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Human Urine as Test Material in ^{1}H NMR-Based Metabonomics: Recommendations for Sample Preparation and Storage

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Metabonomic approaches are believed to have the capability of revolutionizing diagnosis of diseases and assessment of patient conditions after medical interventions. In order to ensure comparability of metabonomic ^{1}H NMR data from different studies, we suggest validated sample preparation guidelines for human urine based on a stability study that evaluates effects of storage time and temperature, freeze-drying, and the presence of preservatives. The results indicated that human urine samples should be stored at or below −25 °C, as no changes in the ^{1}H NMR fingerprints have been observed during storage at this temperature for 26 weeks. Formation of acetate, presumably due to microbial contamination, was occasionally observed in samples stored at 4 °C without addition of a preservative. Addition of a preserving agent is not mandatory provided that the samples are stored at −25 °C. Thus, no differences were observed between ^{1}H NMR spectra of nonpreserved urines and urines with added sodium azide and stored at −25 °C, whereas the presence of sodium fluoride caused a shift of especially citrate resonances. Freeze-drying of urine and reconstitution in D_2O at pH 7.4 resulted in the disappearance of the creatinine CH_2 signal at δ 4.06 due to deuteration. A study evaluating the effects of phosphate buffer concentration on signal variability and assessment of the probability of citrate or creatinine resonances crossing bucket border (a boundary between adjacent integrated regions) led to the conclusion that a minimum buffer concentration of 0.3 M is adequate for normal urines used in this study. However, final buffer concentration of 1 M will be required for very concentrated urines.

The availability of valid data is a fundamental prerequisite for a successful outcome of any study. In order to integrate metabonomic analysis into the physician's arsenal of diagnostic tools, databases comprising multivariate data from disease models are necessary. Such databases should have a predictive or at least confirmatory capability when confronted with data obtained from a patient. To achieve this goal, major precautions are necessary during creation of the database. Conditions under which the data are collected, in particular, the conditions of sample storage and sample preparation, may have a major impact on the content of a multivariate data set. In this paper, generation of valid ^{1}H NMR data from human urine samples is discussed. A set of guidelines for sample preparation for metabonomic studies with human urine is proposed for the first time.

In the past decade, metabonomic approaches have enriched toxicology and diagnostics.[1−4] Metabolic changes in the rat have been studied most extensively.[5−8] In most cases, the metabonomic investigations are conducted using ^{1}H NMR spectroscopy, but LC/MS[9,10] and GC/MS approaches[11] have been pursued. Metabonomic techniques capable of predicting the presence of coronary heart disease have been developed.[12,13] Furthermore, some cancer types have been investigated using metabonomic approaches, e.g., breast cancer and ovarian cancer.[14,15] Biomarkers that have emerged from these studies can be very useful in exploration of the biochemical pathways involved in these diseases. In the studies

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of biomarkers, it is important to ensure that no false biomarkers, resulting for example from sample preparation, are observed. In many previous metabonomic studies, addition of preservatives has been customary as a measure against microbial contamination of the samples, especially when studying rat urine. To our knowledge, this paper addresses for the first time the question of effect of preservatives on the ^{1}H NMR patterns of human urine.

Urine samples having different concentration, pH, and ionic strength will exhibit different ^{1}H NMR chemical shifts of the same metabolite, primarily due to alterations of acid−base equilibria and solute−solute interactions. These variations can cause spurious grouping of samples in chemometric models. To avoid such problems, pH and ionic strength buffering, as well as bucketing of spectral regions (division of the chemical shift axis into buckets, i.e., segments larger than the anticipated chemical shift variations) are standard procedures, but also more sophisticated techniques such as warping and other alignment strategies have been proposed.[16,17] Procedures eliminating differences due to sample conditions are of interest, because current mathematical alignment techniques are unable to handle new or missing signals, signal crossover, or very strongly shifted signals.

