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Identification of 4-deoxythreonic acid present in human urine using HPLC and NMR techniques☆

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ABSTRACT

The ^{1}H NMR spectrum of urine exhibits a large number of detectable and quantifiable metabolites and hence urine metabolite profiling is potentially useful for the study of systems biology and the discovery of biomarkers for drug development or clinical applications. While a number of metabolites (50–100) are readily detectable in urine by NMR, a much larger number is potentially available if lower concentration species can be detected unambiguously. Lower concentration metabolites are thought to be more specific to certain disease states and thus it is important to detect these metabolites with certainty. We report the identification of 4-deoxythreonic acid, a relatively low concentration endogenous metabolite that has not been previously identified in the ^{1}H NMR spectrum of human urine. The use of HPLC and NMR spectroscopy facilitated the unequivocal and non-invasive identification of the molecule in urine which is complicated by extensive peak overlap and multiple, similar resonances from other metabolites such as 3-hydroxybutanoic acid. High-resolution detection and good sensitivity were achieved by the combination of multiple chromatographic fraction collection, sample pre-concentration, and the use of a cryogenically cooled NMR probe.

1. Introduction

Nuclear Magnetic Resonance (NMR) has shown high utility in the area of metabolite profiling and metabolomics or metabonomics [1–8]. Metabolite profiling, which involves the combination of high-resolution spectroscopy with multivariate statistical methods, allows the exploration of subtle differences in sample cohorts by detecting multiple metabolites quantitatively and in parallel [9–12]. Identifiable differences in the spectra that can be related to metabolic variance add to the current knowledge of systems biology and may lead to disease detection or prediction, among a number of applications [7,13,14]. The process of NMR-based biomarker identification is culminated by peak assignment, a task that is usually challenged by the high complexity of the NMR biofluid spectra. The existence of metabolite databases has improved the peak assignment process; nevertheless a close examination of these databases suggest that they are often inadequate for a complete and unambiguous assignment, particularly for molecules that are at low abundance [15].

^{1}H NMR data is by far the most prevalent in NMR-based metabolomics research because of its high sensitivity arising from the high isotopic abundance of ^{1}H and its large gyromagnetic ratio. However, the molecular complexity of biofluids results in overlapping signals that makes peak assignment difficult. Additionally, urine pH and ion concentration has been shown to affect the chemical shift values of peaks, which can be reduced but not completely eliminated [16,17]. These chemical shift variations may potentially lead to errors in peak assignments; consequently it not uncommon to find incorrect peak assignments in the NMR literature. Despite these limitations, however, human urine has served as the sample of choice for numerous metabolomic investigations owing to its relatively ease of collection, low protein content that results in high resolution NMR spectra, and presence of a large number of metabolites in relatively significant concentrations [18]. As an example of the challenges in metabolite identification, consider the assignment of doublets that occur in the range of 1.20–1.25 ppm in the ^{1}H NMR spectrum. The relatively common metabolite 3-hydroxybutanoic acid is known to have a CH_3 (doublet) resonance in that region. However a detailed examination of this peak using selective TOCSY showed read peaks that were not consistent with 3-hydroxybutyrate. The standard approach for identifying small molecule metabolites includes the use of 1D as well as several 2D NMR experiments. However, this approach is challenging for the identification of unknown species in complex mixtures such

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as biofluids because of the degree of spectral overlap. Selective TOCSY can be used to improve the situation by minimizing sample complexity that leads to improved detection [19–21]. However, this approach is typically not sufficient to make an unambiguous assignment of an unknown species.

Owing to the previously outlined issues, a logical choice for simplifying a complex biological matrix such as urine, while concomitantly improving the concentration of analytes for NMR analysis, is chromatographic fractionation. HPLC-NMR methods have been utilized in several metabolomics studies in which the power of the two complimentary techniques has allowed for the characterization of both xenobiotic and endogenous metabolites [22–25].

We have previously demonstrated how hyphenated LC-NMR systems can be used to isolate drug metabolites prior to NMR acquisition with concomitant signal enhancement of up to 90-fold [26,27]. In this work, we apply HPLC and NMR techniques to identify a previously unknown endogenous metabolite, 4-deoxythreonic acid which is sometimes visible in the NMR spectra of urine and has been implicated in the occurrence of type 1 diabetes [31]. Selective TOCSY and two-dimensional experiments were utilized to elucidate the structure of the metabolite *ab initio*. Structural confirmation was obtained by synthesis of the compound followed by spiking experiments which gave a positive match with the peak present in the human urine fraction. The use of HPLC followed by NMR analysis is advantageous because it reduces the complexity of biofluids leading to the ability to detect low concentration metabolites unambiguously.

