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High-Resolution Proton Nuclear Magnetic Resonance Studies of Urine from Asphyxiated Newborn Infants

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ABSTRACT

High-resolution proton nuclear magnetic resonance spectroscopy was used to study human urine obtained from 10 normal babies and twenty babies with various degrees of neonatal asphyxia, respiratory distress syndrome (RDS), and meconium aspiration syndrome (MAS). All sick babies showed different degrees of oxygen deficiency, indicated by an obvious increase of the lactate signal level in the urine spectra. Changes in the concentration of other urinary metabolites produced from the citric acid cycle were also observed. In extremely serious cases, the signals of some of the major components, including citrate, α-ketoglutarate, and succinate, simply disappeared. The spectra of urine, serum, and CSF of an infant suffering from SIDS showed common characteristics of the metabolites.

Index Entries: Asphyxia; fetal distress; meconium aspiration syndrome; urine; ^{1}H-NMR spectroscopy.

INTRODUCTION

High-resolution proton nuclear magnetic resonance (NMR) spectroscopy has been a powerful tool for the structural elucidation of organic compounds ever since its discovery. However, only a small percentage of

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the research has dealt with biological and medical applications. In the past decade, progress in NMR techniques has led to enhanced sensitivity and improved resolution of the spectra, and water suppression strategies have allowed the study of biological fluids that contain more than 90% water. The method is rapid, requires only a small amount of sample, and no pretreatment is necessary. A well-resolved spectrum with plenty of information can be obtained with a modern high-field NMR spectrometer.

The present article is confined to the examination of urine from asphyxiated newborn infants by means of NMR spectroscopy. In addition, normal controls were also studied for comparison. Our purpose is to study the renal involvement in newborn infants with perinatal asphyxia. In the present investigation, we focus on the changes in urinary metabolites in neonates with different degrees of perinatal asphyxia to determine:

1. Whether or not there are disturbances in the urinary metabolites during perinatal asphyxia;
2. What kinds of urinary metabolites are likely to be affected; and
3. Whether the changes in urinary metabolites correlate with the severity of perinatal insult and neurologic outcomes.

EXPERIMENTAL SECTION

^{1}H NMR spectra were measured at a probe temperature of 25°C by using a BRUKER AMX400 spectrometer operating at 400.13 MHz. The spectrometer was equipped with a 16-bit ADC, X32 data system and an array processor.

All urine samples were supplied by the Cheng Kung University, Medical College. Samples were taken from 10 healthy babies, 17 babies with mild asphysia, i.e., meconium aspiration syndrome (MAS) and respiratory distress syndrome (RDS), and 3 babies with severe perinatal asphysia.

Samples for NMR were prepared by addition of 0.05 mL of D20 to 0.45 mL of urine contained in a 5-mm NMR tube.

Usually, the use of the NOESYPR1D pulse sequence gives efficient water-signal suppression. Since the urine samples of newborn infants are usually quite dilute, 128 free induction decays (FIDs) were collected and transformed.

The 90° pulse width for the reverse broad band probe was 7.6 μs. A spectral width of 4800 Hz was used with a data memory of 32,768 points, thus giving a digital resolution of 0.29 Hz. A pulse delay of 0.5 s was used for this experiment.

In the present work, the 2D-COSYPR pulse program was also used in order to identify some of the undefined resonances in the spectra.

Table 1
^{1}H-NMR Chemical Shifts of Metabolites
from the Urine Samples of Newborn Babies

Compound	Symbol	Shift
Valine	Val	1.00 (d); 1.04 (d)
Propylene glycol	Pg	1.15 (d); 3.49 (d); 3.52 (d); 3.89 (m)
Ethanol	Eth	1.20 (t); 3.67 (q)
3-Hydroxybutyrate	Hb	1.21 (d); 2.32 (d); 2.42 (d); 4.16 (m)
Penillic acid	Pa	1.42 (s); 5.12 (s); 5.50 (s); 7.50 (b)
Penicillin G	PG	1.47 (d); 5.52 (s); 7.50 (b)
Alanine	Ala	1.48 (d); 3.78 (q)
Acetate	Ac	1.92 (s)
Acetone	A	2.24 (s)
Lactate	Lac	1.34 (d); 4.12 (q)
Acetoacetate	Ace	2.29 (s); 3.43 (s)
Succinate	Su	2.42 (s)
α-Ketoglutarate	Kg	2.45 (t); 3.03 (t)
Citrate	Cit	2.54; 2.73 (dd)
Dimethylamine	Dma	2.74 (s)
Dimethylglycine	Dmg	2.93 (s)
Creatine	Cr	3.05 (s); 3.94 (s)
Creatinine	Crn	3.06 (s); 4.08 (s)
Pyruvate	Py	2.38 (s)
Glucose	Glu	3.20–4.00 (m); β-4.65 (d); α-5.25 (d)
Taurine	Tau	3.27 (t); 3.44 (t)
Betaine	Bet	3.28 (s); 3.91 (s)
Glycine	Gly	3.57 (s)
Dihydroxy-acetone	Ha	4.43 (s)
Formate	Fm	8.45 (s)

The chemical shifts are referenced to the creatinine signal at 3.06 ppm s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, b = broad.

