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Analysis of Human Urine Using the Absolute*IDQ*™ Kit

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Introduction

Urine is an information rich biofluid that can provide valuable information about the metabolic state of an organism. In contrast to blood, it can be collected non-invasively and has been frequently used for metabolomics study using both mass spectrometric and NMR methods [1]. The concentrations of the metabolites in urine vary considerably. In many cases, 24h-urine is not available, and since the urine volume is strongly influenced by the fluid intake, it can also change in disease states which affect the renal function. Therefore, normalization of the metabolites' concentrations to the creatinine content is a useful approach, which is often applied. Typical normalized concentration values are reported in databases like the human metabolom database (HMDB, www.hmdb.ca) [2].

In this application note, we tested and optimized the AbsoluteIDQ Kit for the analysis of human urine. Creatinine was added as an additional analyte to the metabolite panel of the Kit and its quantification range was determined. This Kit is based on a targeted metabolomics approach [3-4] and can simultaneously identify and quantify a large number of endogenous metabolites from small volumes of biological fluids.
In this study, we analyzed human urine from healthy volunteers (male and female, n=16) and report concentration ranges found for the metabolites with the Kit.

Methods

The AbsoluteIDQ Kit was prepared as described in detail in the User Manual. As an additional first step, 10 µL of a solution of the isotope-labeled internal standard D3-creatinine was added to each well of the 96-well plate, with exception of position A1 (blank well). 10 µL of urine was used as sample amount for each well. The extracts were analyzed using a 4000 QTrap quadrupole mass spectrometer (AB Sciex). The standard flow injection method of the AbsoluteIDQ Kit comprising two 20 µL injections (one for positive and one for negative detection mode) was applied for all measurements. Quantification was achieved by multiple reaction monitoring (MRM) detection in combination with the use of isotope-labeled and other internal standards. The MRM pairs for creatinine and D3-creatinine were added to the acquisition method.

Results and Discussion

Quantification range for creatinine

To evaluate the quantification range for creatinine the metabolite was spiked into pooled human urine and into an artificial matrix (3.5 mM urea, 15 mM phosphate buffer, pH 6) and analyzed with the Kit.

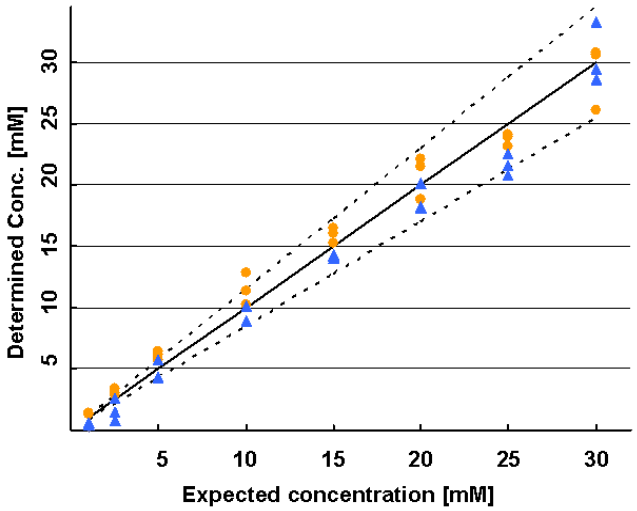

Figure 1: Quantification Range for Creatinine
Creatinine was spiked into a matrix analogue (●) and into pooled urine samples (▲) in the range from 1-30 mM. The graphic exhibits how far the expected concentration and the determined concentration values deviate. The dotted lines indicate 85 % and 115 % of the target values.

As shown in Figure 1, good linearity was observed in concentrations above 3 mM. The analytical parameters for creatinine are listed in Table 1. The observed quantification range encompasses the creatinine concentration commonly found in human urine.

