ELSEVIER

Journal of Chromatography B, 731 (1999) 141–147

JOURNAL OF CHROMATOGRAPHY B

Pilot study of gas chromatographic–mass spectrometric screening of newborn urine for inborn errors of metabolism after treatment with urease

Tomiko Kuhara[a,*], Toshihiro Shinka[a], Yoshito Inoue[a], Morimasa Ohse[a], Xia Zhen-wei[a], Ichiro Yoshida[b], Takahiro Inokuchi[b], Seiji Yamaguchi[c], Masaki Takayanagi[d], Isamu Matsumoto[a]

[a]*Division of Human Genetics, Medical Research Institute, Kanazawa Medical University, 1-1 Daigaku, Uchinada-machi, Kahoku-gun, Ishikawa 920-0293, Japan*

[b]*Research Institute of Medical Mass Spectrometry, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan*

[c]*Department of Pediatrics, Shimane Medical University, 89-1 En-ya-cho, Izumo, Shimane 693-8501, Japan*

[d]*Division of Metabolism, Department of Pediatrics, Chiba Children's Hospital, 579-1 Heta-cho, Midori-ku, Chiba 266-0007, Japan*

Abstract

Gas chromatographic–mass spectrometric (GC–MS) techniques for urinary organic acid profiling have been applied to high-risk screening for a wide range of diseases, mainly for inborn errors of metabolism (IEM), rather than to low-risk screening or mass screening. Using a simplified procedure with urease-pretreatment and the GC–MS technique, which allows simultaneous determination of organic acids, amino acids, sugars and sugar acids, we performed a pilot study of the application of this procedure to neonatal urine screening for 22 IEM. Out of 16 246 newborns screened, 11 cases of metabolic disorders were chemically diagnosed: two each of methylmalonic aciduria and glyceroluria, four of cystinuria, and one each of Hartnup disease, citrullinemia and α-aminoadipic aciduria/α-ketoadipic aciduria. The incidence of IEM was thus one per 1477, which was higher than the one per 3000 obtained in the USA in a study targeting amino acids and acylcarnitines in newborn blood spots by tandem mass spectrometry. Also, 227 cases were found to have transient metabolic abnormalities: 108 cases with neonatal tyrosinuria, 99 cases with neonatal galactosuria, and 20 cases with other transient metabolic disorders. Two hundred and thirty-eight cases out of 16 246 neonates (approximately 1/68) were thus diagnosed using this procedure as having either persistent or transient metabolic abnormalities.

Keywords: Urease

1. Introduction

Urinary water-soluble organic compounds are the end products or intermediates of the catabolism of amino acids, sugars, lipids and many other endogen-

*Corresponding author. Tel.: +81-76-286-2464; fax: +81-76-286-3358.

E-mail address: kuhara@kanazawa-med.ac.jp (T. Kuhara)

0378-4347/99/$ – see front matter
PII: S0378-4347(99)00205-4

ous compounds. Inborn errors of metabolism (IEM), most of which cause severe diseases, can be detected based on the abnormal excretion patterns of these compounds [1,2]. In many of these disorders, pathological consequences can be prevented by appropriate intervention or early treatment of neonates or infants. However, delayed treatment drastically lowers therapeutic effects. For instance, if the treatment for phenylketonuria is started later than 18 days of life [3], irreversible mental retardation results. Early treatment is thus critical for prevention of mental retardation or other irreversible conditions derived from IEM, and early diagnosis is indispensable for achieving timely treatment.

Hence, practical, sufficiently specific, and cost-effective neonatal screening programs for the prevention or significant reduction of clinical symptoms such as mental retardation are currently conducted in most developed countries. All neonates born in Japan are screened for four IEM, congenital hypothyroidism, and congenital adrenal hyperplasia, and as part of this program, 6-month-old infants are also screened for neuroblastoma. Both examination rates are nearly 100%. The IEM currently targeted in Japan include phenylketonuria, maple syrup urine disease, homocystinuria and galactosemia. The program relies primarily on simple bacterial inhibition assays with dried blood spots on filter paper ('the Guthrie tests') [4].