2. Experimental

2.1. Reagents

Deuterium oxide (D_2O, 99.9%) was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA). HPLC-grade acetonitrile (ACN, 99.8%), methanol (MeOH, 99.9%), acetone, butan-2-ol and formic acid (88%) were purchased from Mallinckrodt Baker, Inc. (St Louis, MO) Sodium azide was obtained from Fisher Scientific (Pittsburgh, PA). Crotonic acid, N-methylmorpholine-N-oxidedihydrate, sodium dithionite, ethyl acetate, sodium 3-trimethylsilyl (2, 2, 3, 3-$^2H_4$)-1-propionate (TSP), osmium tetraoxide (2.5%) were obtained from Sigma Aldrich (St Louis, MO). All compounds were used without additional purification. Deionized water was obtained from an EASY pure II UV water purification system (Barnstead International, Dubuque, IA).

2.2. Urine collection and purification

Urine samples were collected from three healthy donors. Sodium azide (0.1%, w/v) was added to the freshly collected urine to prevent bacterial growth, and samples were then purified by centrifugation using Centriprep filters with a nominal molecular weight limit of 10,000 (cat. no. 4321, Millipore, Bedford, MA). The following standard procedure was used: urine samples were pipetted into two filters (15 ml each); the filters were spun at 4000 rpm for 30 min, and the supernatant was then collected. Purified samples were stored at −80 °C. Urine samples were lyophilized to concentrate them by a factor of 6 prior to HPLC separations. All urine samples were collected and processed according to an approved IRB protocol at Purdue University.

2.3. HPLC separation and fractionation

The HPLC system was composed of an LC-10AS Pump and SCL-10A System Controller (Shimadzu Corporation, Kyoto, Japan), 6-port injection valve (Rheodyne, CA, USA), and SPD-10A UV-vis Detector (Shimadzu Corporation, Kyoto, Japan). Fused silica tubes, 125 μm ID, and stainless steel fittings were used as the transfer lines and connectors, respectively (Upchurch Scientific, WA). The HPLC system was operated using Shimadzu EZStart 7.2 software. The analytical separation was performed on a 150 mm × 4.6 mm Hypersil Gold AQ C18 column and a 250 mm × 2.1 mm Beta Basic-18 (Thermo Electron Corporation, MA) connected in series in order to offer a higher loading capacity. The following gradient elution protocol was utilized: 60% (H_2O, 0.1% formic acid)/40% CH_3OH (25 min) ⇒ linear ramp to 20% (H_2O, 0.1% formic acid)/80% CH_3OH (55 min) ⇒ 95% (H_2O, 0.1% formic acid) 5% CH_3OH (10 min). The flow rate was 250 μL/min and a 500 μL injection volume was used. Fractions were collected over 2 min time intervals using a Gilson FC-203B fraction collector. Collected fractions were dried with N_2 gas.

2.4. 1D NMR spectroscopy

The samples for NMR analysis were prepared by reconstituting the dried HPLC fractions in 550 μL of phosphate buffer in D_2O. All samples were prepared in 5-mm tubes with 50 μM TSP for locking and chemical shift referencing, respectively. NMR spectra were obtained on a Bruker Avance DRX 500 MHz spectrometer, equipped with a 5 mm TXI triple resonance z-gradient cryoprobe. All spectra were acquired at room temperature, and were referenced to the TSP methyl peak at 0.00 ppm. Proton spectra were acquired using a 1D NOESY (NOESYPR.1D) pulse sequence incorporating presaturation for residual water suppression during the relaxation delay and mixing times. The relaxation delay and mixing times were set to 2 s and 300 ms, respectively, with a presaturation power of 50 dB in order to achieve complete water peak saturation. 64 FID transients were averaged, resulting in a total acquisition time of 5.15 min. Selective TOCSY experiments utilized the standard pulse sequence, SELDIG.PPR obtained from the pulse program library of Bruker XWINNMR. This consisted of a hard 90° pulse-z-gradient-selective 180°pulse-z-gradient train to achieve selective excitation of the target peak at 1.23 ppm, followed by a DIPSI-2 spin lock. Gaussian-shaped pulsed z-field gradients were 2 ms in duration. The duration of the shaped pulse was 50 ms and the TOCSY mixing time was 60 ms. A total of 256, 16K point FID transients were averaged in each selective TOCSY experiment, resulting in an acquisition time of 45.41 min. Line broadening of 0.10 Hz was used in processing the data.