RESULTS AND DISCUSSION

In the present study, we examined 10 urine samples collected from healthy newborn babies, in order to determine the characteristics of ^{1}H-NMR spectra of normal baby urine. Furthermore, we examined 20 urine samples obtained from infants with different degrees of neonatal therapies. These included severe neonatal asphyxia, RDS, and MAS. The spectral data for all principal components found in the urine samples are listed in Table 1.

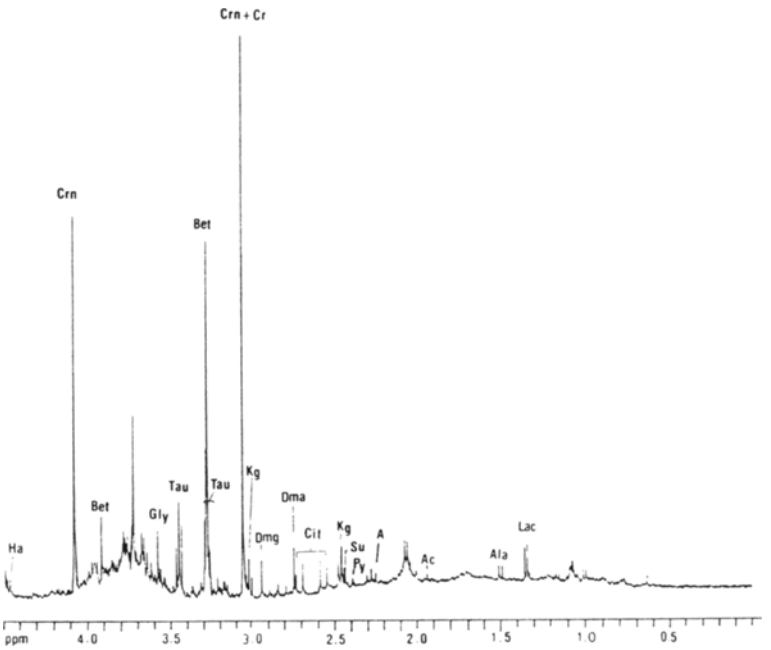

Fig. 1. The ^{1}H-NMR spectrum of the urine of a normal newborn baby (no. 1).

Normal Urine

Figure 1 shows a representative 400-MHz proton NMR spectrum of normal urine obtained from case no. 1. Assignments of the chemical shifts were made by comparison of the spectra obtained for pure compounds and literature values (1–4).

Since all spectra were measured without the use of an external reference compound for the calibration of chemical shifts, the methyl proton signal of creatinine was selected as an internal standard and its chemical shift was set at 3.06 ppm. In this way, the metabolites resonances in this spectrum were found and assigned for lactate, alanine, acetate, acetone, acetoacetate, succinate, α-ketoglutarate, citrate, dimethylamine, dimethylglycine, creatine, creatinine, pyruvate, glucose, taurine, betaine, glycine, and dihydroacetone. The chemical shifts of these compounds are presented in Table 1. Occasionally, a doublet signal for 3-hydroxybutyrate could be recognized at 1.21 ppm in the urine spectra of normal newborns. However, in Fig. 1, the signal was too weak to be observed. The keto compounds, such as 3-hydroxybutyrate, acetone, and acetoacetate, are produced by the oxidation of fatty acids. Normally, the concentrations of these compounds are lower than those of the metabolites produced via the citric acid cycle, so that lower signal intensities are observed than for the components of the citric acid cycle. Pyvuric acid, an intermediate of the glycolysis, is identified by the singlet at 2.38 ppm. In the region between 3.2 and 4.0 ppm, glucose signals appeared as a complex

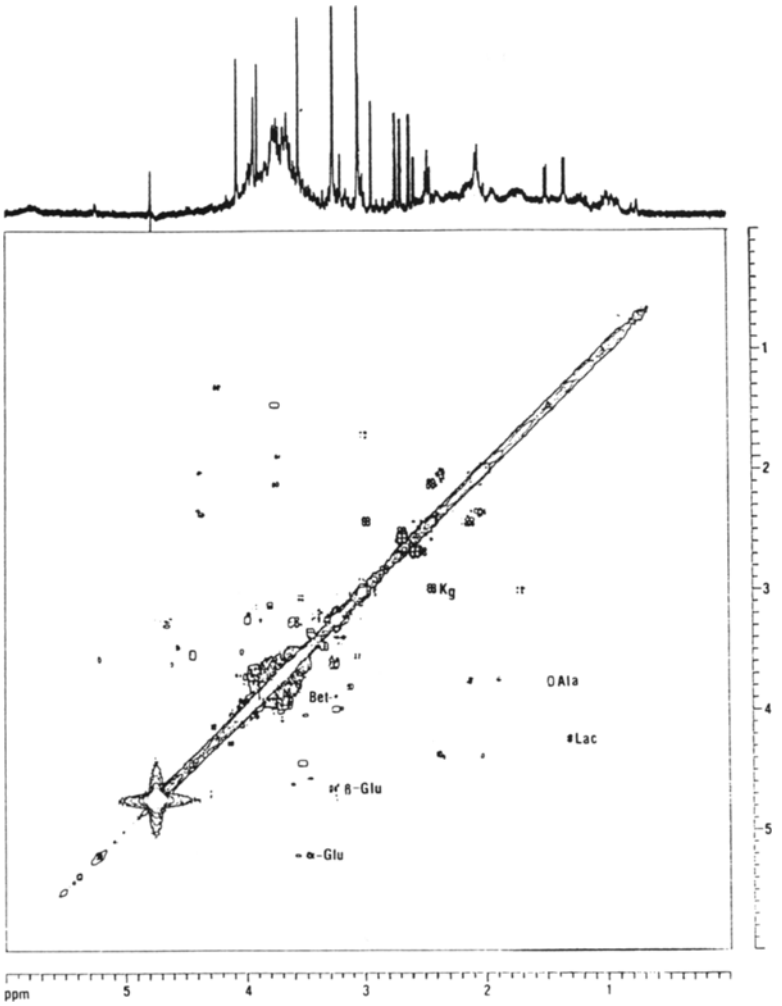

Fig. 2. The COSYPR spectrum of the urine of a normal newborn baby (no. 3).

