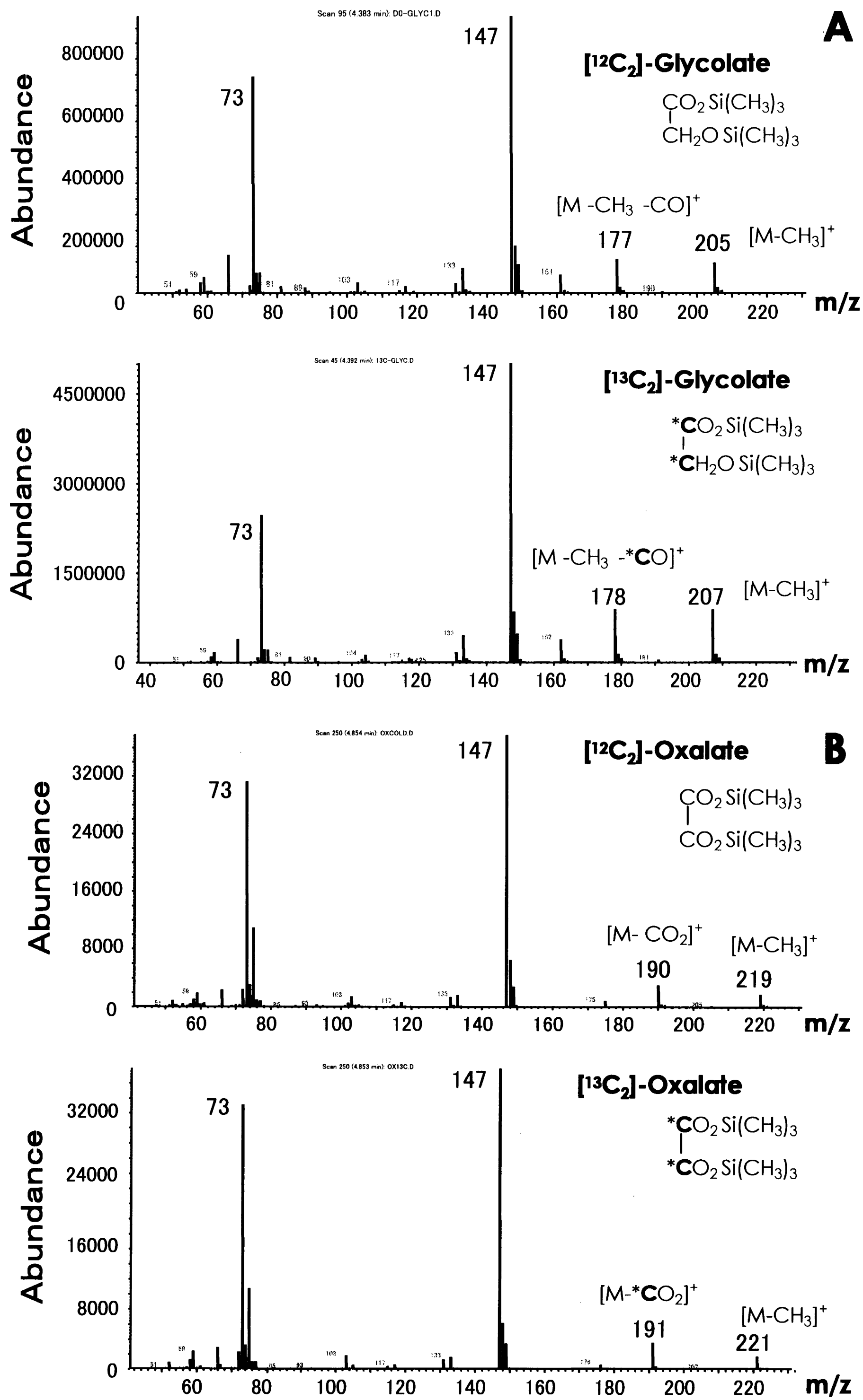

Fig. 1. Mass spectra of 2TMS derivatives of glycolate and [1,2-$^{13}C_2$]glycolate (A), and oxalate and [1,2-$^{13}C_2$]oxalate (B).

