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Stable ^{18}O Labeling Method for Stercobilin and Other Bilins for Metabolomics

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Abstract

Rationale—Bilin tetrapyrroles including stercobilin are unique to mammalian waste; they have been used as markers of source water contamination and may have important diagnostic value in human health conditions. Unfortunately, commercial isotopomers for bilins are not available. Thus, there is a need for isotopomer standards of stercobilin and other bilins for quantification in environmental and clinical diagnostic applications.

Methods—A procedure is described here using $H_2{}^{18}O$ to label the carboxylic acid groups of bilin tetrapyrroles. Reaction conditions as a function of temperature and reagent volume were found to produce a mixture of isotopomers, as assessed by electrospray ionization and Fourier transform ion cyclotron resonance mass spectrometry. Stability as a function of storage time and temperature and in conjunction with solid-phase extraction was assessed.

Results—The highest labeling efficiency was achieved at 70°C for 8 hours, while stable ratio of the isotopmers could be produced at 60 °C for 4 hours. The stability of the isotopic distribution was maintained under storage (room temperature or frozen) for 20 days. It was also stable throughout solid-phase extraction. The high mass accuracy and resolving power of Fourier transform ion cyclotron resonance mass spectrometry enables clear distinction between ^{18}O-labeled bilins from other unlabeled bilins present, avoiding a potential interference in quantitation.

Conclusions—A procedure was developed to label bilins with ^{18}O. The final ratio of the ^{18}O-labeled bilin isotopomers was reproducible and highly stable for at least 20 days under storage. This ratio was not changed in any statistically significant way even after solid phase extraction. Thus a reliable method for producing stable isotopomer ratios for bilins has been achieved.

Keywords

bilins; ^{18}O labeling; tetrapyrroles; stercobilin; ESI; FT-ICR; stable isotope dilution

Stercobilin ($C_{33}H_{46}N_4O_6$, CAS 34217-90-8) is a member of a biological class of mammalian metabolites that are tetrapyrroles called bilins. The bilins are present in both urine and feces. Stercobilin is a product of heme metabolism, formed from the degradation of bilirubin via gut flora.[1] Bilins including stercobilin are potentially useful for human waste detection in public swimming facilities, or as possible biomarkers for diagnostic

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methods.[2–3] Previous work within our group has examined the MS/MS fragmentation pathways of various bilins, including stercobilin, for utility in future metabolomic identification.[4]

The need to quantify stercobilin is motivated from previous research suggesting a depletion of stercobilin within urine samples from autistic children relative to controls.[5] The development of a method to isolate and quantify stercobilin with use of an isotopically-labeled standard would enable comparison of stercobilin levels in urine between subjects with autism and controls, and is an essential step in validation studies of urinary stercobilin for clinical diagnosis of autism.

Analytes may be quantified in mass spectrometry using one of a number of approaches including standard addition, isotopic dilution, and application of internal or external standards.[6] Each of these methods has its own advantages and disadvantages associated with its ability to quantify analytes of interest. Stercobilin standard is commercially available, and an external calibration or standard dilution curve could be constructed with this standard as a means to quantify its presence in biological matrixes. However, these approaches do not take into account possible handling errors during sample preparation or instrumentation errors. In order to take these errors into account, the use of an internal standard (either an isotopomer or an analyte of similar structure to the analyte being investigated) provides for a superior quantification.[7]

Stercobilin extraction from human feces was first performed by a series of several organic reactions that resulted in a low yield of the crystalline structure of stercobilin.[8–9]Recent research has also demonstrated analysis and separation of stercobilin and related tetrapyrrole compounds with high performance liquid chromatography (HPLC) and fluorescent spectroscopy techniques.[10–11] In a recent extensive review of various human urine metabolites, quantification of stercobilin was measured against the amount of creatinine present in the measured sample.[12] Creatinine is often used as a reference to quantify various urinary metabolites in order to take into account the volume of urine expelled during collection.

One method to isotopically label a compound of interest is by substituting an ^{16}O atom for an ^{18}O. One way that this is done in proteomics is by performing a trypsin digest in an $H_2{}^{18}O$ enriched environment to create ^{18}O labeled peptides.[13–14] However, stercobilin is not a protein that can undergo such an enzymatic hydrolysis to incorporate ^{18}O. Instead, small molecules and metabolites such as stercobilin undergo an esterification reaction in which the oxygen atoms available from the carboxylic acid group can be exchanged from ^{16}O to ^{18}O.[15–16] The advantage to using ^{18}O for isotope labeling, in comparison to deuterium labeling, is that a single oxygen exchange will provide a 2 Da mass shift. This is the strategy employed here to produce an ^{18}O-labeled isotopomer of stercobilin.