Since 1975, we have been conducting experiments designed to improve chemical diagnostic procedures by the application of GC–MS analysis of urinary metabolites for IEM, and have conducted high-risk screening at the request of many medical institutions in Japan and other foreign countries [5,6]. As the result of our studies and services, a joint pilot study on the feasibility of neonatal mass screening using GC–MS in Japan was initiated in 1995 at four institutions (Kanazawa Medical University, Kurume University Medical School, Shimane Medical University, and Chiba Prefectural Children's Hospital) in cooperation with Yokogawa Analytical Systems Inc., Shimadzu Seisakusho Ltd., and JEOL Ltd.

We developed a simplified procedure combining sample pretreatment with urease and the GC–MS technique, as previously described [7], which enables accurate chemical diagnoses of various IEM. In this paper, we report a pilot study carried out at the four institutions and discuss the usefulness of this procedure.

2. Experimental

Urine specimens from neonates, taken on day 5–7 with informed consent of parents when blood was also taken for the Guthrie test, were absorbed onto filter paper and dried with air, and then sent to the institutions, where they were eluted with water. Urine sample preparation and GC–MS analysis were performed as follows, according to the method described [7]. The eluate (0.1 ml) from dried urine filter paper was incubated with urease type C-3 from Sigma (St. Louis, MO, USA) for 10 min to remove urea. Internal standards of 2,2-dimethylsuccinate, heptadecanoate, 3-hydroxymyristate, d_3-creatinine (100 nmol), and deuterium-labeled amino acids each 50 nmol were added. After deproteinization with ethanol (final concentration 90%), centrifugation to remove any precipitate, and evaporation to dryness, the residue was trimethylsilylated with 0.1 ml of BSTFA and TMCS (10:1) for 30 min at 80°C. Aliquots of 0.5–2 μl of the derivatized extract were injected into a GC–MS apparatus using an automatic injection mode with a split ratio of 1:10–1:50. The conditions for GC–MS were the same as described previously [7].

3. Results and discussion

The initial 22 target diseases were methylmalonic acidemia, propionic acidemia, isovaleric acidemia, glutaric aciduria type I, β-methylcrotonylglycinuria, β-hydroxy-β-methylglutaric aciduria, alkaptonuria, multiple carboxylase deficiency, β-ketothiolase deficiency, nonketotic hyperglycinemia, glutaric aciduria type II, ornithine transcarbamylase deficiency (strictly speaking, four urea cycle disorders can be screened; for their differential diagnosis, the quantification of arginine and citrulline are required, while in our method this is difficult), α-aminoadipic-α-ketoadipic aciduria, tyrosinemia, cystinuria, lysinuria, glyceroluria, maple syrup urine disease, galactosemia, phenylketonuria, hyperphenylalaninemia, homocystinuria, and neuroblastoma.

These diseases were targeted for reasons such as severity of illness and effectiveness of early treatment. Urine was also examined for neuroblastoma, which is not an IEM.

Chamberlin et al. [8] reported in 1987 that urine filter paper was generally more useful than blood spot filter paper, except for diseases where very hydrophobic compounds accumulate. We also made a comparison of the results of GC–MS analysis using urine filter paper with those using urine, serum or blood filter paper. As shown in Fig. 1, the urine eluted from dried filter paper gave a marked increase of methylmalonate, while the serum did not in a patient with benign methymalonic aciduria. Blood spots on filter paper from the same patient did not show any signal on tandem mass spectrometry (MS–MS) conducted by Roe and Sweetman (not published), showing the difficulty of detecting propionylcarnitine, which is the target compound for methylmalonic aciduria with blood spot. This may, however, have been due to the weakness in the block of the methylmalonate metabolism in the patient. Urine and serum from a patient with propionic acidemia were analyzed and the results are shown in Fig. 2. Methylcitrate and 3-hydroxypropionate were detected in large amounts in urine but only the former in lower amounts in the serum of the patient. Uracil and/or orotate were targeted for the deficiency of the enzymes involved in the urea cycle, except carbamoylphosphate synthase and *N*-acetylglutamate synthase. In Fig. 3, the data of urine and serum are compared for a patient with ornithine carbamoyltransferase deficiency. Based on information on the levels of citrulline and arginine obtained by conventional amino acid analysis (not GC–MS), this patient was chemically diagnosed as having ornithine carbamoyltransferase deficiency.