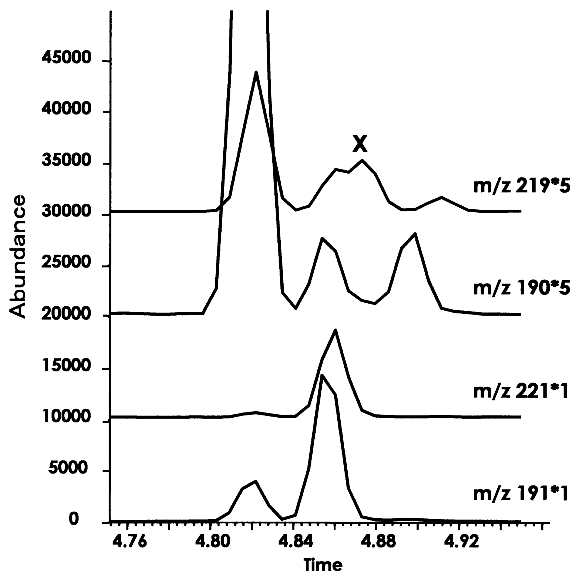

Fig. 2. SIM chromatogram of plasma oxalate from a healthy subject (2 μ*M*). The target ions at 4.85 min were *m*/*z* 190 and *m*/*z* 219 for oxalate 2TMS derivatives and *m*/*z* 191 and *m*/*z* 221 for [1,2-$^{13}C_2$]oxalate 2TMS derivatives, an internal standard. An unknown peak (X) appeared at the retention times of interest on the ion chromatogram of *m*/*z* 219.

uremic patients. All uremic patients exhibited significantly higher plasma oxalate levels compared with the healthy subjects.

To evaluate the impact of the LTx and dialysis, the plasma levels of glycolate and oxalate in two PH1 patients taken pre- and post-LTx, and pre- and post-dialysis are compared in Table 2. The levels of plasma glycolate in patient 1 and patient 2 were dramatically decreased from 772 to 60 μ*M* (one-thirteenth) and from 902 to 44 μ*M* (one-twentieth), respectively, while those of oxalate were notably decreased but less markedly than those of glycolate from 138 to 57 μ*M* (two-fifths) and from 122 to 63 μ*M* (a half), respectively. Furthermore, the initial measurement values of post-LTx oxalate of these patients were almost equal to the post-dialysis values that were obtained prior to LTx.

The average decrement rates owing to dialysis in the PH1 patients for plasma glycolate were 24±4.9% (range 19–28%) prior to LTx and 62±10.0% (range 53–73%) post-LTx, while for plasma oxalate they were 39±7.8% (range 31–46%) prior to LTx and 50±8.5% (range 40–61%) post-LTx.

4. Discussion

We were able to prepare and measure plasma glycolate and oxalate simultaneously using the stable isotope dilution GC–MS method. With the present

Table 1
Intra- and inter-assay precision for determination of oxalate and glycolate in plasma

Intra-assay (nmol/0.5 ml)	Measurements (*n*)	Mean (nmol/0.5 ml)	SD (nmol/0.5 ml)	C.V. (%)
Oxalate added				
2	4	4.4	0.4	9.8
5	4	6.9	0.6	8.0
50	4	72.4	1.6	2.2
Glycolate added				
2	4	5.1	0.4	8.2
5	4	8.5	0.3	3.3
50	4	58.6	1.8	3.1
Inter-assay (nmol/0.5 ml)	Preparations (*n*)	Mean (nmol/0.5 ml)	SD (nmol/0.5 ml)	C.V. (%)
Oxalate added				
50	5	73	1.9	2.6
Glycolate added				
50	5	59.6	1.4	2.4