EXPERIMENTAL

Materials

The following materials were used in the synthesis of ^{18}O labeled stercobilin and determination of stability studies: stercobilin hydrochloride (Frontier Scientific, Logan, UT), isotopically labeled water (^{18}O, 97%) (Cambridge Isotope Laboratories, Inc., Tewksbury, MA), methanol, HPLC grade ≥99.9% (Sigma-Aldrich, St. Louis, MO), acetonitrile, HPLC grade ≥99.9% (Sigma-Aldrich, St. Louis, MO), ammonium hydroxide (J.T. Baker, Central Valley, PA), trifluoroacetic acid (TFA) and formic acid (FA, Fisher Scientific, Fair Lawn, NJ), water filtered using Thermo Barnstead Nanodiamond™ filtration system (San Jose, CA), and Oasis © MAX 1cc 30mg extraction cartridges (Waters, Milford, MA).

Instrumentation

All samples were analyzed using a Hewlett-Packard 1050 series HPLC (Waldbronn, Germany) coupled to a 12T Bruker Solarix Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Billerica, MA). Mobile phase consisted of 20:80 (v/v) acetonitrile (ACN):H_2O with 0.1% FA. Flow rate was set to 100 μl/min and sample injection size was 20 μL. All samples were analyzed with electrospray ionization (ESI) in positive ion mode using a 2M word data set for high resolution. All samples were injected five times for statistical purposes.

Method Development

Reaction Time Dependence—Method development for this procedure was initially based from work performed by Bergmann to create an isotopic standard for Fumonisin B_1.[16] The following reaction conditions were compared for optimizing ^{18}O labeling: reaction time, incubation temperature conditions and volume of $H_2{}^{18}O$. Reproducibility in labeling efficiency was evaluated by highest yield of isotopically-labeled compounds with an acceptable (≤ 10%) RSD, with the goal being that a satisfactory condition could be developed in under eight hours. Each optimization method was performed three times for comparison of reproducibility. For the first set of experiments, ca. 5×10^{-10} moles of stercobilin was reacted with $H_2{}^{18}O$ with freshly prepared 1 μL 5%(v/v) TFA in 5 μL of $H_2{}^{18}O$ in a 300 μL glass vial insert loaded into Agilent autosampler vials and screw cap lids and incubated in a water bath at 50°C for either 4 or 8 hours. Eventually, higher reproducibility was found using 10 μl of $H_2{}^{18}O$ (see below). Following the reaction, samples were dried using N_2 gas and reconstituted with 200 μL of 20:80 (v/v) ACN:H_2O. Samples were then measured on the FT-ICR to determine the ratio of each oxygen labeled species.

Reaction Temperature and Volume Dependence—A temperature and volume dependence study was conducted to determine if increasing the incubation temperature or increasing the amount of $H_2{}^{18}O$ reactant would increase the ratio of ^{18}O labeled stercobilin species. Each sample contained ca. 5×10^{-10} moles of stercobilin in 1 μL in 5% (v/v) TFA solution. To this, 5 μL of $H_2{}^{18}O$ was added, capped and incubated for 4 or 8 hours at either 50°C, 60°C or 70°C. Following the reaction, samples were dried using N_2 gas and reconstituted with 200 μL of 20:80 (v/v) ACN:H_2O. For the volume dependence study, either 5 μL or 10 μL of $H_2{}^{18}O$ was reacted at 60°C or 70°C for 4 or 8 h.

Storage Stability Study—The stability of the ^{18}O labeled stercobilin product was measured under different storage conditions. In both conditions, ^{18}O labeled stercobilin was kept in 1 mL of 20:80 (v/v) ACN:H_2O storage solution. The two samples to be measured were either stored at room temperature (~25°C) or at −4°C. Samples were measured using FT-ICR coupled with HPLC; the mobile phase was 20:80 ACN:H_2O with 0.1% formic acid, flow rate was set at 100 μL/min with a 20 μL sample injection. Sample measurements were repeated five times on each sampling day. The ratio of each species was then calculated to compare any back labeling exchanges (^{18}O exchanging back to ^{16}O).

SPE Stability Study—To determine if any back labeling exchange occurs during SPE, a sample of 2ppb $^{18}O_3$ stercobilin (from 60 °C, 4 hr exchange) is made in 1 mL 20:80 ACN:H_2O. A second sample is made of the same concentration and is processed through the following SPE analysis using a Waters Oasis© MAX cartridge: 1) conditioned with 3mL of methanol, 2) equilibrated with 3 mL of H_2O, 3) sample loading (~1 mL/min), 4) sample elution with 1.2 mL 5% (v/v) formic acid in methanol, 5) dried down with N_2 gas and reconstituted in 1 mL of 20:80 ACN:H_2O. Both samples were measured using the FT-ICR via 20 μL direct injection. Samples were compared to one another using the relative ^{18}O labeled ratios (m/z 595/597/599/601/603).

Urinalysis—Urine samples collected from volunteers were analyzed via SPE and mass spectrometry to determine concentration of stercobilin. Aliquots containing 1mL of urine were diluted with 5mL of water and spiked with 2 ppb of ^{18}O labeled-stercobilin from the 70 °C, 8 hour reaction. SPE analysis was performed using Waters Oasis© MAX cartridge under the following conditions: 1) conditioned with 3mL of methanol, 2) equilibrated with 3 mL of H_2O, 3) sample loading (~1 mL/min), 4) sample elution with 1.2 mL 5% (v/v) formic acid in methanol, 5) dried down with N_2 gas and reconstituted in 1 mL of 20:80 ACN:H_2O. Urine samples were then analyzed using FT-ICR via 20 μL direct injection. The optimized procedure for quantification of bilins in urine is outlined in Scheme 1.