In the present pilot study, 16 246 newborns were examined in the four institutions. Kanazawa Medical University analyzed 5844 urine samples, and found eight abnormal cases: two of methylmalonic aciduria, one each of glyceroluria, Hartnup disease and α-aminoadipic/α-ketoadipic aciduria, and three of cystinuria; the estimated frequency was 1/731. Kurume University analyzed 6910 samples, and found three abnormal cases: one each of glyceroluria, cystinuria and citrullinemia: the estimated frequency was 1/2303. Shimane Medical University and Chiba Children's Hospital analyzed 2406 and 1086 samples, respectively, and did not encounter

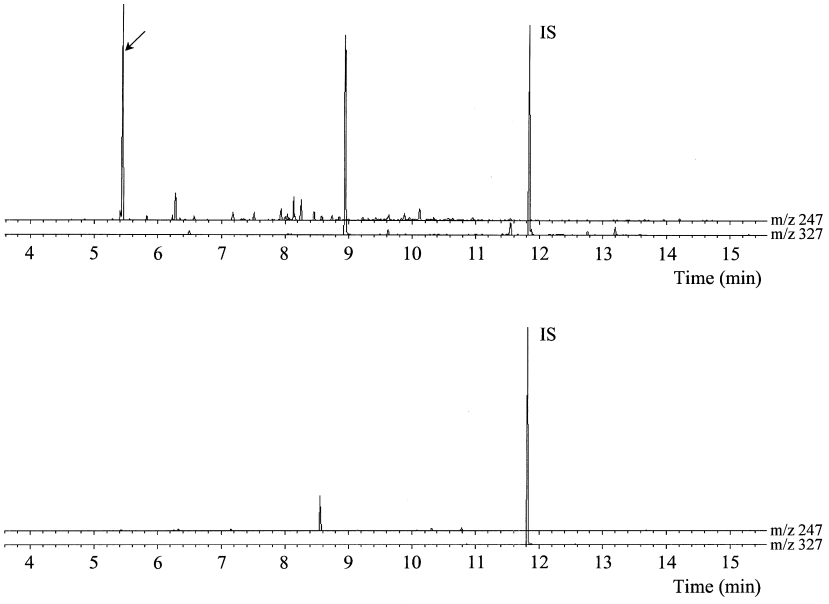

Fig. 1. Mass chromatograms of trimethylsilyl (TMS) derivatives of metabolites in urine (top) and serum (bottom) from a patient with benign form, at present, of methylmalonic acidemia. Ion of *m*/*z* 247 at 5.45 min is due to methylmalonate (di-TMS). The ion at *m*/*z* 327 at 11.8 min is due to *n*-heptadecanoate (mono-TMS) used as an internal standard: 50 nmole was spiked in 0.1 ml urine or serum.

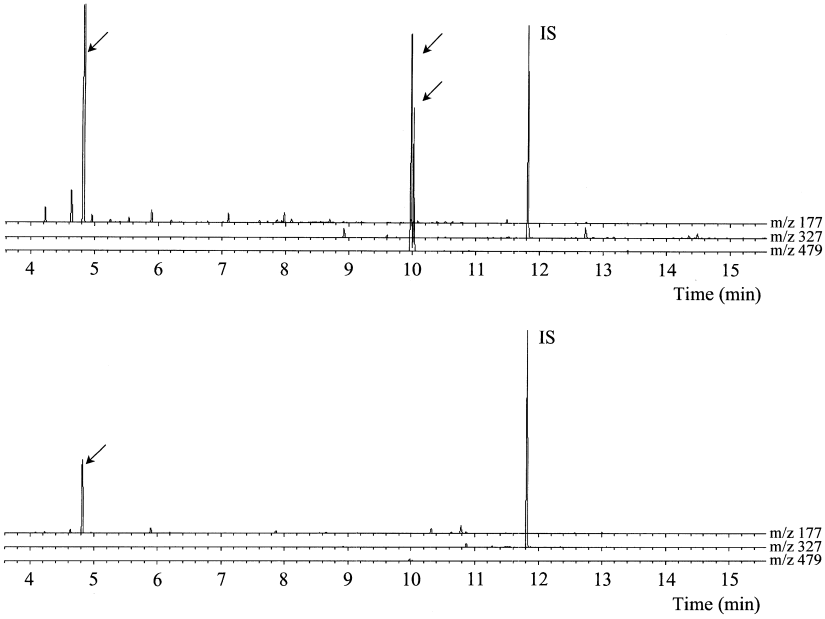

Fig. 2. Mass chromatograms of trimethylsilyl (TMS) derivatives of metabolites in urine (top) and serum (bottom) from a patient with propionic acidemia. Ion of m/z 177 at 4.8 min and that of m/z 479 at 9.98 and 10.02 are due to 3-hydroxypropionate (di-TMS) and diastereomers of methylcitrate (tetra-TMS), respectively. The ion of m/z 327 at 11.8 min is due to *n*-heptadecanoate (mono-TMS) used as an internal standard: 50 nmole was spiked in 0.1 ml of urine or serum.