Normal human urine samples are expected to have pH values in the range 5.5−6.5. However, pH of urine often changes, e.g., under physiological stress leading to an extended pH range of 4.6−8.0, and hence, it is important to use a buffer to minimize the variation of NMR chemical shifts. The majority of studies use urine buffering to minimize these differences, but the buffer concentrations used vary considerably, and there are apparently no studies documenting optimal buffer concentration. The added buffer must provide a sufficient buffer capacity not only to guarantee as constant a pH as possible over the entire series of samples but also to minimize possible chemical shift variations resulting from differences in ionic strength. Previous studies have shown that ionic strength of normal human urine differs by a factor of ∼10.[18] On the other hand, use of excessive buffer concentrations is counterproductive with respect to ^{1}H NMR data quality due to magnetic susceptibility effects, altered sample relaxation properties, and absorption of radio frequency radiation by the sample. In this work, effects of buffer concentration are evaluated by monitoring resonances of creatinine and citrate, i.e., compounds that give resonances sensitive to pH variations around neutral pH.[19]

Stability of urine frozen at ∼ −20 °C or below is generally considered good. However, storage at −80 °C is also common, but a possible advantage of this additional temperature lowering is unclear. None of previous studies assessed the effect of storage temperature in the context of metabonomics, i.e., monitoring of all detectable metabolites simultaneously.[20−24] Herein, we present results of investigations of effects of urine storage temperature, as well as effects of the presence of preservatives, on ^{1}H NMR patterns of human urine, as assessed by principal component analysis (PCA).

EXPERIMENTAL SECTION

Sample Collection. Urine was collected from healthy male volunteers receiving a normal diet, 25−58 years old, and was processed immediately after collection.

Chemicals. Water purified by deionization and membrane filtration (0.22 μm) on a Millipore system was used for preparation of all solutions. Preservatives were added from stock solutions in water containing 0.3% w/v NaN_3 or 3.0% w/v NaF. Buffer solutions (0.1, 0.2, 0.5, and 1.0 M, pH 7.4) were prepared from $Na_2HPO_4{\cdot}2H_2O$ or $K_2HPO_4{\cdot}3H_2O$ and KH_2PO_4 in D_2O (99.9 at. % deuterium). Sodium 3-trimethylsilylpropionate-d_4 (TSP, 99 at. % deuterium) was added to the buffers typically at a concentration of 0.3 mM. Final adjustment of the pH was carried out with HCl or KOH solutions using a pH-meter equipped with a glass combination electrode.

Assessment of Effect of Buffer Concentration. Urine from three donors was collected and analyzed after addition of phosphate buffer at four different concentrations. Thus, 0.5 mL of urine was mixed with 0.25 mL of a buffer, the sample was centrifuged at 3000*g* for 15 min, and aliquots of 0.6 mL were transferred to 5-mm NMR tubes for ^{1}H NMR analysis. Final buffer concentrations were 0.03, 0.07, 0.17, or 0.33 M, and final TSP concentration was 0.1 mM. Each analysis was preformed in triplicate using independently prepared samples. Separate urine samples from the same donors were concentrated by a factor of 10 by freeze-drying, and 0.5 mL aliquots of the concentrated urines were mixed with 0.75 mL of a buffer and prepared for ^{1}H NMR analysis as above. Final buffer concentrations were 0.1, 0.2, 0.5, or 1.0 M. In another series of experiments, urine from nine donors was analyzed at six different buffer concentrations. Thus, 0.4-mL urine aliquots were mixed with 0.2 mL of a buffer solution to give final buffer concentrations of 0.0, 0.000 33, 0.0033, 0.033, 0.18, or 0.33 M. The samples were centrifuged at 10000*g* for 5 min, 0.55-mL portions were transferred to 5-mm NMR tubes, and the tubes were stored at −25 °C until the NMR analysis. Each analysis was performed in triplicate except for the freeze-dried samples, which were analyzed in duplicate.

Assessment of Preservatives and Long-Term Sample Storage Temperature. Urine samples from four donors were each divided into four portions, A−D, and each of these samples was further subdivided into 27 samples of 0.5 mL each. Nine of the latter were mixed with 0.25 mL of water each, nine had added 0.25 mL of the stock solution of NaN_3, and the remaining nine samples had added 0.25 mL of the stock solution of NaF. Samples A−C were stored at 4, −25, and −80 °C, respectively, whereas samples D were freeze-dried and the residues stored at −80 °C. Freezing of the samples was initiated immediately after the samples were drawn and additions of the appropriate solvents were performed. One of the nine samples in each group was analyzed by ^{1}H NMR after storage for 0, 1, 2, 3, 4, 6, 10, 14, and 26 weeks, respectively. This corresponds to 48 samples analyzed at each of

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the nine time points (four donors, four storage conditions, two preserving agents, and one control). For the analysis, each of the samples was allowed to thaw at room temperature, mixed with 0.375 mL of 1.0 M buffer (the freeze-dried samples were dissolved in 1.125 mL of the 0.33 M phosphate buffer), centrifuged at 10000*g* for 10 min, and analyzed by ^{1}H NMR spectroscopy after transferring of 0.6-mL aliquots to 5-mm NMR tubes.