multiplet. However, α and β anomers showed doublets at 5.24 and 4.65 ppm, respectively.

Further identification of uncertain signals was achieved by means of 2D spectroscopy. Figure 2 represents a 2D-COSYPR spectrum of urine collected from case no. 2. The crosspeaks indicate the correlation between the adjacent protons via scalar coupling in the same molecule. Correlations were found for α-ketoglutarate (2.45, 3.03 ppm), alanine (1.48, 3.78 ppm), lactate (1.34, 4.12 ppm), and a long-range coupling between nuclei resonating at 3.28 and 3.91 ppm was assigned for betaine. Betaine is not usually excreted by adults. Iles and coworkers (*8,13,15*) indicated that the presence of high levels of betaine in the urine of newborn babies may come from the degradation of choline or from damage of cells in the renal medulla.

Table 2
The Relative ^{1}H-NMR Intensity Ratios
of Some Major Urinary Metabolites to the Creatinine Peak

Case no.	Diagnosis	lac/crn	cit/crn	bet/crn	gly/crn	glu/crn
1	Normal	0.06	0.05	0.63	0.12	0.11
2	Normal	0.10	0.19	1.70	0.44	0.12
3	Normal	0.13	0.17	1.70	0.46	0.20
4	Normal	0.07	0.11	2.04	0.49	0.15
5	Normal	0.09	0.21	1.39	0.03	0.22
6	Normal	0.10	0.18	1.09	0.16	0.16
7	Normal	0.10	0.08	1.58	0.54	0.16
8	Normal	0.05	0.08	0.94	0.44	0.14
9	Normal	0.10	0.10	1.08	0.17	0.22
10	Normal	0.03	0.03	0.89	0.24	0.13
11	MAS, RDS, asphyxia	28.57	0.00	1.43	1.00	0.71
12	SIDS, asphyxia	26.40	0.00	2.00	1.00	5.60
13	MAS, asphyxia	8.59	0.12	2.41	0.29	0.23
14	Asphyxia	2.47	0.00	0.84	0.61	0.53
15	Asphyxia	0.64	0.20	1.18	1.51	0.58
16	PM, asphyxia	0.29	0.21	2.31	1.35	3.77
17	PM, asphyxia	0.22	0.46	6.53	1.00	3.00
18	Asphyxia	0.16	0.00	2.00	1.23	1.06
19	RDS	0.35	0.21	2.30	0.68	0.17
20	RDS	0.13	0.07	1.15	0.75	0.35
21	MAS, RDS	0.12	0.07	0.91	0.16	0.17
22	RDS	0.11	0.10	1.33	0.44	0.73
23	RDS	0.10	0.12	1.39	0.31	0.59
24	MAS	0.11	0.04	1.85	0.43	0.91
25	MAS	0.11	0.00	1.31	0.30	0.17
26	MAS	0.10	0.06	0.68	0.19	0.25
27	MAS	0.09	0.00	2.06	0.66	0.47
28	MAS	0.08	0.04	1.16	0.18	0.21
29	MAS	0.07	0.16	1.52	0.57	0.17
30	MAS	0.04	0.00	1.31	0.26	0.34

[a]The average intensity of the doublet of lactate at 1.34 ppm, and the average intensity of two doublets of citrate were used. The signal intensity of betaine was referred to the resonance at 3.28 ppm. For glycine and glucose, an average value was taken from the respective multiplets.

MAS: meconium aspiration syndrome; RDS: respiratory distress syndrome; SIDS: sudden infant death syndrome; PM: premature.

In order to evaluate the relative quantities of some major components in urine samples, we calculated the ratios for the signal intensities of lactate, citrate, betaine, glycine, and glucose to creatine. The results are presented in Table 2. As can be seen, the lactate amount increased in the presence of oxygen deficiency, and therefore, the lac/crn ratio might give an indication of the severity of fetal distress syndrome.

For all spectra of the urine of healthy newborns, a lac/crn ratio between 0.03 and 0.15 was found. These values are far less than those found for asphyxiated newborns (nos. 11–18), namely, lac/crn ratios from 0.16 to 28.57. The lac/crn ratios lie in the range 0.10–0.35 for newborns with RDS (nos. 19–23) and between 0.04 and 0.11 for those babies with MAS (nos. 24–30). These results agreed quite satisfactorily with the clinical status for the severity of neonatal hypoxia. It is quite normal to observe that the urinary lactate content of some healthy newborns is slightly larger than of those with MAS. The emotional response of a healthy baby under physiological stimulus may cause fast heart beats and irregular breathing, thus leading to a temporary oxygen deficiency.