Data Analysis—All data analysis was performed using Bruker Data Analysis software for extracting mass spectra and relative peak intensities for the isotopically labeled species. Statistical analysis and data graphing was performed using SigmaPlot© software.

RESULTS AND DISCUSSION

Reaction Time Dependence

Table I summarizes the results for each reaction condition tested. Figure 1 a and b are the typical positive ion mode ESI FT-ICR mass spectra of stercobilin in the region around the $(M+H)^+$ ion before (a) and after (b) isotope labeling. The standard deviations for the percentage of each ^{18}O labeled species is also given in Table I under each condition used. With regards to the incubation time-dependence study performed at 50 °C, as shown in Table I, allowing stercobilin to react for 8 hours versus 4 hours results in a smaller percentage of the starting stercobilin material at *m/z* 595 (4.0% vs. 10.6%), and there is a greater ratio of three- and four-oxygen labeled species (601 and 603 *m/z* respectively); after 4 hr, the $^{18}O_3$ is 29.7% and becomes 35.4% after 8 hr, and likewise after 4 hr the $^{18}O_4$ is

17.7% and increases to 28.8% after 8 hr; thus, after 8 hr, an overall labeling efficiency of 68.7% is obtained . Additional parameters including temperature and volume of $H_2{}^{18}O$ were monitored to determine if improved ^{18}O labeling can occur using higher reaction temperature or with higher volumes of $H_2{}^{18}O$.

Reaction Temperature and Volume Dependence

As shown in Table I, labeling stercobilin at either 60°C or 70°C at 4 hr or 8 hr produced a greater level of the higher ^{18}O labeled species. While the initial amount of stercobilin present is the same in all cases, the reaction with the maximum labeling occurs using 10μL of $H_2{}^{18}O$ (vs. 5μL) at 70°C for 8 hr.. As shown in Table I, under these conditions, the labeling extent was ca. 2.3% $^{18}O_0$:8.2% $^{18}O_1$:20.6% $^{18}O_2$:36.8% $^{18}O_3$:32.7% $^{18}O_4$, thus an overall labeling efficiency of 72.6%. This is modestly higher than at 50 °C for an 8 hr reaction using only 5 μL of $H_2{}^{18}O$. As noted from Table I, the highest reproducibility in labeling occurs for reactions taking place at 60 °C for 4 hr with 10μL $H_2{}^{18}O$, which was then used in the storage stability and SPE stability studies below. In general, there was better reproducibility when 10 μL of $H_2{}^{18}O$ was used instead of 5 μL

Storage Stability Study

Figure 2 a and b show the results of the storage stability under room temperature and −4 °C storage conditions, respectively. At day 20 for the storage conditions at −4°C, there is a larger standard deviation compared to shorter storage times. These measurements were then compared to day 11's measurements using a t-test at 95% confidence interval. Based on the calculated results at the 95% confidence interval, there is no statistical change in the ratio of each ^{18}O labeled species over the 20 day time period. These reported results compare to the work performed by Bergmann, in which their ^{18}O labeled fumonisin B_1 was stable for 112 days stored in 60:40 ACN:H_2O at 4°C.[16] As part of the future work for this project, additional data points for long term storage stability need to be made to determine the absolute longest storage time capabilities. Nevertheless, it is clear that the isotopic labeling ratio of stercobilin is stable over a 20 day period.

SPE Stability Study

A final study to determine the stability and suitability of the ^{18}O labeled stercobilin as a viable surrogate standard during solid phase extraction (SPE) was conducted. One potential problem in using ^{18}O labeled stercobilin is that the labeled oxygen atoms are located on a labile position, so there is the potential for back exchange to occur during SPE. Although the SPE method occurs at room temperature, well below the incubation temperature needed to label stercobilin, two steps in the SPE method occur under basic and acidic conditions, which may induce back labeling of the ^{18}O labeled species. A t-test was performed at 95% confidence level to determine if SPE causes any back exchange by examination of the relative ratios of the unlabeled, singly-, doubly-, triply-, and quadruply-labeled isotopomer whether SPE has been performed or not. As shown in Table II, each calculated t-value was lower compared to that of the t-table for every isotopic species; thus each value measured is not statistically different whether or not SPE is employed. Therefore, back labeling does not occur to any statistically significant extent during the SPE process. This is reassuring

because SPE may need to be employed for quantification of stercobilin with biological specimens.