Assessment of Preservatives and Short-Term Sample Storage Temperature. Urine samples (0.5 mL) from one donor had added 0.25 mL of water or 0.25 mL of the stock solutions of NaF and were stored at 4, −25, or −80 °C or were freeze-dried and the residues stored at −80 °C. One sample from each group was analyzed as described above after 0, 1, 2, 3, 4, 5, 6, and 7 days.

NMR Analysis. The ^{1}H NMR spectra were acquired at 296.2 K with a Bruker Avance 400-MHz spectrometer operating at 400.13 MHz for ^{1}H and equipped with a 5-mm dual ^{1}H/^{13}C normal-configuration probe or at 300.0 K on a Bruker Avance 600-MHz spectrometer operating at 600.13 MHz for ^{1}H, using a 5-mm triple resonance (^{1}H/^{13}C/^{15}N) inverse-configuration probe equipped with *z*-gradient. The 1D NOESY pulse sequence with presaturation during relaxation delay (2 s) and mixing time (0.1 s) was used for suppression of water resonance. A total of 512 transients were accumulated with a spectral width of 20 ppm, typically collecting 64k data points. The data were apodized with an exponential function using a line broadening of 1.0 Hz prior to Fourier transformation. Phase corrections were performed manually. Assignment of metabolite resonances was performed by comparison with published literature data.[18]

Data Reduction and Analysis. All spectra were calibrated to internal TSP (δ 0.00), bucketed (i.e., segmented into equal-sized regions or buckets for determination of the integrated signal intensity in each bucket), and normalized to total integral area of the spectrum using MatLab ver. 7.0 software (MathWorks, Natick, MA) with in-house written scripts. A bucket size of ∼0.04 ppm was used resulting in 239 buckets/spectrum. The spectral region containing residual water and urea resonances (4.4−6.2 ppm) was excluded from the analysis. Nonbucketed (full resolution) data were used in the assessment of effects of buffer concentration; the digital resolution of these data was changed to 1.0×10^{-4} ppm/point in the first series of buffer experiments and to 2.5×10^{-4} ppm/point in the second series. Univariate data analysis was accomplished with Microsoft Excel 2002. PCA calculations[25] were carried out using Unscrambler ver. 9.2 software (CAMO Software, Oslo, Norway). Analyses were performed on centered data. Cross-validation using the leave-one-out procedure was used.

RESULTS AND DISCUSSION

Effect of Buffer Concentration on ^{1}H NMR Spectra of Human Urine. Effects of final buffer concentration were assessed in two series of experiments. In the first series, phosphate buffer was added to three different urine samples at four final buffer concentrations in the range 0.03−0.33 M, keeping the final concentration of TSP (internal chemical shift standard) at a constant value of 0.1 mM. In another series of experiments, three different urine samples were concentrated 10-fold, and phosphate buffer was added to obtain four different final buffer concentrations in the range of 0.1−1.0 M, with the same final TSP concentration. Resonances of creatinine (δ around 3.05 and 4.06) and citrate (δ around 2.67) were used as a probe for assessment of the buffer concentration effect. The results are shown in Table 1.

Table 1. Standard Deviations for Interpersonal Chemical Shift Variations of Citrate and Creatinine Resonances in Human Urine at Various Buffer Concentrations (pH 7.4)

		standard deviation (ppm) of chemical shift variations		
series[a]	final buffer concn (mM)	citrate resonance, δ 2.67	creatinine resonance, δ 3.05	creatinine resonance, δ 4.06
1	0.033	1.53×10^{-3}	5.77×10^{-4}	1.00×10^{-3}
1	0.066	1.15×10^{-3}	1.00×10^{-3}	5.77×10^{-4}
1	0.166	1.15×10^{-3}	5.77×10^{-4}	5.77×10^{-4}
1	0.333	5.77×10^{-4}	5.77×10^{-4}	5.77×10^{-4}
2	0.100	7.51×10^{-3}	4.73×10^{-3}	8.39×10^{-3}
2	0.200	6.66×10^{-3}	3.61×10^{-3}	5.03×10^{-3}
2	0.500	3.79×10^{-3}	2.52×10^{-3}	3.51×10^{-3}
2	1.000	3.46×10^{-3}	1.15×10^{-3}	1.53×10^{-3}

[a] Series 1: nonconcentrated urine, $n = 3$. Series 2: 10-fold concentrated urine, $n = 3$.

