Chulalongkorn Medical Journal

Volume 43
Issue 1 *January 1999*

Article 3

1-1-1999

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Proton NMR spectroscopic analysis of urinary matabolytes in thalassemia

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Poshyachinda M, Saesow N. Proton NMR spectroscopic analysis of urinary matabolytes in thalassemia. Chula Med J 1999 Jan; 43(1): 15-28

Background : *Only few studies have been reported in the literature regarding excretion of β-aminoisobutyric acid (β-AIBA) and other amino acids in the urine of patients with thalassemia. Since there is a high prevalence of thalassemia in Thailand it is of interest to apply Proton Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy to study urinary excretion of amino acids and other metabolyte products in the urine of these patients.*

Objectives : *1. To determine amino acids and other metabolyte products in the urine of thalassemia patients in correlation with clinical severity and kidney function*

2. To demonstrate the application of ^{1}H-NMR spectroscopy in medicine

Setting : *Division of Nuclear Medicine, Department of Radiology, Faculty of Medicine, Chulalongkorn University*

Research design : *Prospective study*

Materials : *Twenty patients with β-thalassemia/Haemoglobin E (β-thal/HbE) and 18 patients with thalassemia Haemoglobin H disease (Hb H disease) were studied. Twenty normal healthy subjects were also studied as a control group. ^{1}H-NMR spectroscope equipment was used for urinary analysis.*

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Methods : *Haemoglobin concentration and kidney function were determined in all patients. Twenty-four hour urine was collected from all patients and normal subjects and analysed for amino acids and other metabolytes by using ^{1}H-NMR spectroscopy.*

Results : *All patients with β-thal/HbE demonstrated hyperexcretion of β-AIBA in their urine ranging from 27.4 to 754.7 μmol/24h/kg body wt.(median = 105.4). Splenectomized patients had significantly higher amounts of urinary β-AIBA than nonsplenectomized patients. There was no correlation of β-AIBA excretion with clinical severity or hemoglobin level. Urinary β-AIBA was detected in 11 of the 18 patients with Hb H disease but in much smaller amounts ranging from 22.8 to 155 μmol/24h/kg body wt. (median =64.0) Only 2 normal subjects had trace amounts of β-AIBA in their urine.*

Conclusion : *^{1}H-NMR spectroscopy provides a fast, simple and non-invasive method for identification of urinary compounds. This study demonstrated that patients with β-thal/Hb E excreted significantly higher quantities of β-AIBA in their urine than patients with Hb H disease. Urinary β-AIBA in splenectomized patients is significantly higher than for non-splenectomized patients. Generalized tissue destruction, apart from removal of normoblasts from the circulation by other tissues, may account for higher excretions of β-AIBA in splenectomized patients and may reflect poor prognosis.*

Key words : *Thalassemia, Haemoglobinopathy, β-aminoisobutyric acid, Urinary metabolites, ^{1}HNMR spectroscopy.*

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Received for publication. September 15, 1998.

มาคุ้มครอง โปษยะจินดา, นุ้ย แซ่โซว. การวิเคราะห์เมตาบอไลท์ในปัสสาวะของผู้ป่วยธาลัสซีเมียด้วยโปรตอน เอ็นเอ็มอาร์ สเปคโตรสโคปี. จุฬาลงกรณ์เวชสาร 2543 ม.ค; 43(1): 15-28

ปัญหา : *มีการศึกษาน้อยมากเกี่ยวกับการขับถ่ายกรดเบต้า-อะมิโนไอโซบิวทิริค (เบต้า-เอไอบีเอ) และกรดอะมิโนตัวอื่น ๆ ทางปัสสาวะของผู้ป่วยธาลัสซีเมีย เนื่องจากอุบัติการของโรคธาลัสซีเมียค่อนข้างสูงในประเทศไทย จึงเห็นว่าน่าจะทำการศึกษาการขับถ่ายกรดอะมิโนและเมตาบอไลท์ตัวอื่น ๆ ในปัสสาวะของผู้ป่วยเหล่านี้โดยใช้เครื่องโปรตอนเอ็นเอ็มอาร์ สเปคโตรสโคปี*