The glucose levels of some of the normal newborns were quite low, with a glu/crn < 0.20. It is believed that an infant would lose heat shortly after delivery, causing a slight drop in body temperature. Restoration of normal body temperature could cause a decrease in glucose level.

Asphyxia

Figure 3 demonstrates a proton spectrum of urine obtained from case no. 11, who presented to the hospital with a variety of asphyxial disorders. This spectrum is quite characteristic for severe hypoxic, ischemic asphyxiated newborns. The lactate concentration was extraordinary high, showing a calculated lac/crn ratio of 28.57, which was the highest value among all the measured spectra. This result indicated that the newborn was desperately oxygen-deficient. The oxidative decarboxylation pathway was inefficient, and glycolysis played an important role in ATP production. Therefore, the pyruvic acid was converted into lactic acid. Further observation showed that the resonances of metabolites produced by the citric cycle, i.e., citrate, succinate, and α-ketoglutarate, which generally appeared in the spectral region 2.4–3.0 ppm, were scarcely detectable. The signals of dimethylamine and dimethylglycine were also absent. Betaine, glycine, creatine, creatinine, and glucose levels were reduced enormously. However, small peaks for valine (1.00 ppm) and alanine (1.48 ppm) were visible in this spectrum.

Figure 4 shows three ^1H NMR spectra of urine, cerebrospinal fluid (CSF), and serum collected from a very sick infant (no. 12) who suffered from hypoxia, asphyxia, seizure, and later died. All three spectra have the same visual characteristics. An extremely high lactate signal is observed in these urine spectra, as well as in CSF and serum spectra. The lac/crn ratios for urine, CSF, and serum were 26.2, 40.5, and 45.6, respectively. As can be seen, the lactate level was highest for serum, and the next highest was for CSF. However, the lac/crn ratio of 26.2 observed for urine is already as astonishingly high value when compared with the normal control.

The major components identified in urine, CSF, and serum were quite similar. In the spectral region 2.4–3.0 ppm, the signals for citrate, succinate, α-ketoglutarate, dimethylamine, and dimethyl glycine were all

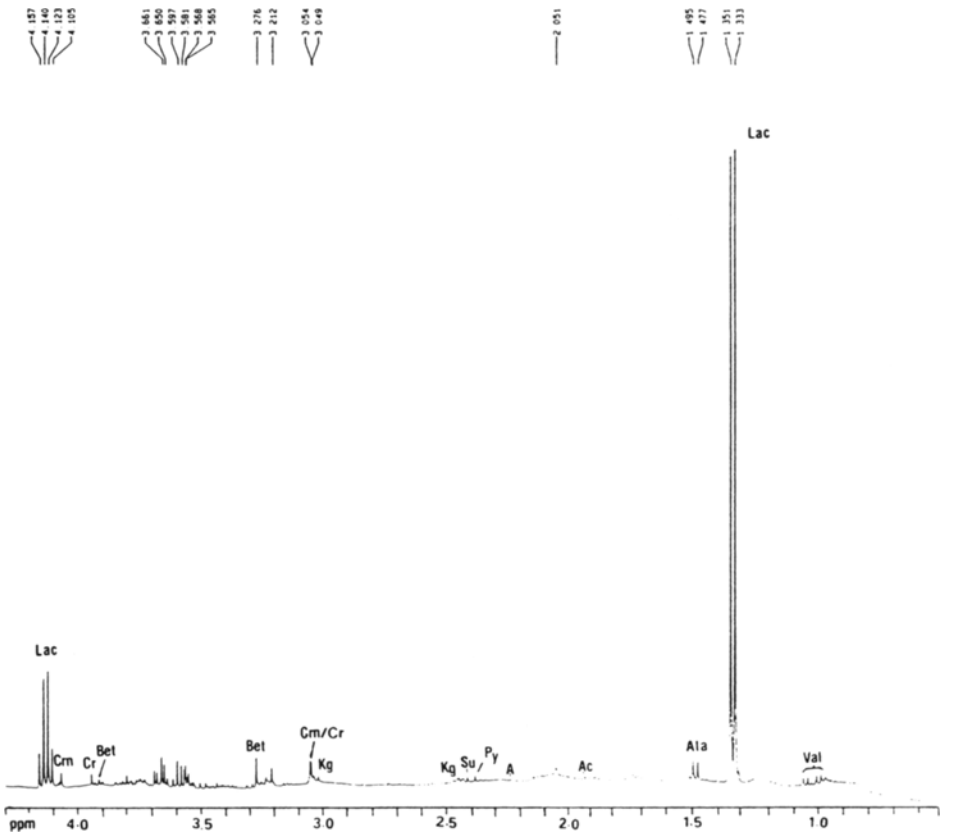

Fig. 3. The urine spectrum of a baby with MAS, RDS and serious asphyxic disorders (no. 11).