2.5. 2D NMR spectroscopy

Following the identification of the fraction of interest using 1D proton NMR, several two-dimensional experiments were performed, including ^1H–^1H double quantum filtered correlation spectroscopy (DQF-COSY), ^1H–^1H total correlation spectroscopy (TOCSY), sensitivity enhanced heteronuclear and multiplicity edited ^1H–^{13}C heteronuclear single quantum correlation (edited HSQC) and ^1H–^{13}C gradient enhanced heteronuclear multiple bond correlation (HMBC) experiments. For the DQF-COSY experiments, a sweep width of 5483 Hz was used in both dimensions, 512 t_1 increments were acquired and zero filled twice to make 2048 spectral data points in each dimension. The number of transients per t_1 increment was 32, and the relaxation delay was 3 s. Phase sensitive data were obtained using the TPPI method. 2D-TOCSY data were obtained in echo-antiecho mode. A spectral width of 5485 Hz was used in both dimensions. The number of t_1 increments was 512, and 32 transients (each 2048 data points) were acquired per t_1 increment. Both ^1H–^{13}C multiplicity edited HSQC and HMBC experiments were performed with spectral widths of 5483 Hz and 29,000 Hz in the ^1H and ^{13}C dimensions, respectively. The number of t_1 increments was 256 and 128 transients (each 2048 data points)

were acquired per t_1 increment. The recycle delay was set to 1.5 s. Phase-sensitive data for the edited HSQC experiment was obtained using echo-antiecho mode. For the HMBC experiment, NMR data were obtained in magnitude mode without proton decoupling in the t_2 dimension. The resulting NMR data were zero filled to 1024 points in the t_1 dimension and double Fourier transformed after multiplying by a squared sine-bell window function shifted by $\pi/2$ along both dimensions.

2.6. Synthesis of 4-deoxyerythronic acid and 4-deoxythreonic acid

The target compounds were synthesized following the methods developed by Armstrong et al. [28] 4-deoxythreonic acid (compound **1**) was synthesized by the addition of an aqueous solution of osmium tetraoxide (2.5%, 3 μL) to a solution of crotonic acid (0.1 g) and N-methylmorpholine N-oxide dihydrate (0.2 g) in a mixture of water (400 μL), acetone (600 μL), and butan-2-ol (100 μL). The mixture was stirred overnight. Sodium dithionite, (0.02 g) was added, stirred for 2 min and extracted with diethyl ether (2 × 20 ml). The ethereal extract was concentrated under reduced pressure [28]. 4-Deoxyerythronic acid (compound **2**) was prepared by the addition of 30% hydrogen peroxide (1.4 equiv.) to a solution of crotonic acid (0.1 g) and 90% formic acid (500 μL). The mixture was stirred at 70 °C for 3 h and then left overnight at room temperature. The solution was then concentrated under reduced pressure.

3. Results and discussion

A typical urine spectrum can be seen in Fig. 1, with a number of doublets apparent in the 1.0–1.5 ppm region, as indicated in the expanded region. Of particular interest is the peak at 1.23 ppm, which is in the region where 3-hydroxybutyrate has been observed. However, a selective TOCSY experiment, using an excitation centered at 1.23 ppm (Fig. 2A) indicates that the species of interest is not 3-hydroxybutyrate shown in Fig. 2B. In order to identify this metabolite, it was necessary to fractionate and concentrate the urine sample prior to NMR analysis.

Fig. 3 shows a chromatogram of urine separated by reverse-phase chromatography. Even under optimum conditions it was impossible to obtain baseline separation of the region that extends from 12 to almost 40 min. In order to determine the region of interest, we collected eight urine fractions using 10 min time inter-