Urinalysis

A urinalysis study was preformed to utilize the isotopically labeled stercobilin for quantification of stercobilin in the urine samples. Our goals are to use this method for quantification of stercobilin in control versus autistic urine samples as a means to determine autism presence in future subjects. Using two female (F) and three male (M) subjects (Health Sciences Institutional Review Board #621930), samples were prepared and analyzed as described in the methods section of this paper. Stercobilin in the urine samples was quantified using the $^{18}O_4$-stercobilin peak at *m/z* 603. Table III reports the quantified stercobilin levels found in each subject's urine. These preliminary results demonstrate that on average, females excrete a lower amount of stercobilin in comparison to males. However, further examination with a larger sample population will be performed to determine average amounts of stercobilin in female and male populations.

Benefit for high mass spectrometry resolving power in bilin labeling

One particular benefit for the project is the use of high resolution mass spectrometry. It is very imperative, especially with the isotopic labeling, that the *m/z* peaks are analyzed using high resolution. FT-ICR MS has the benefit of having resolving power capabilities of 100,000 FWHM or more.[6] During the isotopic labeling reaction of stercobilin-^{16}O to stercobilin-$^{18}O_n$, there is concurrent isotopic labeling of i-urobilinogen ($C_{33}H_{44}N_4O_6$, CAS 14684-37-8). I-urobilinogen (sometimes also known as mesobilirubinogen) is structurally similar to stercobilin, and possesses two carboxylic groups that are available for ^{18}O exchange. Furthermore, because of its close structural similarity to stercobilin, urobilinogen is present in the commercial standard and cannot be easily removed. Figure 3 compares the structural similarities of stercobilin (A) to urobilinogen (B). After the ^{18}O exchange, there is residual stercobilin that has not been labeled with a corresponding *m/z* of 595.34901. When urobilinogen has one ^{18}O exchange, its corresponding *m/z* is 595.330331. The mass difference between unlabeled stercobilin and urobilinogen-$^{18}O_1$ is 18.7 mDa. To resolve these two peaks requires an instrument capable of a resolving power at 31,900. FT-ICR MS is one of a few instruments that have this capability, along with Orbitraps, reflectron time-of-flight, and magnetic sector mass analyzers. As shown in Figure 4, these two peaks are distinguished with a resolving power of 161,000. The high resolving power paired with high mass accuracy allows us to *unequivocally* denote an *m/z* peak to a particular compound. Thus, when analyzing urine, feces or pool water samples containing stercobilin, with FT-ICR MS it will be simple to distinguish the urobilinogen-$^{18}O_1$ peak from unlabeled stercobilin.

CONCLUSIONS

We have developed a method for labeling stercobilin using $H_2{}^{18}O$. Our results indicate that the method demonstrates high batch-to-batch reproducibility, as well as also high stability during storage and SPE processing. Using this labeled isotomer as an internal standard, future work will demonstrate its utility for quantification of stercobilin in various

environmental and biological matrices as an in-house prepared surrogate standard. This isotopic labeling method also has promise to produce other surrogate standards for similar bilin tetrapyrrole structures of importance in clinical diagnosis and environmental monitoring.

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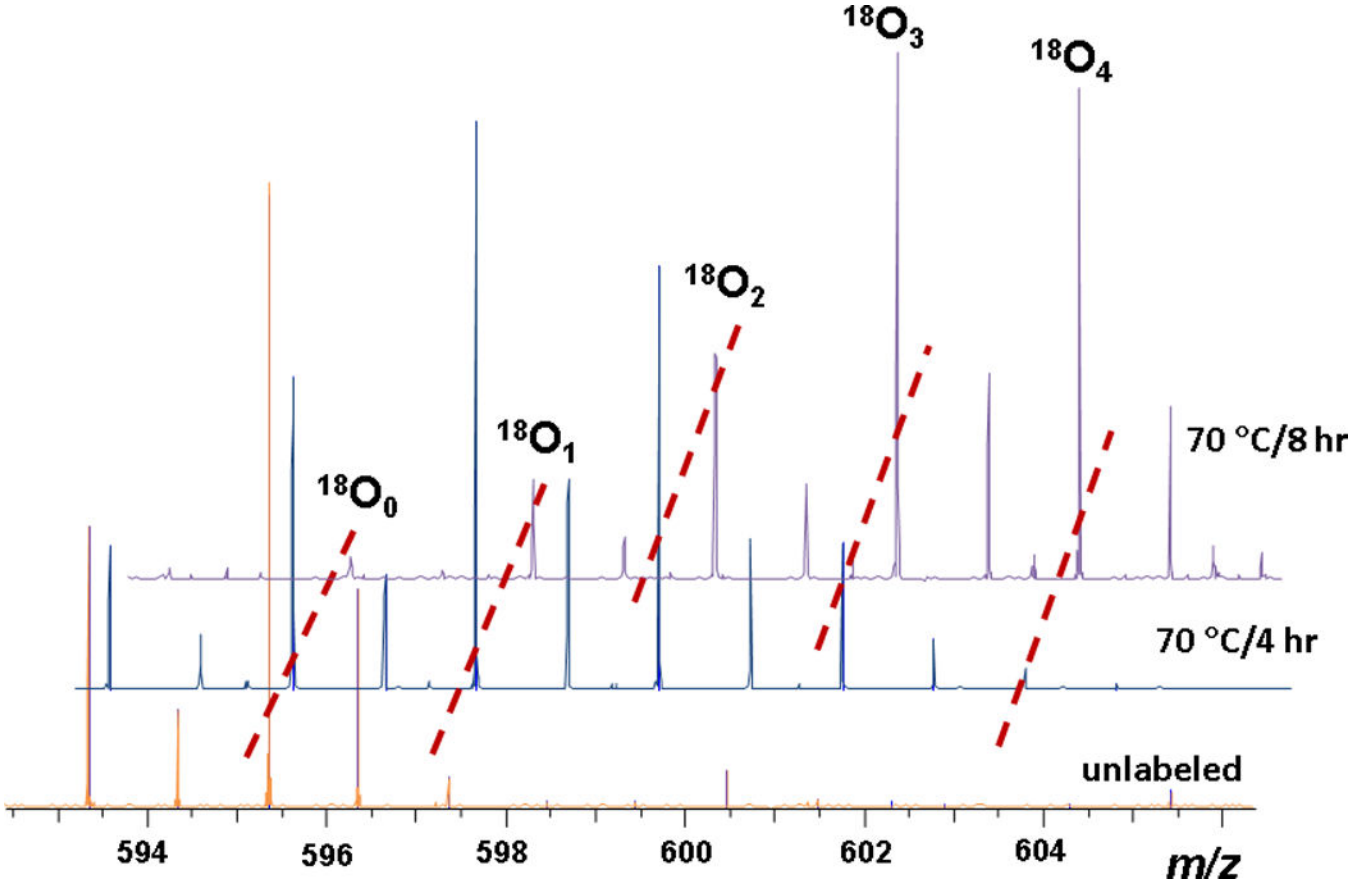