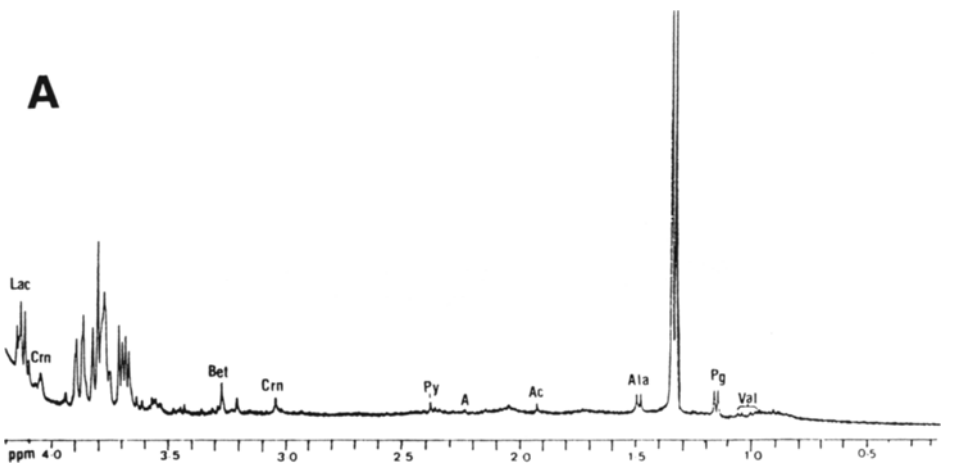

Fig. 4. (**A**) The urine spectrum of a very sick baby with hypoxia, asphyxia, seizure, and SIDs (no. 12).

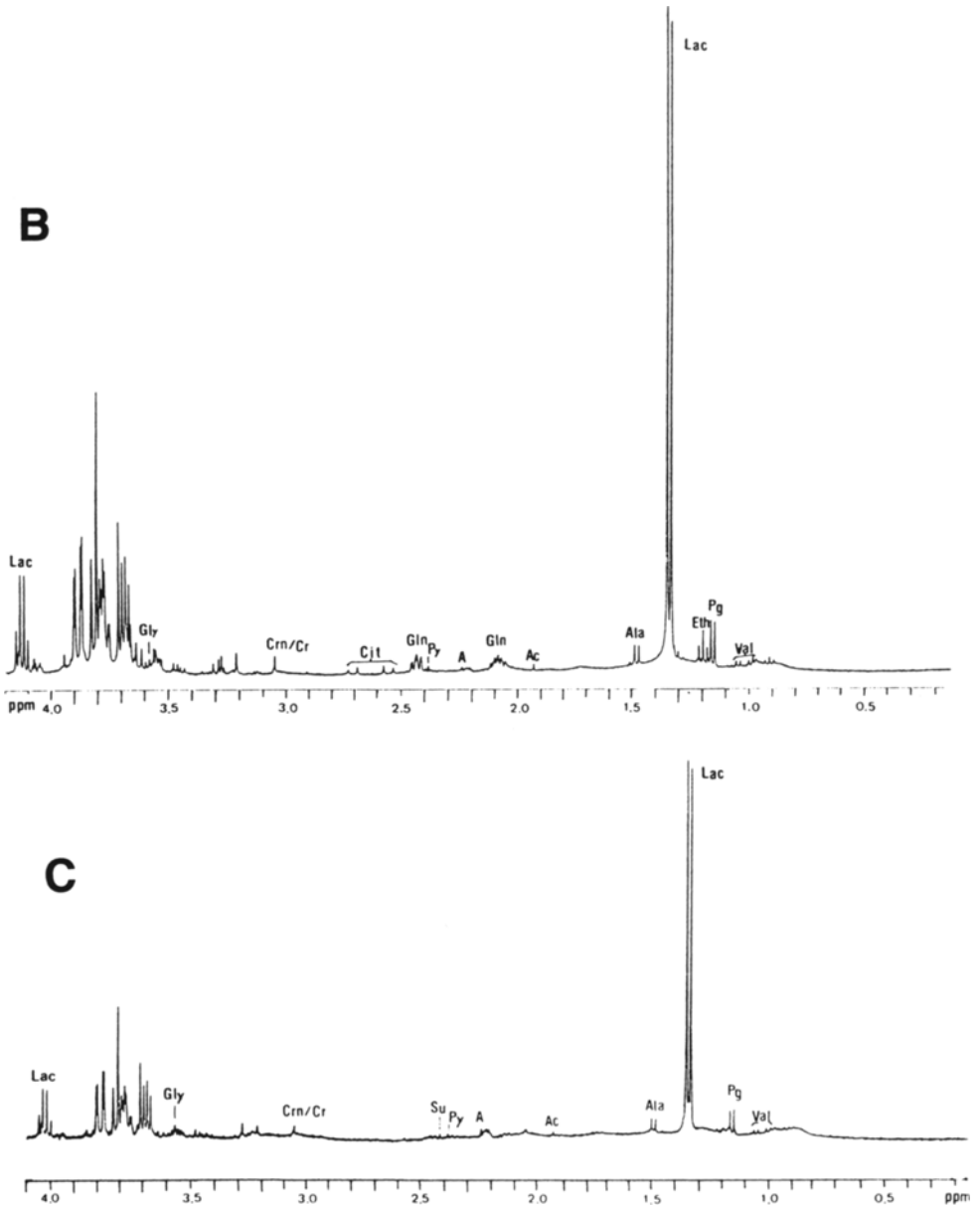

Fig. 4. (continued). (**B**) The CSF spectrum. (**C**) The serum spectrum from the same baby.

missing. A trace of citrate signal could still be observed in the CSF spectrum (Fig. 4B). In addition, a weak triplet at 1.21 ppm was tentatively assigned to the methyl protons of ethanol. Betaine, glycine, creatine, and creatinine signal intensities were also reduced. Here again, valine, alanine, acetylacetate, and acetone signals were still present. The resonance at 1.16 ppm was that of propylene glycol, which had been fed to the sick