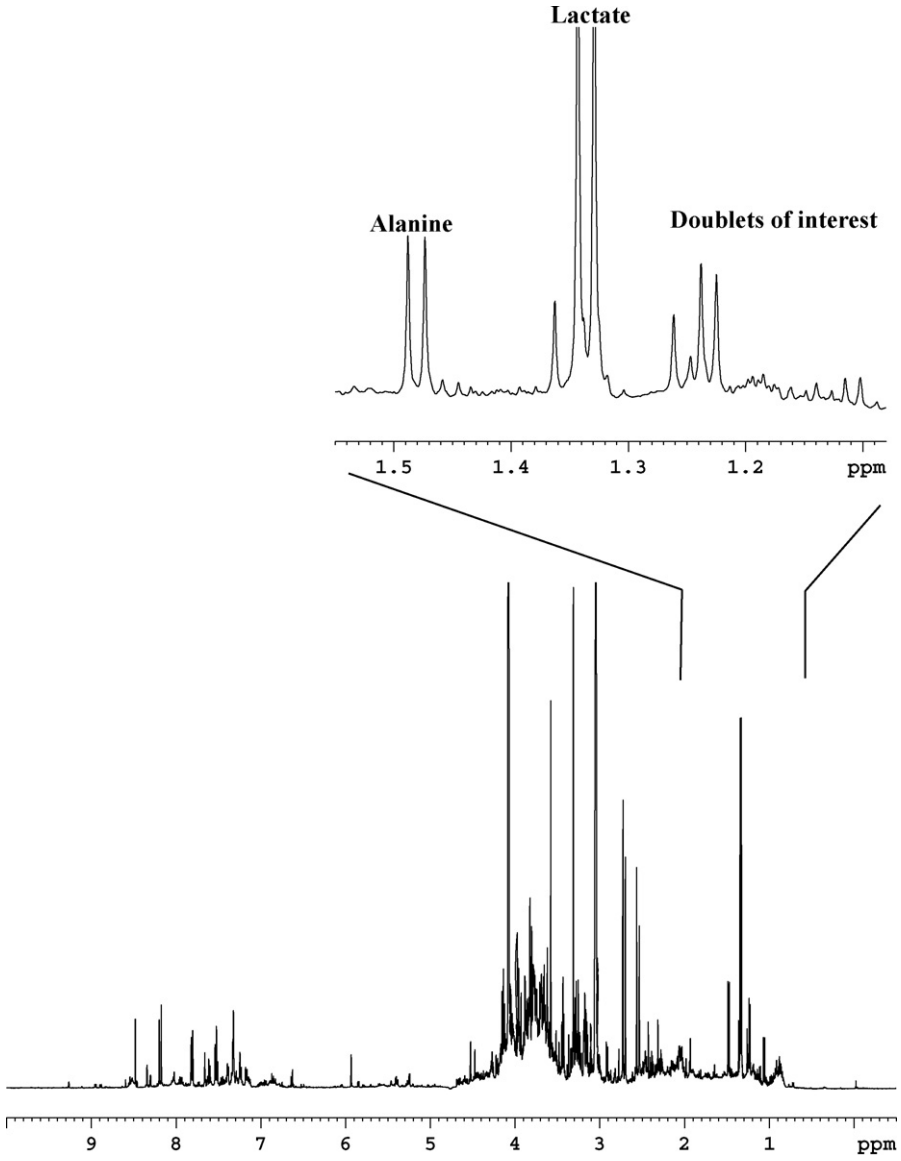

Fig. 1. NMR spectra of a normal human urine. Insert shows the expanded 1.1–1.5 ppm region where a number of doublets appear.

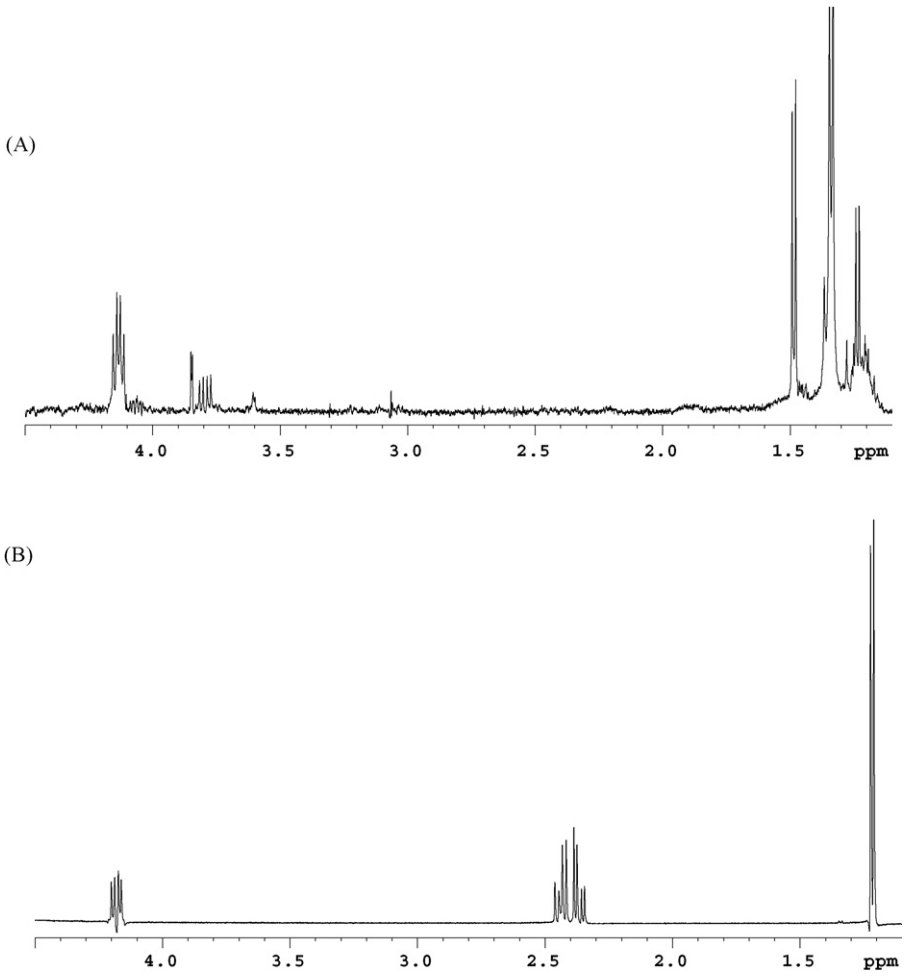

Fig. 2. (A) Selective TOCSY spectrum of region of interest from whole urine showing doublet at 1.23 ppm and a number of other doublets. (B) Selective TOCSY spectrum of 3-hydroxybutanoic acid.

vals corresponding to the entire span of the chromatogram. This procedure was repeated five times using an injection volume of 500 μL each. These fractions were dried and reconstituted in pH 7.0 phosphate buffer. ^{1}H NMR spectra were collected for the individual fractions in order to identify the region that contained the metabolite. From this process it was determined that the first fraction contained the metabolite of interest. The chromatography was then repeated, but this time 2 min collection intervals were used to sub divide the first fraction. A total of 4 sub-fractions were collected, from which it was again determined that the second sub-fraction contained the highest concentration of the unknown metabolite of interest. Even though highly concentrated metabolites such as lactate and alanine co-eluted with the analyte of interest, the NMR spectrum of this fraction (shown in Fig. 4A) was much simpler than the original (whole) urine spectrum of Fig. 1, and resulted in an improved spectral resolution. Consequently, all subsequent NMR experiments were performed using this sub-fraction.