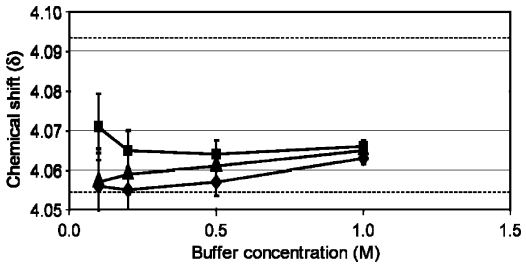

Figure 1. Dependence of chemical shifts of creatinine resonance around δ 4.06 on final buffer concentration for 10-fold concentrated urine from three different donors. Dotted lines show bucket limits.

Since most of the measurements were performed using three independently prepared samples (see Experimental Section), the standard deviations for repeats could be calculated. The standard deviations for repeated measurements with normal urine were on average 6.41×10^{-5} ppm, and those for the measurements with concentrated urines 8.63×10^{-4} ppm. It is apparent that standard deviations for interpersonal chemical shift variations of citrate and creatinine resonances are larger than those of the repeats (Table 1), as expected for urine samples with different composition. The relatively largest deviations were observed for interpersonal variations of the creatinine resonance around δ 4.06 in the concentrated urines (Table 1, Figure 1). Assuming that the 10-fold concentrated urine represents the upper limit of urine concentration that can be encountered in metabonomic investigations,[18] it can be concluded that a buffer concentration of 1.0 M is needed to maintain sufficiently constant chemical shift of this resonance. *F*-Tests emphasized these results proving different variances ($\alpha = 0.05$, $p_{\text{one-tail}} = 0.032$) between the standard deviations calculated for the creatinine signal around δ 4.06 in the concentrated urine for 1.0 and 0.1 M buffer concentrations. Similar *F*-test results were obtained for the creatinine signal around δ 3.05 and the citrate signal around δ 2.67, but at a lower level of significance. At lower buffer concentration, the signals

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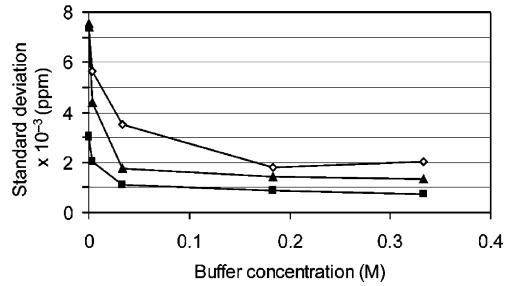

Figure 2. Standard deviations of the chemical shifts of citrate (CH_2 group, open prisms) and creatinine (CH_3 group, filled squares; CH_2 group, filled triangles) as a function of final buffer concentration in nonconcentrated urines.

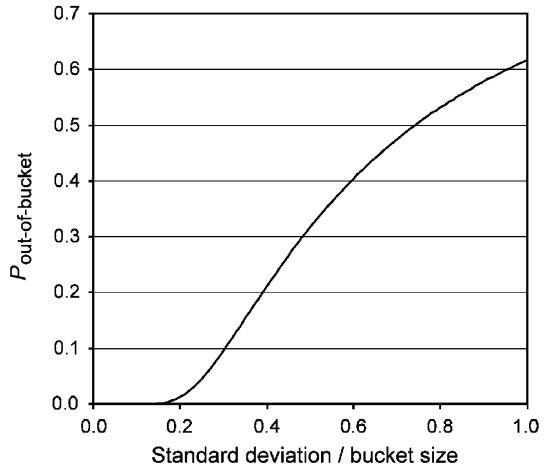

Figure 3. Calculated probability of a ^{1}H NMR signal placed in the center of the bucket to shift to the neighboring bucket due to chemical shift variability, expressed as standard deviation.

can approach, and possibly even cross, the bucket border (the boundary between adjacent integrated regions) leading to erroneous results (Figure 1). For nonconcentrated urines, a final buffer concentration of 0.33 M appeared adequate (Table 1). For the concentrated as well as the nonconcentrated urine, increased buffer concentration resulted in smaller *p*-values, proving a larger degree of stability of the NMR signals at the optimal buffer concentration.