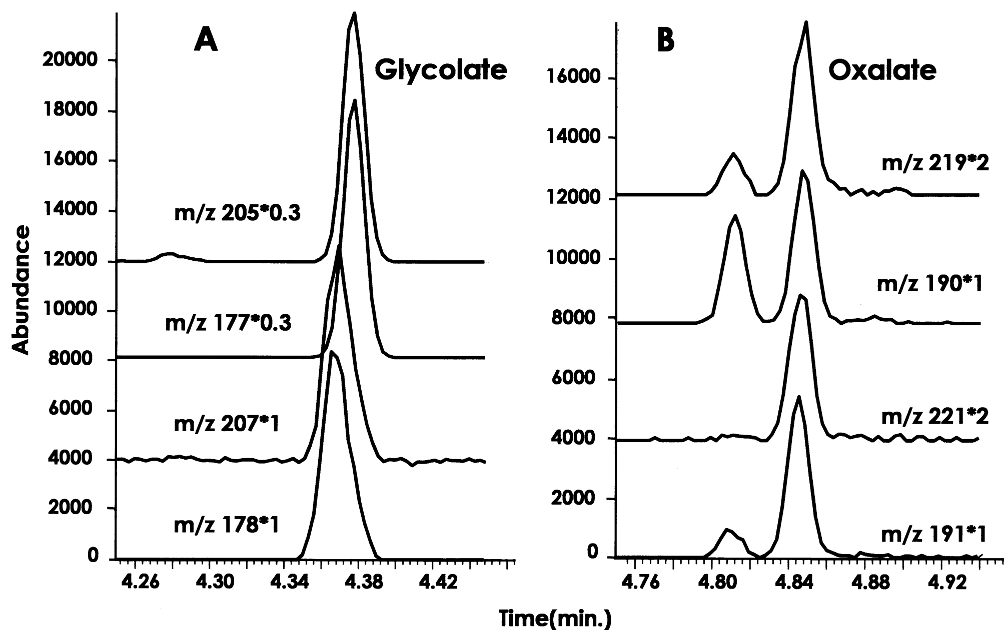

Fig. 3. Mass chromatograms of target ions of glycolate (A) and oxalate (B) extracted from a plasma sample of PH1 patient 1. The target ions at 4.38 min were m/z 205 and m/z 177 for glycolate 2TMS derivatives and m/z 207 and m/z 178 for [1,2-$^{13}C_2$]glycolate 2TMS derivatives, an internal standard. The target ions at 4.85 min were m/z 190 and m/z 219 for oxalate 2TMS derivatives and m/z 191 and m/z 221 for [1,2-$^{13}C_2$]oxalate 2TMS derivatives, an internal standard.

procedure, results of plasma glycolate and oxalate are provided within 2 h. In almost all previous reports, plasma glycolate and oxalate were measured using separate preparation and/or measurement techniques (Table 3). These methods require many more time-consuming manipulations than the present procedure. The preparation step for the ion chromatography for oxalate [5] was comparatively simple. However, one had to measure glycolate in another chromatographic procedure [4,16]. With enzyme-spectrophotometric liquid chromatography [2,3,11], the preparation step itself requires more than 2 h.

The normal values of plasma glycolate and oxalate in healthy subjects, which have been reported in different studies using various methods, are shown in Table 3. Many authors have pointed out that spontaneous oxalate generation occurs in serum soon after blood is drawn, due both to ascorbate degradation and enzyme-promoted reactions [7–9,15]. Strong acidification of plasma just after sample collection has been suggested to prevent the in vitro ascorbate degradation. Indeed, working at a pH close to 1, we obtained for healthy subjects oxalate concentrations averaging 2.8±1.1 μM (mean±SD, n=8). We also obtained glycolate concentration values for healthy subjects using the same measurement procedure averaging 7.5±2.4 μM (mean±SD, n=8). The glycolate level we obtained was very close to the levels measured by other authors (Table 3).