Figure 1.

Comparison of positive ion ESI-FT-ICR mass spectra of stercobilin before and after isotopic labeling at 70 °C..

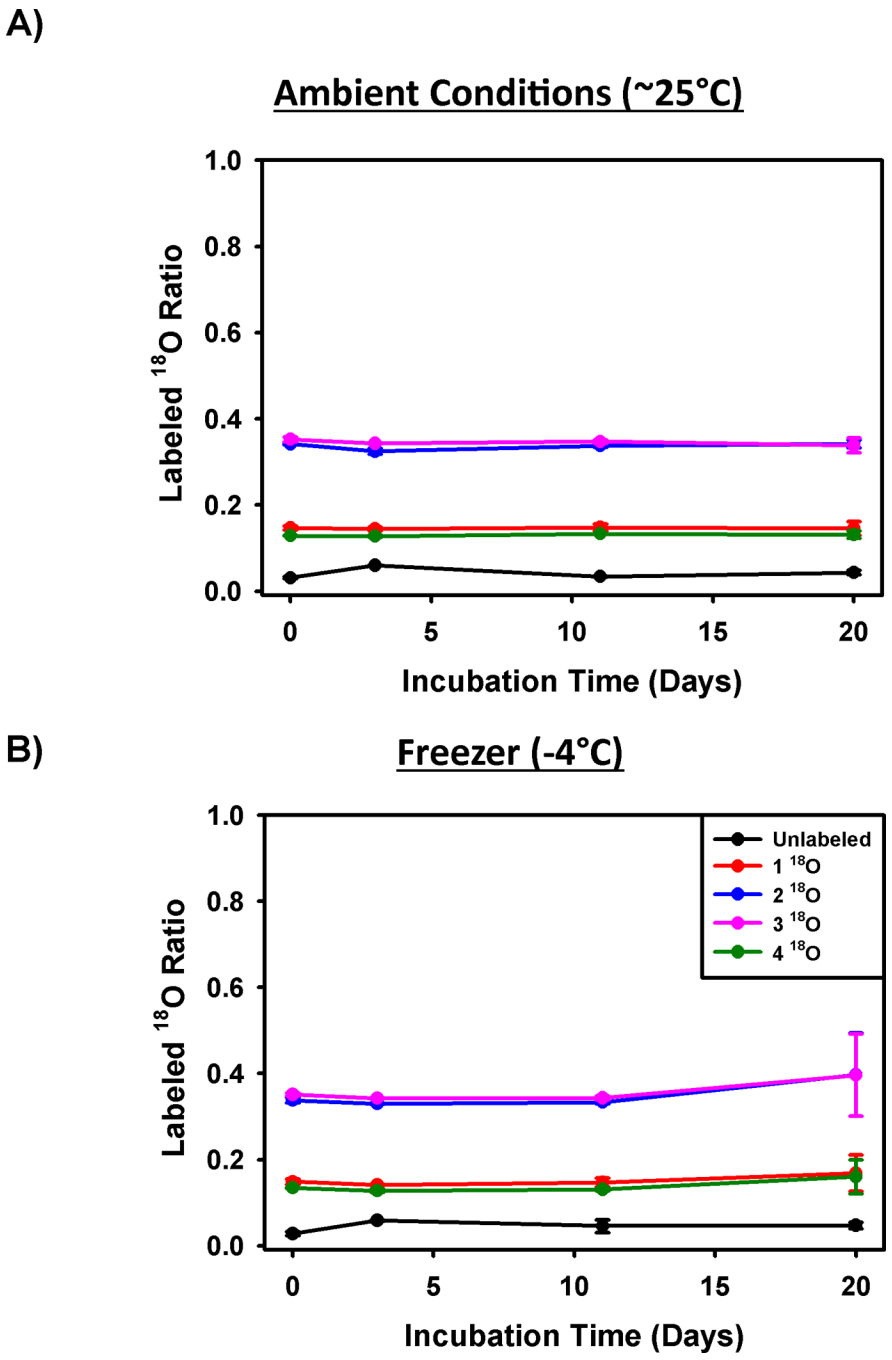


Figure 2.
Storage stability comparison of isotopically labeled stercobilin at room