วัตถุประสงค์ : *1.เพื่อตรวจหากรดอะมิโนและเมตาบอไลท์อื่น ๆ ในปัสสาวะของผู้ป่วยธาลัสซีเมียโดยดูความสัมพันธ์กับความรุนแรงของโรคและการทำงาน ของไต 2. เพื่อแสดงให้เห็นถึงการใช้ประโยชน์ของโปรตอน เอ็นเอ็มอาร์สเปคโตรสโคปีในการแพทย์*

สถานที่ที่ทำการศึกษา : *สาขาเวชศาสตร์นิวเคลียร์ ภาควิชารังสีวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย*

รูปแบบการวิจัย : *การศึกษาไปข้างหน้า*

ผู้ป่วยที่ได้ทำการศึกษา : *ผู้ป่วยที่เป็นโรค เบต้า-ธาลัสซีเมีย/ฮีโมโกลบิน อี จำนวน 20 ราย ผู้ป่วยที่เป็นโรคธาลัสซีเมีย ฮีโมโกลบินเอช (โรคฮีโมโกลบินเอช) จำนวน18 ราย และคนปกติเพื่อเป็นกลุ่มควบคุม 20 ราย*

วิธีการศึกษา : *ตรวจวัดระดับฮีโมโกลบินและตรวจวัดการทำงานของไตในผู้ป่วยทุกคนเก็บปัสสาวะของผู้ป่วยธาลัสซีเมียและคนปกติ 24 ชั่วโมง แล้วนำไปวิเคราะห์หากรดอะมิโนและเมตาบอไลท์ตัวอื่น ๆ โดยใช้เครื่องโปรตอนเอ็นเอ็มอาร์ สเปคโตรสโคปี*

ผลการศึกษา : *ผู้ป่วยเบต้า-ธาลัสซีเมีย/ฮีโมโกลบิน อีขับ เบต้า-เอไอบีเอออกมาในปัสสาวะมากกว่าปกติทุกราย โดยมีค่าระหว่าง 27.4 ถึง 754.7 ไมโครโมล/24 ชั่วโมง/กิโล ของน้ำหนักตัว (ค่ามัธยฐาน = 105.4) ผู้ป่วยที่เคยตัดม้ามออกขับถ่ายเบต้า-เอไอบีเอ มากกว่ากลุ่มผู้ป่วยที่ไม่เคยตัดม้ามออก ไม่พบความสัมพันธ์ระหว่างการขับเบต้า-เอไอบีเอ กับความรุนแรงของโรคหรือ ระดับฮีโมโกลบิน ตรวจพบเบต้า-เอไอบีเอ ในปัสสาวะของผู้ป่วยธาลัสซีเมีย ฮีโมโกลบินเอช 11 ใน 18 ราย (61%) โดยมีค่าน้อยกว่ามากคือระหว่าง 22.8 ถึง 155 ไมโครโมล/24 ชั่วโมง/กิโลของน้ำหนักตัว (ค่ามัธยฐาน = 64.0)*

วิจารณ์และสรุป : *โปรตอน เอ็นเอ็มอาร์ สเปคโตรสโคปี เป็นการตรวจที่ง่าย ไม่รบกวนผู้ป่วย ใช้สำหรับตรวจวิเคราะห์สารประกอบต่าง ๆ ในปัสสาวะ การศึกษานี้ได้แสดงให้เห็นว่าผู้ป่วย เบต้า ธาลัสซีเมีย/ฮีโมโกลบิน อี ขับถ่ายเบต้า-เอไอบีเอลงมาในปัสสาวะมากกว่าผู้ป่วยที่เป็นโรคฮีโมโกลบินเอช และพบว่าผู้ป่วยที่เคยตัดม้ามมีการขับถ่าย เบต้า-เอไอบีเอ มากกว่าผู้ป่วยที่ไม่เคยตัดม้าม ทั้งนี้นอกจากการที่เนื้อเยื่ออื่น ๆ กำจัดเม็ดเลือดแทนม้ามแล้ว อาจเป็นเพราะมีการทำลายเนื้อเยื่ออื่น ๆ ด้วย ดังนั้นถ้ามีการขับ เบต้า-เอไอบีเอ ในปัสสาวะมากอาจสะท้อนถึงความรุนแรงของโรค*