Our simultaneous measurement of glycolate and oxalate provided evidence of hepatic AGT activity and oxalate calcium accumulation in PH1 patients. Our method confirmed a dramatic decrease in plasma glycolate levels just after LTx, indicating that the liver transplantation was successful. Blood oxalate values, however, changed little in comparison with glycolate. The effect of the LTx was long-lasting in both patients. The activity of AGT in the liver graft was maintained well. Except for the oxalate value on the 60th day after LTx for patient 2, the levels of glycolate and oxalate following LTx were always lower than the levels at the end of dialysis prior to

Table 2
Plasma levels of oxalate and glycolate in primary hyperoxaluria type I (PH1) patients, uremic patients, donors, and healthy subjects

Patient	Days from transplant	Transplant dialysis	Plasma levels (μ*M*)		Decrement rate (%)	
			Oxalate	Glycolate	Oxalate	Glycolate
Patient 1	−11	Pre-D[a]	145	690		
	−6	Pre-D	113	419		
	−6	Post-D[b]	35	78	31	19
	−4	Pre-D	138	772		
	−4	Post-D	56	199	41	26
	1	Post-LTx[c]	57	60	41	8
	2	Pre-D	38	23		
	7	Pre-D	35	13		
	10	Pre-D	33	9		
Patient 2	−5	Pre-D	138	1395		
	−5	Post-D	64	392	46	28
	−1	Pre-D	122	902		
	0	Post-LTx	63	44	52	5
	7	Pre-D	61	14		
	7	Post-D	37	9	61	61
	112	Pre-D	85	11		
	112	Post-D	34	8	40	73
	261	Pre-D	87	14		
Patient 3		Pre-D	218	723		
Uremic patient 1		Pre-D	62	14		
Uremic patient 2		Pre-D	50	14		
Donor 1			3	8		
Donor 2			5	3		
Healthy subjects ($n=8$)			2.8±1.1	7.5±2.4		

[a] Pre-dialysis.
[b] Post-dialysis.
[c] Post-liver transplant.

LTx. These results show that the living-relative LTx was an effective treatment for these PH1 patients.

The glycolate and oxalate decrement rates of our patients pre- and post-dialysis prior to LTx were close to the values of the PH1 patients reported by previous authors who used other methods [3,6,11]. Our study also found that the glycolate and oxalate plasma levels at the beginning of dialysis returned to almost the same levels by the time of the next scheduled dialysis. The decrement rates of glycolate owing to dialysis of PH1 patient 2 following LTx were slightly greater than they had been before LTx. This effect may have been because the glycolate values were already lower before dialysis than they had been prior to LTx. Following LTx, the glycolate and oxalate levels of the PH1 patients were similar to those of uremic patients. It seems that these levels will not change if the patients excrete oxalate only by dialysis. When we began this study, it had been planned to perform a KTx when the level of oxalate of the PH1 patients fell to a significantly lower level. At the moment, LTx remains the only long-term cure for pyridoxine-resistant PH1. The relative merits and long-term outcome of LTx alone, simultaneous and sequential LTx/KTx [14], and KTx alone [13] in PH1 have been debated extensively [1]. It appears that it is still difficult to judge the best time to perform KTx when the patients have renal failure. Early chemical diagnosis is again very important. We will draw on the present experience in planning the

Table 3
Reported concentrations of blood oxalate and glycolate in PH1 patients and normal subjects

Year, reference	Normal subjects			PH1 patients			Method		Specimen
	Oxalate	Glycolate	*n*	Oxalate	Glycolate	*n*	Oxalate	Glycolate	
1988 [8]	1.25±0.47		23				Double enzymic LC		Plasma
1989 [5]	21.02±8.3		15				IC		Serum
1990 [15]	6.75±2.62		18				Radioisotopic dilution IC		Plasma
1991 [2]	2.5±0.7		21				Enzyme-LC		Plasma
1991 [3]		8.00±2.04	14		153.8±96.2 (91.4–297.0)	4		Enzyme-LC	Plasma
1991 [11]	6.1±1.6	7.8±1.7	12	162.0±24.0	1536±1062	4	IC	Enzyme-LC	Plasma
1992 [6]	2.4±0.6	7.9±2.4	19[b]	59.5±72.0	199±147	8	IC	Enzyme-LC	Plasma
1993 [4]									
	2.0±0.2[a]	4.7±0.4[a]	14[c]				IC	IC	Plasma
	1.8±0.1[a]	4.4±0.3[a]	25[d]						
1982 [7]	2.8±1.1		22	132		1	GC (I.S.: malate)		Plasma
1988 [9]	4.70±1.44		11				GC–MS SIM (I.S.: [$^{13}C_2$]oxalate)		Plasma
1996 [10]	1.53	UD					GC–MS (I.S.: 3-phenylbutyrate)		Plasma
Present procedure	2.8±1.1 (1.1–5.0)	7.5±2.4 (2.9–11.0)	8	160±50 (113–218)	833±278 (419–1395)	3	GC–MS SIM (I.S.: [$^{13}C_2$]oxalate, [$^{13}C_2$]glycolate)		Plasma