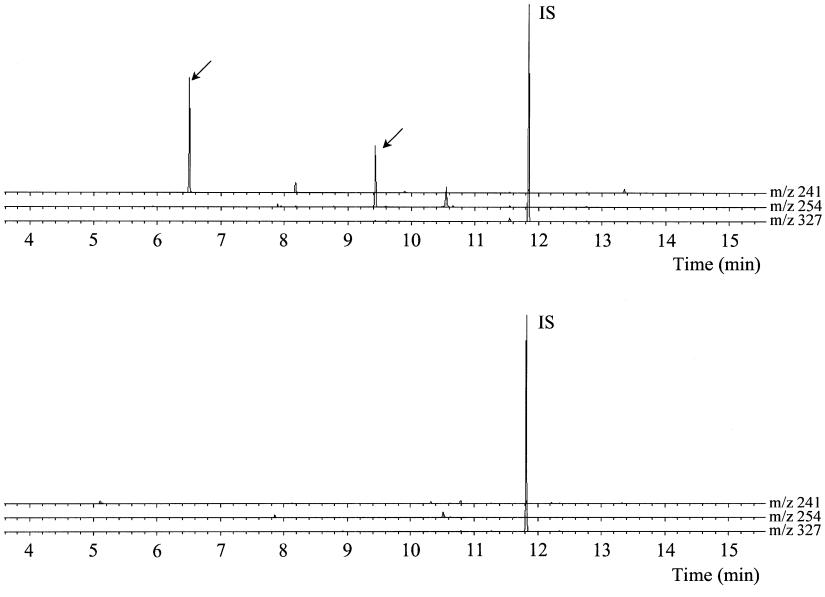

Fig. 3. Mass chromatograms of trimethylsilyl (TMS) derivatives of metabolites in urine (top) and serum (bottom) from a patient with ornithine carbamoyltransferase deficiency. Ion of m/z 241 at 6.5 min and that of m/z 254 at 9.43 min are due to uracil (di-TMS) and orotate (tri-TMS), respectively. The ion of m/z 327 at 11.8 min is due to *n*-heptadecanoate (mono-TMS) used as an internal standard: 50 nmole was spiked in 0.1 ml urine or serum.

any abnormal cases. Of 16 246 newborns examined, 11 cases were chemically diagnosed as having metabolic disorders. The incidence was one per 1477, which is higher than the one per 3000 obtained in the pilot study in Pennsylvania and North Carolina which targeted amino acids and acylcarnitines in blood spots on filter paper by tandem mass spectrometry [9]. A follow-up study of a neonate with α-aminoadipic/α-ketoadipic aciduria detected in this study has been reported [10].

As shown in Table 1, a total of 227 transient abnormalities were detected by the four groups: 91 cases in Kanazawa Medical University, 54 cases in Kurume University, 81 cases in Shimane Medical University, and one case in Chiba Children's Hospital: 108 cases with tyrosinuria, 99 cases with galactosuria, and 20 cases with other transient metabolic disorders. Two hundred and thirty-eight cases out of 16 246 neonates (approximately 1/68) were diagnosed as having either persistent or transient metabolic abnormalities. Cases of transient neonatal tyrosinemia were found at higher frequency in premature neonates than mature ones supporting the idea that the activity of *p*-hydroxyphenylpyruvate dioxygenase increases 24 h after birth. In patients with galactosemia, tyrosinemia or other diseases, the increase of target metabolites was far more marked than in those with transient neonatal forms.