A selective TOCSY pulse sequence was utilized to irradiate the doublet at 1.23 ppm in order to determine the corresponding proton signals that are associated with the unknown molecule. It can be observed from Fig. 4B that the selective TOCSY read peaks are comprised of a doublet at 3.85 ppm and a multiplet at 4.06 ppm. This result confirmed that indeed the peak of interest was not that of 3-hydroxybutanoic acid, which has a doublet at 1.20 ppm and two multiplets at 2.31 ppm and 4.13 ppm.

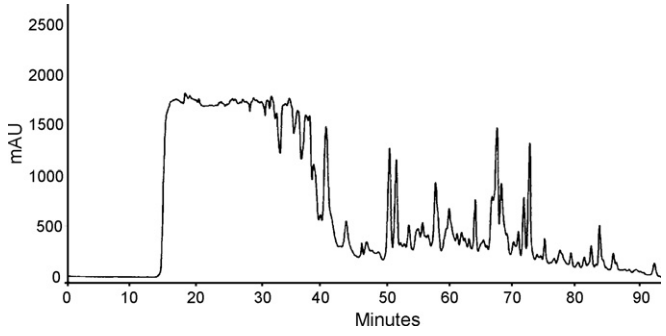

Fig. 3. Chromatogram of the urine separation under reverse-phase conditions.

3.1. Structure elucidation

A CH–CH–CH_3 fragment of the metabolite was tentatively identified by tracing the cross peak positions along both frequency dimensions in the DQF-COSY and TOCSY spectra, taking into account the signals in the 1D ^{1}H NMR spectra. Multiplicity edited HSQC and HMBC spectra facilitated the assignment of the carbon chemical shifts for the directly attached protons as well as the carbon multiplicity. The three proton attached carbons (see Fig. 5 and Table 1) were identified in the HSQC spectrum based on the corresponding proton chemical shifts obtained from the analysis of the DQF-COSY spectrum. The sign of all the three cross peaks in the HSQC spectrum was negative thus confirming that