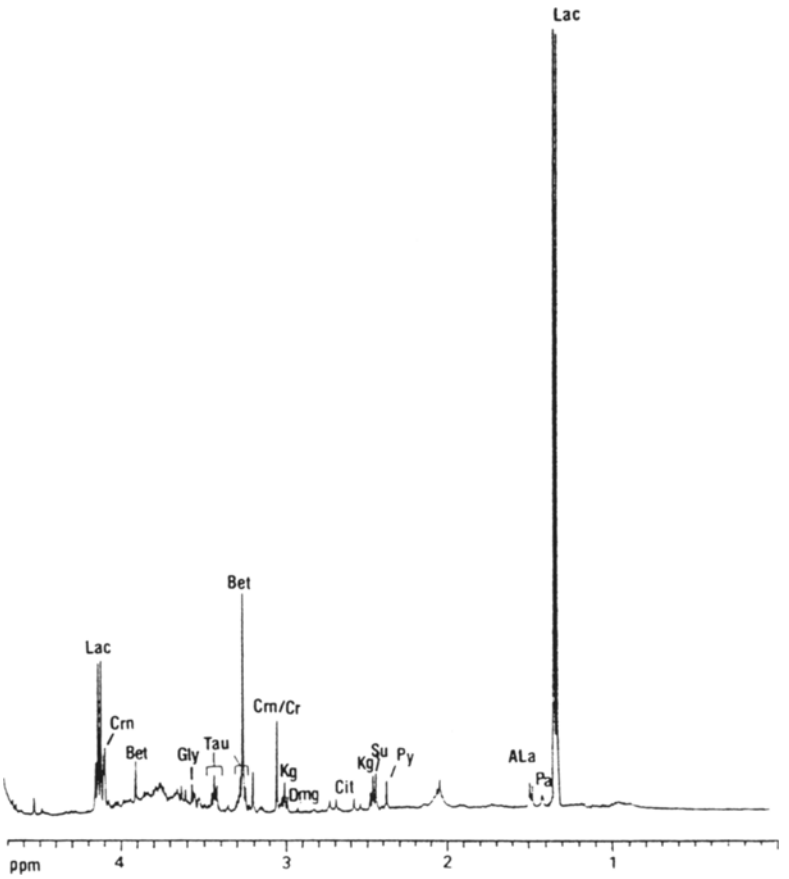

Fig. 5. The urine spectrum of a baby with MAS and asphyxia (no. 13).

baby before the samples were taken. The glucose signals showed a simple pattern. The individual protons were assigned in a straightforward manner.

Figure 5 presents the urine spectrum of a newborn (no. 13). The clinical reports indicated severe asphyxia and MAS, a low apgar score of 1-6 was detected, and the glucose concentration was extremely low, which indicated sugar deficiency. For this case, a lac/crn ratio of 8.6 was obtained, and the betaine signal was notably high. The glucose signal levels were very low, in good agreement with the clinical report. The glycine signal was buried in the hump formed by the glucose multiplets. However, unlike case nos. 11 and 12, a detectable amount of taurine was found at 3.27 (t) and 3.44 ppm (t). Citrate, succinate, α-ketoglutarate, and pyvurate resonances were also visible. Additionally, dimethylamine, dimethylglycine, glycoprotein, and alanine signals were also present.

Figure 6 presents two spectra of urine obtained from the same baby. The samples were collected at different times. Figure 6A (no. 17) showed signals at 1.42 (s), 5.12 (s), 5.50 (s), and 7.50 ppm(m), which were assigned to the resonances of penillic acid, a degradation product of penicillin G, which