Beta aminoisobutyric acid (β-AIBA) is considered to be a product of the catabolism of thymine and valine. Excretion of large amounts of β-AIBA in the urine of patients with thalassemia major was first observed by Hillcoat.[1] The amount excreted suggests a correlation with the haematological status of the patients,[2] and the excretion significantly dropped following splenectomy.[2-3] Further study of urine β-AIBA and other amino acid excretions by patients with β - thalassemia had been reported using ion exchange chromatography as an aid in the quantitative analysis.[4]

Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy is a powerful tool for both qualitative and quantitative analysis of organic compounds. Despite the drawback of expensive, sophisticated instrumentation, ^{1}H-NMR spectroscopy offers some unique advantages for clinical chemistry: it permits rapid, specific, nondestructive measurement of several compounds simultaneously, including some that may be inconvenient to measure by conventional means.[5] ^{1}H-NMR spectroscopy has been used to measure metabolytes, drugs, and toxic agents in body fluids. Over the last decade, this technique has been applied to serum, urine, cerebrospinal fluid and other fluids.[6-8] Improvements in nuclear magnetic resonance technology are generating an expanding variety of medical applications.[9-13]

Since thalassemia is highly prevalent in Thailand,[14] it is of interest to apply proton nuclear magnetic resonance spectroscopy to study urinary excretion of amino acids and other metabolyte products in thalassemic patients in correlation with clinical severity and kidney function.

Materials and Methods

Twenty patients with β- thalassemia/Haemoglobin E (β-thal/Hb E) and 18 patients with thalassemia Haemoglobin H disease (Hb H disease) without kidney diseases or urinary tract infections as judged by urine analysis and urine culture were included in the study. The other inclusion criterion was that the age of the patients had to range from 8-16 years old. Twenty normal healthy subjects with the same age range were recruited as a control group.

Diagnosis of thalassemia was based upon complete haematological examination including electrophoresis to identify specific types of abnormal haemoglobins. The clinical severity was classified into three groups. Group I, mild severity, referred to patients who were able to maintain haemoglobin concentrations around 8-10 g per dl without blood transfusion. Group II, moderate severity, was patients whose haemoglobin range from 4-7 g per dl and blood transfusion was regularly required about 3-5 times per year. Group III was markedly severe patients who sustained very low haemoglobin concentrations, usually below 5 g per dl and monthly blood transfusions were necessary for their daily activities.

Endogenous creatinine clearance, serum BUN and creatinine were performed in all patients. The analysis for urinary components in all cases was carried out on an aliquot of the 24-hour urine collection. Five-tenths milliliters of untreated urine sample plus 0.04 milliliters of deuterium (D_2O) were put into a NMR sample tube. The D_2O was used as an internal lock of the magnetic field. DSS 3-(trimethylsilyl) propanesulfonic acid sodium salt was added to a sample tube and used as an internal standard for chemical shift and signal intensity. ^{1}H-NMR spectra were obtained

from a JEOL JNM-FX90 NMR spectrometer operating at 90 MHz. The water proton was irradiated using the gated homonuclear decoupling mode in order to suppress the proton signal of water.[15] Ninety degree pulses (length: 34 μsec) were applied 200 times with recycle of 10 seconds, and 8 K data points were used for the spectral width of 2 KHz.

A few urine samples from each studied group were lyophilized prior to NMR spectroscopic analysis in order to compare to untreated urine. Morning urine in all patients with β-thal/Hb E were also analysed for comparison with 24-hour urine.

All clinical studies were approved by the hospital committee for ethical aspects.

Results

The age and sex of patients and normal controls are summarized in Table 1. The majority of patients in the β-thal/Hb E group were clinically severe and moderately severe, only 2 cases were clinically mild. Patients in the Hb H disease group were mainly mild to moderate clinical severity, only one case was clinically severe.

Eleven of 20 patients in the β-thal/Hb E group had been splenectomized ranging from 4 months to 9 years with an average of 4.2 years. None of the 18 patients with Hb H disease were subjected to splenectomy. The typical ^{1}H-NMR spectra of lyophilized urine from normal healthy subject demonstrated multiple urinary matablolytes including creatinine, creatine, citrate, and hippurate. Urine specimens without pretreatment revealed similar spectra to the lyophilized urine but with less intensity of signals (Fig. 1a and b). Two of the 20 normal subjects exhibited traces of signals of chemical shift at 1.14 and 1.22 parts per million (ppm).