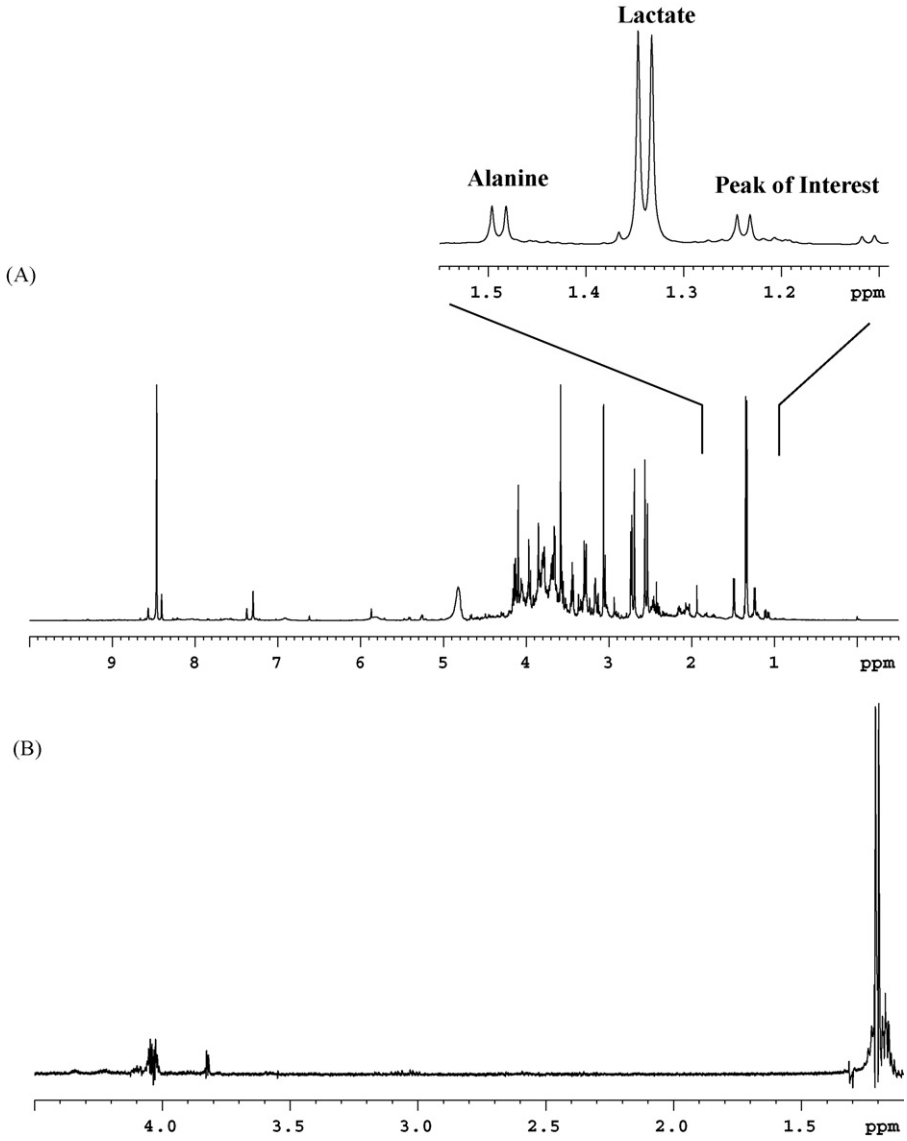

Fig. 4. (A) 1D ^{1}H NMR spectrum of the urine fraction of interest (i.e. containing an unknown doublet) showing the expanded view of the region containing the unidentified peaks of interest. (B) Selective TOCSY spectrum of the molecule of interest showing all read peaks. The excitation was centered at 1.23 ppm.

all the protonated carbons belong to CH or CH_3 groups. The long-range couplings among CH and CH_3 groups observed in the HMBC spectrum matched with the CH–CH–CH_3 moiety of the metabolites. From these assignments, the tentative proton assignments of the DQF-COSY spectra were confirmed. However the carboxylic acid carbons were not readily observable in both HSQC and HMBC spectra. This is likely due to issues arising from the relatively low concentration of the metabolite. Nevertheless, based on the observable signals, the molecule was predicted to be 2,3-dihyroxybutanoic acid.

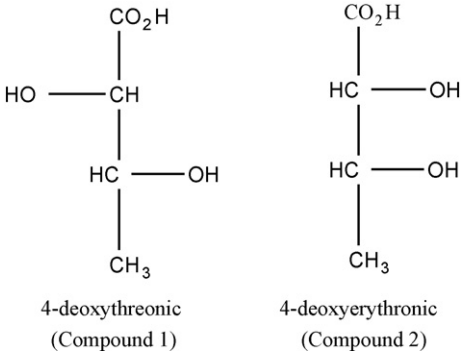

Fig. 5. Structures of the two possible diastereomers for 2,3-dihydroxybutanoic acid. Deoxythreonic acid is labeled as compound **1** while deoxyerythronic acid is labeled as compound **2**.

3.2. Structural confirmation by spiking of the synthesized molecule

In order to confirm our findings, we used two previously published protocols for the synthesis of the two possible diastereomers for the predicted molecule [28]. The 1D ^{1}H NMR of both molecules showed an interestingly close similarity for the two possible diastereomers of this molecule in terms of the distribution of the signals. We could use NMR to differentiate the two molecules owing to the differences in chemical shifts and scalar coupling constants from the 1D ^{1}H NMR spectra. Other approaches would have required tedious and possibly time-consuming chromatographic separation in order to resolve the two molecules [29,30].

Table 1
^{1}H and ^{13}C chemical shift assignments for the two isomers. Proton and carbon NMR spectra were referenced to the TSP peak at 0.00 ppm. All samples were measured at biological pH 7.0.

Compound **1** (4-deoxythreonic acid)			Compound **2** (4-deoxyerythronic acid)		
Carbon/proton type	^{1}H chemical shift (ppm)	^{13}C chemical shift (ppm)	Carbon/proton type	^{1}H chemical shift (ppm)	^{13}C chemical shift (ppm)
COOH	–	178.68	COOH	–	175.36
CH	3.85	75.91	CH	4.22	74.12
CH	4.06	68.57	CH	4.08	68.30
CH_3	1.24	18.26	CH_3	1.14	16.18

To demonstrate that we were actually observing compound **1** and not compound **2** in the urine fraction sample, a selective TOCSY was performed by irradiating the doublet at 1.23 ppm before and after spiking urine fraction with an aliquot of the synthesized product. As can be seen in Fig. 6B, the selective TOCSY spectrum showed a close similarity for the read peak spectrum with that of Fig. 4B, which shows the spectrum before spiking, and hence provides strong structural confirmation. These results were further confirmed by performing DQF-COSY and HMBC experiments (see supplemental information Figures S1 and S2) for both isomeric synthetic products. The results are summarized in Table 1, in which we provide a complete list of ^{1}H and ^{13}C chemical shifts for both metabolites. This is the first report of the complete NMR chemical shifts of this metabolite in human urine. A further step would be to resolve the actual stereochemistry at the two chiral carbon atoms, and potentially the relative enantiomeric ratios in the urine samples. This could be accomplished using Mosher ester analysis [29]. However, given the low concentration of the metabolite observed in urine, and the time consuming nature of the experiments we have chosen to leave these determinations to future work.