In order to study the effects of buffer concentration further, a larger study was conducted adding buffer to plain (nonconcentrated) urines at six different concentrations in the range 0−0.33 M (see Experimental Section). The results show that the standard deviations for creatinine and citrate resonances at 0.33 (final buffer concentration) and 0.18 M (the next highest concentration) are virtually identical, but the standard deviations increase as the buffer concentration decreases (Figure 2). The standard deviations obtained in this series of experiments (Figure 2) correspond very well to those obtained in the previous series (Table 1), thus validating the conclusions.

The importance of using an appropriate buffer concentration is illustrated by a calculation of probability of a signal crossing the bucket border (Figure 3). The graph represents the normal probability function:

$$f(x) = \frac{1}{\sqrt{2\pi\sigma}} e^{-(x-\mu)^2/2\sigma^2}$$

In this equation, σ and μ are the standard deviation of the measurement and the bucket mean, respectively. In the present case, $\mu = 0$, corresponding to the resonance frequency placed in the center of the bucket, and thus, the probability of the resonance to fall outside the bucket is

$$P_{\text{out-of-bucket}} = 1 - \int_{-\text{bucket}/2}^{+\text{bucket}/2} \frac{1}{\sqrt{2\pi\sigma}} e^{-(x-\mu)^2/2\sigma^2} dx$$

The above calculation assumes that the width of the NMR signal is small compared to the size of the bucket. Furthermore, it is assumed that the distribution of the NMR signals around the center of the bucket follows normal distribution. The calculation shows that, for a bucket size of 0.04 ppm and insufficiently buffered urines (Table 1, Figure 3), a creatinine or citrate signal falls out of the bucket 1−2 times in 100 measurements. This diminishes to below 1 case out of 100 for concentrated urines buffered at 1.0 M and virtually to zero in nonconcentrated urines buffered to the final buffer concentration of 0.18 or 0.33 M. However, in reality, the NMR signals are distributed evenly over the entire bucket area and are not normally distributed around the center of the bucket, leading to underestimation of the predicted out-of-the-bucket probability in the above calculations. On the other hand, the calculations performed for creatinine and citrate resonances presumably represent the worst case, as those signals exhibit the largest pH-dependent variations in solutions around pH 7. From the above results, it may be recommended that the final buffer concentration of 0.3 M is used in routine work, as it gives some safety margin relative to 0.18 M buffer that gave sufficient stabilization of creatinine and citrate resonances in normal urines used in this work. Only in cases when very concentrated urines are expected, 1.0 M buffer is preferable. The line widths of the observed ^{1}H NMR signals, defined by effective transverse relaxation time (T_2*), have in the present work been constant over the entire buffer concentration range, demonstrating that addition of buffers in the concentrations described above does not compromise quality of the spectra.

Effect of Storage Time, Storage Temperature, and Preservatives. To assess the effects of storage time, storage temperature, freeze-drying, and the presence of preservatives, two series of experiments were conducted. The long-term study evaluated urine storage at 4, −25, and −80 °C and freeze-drying followed by storage at −80 °C, in each case with and without a preservative (NaN_3 or NaF), over a period of 26 weeks. The short-term study evaluated the same conditions every day over a period of 7 days. Urine samples were frozen immediately after collection, and no evaluation of possible influence of differences in the time interval between the collection and freezing was performed. Only NaF was used as the preservative in the short-term experiment, as no differences between NaN_3 and controls were observed in the long-term study.