Table 1. Summary of studied groups.

	Number of cases	Sex	Age (Yr)	
		F/M	Mean ± S.D.	Range
Normal controls	20	6/14	11.5 ± 2.6	8-15
β-thal/HbE	20	7/13	13.1 ± 2.2	8-16
Hb. H Disease	18	6/12	11.2 ± 2.4	8-14

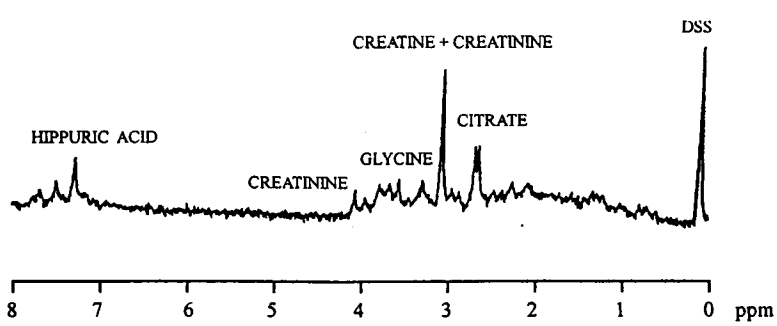

Figure 1 a. 90 MHz ^{1}H-NMR spectrum of lyophilized urine from normal healthy subject.

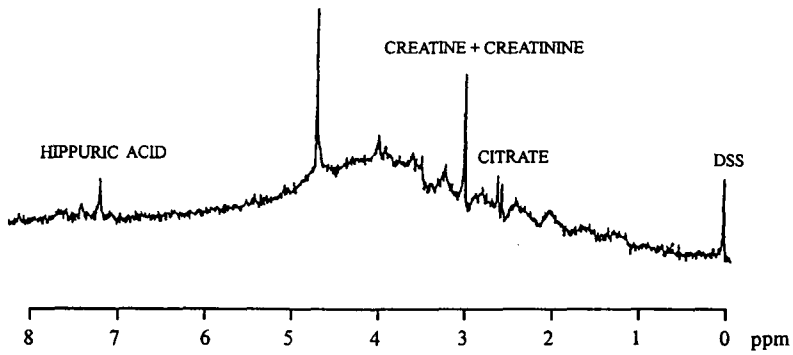

Figure 1 b. 90 MHz ^{1}H-NMR spectrum of untreated urine from the same subject.

All twenty patients with β-thal/Hb E demonstrated intense abnormal resonance at 1.14 and 1.22 ppm. Fig. 2a and b illustrate typical spectra from lyophilized and untreated urine in a patient with β-thal/Hb E. The striking resonance at 1.14 and 1.22 ppm. was eventually identified to be beta-aminoisobutyric acid (β-AIBA). Identification of β-AIBA was performed by adding standard β-AIBA into the urine and then repeating the ^{1}H-NMR spectroscopic analysis (Fig. 3a and b). Morning urine from 20 patients in this group all demonstrated urinary β-AIBA as in 24-hour urine samples (Fig. 4a and b).

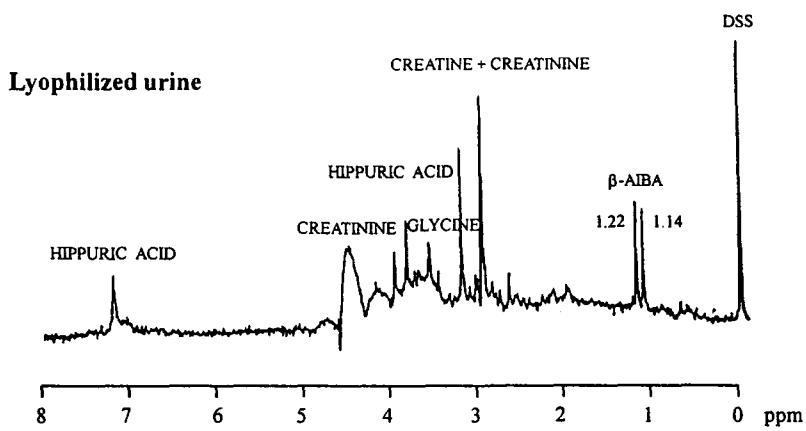

Figure 2 a. ^{1}H-NMR spectrum of lyophilized urine from a patient with β-thal/Hb E.