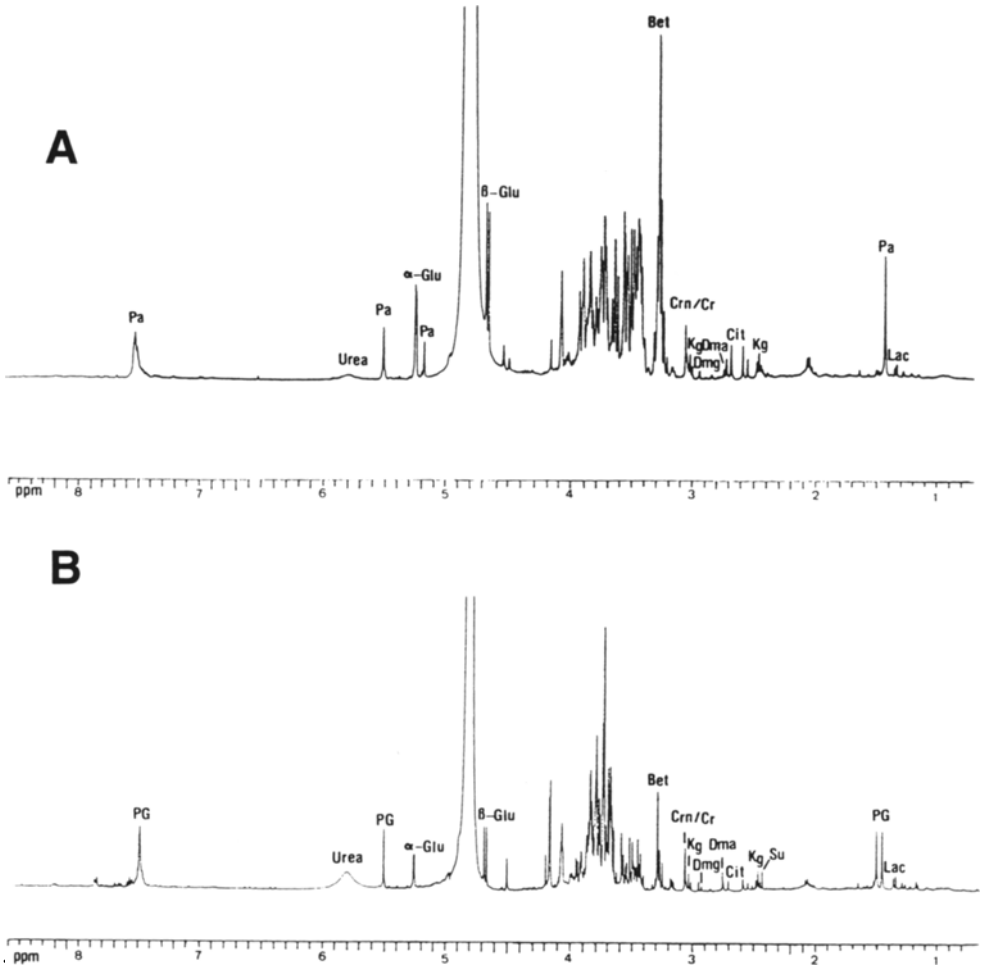

Fig. 6. (**A**) The urine spectrum of an asphyxic premature baby (no. 17) (**B**) The urine spectrum of the same baby from which the specimen was collected 1 wk later (no. 16).

was given to the sick baby before the sample was taken. Figure 6B (no. 16) was recorded for a specimen taken from the same infant 1 wk later. Here, the resonances of penicillin G can still be seen as a doublet at 1.47 ppm for the methyl group. The lac/crn ratios for cases 17 and 16 were 0.22 and 0.29, respectively. The glucose levels were high for both spectra.

Respiratory Fetal Distress

The spectra shown in Fig. 7 (case no. 19) and Fig. 8 (case no. 21) were acquired for urine collected from two newborns who presented fetal distress after birth. For case no. 19, the lac/crn ratio was 0.35, which was the highest lactate level among this class of patients and which indicated an oxygen deficiency. However, the glucose level glu/crn = 0.17 is low.

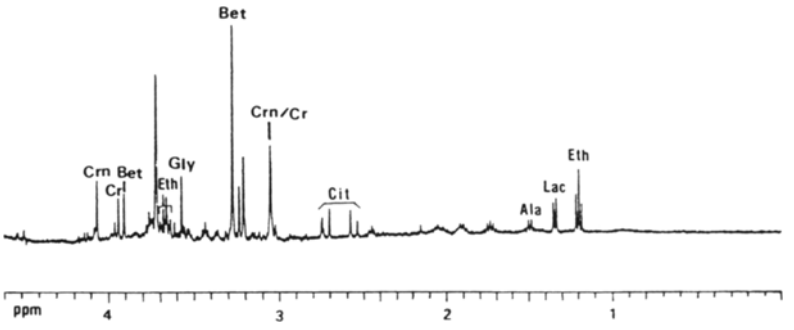

Fig. 7. The urine spectrum of a newborn baby with RDS (no. 19).

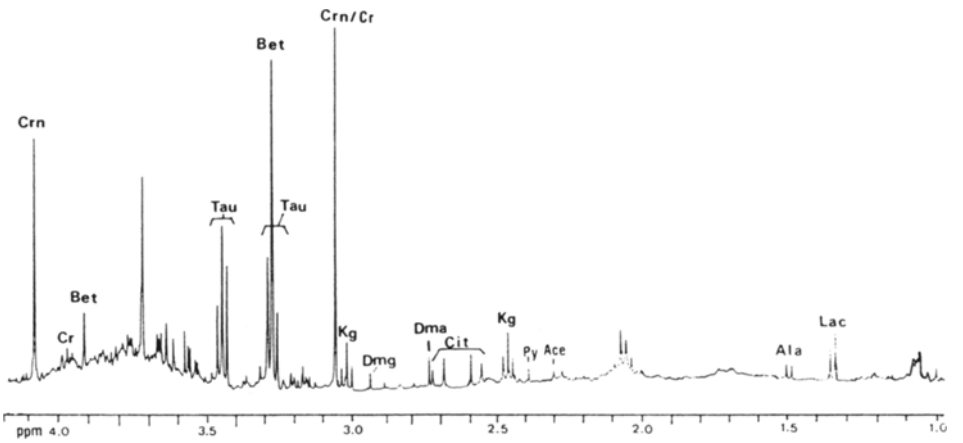

Fig. 8. The urine spectrum of a newborn baby with MAS and RDS (no. 21).

The resonances for citrate were at 2.54 and 2.73 ppm as a doublet of doublets with cit/crn = 0.21. The succinate signal was absent. The signals of the rest of the intermediates in the citric acid cycle, such as α-ketoglutarate, taurine, dimethylamine, and dimethylglycine, were scarcely observable. Moreover, the keto compounds, such as 3-hydroxybutyrate, acetoacetate, and acetone, were also undetectable. A triplet at 1.20 ppm and a quartet at 3.67 ppm were unambiguously assigned to the resonances of ethanol, the presence of which is the result of contamination. The betaine signal is the largest signal in the entire spectrum, with a bet/crn = 2.3. It is also the primary spectral characteristic for this class of patients.