For analyzing organic acids, GC–MS has been proven by several institutions in various countries to

Table 1
Cases detected as having transient metabolic abnormalities in four institutions ('95.7–'98.6)

Disease/abnormality	Cases	5 SD≤ 10 SD	10 SD≤
Kanazawa Medical University			
Transient neonatal tyrosinuria[a]	23	1	22
Transient neonatal galactosuria[b]	50	38	12
Cystinuria[c]	4	1	3
Glyceroluria[d]	4	1	3
Lactic aciduria[e]	4	2	2
Hyperphenylalaninuria[f]	2	–	2
Hyperglycinemia[g]	1	–	1
Hyperlysinuria[h]	3	2	1
Sub-total	91	45	46
Kurume University			
Transient neonatal tyrosinuria[a]	33	–	33
Transient neonatal galactosuria[b]	21	15	6
Sub-total	54	15	39
Shimane Medical University			
Transient neonatal tyrosinuria[a]	52	52	–
Transient neonatal galactosuria[b]	28	28	–
Glyceroluria[d]	1	–	1
Sub-total	81	80	1
Chiba Children's Hospital			
Glyceroluria[d]	1	–	1
Sub-total	1	–	1
Total: with transient abnormality	227		
Total: screened	16 246		
Incidence	1/72		

Abnormalities were expressed as n in means+n SD of *p*-hydroxyphenyllactate[a], galactose[b] cystine[c], glycerol[d], lactate[e], phenylalanine[f], glycine[g], lysine[h].

be the most efficient method for chemical diagnosis of IEM for high-risk individuals [1,2,5,6,8]. It is also used for the secondary screening or scrutiny of positive cases detected by current neonatal mass screening with the Guthrie test. For low-risk screening of large populations, however, only a few projects presently implement GC–MS [11]. A mass screening program for neuroblastoma at 3 weeks of age [12] in Quebec adopted GC–MS analysis with urine filter paper after organic solvent extraction under acidic conditions. This program has been further extended to the screening of acidic markers for 20 or more different metabolic conditions [11].

In 1991, Shoemaker et al. reported that urinary organic acids, amino acids and sugars can be analyzed simultaneously by GC–MS after excessive urea in the urine is degraded with urease and removed [19]. Shoemaker's procedure, however, takes several hours, needs skilled technicians, and is not very practical. We therefore simplified this method for use in multiple sample analysis [7]. As a result, rapid, practical and simultaneous analysis of amino acids, sugars, sugar alcohols and nucleic acid bases in addition to organic acids became possible. We have further improved the procedure for potential use in neonatal mass screening. We adopted a stable isotope dilution method using not only d_3-creatinine but also stable-isotope-labeled amino acids as internal standards. This new procedure takes 1 h for pretreatment of one sample, or 3 h for a batch of 30 samples, as described [7,20]. In mass screening, many specimens must be treated, and therefore a fully automatic pretreatment system is desirable. We are now developing automated sample preparation using HP Prep-Station. When the whole analytical process is fully automated in an appropriate diagnostic program in the near future, it will be possible to analyze 60 samples in a day using one GC–MS instrument, and thus to analyze about 15 000 samples annually, based on 250 workdays a year.

Our new diagnostic procedure [7] can be used to simultaneously analyze amino acids, organic acids, sugars, sugar alcohols, and nucleic acid bases [13,14]. Therefore, it is a highly comprehensive diagnostic tool for a wide range of metabolic disorders. Unlike Millington's method [15] or Rashed's method [16], our method allows accurate chemical differentiation (differential diagnosis) between methylmalonic aciduria and propionic acidemia in a single analysis. In methylmalonic aciduria, which is caused by abnormally reduced activity of methylmalonyl-CoA mutase and is the most frequently observed disorder among the organic acidemias, methylmalonate is the target, and sometimes also methylcitrate and 3-hydroxypropionate. In propionic acidemia, methylcitrate, 3-hydroxypropionate and propionylglycine are targets. Analysis of acylcarnitines and amino acids in blood spots using MS–MS is now completely controlled by computer, and the analytical time per specimen is only a few minutes [17]. These methods are truly revolutionary compared to conventional chemical diagnosis by urine GC–MS analysis [1,2], which requires time for sample pretreatment and MS measurement. Recently, more rapid and accurate methods have been developed, and the use of MS–MS for mass screening of blood spots could be an efficient method with a reasonable cost [18]. Those methods are, however, limited to narrow classes of analytes compared with our GC–MS method [7]. The other methods permit high-speed analyses, but it might be more appropriate to refer to them as tools for screening than for chemical diagnosis. For screening, the MS–MS method is quicker than our GC–MS method. However, at the level of conclusive diagnosis, our method may be quicker. This is because our method, in most cases, gives almost conclusive diagnoses of IEM [7,19,20]. For instance, in the case of MSUD, our method enables not only the determination of the branched-chain amino acids, leucine, valine, and isoleucine, using deuterium-labeled leucine as an internal standard, but also the determination of the corresponding branched-chain α-hydroxy acids. In addition, it can be tested at the same time whether metabolites derived after the oxidative decarboxylation of the branched chain α-oxo acids are increased. For galactosemia, galactose, galactitol and galactonate are also our target compounds.