temperature (a) and at freezing (−4 °C) (b) conditions.

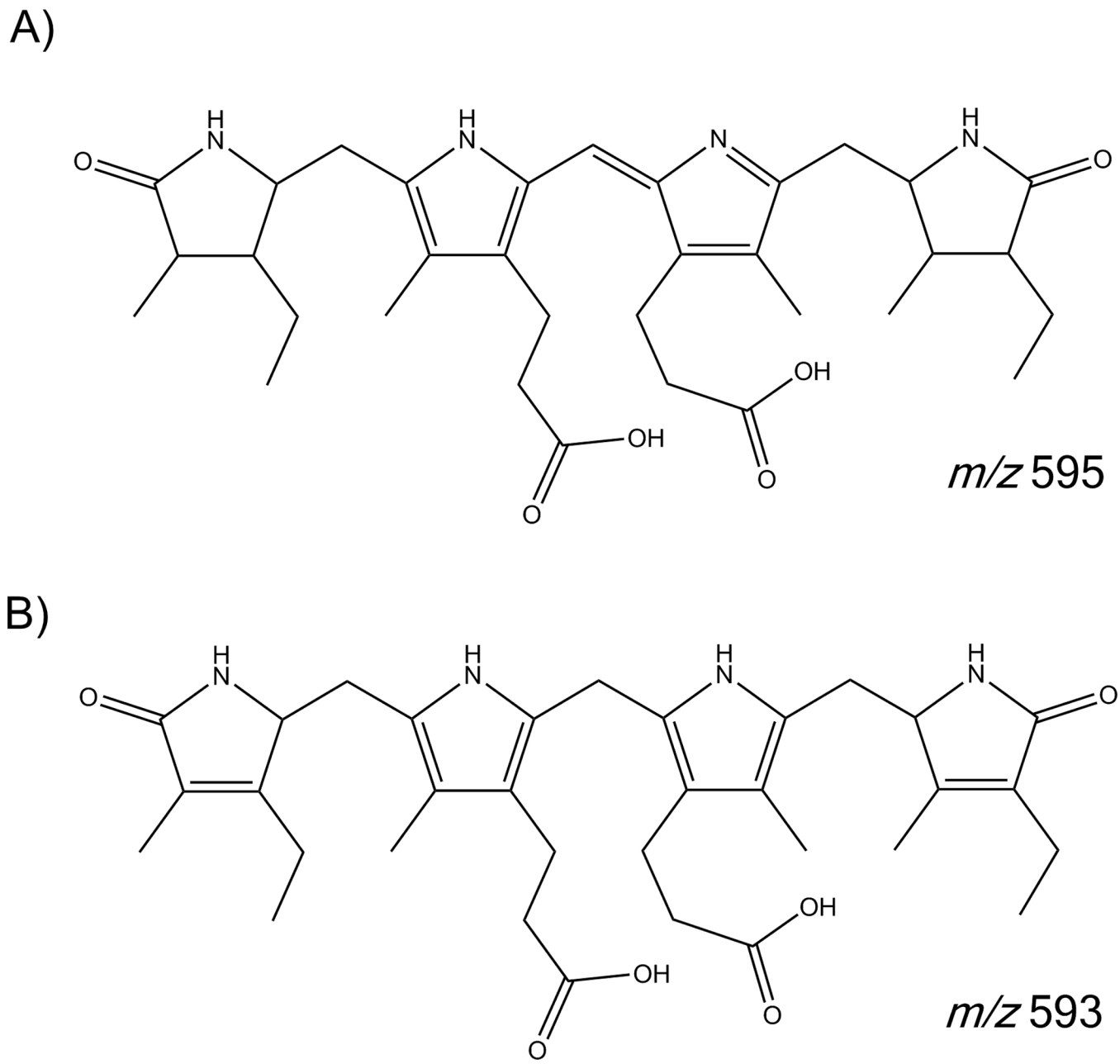


Figure 3.
Structural comparison of stercobilin (a) and urobilinogen (mesobilirubinogen) (b) present in commercial stercobilin hydrochloride standard.