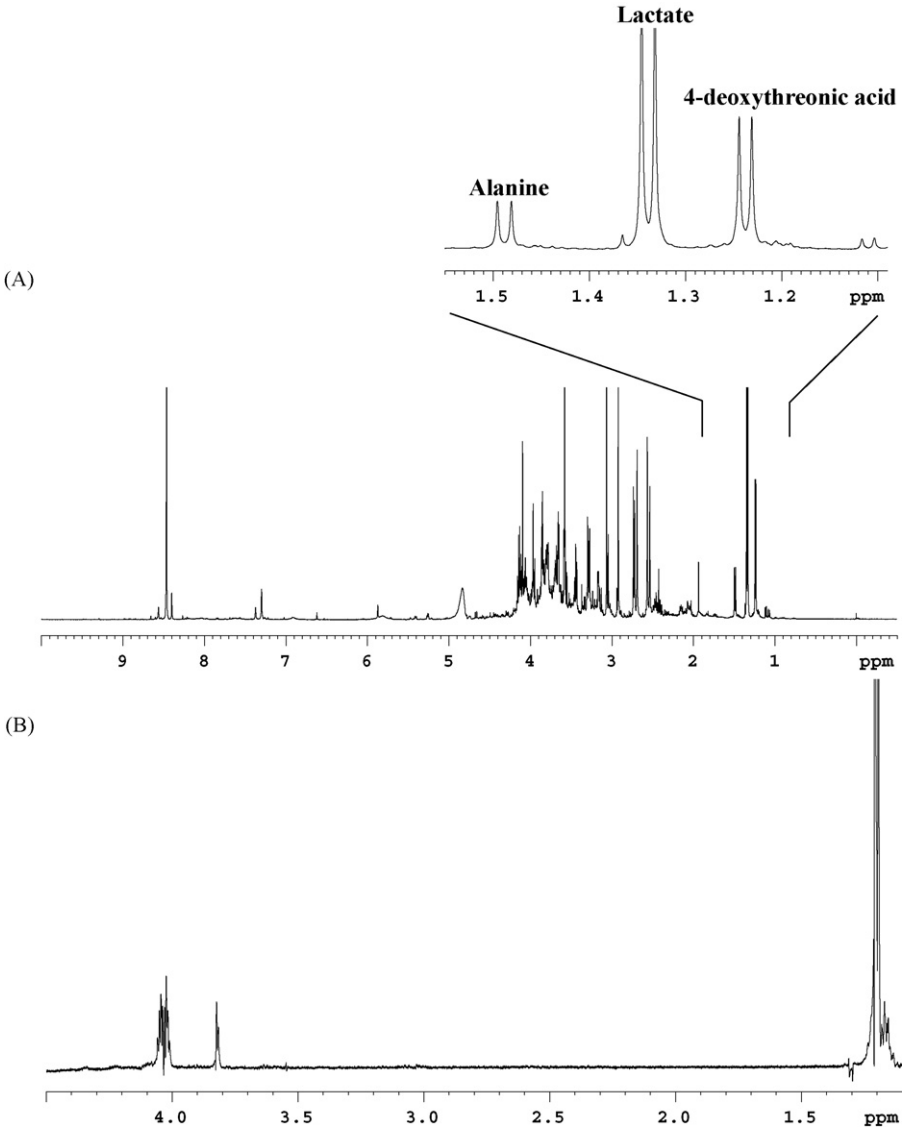

Fig. 6. (A) 1D ^{1}H NMR spectrum of a urine fraction spiked with synthesized compound **1** and an expanded view showing a difference in the intensity of the peak of interest as compared to Fig. 4A. (B) Selective TOCSY spectrum (excitation centered at 1.23 ppm) after spiking. The read peaks are very consistent with those observed before spiking in Fig. 4B. Spectrum (B) was plotted with the same scale as in Fig. 3 in order to allow for direct comparison of their intensities.

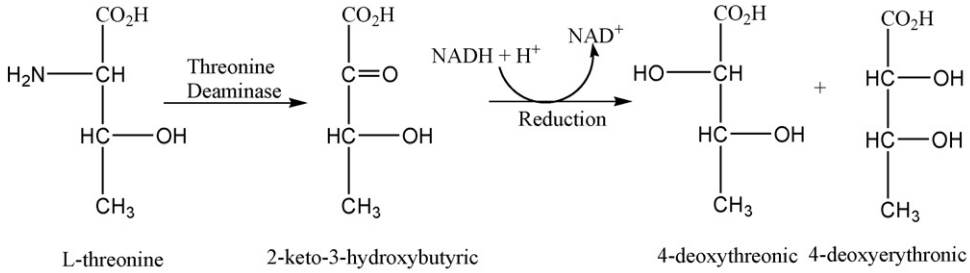

Fig. 7. (A) Metabolic pathway showing the production of the two possible isomers of 2,3-dihydroxybutanoic acid (compound **1** and compound **2**). The latter was not observed by NMR in the urine samples.