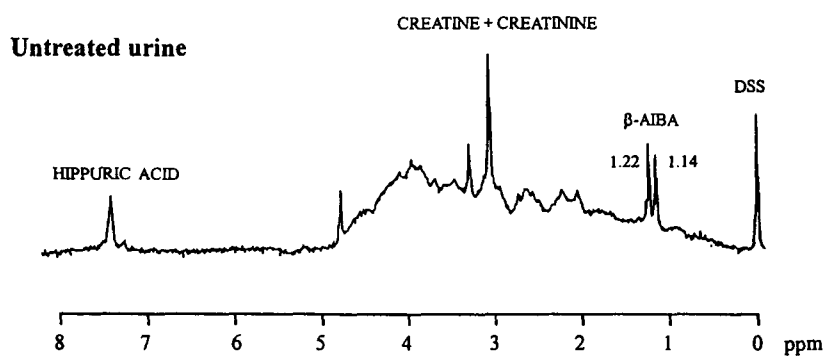

Figure 2 b. ^{1}H-NMR spectrum of untreated urine from same patient with β-thal/Hb E.

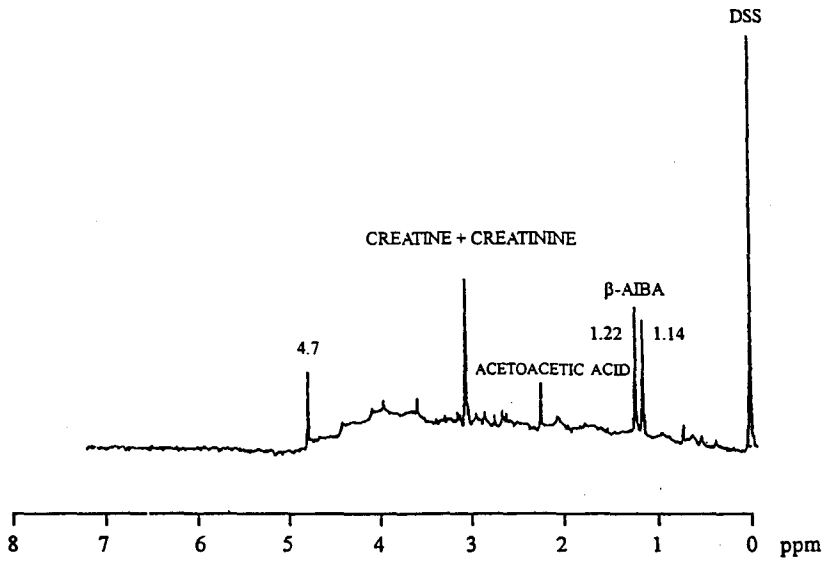

Figure 3 a. ^{1}H-NMR spectrum of urine from a patient with β-thal/Hb E.

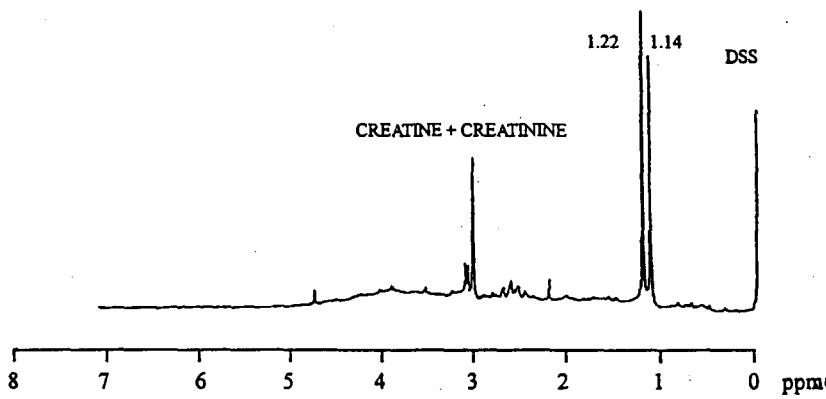

Figure 3 b. ^{1}H-NMR spectrum of urine from the same patient after adding 1 mg. of standard β-AIBA.