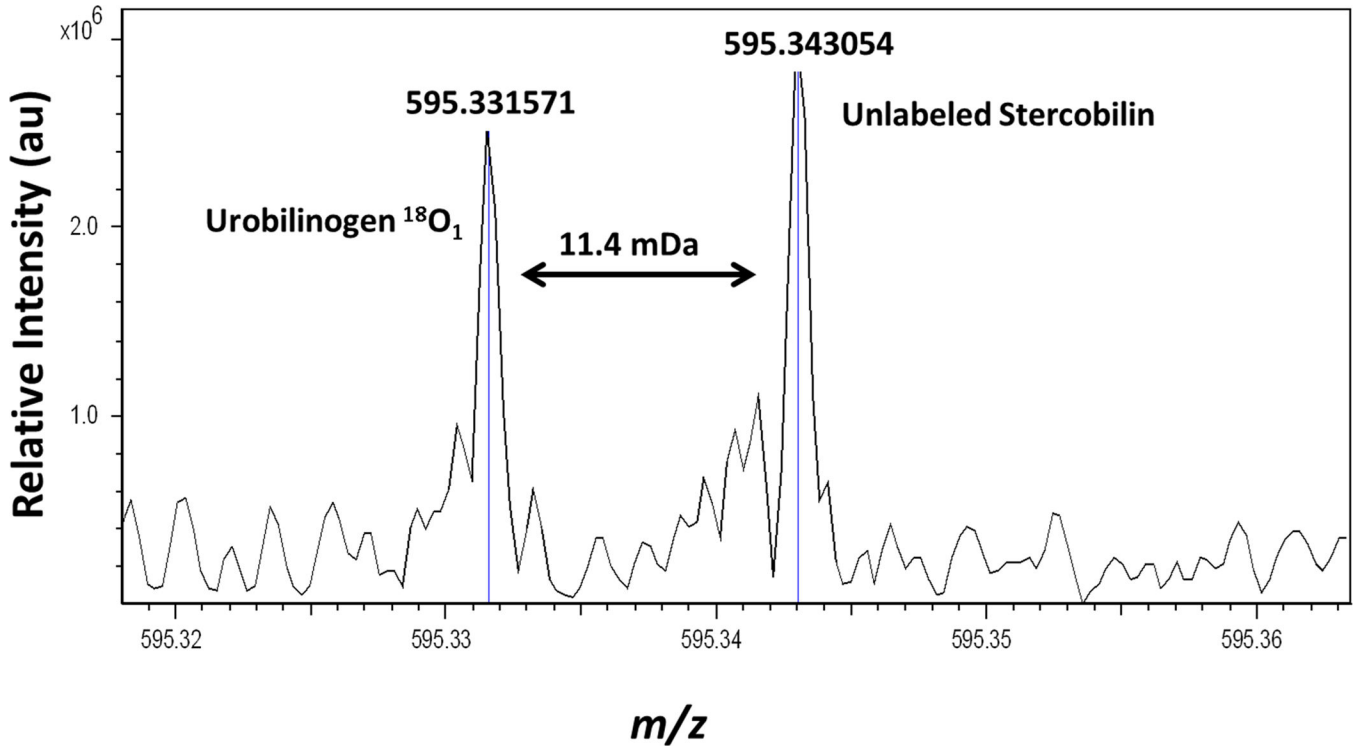
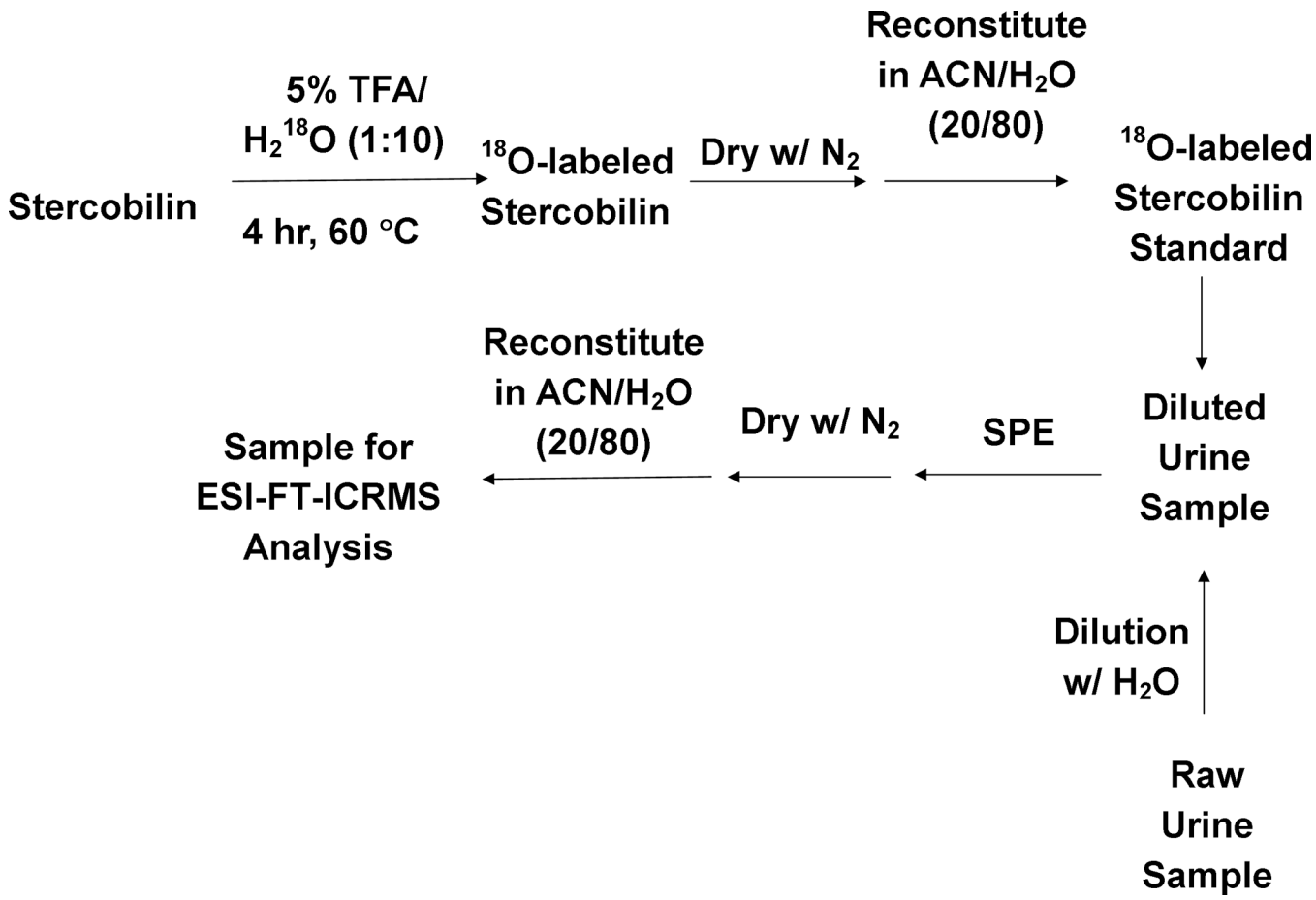