The two molecules, compound **1** and compound **2**, have been previously reported as potential biomarkers for type 1 diabetes using hyphenated GC-MS techniques [30–32]. A pathway for the likely production of these two metabolites has been postulated using an animal model, in which it was suggested that these molecules were products of L-threonine metabolism. L-Threonine is a ketogenic amino acid, hence its catabolism yields products that are able to enter into energy producing metabolic pathways such as the Krebs cycle [33]. Threonine has three major routes of degradation, two of which produce acetyl-COA that is a major source of carbon atoms in the Krebs cycle, while the third route produces a precursor for the formation of isoleucine [32]. Despite the knowledge of these metabolic routes, there exists the possibility of another metabolic pathway involving threonine as its source that is expected to produce 4-deoxythreonic acid [31]. This involves a deamination step that is catalyzed by the enzyme threonine deaminase which results in the production of 2-keto-3-hydroxybutytrate followed by the action of a reductase to form compound **1** or compound **2** (Fig. 7). We did not observe signals arising from compound **2** in our data, however. This could be due to a stereo-specificity of this pathway in human metabolism. Alternatively, perhaps its production in a relatively healthy human subject results in quantities that are well below NMR detectable levels.

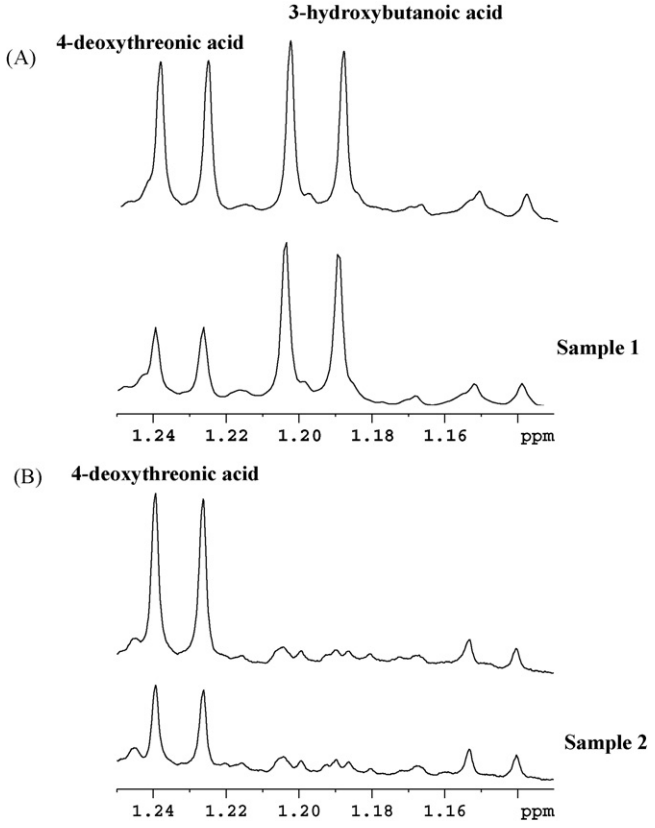

Fig. 8. ^{1}H NMR spectrum of urine obtained from (A) donor 1 and (B) donor 2, respectively. Spectra were obtained before (lower) and after (upper) spiking with compound **1** for each donor.

In order to demonstrate that the identified metabolite was a normal and endogenous human urinary metabolite, urine was obtained from two additional donors who have different dietary patterns. The 1D ^{1}H NMR spectrum of both samples showed the presence of compound **1** as can be seen in Fig. 8A and B. A broader study would be needed to establish the relationship between the up-regulation of compound **1** and diabetes. Additionally, owing to the issues that were encountered during the chromatographic method development process, we recommend that other chromatographic methods such as hydrophilic interaction chromatography (HILIC) be investigated, since it is expected to offer better separation of the highly polar metabolites that are normally found in urine samples [34]. We plan to investigate this approach in future metabolomic investigations using HPLC-NMR.

4. Conclusions

HPLC and NMR techniques were utilized to identify a previously unidentified human urine metabolite, 4-deoxythreonic acid, that is visible in some urine NMR spectra. The use of this approach further enhances the ^{1}H NMR spectral resolution of normal human urine. 4-Deoxythreonic acid appears to be a component in normal human urine; however, 4-deoxyerythronic acid is either absent or present in very low concentration. The approach used here may be further improved by the use of HILIC columns that are expected to provide better separation of polar metabolites. The identification of 4-deoxythreonic acid is expected to provide the basis for further studies involving larger sample sets in order to establish its possible role as a disease marker.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2009.06.007.

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