Figure 8 represents a spectrum acquired for case no. 21, both RDS and MAS being detected for this baby. The lac/crn ratio was 0.12. The strongest signals found were those of creatinine and betaine. However, the two triplets for taurine were easily identified at 3.27 and 3.44 ppm. In addition, the two triplets for α-ketoglutarate were also seen at 2.46 and 3.03 ppm. Moreover, the resonances for dimethylamine and dimethylglycine, pyruvate, and acetoacetate were also observed.

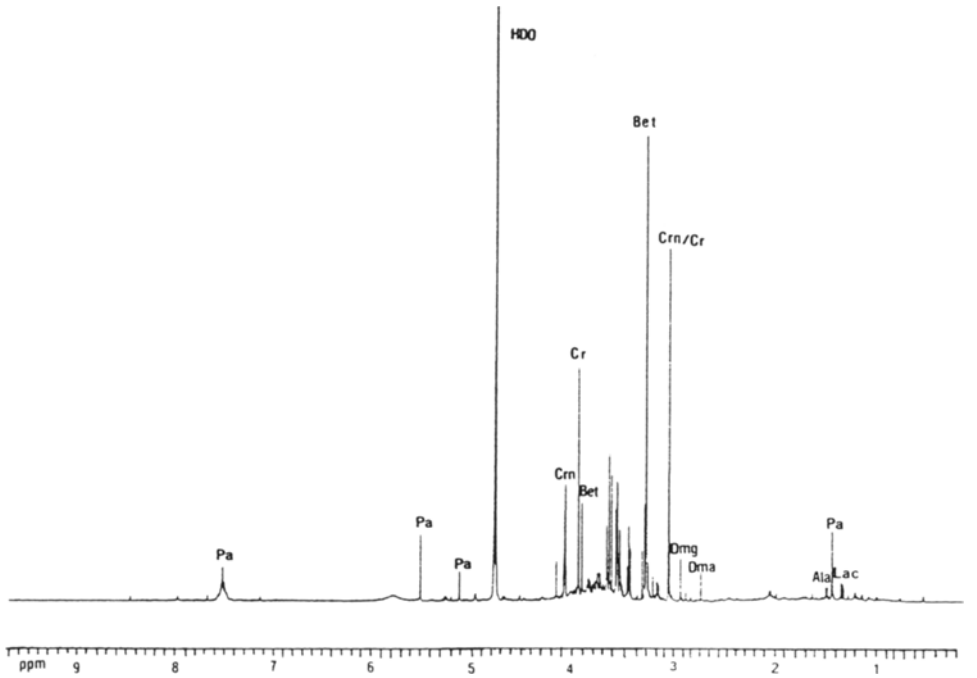

Fig. 9. The urine spectrum of newborn baby with MAS (no. 30).

Meconium Aspiration Syndrome

Figure 9 shows the urine spectrum taken for case no. 30, who was shown to have mild MAS. The characteristics of this spectrum can be summarized as follows: first, the lactate signal at 1.34 ppm was almost the same as that of normal babies, suggesting that a lactate increase does not necessarily exist for all babies with MAS. A lac/crn value of 0.04 was found for this spectrum. Second, the concentrations of metabolites from energy production processes, i.e., succinate, citrate, and α-ketoglutarate, were obviously reduced during asphyxia. For example, in Fig. 9, these signals cannot be found. Furthermore, dimethylamine and dimethylglycine signals were also absent. The signal of creatinine at 3.06 ppm was still dominant.

In the spectrum for case no. 30, the glycine and taurine signals could still be recognized. The glucose signals were extremely weak, which indicated a sugar deficiency.

In the spectra of urine collected from neonatal infants with mild to moderate MAS, case nos. 23–30, certain characteristic features could be observed: low lactate, alanine, dimethylamine, and dimethylglycine levels. Traces of succinate, citrate, and α-ketoglutarate were detected, together with a low level of glucose. Intense signals were observed for creatine, creatinine, betaine, and glycine. From these spectra, we observed

other resonances at 1.42, 5.10, 5.50, and 7.50 ppm, which were assigned to penillic acid, the degradation product of penicillin G.

CONCLUSIONS

From the present investigation, we conclude that metabolic disturbances in neonatal asphyxia can be characterized by ^1H NMR spectra. However, an increase in lactate in neonatal urine with MAS did not occur in all cases, suggesting that hypoxia does not necessarily accompany MAS. For the spectra measured for RDS, the lactate/creatine ratio remained fairly constant in the urine spectra. Since penillic acid signals were present in these spectra, a regulation of the citric acid cycle metabolism with penicillin G could have been helpful in reducing the severity of hypoxia.

All spectra revealed a decrease of succinate, citrate, α-ketoglutarate, dimethylamine, dimethylglycine, and glucose levels for perinatal asphyxia. However, creatine, creatinine, and betaine levels remained constant for all urine samples.

It has been demonstrated that NMR spectra of urine can be employed to detect the metabolite changes for asphyxia. A lactate/creatine ratio larger than 2 already indicates that this infant suffers from severe asphyxia.

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