Figure 4.

Positive ion mode ESI demonstrating the resolving capability of the 12T FT-ICR to resolve $^{18}O_1$ labeled urobilinogen from unlabeled stercobilin.



Scheme 1.

Outline of method for synthesis isotopic standard for internal standard added to urine specimens for bilin quantification.

Table I

Comparison and summary for methods used to isotopically label stercobilin with ^{18}O.

Reaction Conditions				% ^{18}O Label (STDEV)				
5% TFA (μL)	$H_2{}^{18}O$ (μL)	T (C °)	t (h)	Unlabeled *m/z* 595	$1^{18}O$ *m/z* 597	$2^{18}O$ *m/z* 599	$3^{18}O$ *m/z* 601	$4^{18}O$ *m/z* 603
1	5	50	4	10.6 (1.0)	17.0 (3.1)	25.0 (4.1)	29.7 (1.5)	17.7 (4.8)
1	5	50	8	4.0 (0.9)	10.0 (2.2)	21.8 (0.9)	35.4 (2.1)	28.8 (2.0)
1	5	60	4	2.4 (0.8)	8.0 (1.5)	19.7 (4.2)	34.8 (6.8)	35.2 (12.4)
1	5	70	4	2.3 (0.5)	8.4 (0.6)	21.5 (1.6)	39.3 (0.4)	28.4 (2.3)
1	10	60	4	6.2 (1.2)	12.2 (1.3)	28.2 (5.8)	37.9 (2.7)	16.0 (1.9)
1	10	70	4	12.4 (7.5)	24.2 (12.6)	31.3 (3.7)	23.8 (12.2)	8.3 (6.3)
1	10	70	8	2.3 (2.6)	8.2 (4.2)	20.6 (7.9)	36.8 (2.0)	32.7 (13.9)

Table II

t-test results for isotopically-labeled standard processed by SPE analysis versus control labeled species.

	No label		**$^{18}O_1$**		**$^{18}O_2$**		**$^{18}O_3$**		**$^{18}O_4$**	
$(M+H)^+$	***m/z* 595 Control**	***m/z* 595 SPE**	***m/z* 597 Control**	***m/z* 597 SPE**	***m/z* 599 Control**	***m/z* 599 SPE**	***m/z* 601 Control**	***m/z* 601 SPE**	***m/z* 603 Control**	***m/z* 603 SPE**
Average Ratio	0.119	0.175	0.181	0.152	0.215	0.247	0.349	0.313	0.137	0.113
STD	0.035	0.048	0.021	0.051	0.044	0.081	0.030	0.065	0.054	0.062
t_{calc}	1.411		1.754		0.130		1.177		0.286	
t (CI95%)	2.069		2.069		2.069		2.069		2.069	
Acceptable (Y/N)	Y		Y		Y		Y		Y	

Table III

Urinalysis results using isotopically-labeled stercobilin for quantification

Urinalysis Subject	Quantified stercobilin (ppb)
F1	2.0
F2	0.7
M1	4.4
M2	0.8
M3	2.7