The values of oxalate and glycolate are shown as mean±SD, unless otherwise indicated. UD, undetectable.
[a] Mean±S.E.
[b] Age 32.5±12 years.
[c] Male (age 40±10 years).
[d] Female (age: 39±10 years).

treatment of future PH1 patients. Glycolate is an indicator in LTx, and oxalate will become an indicator in KTx. We believe that our method can be applied for both LTx and KTx.

We conclude that this stable isotope dilution GC–MS procedure is specific and suitable for the differential chemical diagnosis of hyperoxaluria and monitoring plasma glycolate and oxalate levels in patients with ESRF due to PH1 who want receive a sequential LTx/KTx. This procedure will be used to obtain evidence of hepatic AGT activity and a patient's calcium oxalate accumulation.

Acknowledgements

This study was partly supported by a grant for Child Health and Development (13C-4) from the Ministry of Health, Labor and Welfare of Japan.

References

[1] C.J. Danpure, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, 8th ed, McGraw-Hill, New York, 2001, p. 3323, Chapter 133.
[2] D.M. Wilson, R.R. Liedtke, Clin. Chem. 37 (1991) 1229.
[3] M. Petrarulo, M. Marangella, F. Linari, Clin. Chim. Acta 196 (1991) 17.
[4] L. Hagen, V.R. Walker, R.A. Sutton, Clin. Chem. 39 (1993) 134.
[5] L. Poloti, R. Chiaraluce, V. Consalvi, N. Cerulli, R. Scandurra, Clin. Chim. Acta 184 (1989) 155.
[6] M. Marangella, M. Petrarulo, C. Vitale, D. Cosseddu, F. Linari, J. Urol. 148 (1992) 986.
[7] B.G. Wolthers, M. Hayer, Clin. Chim. Acta 120 (1982) 87.
[8] J. Costello, D.M. Landwehr, Clin. Chem. 34 (1988) 1540.
[9] N.C. France, T.K. McGhie, M.R. Wallace, J. Chromatogr. B 433 (1988) 1.
[10] K.J. Van Acker, F.J. Eyskens, M.F. Espeel, R.J.A. Wanders, C. Dekker, I.O. Kerckaert, F. Roels, Kidney Int. 50 (1996) 1747.

[11] M. Marangella, M. Petrarulo, O. Bianco, C. Vitale, P. Finocchiaro, F. Linari, Kidney Int. 39 (1991) 149.
[12] M.F. Gagnadoux, F. Lacaille, P. Niaudet, Y. Revillon, P. Jouvet, D. Jan, G. Guest, M. Charbit, M. Broyer, Pediatr. Nephrol. 16 (2001) 946.
[13] C.G. Monico, D.S. Milliner, Liver Transpl. 7 (2001) 954.
[14] J. Rogers, J. Bueno, R. Shapiro, V. Scantlebury, G. Mazariegos, J. Fung, J. Reyes, Transplantation 72 (2001) 1666.
[15] M. Petrarulo, M. Marangella, Clin. Chim. Acta 189 (1990) 249.
[16] T.R. Wandzilak, L.E. Hagen, H. Hughes, R.A.L. Sutton, L.H. Smith, H.E. Williams, Kidney Int. 39 (1991) 765.