Table 1: Analytical performance for creatinine

Analyte	*LOD [mM]*	*LLOQ [mM]*	*ULOQ [mM]*
Creatinine	0.5	3	30

Ion suppression effects / zero samples

The internal standards (IS) of the Kit are essential for quantification. Therefore, the signal intensities of the MRM pairs of the IS in human urine samples were tested. For most amino acids, the intensities of the IS were mainly in a similar range as in plasma, but for the amino acids

Arg, Tyr and Val the signal intensities were considerably lower in a number of tested urine samples, but still high enough for a reasonable quantification. The IS for the amino acid Orn exhibited unusually high ion suppression in most urine samples, and therefore this metabolite was removed from the analysis. Furthermore, a matrix analogue (3.5 mM urea, 15 mM phosphate buffer, pH 6) containing a similar urea and salt concentration as urine was tested as zero sample and is recommended for the analysis of urine samples. The zero samples are used to calculate the limit of detection (LOD). The LOD is defined as the median value of the zero samples multiplied by three.

MetIQ Software: A new operating procedure (OP, KIT-1-40-5) was created for the MetIQ software, which is essential to run the Kits. In the OP, the metabolite creatinine was added and the metabolites Orn and PC ae 40:0 (unusual high LOD) were removed. The Kit user can obtain the new OP as software patch together with the modified Analyst™ acquisition methods.

Table 2: Amino acids, creatinine and hexose concentration in urine

Metabolite	LOD [µM]	Conc. range [µM]	[µM/mM Creatinine]
Arg	2	4-28	1-3
Gln	8	55-684	17-85
Gly	6	82-2110	27-454
His	1	55-1100	12-112
Met	2	2-22	1-3
Phe	1	5-64	1-5
Pro	2	4-36	1-3
Ser	4	43-485	11-50
Thr	3	17-230	5-24
Trp	13	10-92	3-11
Tyr	3	9-149	2-15
Val	2	5-54	1-6
xLeu	2	9-88	2-9
Hexose	20	151-1748	48-162

Concentration ranges / Creatinine normalization

Tables 1-4 summarize the obtained data for the human urine samples. Each sample was measured six times and median values are shown in the tables. The LOD values were obtained using the urine matrix analogue analyzed over several kit plates. The concentration ranges are reported if the concentration of the respective metabolite was above LOD in at least 25% of the human urine samples.

The concentration ranges were compared to the ranges after applying creatinine normalization. By using the normalization the spread of the ranges decrease considerably, as seen in Table 2. The data in the table should give an overview of what concentration might be expected for the individual metabolites using the Kit. However, it should be noted that metabolite concentrations in human urine samples can vary dramatically since factors like age, gender, environment and medical conditions influence the metabolome significantly.

Amino acids, hexose and creatinine

All amino acid concentrations of urine samples were in the µM range and clearly above LOD. The hexose concentration was in the µM to mM range. With the exception of one sample, all creatinine values are in the linear quantification range of the assay.

Table 3: Acylcarnitine concentrations in urine

Metabolite	LOD [µM]	Conc. range [µM]	[µM/mM Creatinine]
C0	3.2	12-235	3.4-13.9
C2	0.09	4-64	1.3-8.2
C3	0.03	0.26-4	0.078-0.26
C3-DC/C4-OH	0.03	0.05-0.34	0.013-0.05
C3-DC-M/C5-OH	0.06	0.23-2.5	0.076-0.14
C3-OH	0.04	0.02-012	0.004-0.013
C3:1	0.02	LOD-0.03	0.001-0.004
C4	0.03	1.5-16	0.202-1.2
C4:1	0.02	0.04-0-42	0.012-0.051
C4:1-DC / C6	0.12	0.15-1.1	0.038-0.17
C5	0.04	0.45-7.1	0.18-0.39
C5-DC / C6-OH	0.03	0.15-1.45	0.041-0.088
C5-M-DC	0.06	0.12-0.66	0.021-0.14
C5:1	0.05	0.14-2.1	0.059-0.13
C5:1-DC	0.04	0.08-0.77	0.019-0.056
C6:1	0.03	0.03-0.18	0.007-0.030
C7-DC	0.05	0.08-1.0	0.020-0.088
C8	0.16	LOD-1.5	0.043-0.089
C8:1	0.02	0.31-6.6	0.079-0.83
C9	0.03	0.53-12.9	0.133-1.4
C10	0.14	LOD-0.50	0.022-0.053