Decomposition into principal components by PCA of the data from the long-term study revealed, not surprisingly, that the major source of variation was the origin of urine (interpersonal variation), as illustrated by the PCA score plot shown in Figure 4A (R^2 = 0.98, Q^2 = 0.97). Freeze-drying appeared to be the second-largest source of variation. Subsequent PCA modeling on samples from individual donors indicated a small difference according to

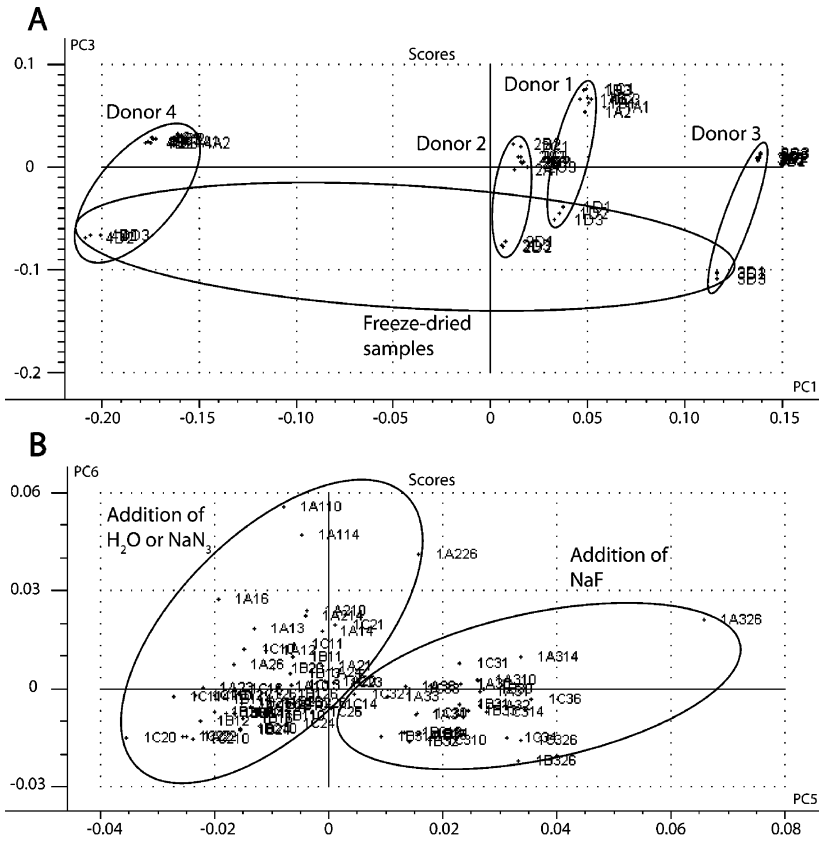

Figure 4. (A) PCA score plot for urine stability during storage for 26 weeks ($R^2 = 0.98$, $Q^2 = 0.97$). Codes: The first numeral designates donor (1−4), the middle letter designates storage conditions (A, B, and C, storage at 4, −25, and −80 °C, respectively; D, freeze-drying, storage at −80 °C, and reconstitution with D_2O), and the third numeral designates preservatives (1, no preservative; 2, NaN_3; 3, NaF). (B) PCA score plot (donor 1) showing typical effects of preservation ($R^2 = 1.00$ $Q^2 = 0.99$). Codes as in (A). In addition, the last one or two numerals designates sampling time (0, 1, 2, 3, 4, 6, 10, 14, or 26 weeks, respectively).

preservation (Figure 4B). The loading plots enabled useful information regarding differentiation among groups to be extracted from the raw data.

Thus, appearance of acetate, presumably caused by bacterial contamination, was observed in the long-term storage experiment in one sample stored at 4 °C. The formation of acetate was paralleled by a decrease of the intensity of citrate resonance. Furthermore, a decrease of the hippurate concentration was observed in the samples stored at 4 °C without preservation after 1 week, apparently due to degradation to benzoic acid and glycine (δ 3.57), both observed to increase concomitantly. Histidine resonances (δ 7.12 and 7.96) were also observed to decrease, but only after 14 weeks at 4 °C and without preservation. Since the exposure of the sample to D_2O was very similar for all samples, the observed decrease of histidine resonances is believed to be due to degradation. These findings demonstrate that pronounced changes can occur in nonfrozen urine samples in the absence of preservatives, even though human urine is normally considered sterile. The experiments with storage at 4 °C with NaF or NaN_3 showed that it is possible to extend the stability of urine at this temperature using the preservatives. However, long-term storage (above 1 week) of nonfrozen human urine samples is not recommended.