We believe that this study has provided valuable information on early diagnosis and treatment of IEM. This method, technically practical yet comprehensive from the metabolic point of view, could become well established for mass screening of all age groups ranging from neonates to the elderly.

Acknowledgements

This study was supported in part by grants of the JAMW Ogyaa Donation Foundation and project research from High-Technology Center of Kanazawa Medical University (H97-P3, H98-P3). We are greatly indebted to Dr. Shoichi Sakamoto (Professor Emeritus, The University of Tokyo) for his continuing interest and encouragement. Special acknowledgement must be made to Professors Roe and Sweetman (Baylor University Medical Center), who demonstrated the absence of propionylcarnitine in blood spots from a patient with benign methylmalonic acidemia using MS–MS. We also express deep gratitude to the persons in charge at the apparatus manufacturers, and Miki Osabe for preparation of the manuscript.

References

[1] S.I. Goodman, S.P. Markey, Diagnosis of Organic Acidemias by Gas Chromatography–Mass Spectrometry, Alan R. Liss, New York, 1981.
[2] R.A. Charlmers, A.M. Lawson, Organic Acids in Man, Chapman and Hall, London, 1982.
[3] C.R. Scriver, L.E. Rosenberg, Amino Acid Metabolism and its Disorders, Vol. X in the Series of Major Problems in Clinical Pediatrics, W.B. Saunders, Philadelphia, PA, 1987, p. 290.
[4] R. Guthrie, in: T.P. Carter, A.M. Wiley (Eds.), Genetic Disease: Screening and Management, Alan R. Liss, New York, 1986, p. 319.
[5] I. Matsumoto, T. Kuhara, Mass Spectrom. Rev. 6 (1987) 77.
[6] I. Matsumoto, T. Kuhara, in: D.M. Desiderio (Ed.), Mass Spectrometry; Clinical and Biomedical Applications, Vol. 1, Plenum Press, New York, 1992, p. 259.
[7] I. Matsumoto, T. Kuhara, Mass Spectrom. Rev. 15 (1996) 43.
[8] B.A. Chamberlin, C.C. Sweeley, Clin. Chem. 33/4 (1987) 572.
[9] D.H. Chace, S.L. Hillman, D.S. Millington, S.G. Kahler, B.W. Adam, H.L. Levy, Clin. Chem. 42 (1996) 349.
[10] H. Peng, T. Shinka, Y. Inoue, H. Mitsubuchi, J. Ishimatsu, M. Yoshino, T. Kuhara, Acta Paediatr., in press.
[11] M. Tuchman, M.T. McCann, P.E. Johnson, B. Lemieux, Pediatr. Res. 30 (1991) 3.
[12] M. Tuchman, B. Lemieux, C. Auray-Blais, L.L. Robinson, R. Giguere, M.T. MacCann, W.G. Woods, Pediatr. 85 (5) (1990) 765.
[13] C. Ning, T. Kuhara, I. Matsumoto, Clin. Chim. Acta 247 (1996) 197.
[14] K. Suzuki, S. Kobayashi, K. Kawamura, T. Kuhara, R. Tsugawa, Int. J. Urol. 4 (1997) 304.
[15] D.S. Millington, N. Kodo, D.L. Norwood, C.R. Roe, J. Inherit. Metab. Dis. 13 (1990) 321.
[16] M.S. Rashed, P.T. Ozand, M.P. Bucknall, D. Little, Pediatr. Res. 38 (1995) 324.
[17] D.H. Chace, S.L. Hillman, D.S. Millington, S.G. Kahler, C.R. Roe, E.W. Naylor, Clin. Chem. 41 (1995) 62.
[18] L. Sweetman, Clin. Chem. 42 (1996) 345.
[19] J.D. Shoemaker, W.H. Elliott, J. Chromatogr. 562 (1991) 125.
[20] T. Kuhara, T. Shinka, Y. Inoue, M. Matsumoto, C. Zhang, C. Ning, T. Furumoto, I. Matsumoto, Proceedings of International Society for Neonatal Screening, Boston, 1997, p. 123.