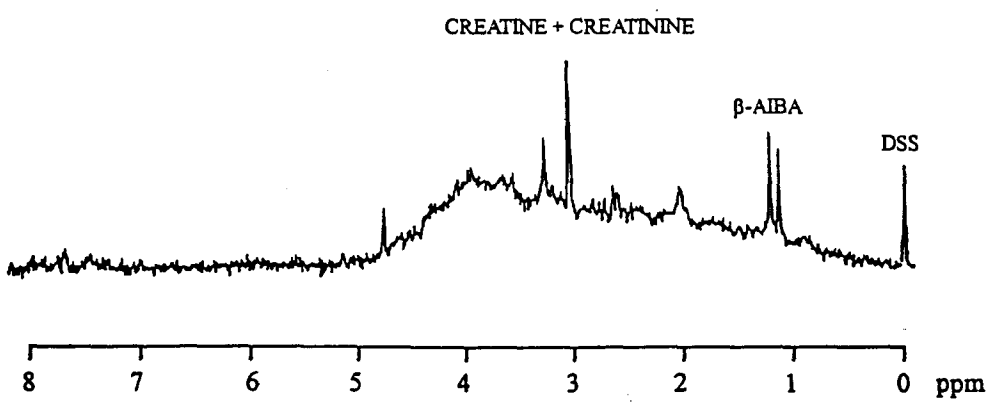

Figure 4 a. ^{1}H-NMR spectrum of 24-hour urine from a patient with β-thal/Hb E.

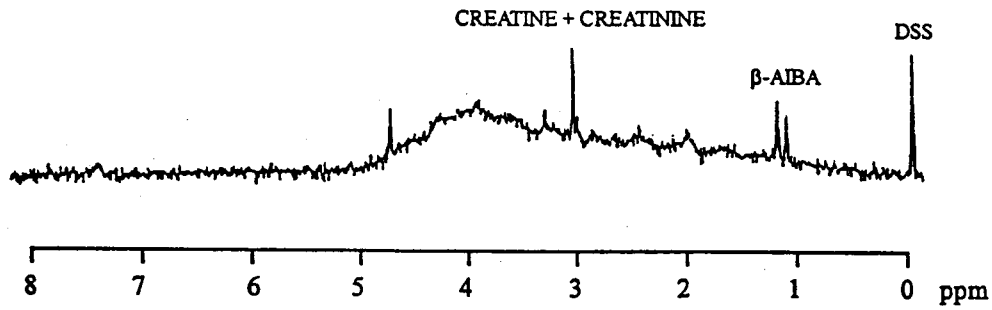

Figure 4 b. 1H-NMR spectrum of morning urine from the same patient.

^{1}H-NMR spectra of urine from 11 of the 18 patients (61%) with thalassemia Hb H disease demonstrated β-AIBA while the remaining 7 patients showed normal spectra.

Estimation of urinary β-AIBA concentrations was attempted by comparing the intensity of the β-AIBA signal with those of the signals of known concentrations of DSS and a series of known standard of β-AIBA (0.25-2 mg/ml). The amount of urinary β-AIBA above 0.025 mg/ml (0.24 μmol/ml) was regarded as positive while below this level can hardly be measured and was considered as trace.

To test the accuracy in estimation, the ^{1}H-NMR spectrum of a urine sample was recorded and estimated and then the standard amount of β-AIBA (1 mg) was added to the same sample and the spectrum was re-recorded under the same conditions. The recovery rate was found to be over 90 per cent.

The results of the studies in the thalassemic patients are summarized in Tables 2 and 3. Urinary excretion of β-AIBA in patients with β-thal/HbE was ranged from 27.4 to 754.7 μmol /24h/kg body wt. (median = 105.4). Eleven of 18 patients with Hb H disease excreted urinary β-AIBA with the range of 18.3 to 155 μmol/24h/kg body wt (median = 64.0) while the rest of patients had undetectable urinary β-AIBA Patients in the β-thal/Hb E group showed obviously higher rates of excretion of β-AIBA as compared to patients with Hb H disease, and the haemoglobin level was significantly lower ($p<0.001$).