No differences between ^{1}H NMR patterns were observed between urine samples stored at −25 and −80 °C. However, the long-term study demonstrated changes of the spectra according to the kind of preservative added. Thus, the presence of 1.0% NaF resulted in a separate sample clustering as compared to nonpreserved samples or samples preserved with 0.1% NaN_3 (Figure 4B). The corresponding loading plot and associated raw data demonstrated that the difference is caused by a shift of citrate resonances in combination with other, minor effects. Shift of the citrate resonances is expected due to the binding of fluoride ion to metal ions, such as Ca^{2+} and Mg^{2+}, present in urine. These metal ions form complexes with a variety of compounds, notably citrate, resulting in variations of chemical shifts. The addition of NaF was observed to move the resonance of citrate by up to 0.008 ppm toward lower chemical shifts. These apparent complexation effects suggest the possibility of stabilization of citrate chemical shift by addition of NaF or another complexing agent, especially when the concentration of metal ions is expected to be substantial and varying between samples. Further studies of this subject are required. It should be emphasized that the preservation of urine with 0.1% NaN_3 gave ^{1}H NMR patterns identical to those observed for nonpreserved urines (stored at −25 and −80 °C). The significance of this finding is twofold. First, human urine samples stored frozen at −25 °C or below for up to 26 weeks do not appear to require preservation. Second, the presence of NaN_3 did not cause any observable changes in the metabonome composition, despite the reactive nature of azide (a powerful nucleophile and potential participant in redox and complexation reactions). Use of this preserving agent is especially recommended when working with animal urine, where the risk of bacterial contamination is substantially higher than with human urine.

Analysis of data from the short-term storage study (1-week storage) indicated minor differences in ^{1}H NMR patterns caused by the presence of NaF, consisting mainly of changes of citrate chemical shift. The results of this experiment are thus in agreement with the results of the long-term experiment described above.

Effects of Freeze-Drying. The observed changes of the metabonome composition due to freeze-drying appeared as another direction in the score plot, indicating a different source of variation compared to that caused by donors (Figure 4A). These changes are presumably due in part to difficulties with complete redissolution of the freeze-dried urine residues and potentially also to possible removal of volatile metabolites. The observed decrease of intensity of creatinine and hippurate resonances is attributable to incomplete redissolving of these abundant metabolites. The creatinine CH_2 signal at δ 4.06 disappeared almost completely in freeze-dried samples reconstituted with the D_2O-based buffer, evidently due to deuteration at this position. Since deuteration of creatinine is subject to general-acid as well as general-base catalysis,[18,26] it is expected to be fast in a complex solution such as urine, especially at high urine and buffer concentrations. Other minor changes observed in the freeze-dried samples were not analyzed in detail, but may be important in another context. The short-term storage stability study showed that the changes were observed already in the first measurement with the freeze-dried samples. Thus, the changes in the ^{1}H NMR profiles are due to freeze-drying and reconstitution themselves and not to prolonged storage of the freeze-dried samples. Therefore, although freeze-dried urine samples are expected to have excellent long-term stability, and redissolving in D_2O makes water peak suppression easier, the process introduces alteration to metabolite profiles, in particular due to deuteration of enolizable metabolites via buffer-catalyzed exchange.

CONCLUSIONS

Growing attention to quality assurance of studies employing ^{1}H NMR-based metabolomics has recently been reflected in numerous investigations of baseline data variability,[27−29] data preprocessing methods,[30−33] analytical reproducibility,[34] and metabolite identification.[35,36] The results of this study emphasize the importance of a validated sample preparation and storage protocol. The following recommendations for human urine sample preparation can be derived from the results described above: (1) Standard human urine samples should be buffered to a final phosphate buffer concentration of 0.3 M when the bucket size of 0.04 ppm is used; if highly concentrated urine samples are expected, the final buffer concentration should be increased to 1.0 M. (2) No preservatives are necessary if urines are stored at or below −25 °C; no beneficial effect of lowering the storage temperature to −80 °C was observed. (3) If the addition of preservatives is deemed necessary, e.g., because of temporary storage at 4 °C or if sterility cannot be assured, 0.1% sodium azide (final concentration) is satisfactory and preferred over preservation with sodium fluoride. (4) Freeze-drying and reconstitution with D_2O is not recommended because of the changes to ^{1}H NMR patterns introduced by this operation.

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