Table 2. Clinical data and urinary β-AIBA in β-thal/Hb E patients.

Case No.	Age Sex	Duration after Splenectomy (Yr)	Hb g/dl	Urine pH	β-AIBA μmol/24h/kg body wt.
1	12/M	1	4.8	7.0	265.4
2	12/M	1/3	6.0	6.5	163.6
3	14/M	8	6.2	6.5	27.5
4	11/M	5	6.2	8.0	51.5
5	14/M	2	6.3	6.5	133.8
6	16/M	3	7.5	6.3	249.7

Table 2. (ต่อ)

Case No.	Age Sex	Duration after Splenectomy (Yr)	Hb g/dl	Urine pH	β-AIBA μmol/24h/kg body wt.
7	9/M	3	7.5	6.5	186.6
8	16/F	2	7.9	6.5	27.4
9	13/F	8	6.3	6.2	754.7
10	15/F	6	6.6	7.6	85.8
11	11/M	9	7.2	6.3	136.8
12	15/M	-	6.9	6.2	119.4
13	12/F	-	6.2	7.0	261.0
14	15/M	-	6.2	6.2	64.4
15	14/M	-	6.9	6.5	83.7
16	13/F	-	7.5	6.2	155.3
17	15/M	-	6.6	6.5	36.7
18	8/M	-	7.2	6.5	35.1
19	12/F	-	7.2	6.5	91.5
20	15/F	-	7.6	7.0	51.7
Median	13.5	3.0	6.75	6.5	105.4

Table 3. Clinical data and urinary β-AIBA in patients with Hb H disease.

Case No.	Age sex	Hb g/dl	Urine pH	β-AIBA μmol/24h/kg body wt.
1	9/M	6.3	6.2	18.3
2	11/F	5.4	6.5	88.0
3	13/F	5.6	7.0	64.0
4	12/M	7.6	6.0	0
5	15/F	7.4	8.5	0
6	9/M	10.3	5.8	85.4
7	10/M	9.1	6.5	22.8
8	11/M	8.2	5.8	115.3
9	8/F	7.9	6.3	23.5
10	12/M	8.1	7.5	0
11	13/M	9.0	6.4	0
12	13/F	9.7	6.5	0
13	14/M	9.2	6.7	49.3

Table 3. (ต่อ)

Case No.	Age sex	Hb g/dl	Urine pH	β-AIBA μmol/24h/kg body wt.
14	8/M	9.2	6.7	155.0
15	10/M	9.7	6.7	102.4
16	12/M	9.8	6.2	53.6
17	8/M	9.7	6.5	0
18	14/F	11.4	6.2	0
Median	11.5	9.0	6.5	64.0

Urinary β-AIBA in the 11 splenectomized patients with β-thal/Hb E was significantly higher than in the 9 non-splenectomized patients ($p<0.007$), as shown in Table 4.

There was no hyperexcretion of other amino acids except glycine which was found in 3 patients in each group. Excretion of taurine could be demonstrated in only 3 patients with β-thal/Hb E. A quantitative measurement was not attempted.

The serum BUN, creatinine and glomerular filtration rate (GFR) in both groups of patients were within normal limits, except 3 cases from the βthal/Hb E group and one case from the Hb H disease group had decreased GFR (Table 5).

Table 4. Urinary excretion of β-AIBA in β-thal/Hb E.

β-thal/Hb E	Age Yr	Hb g/dl	Urine pH	β-AIBA μmol/24h/kg body wt.
SPLENECTOMIZED PATIENTS (11 cases)				
Range	9 - 16	4.8 - 7.9	6.3 - 8.0	27.4 - 754.7
Median	12	6.3	6.5	136.8
NON-SPLENECTOMIZED PATIENTS (9 cases)				
Range	8 - 15	6.2 - 7.6	6.3 - 7.6	35.1 - 261.0
Median	14	6.9	6.5	83.7

Table 5. Glomerular function in thalassemic patients.

		β-thal/Hb E	Hb H disease
BUN (mg/dl) :	Range	6.4 - 20.0	8.0 - 13.2
	Median	12.6	9.8
Creatinine (mg/dl) :	Range	0.3 - 0.85	0.40 - 1.05
	Median	0.5	0.6
GFR (ml/min/1.73 m^2) :	Range	54 - 236	45 - 193
	Median	101	94

Discussion

Proton NMR spectrosopy has several advantages over other biochemical techniques. It is simple, fast and noninvasive. There has been increasing interest in application of NMR spectroscopy as an investigative tool in medicine. [9-13] By means of proton NMR spectroscopy, urinary metabolites are readily demonstrated without pretreatment of urine samples.[8,16] But from the present study, lyophilized urine provided considerable distinct chemical shifts.

Spectra of normal urine in our study are similar to other reports, and only 2 subjects revealed traces of urinary β-AIBA. Excretion of β-AIBA by normal individuals is very low but increased excretion of this amino acid is fairly common in orientals.[3,4] Since β-thal/Hb E and Hb H disease are highly prevalent in Thailand, it is expected that excretion of β-AIBA should be higher in our normal controls but the number of subjects included in this study may have been too small.

Hyperexcretion of β-AIBA was found in all patients with β-thal/Hb E, but with wide variation in the amount of excretion. There was no correlation between the amount of excretion and the clinical severity or haemoglobin level. In contrast to other reports which found that after the removal of the spleen, little or no excretion of β-AIBA was observed,[2-4] the present study showed significantly higher amounts of urinary β-AIBA in splenectomized patients as compared to nonsplenectomized patients ($p<0.007$). No significant differences in the hemoglobin levels or the urine pH between the splenectomized and nonsplenectomized groups was found.

The amount of urinary β-AIBA in patients with Hb H disease is obviously less than in patients with β-thal/Hb E. Among these, β-AIBA was not detected in 7 patients (39%). It is well established that the clinical course of thalassemia Hb H disease is much less severe than β-thal/Hb E disease. In our study most of the patients in Hb H disease group had clinically mild to moderate severity, while patients in the β-thal/Hb E groups had clinically moderate to marked severity.

It is of interest that morning urine could demonstrate β-AIBA as well as 24-hour urine since it may be useful in screening thalassemia patients, particularly those with β-thal/Hb E. Although it was reported that some iron deficiency patients excreted urinary β-AIBA but with much less quantity.[3] However it will not be helpful for screening patients with Hb H disease since urinary β-AIBA may be undetectable in this group.

Small numbers of patients demonstrated hyperexcretion of taurine and glycine, and this may due to the fact that in order to irradiate water resonance, those amino acid protons were also irradiated. Therefore, lyophilized urine should be used for a better analysis unless high resolution ^{1}H-NMR spectroscopy is used.

The excretion of β-AIBA is always associated with extensive cellular breakdown, such as may occur in leukemia and carcinoma following radiation and after operations, and thymine is the more likely source of β-AIBA.[3,4] The most likely source of β-AIBA and taurine found in the urine of thalassemic patients is from the destruction of circulation normoblasts by the spleen, therefore it is suggested that the excretion of β-AIBA is a good index of the number of normoblast nuclei trapped and broken down in the spleen.[3] Tissues other than the spleen retain the ability to remove normoblasts from the circulation in adult patients post splenectomy, tissue destruction may involve other tissues besides erythron. [3,4] Our study suggests that

the high excretion of β-AIBA in the urine of splenectomized patients may derive from generalized tissues destruction apart from the removal of circulatory normoblasts by other tissues and this may reflect a poor prognosis.

Conclusion

^{1}H-NMR spectroscopy provides a fast, simple and noninvasive method for identification of urinary compounds. In this study, β-thal/Hb E. disease patients excreted significantly greater amounts of β-AIBA in their urine than did patients with Hb H disease, and splenectomized patients have significantly higher amounts of urinary β-AIBA than nonsplenectomized patients.

Acknowledgements

This work was supported by a Rachadapisek-sompoj Research grant of the Faculty of Medicine, Chulalongkorn University. We are grateful to Drs. Chulee Mitrakul and Dhevy Watana, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, for their kind assistance in providing patient urine samples and clinical data. The authors also wish to thank Miss Wanida Jinsart of the Scientific and Technological Research Equipment Center, Chulalongkorn University for her kind assistance in analysis of specimens by ^{1}H-NMR spectroscopy.

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