Metabolite	LOD [µM]	Conc. range [µM]	[µM/mM Creatinine]
C10:1	0.19	0.26-3.2	0.077-0.33
C10:2	0.05	0.08-1.3	0.022-0.32
C12	0.06	0.07-0.36	0.014-0.049
C14:1-OH	0.01	0.01-0.04	0.001-0.004
C14:2	0.01	0.01-0.04	0.001-0.004
C14:2-OH	0.01	0.01-0.04	0.001-0.004
C16	0.02	LOD-0.04	0.002-0.005

Acylcarnitines

A high number of acylcarnitines (28) were detected in the urine of the healthy volunteers, as shown in Table 3. Therefore, it can be expected that in samples of patients most acylcarnitines will be above LOD. The concentration of acylcarnitines can significantly increase as a result of enzymatic disorders or in certain disease states. The ranges for many acylcarnitines are generally higher compared to the values normally found in plasma samples, thus the analysis of urine sample should give information on a broad range of different acylcarnitines.

Table 4: Glycerophospholipid concentrations in urine

Metabolite	LOD [nM]	Conc. range [nM]	[nM/mM Creatinine]
PC aa C28:1	43	LOD-100	3.5-19.8
PC aa C34:1	100	LOD-293	2.5-32.8
PC aa C34:4	7	LOD-33	0.4-8.1
PC aa C36:1	27	LOD-117	1.2-16.7
PC aa C36:3	50	LOD-125	0.8-16.7
PC aa C38:6	17	LOD-57	0.5-6.9
PC aa C40:3	4	LOD-8	0.1-1.0
PC aa C42:5	13	LOD-17	0.3-4.2
PC ae C32:1	7	LOD-17	0.1-2.3
PC ae C34:1	13	LOD-73	0.3-10.3
PC ae C34:2	13	LOD-37	0.2-5.2
PC ae C36:2	10	LOD-33	0.3-4.7
PC ae C36:4	13	LOD-20	0.4-4.8
PC ae C38:1	7	LOD-11	0.2-2.9
PC ae C38:2	10	LOD-17	0.3-2.7
PC ae C38:6	13	LOD-128	4.7-18.4
PC ae C40:2	7	LOD-12	0.3-5.0
PC ae C42:3	3	LOD-8	0.2-2.7
lysoPC a C16:1	17	LOD-56	0.9-4.0
lysoPC a C17:0	3	LOD-89	0.7-4.7
lysoPC a C18:0	23	LOD-86	1.8-8.0
lysoPC a C18:1	63	LOD-132	3.4-11.6
lysoPC a C18:2	37	LOD-60	2.2-9.8
lysoPC a C20:4	13	LOD-43	1.2-4.3

Glycerophospholipids and Sphingomyelins

In Tables 4 and 5, the concentrations of glycerol-phospholipids and sphingomyelins are shown. The concentration ranges are in the nM range and much lower than in plasma samples, as expected. Despite the low concentrations, a good number of lipids were detected at concentrations above LOD in the assay. It can be anticipated that in certain disease states even higher lipid levels might be detected.

Table 5: Sphingomyeline concentrations in urine

Metabolite	LOD [nM]	Conc. range [nM]	[nM/mM Creatinine]
SM C16:0	43	LOD-435	3.4-61.5
SM C16:1	10	LOD-37	0.6-3.8
SM C18:1	3	LOD-16	0.1-2.1
SM C20:2	3	LOD-18	0.1-1.1
SM C22:3	7	LOD-29	0.2-3.2
SM (OH) C22:1	10	LOD-55	1.7-5.6
SM (OH) C22:2	3	LOD-13	0.2-4.2
SM C24:1	23	LOD-101	1.7-14.3
SM (OH) C24:1	7	LOD-38	0.4-5.3
SM C26:0	7	LOD-33	0.4-4.6

Conclusion

The results presented in this application note reveal that the AbsoluteIDQ Kit can be applied to urine samples successfully. This should allow for further targeted metabolomics studies using this important and easily accessible biofluid. Since the Kit also allows the quantification of the normalization parameter creatinine, its results would be easily comparable with relative concentrations obtained by alternative standard metabolite assays. The broad coverage of acylcarnitine species holds the potential to receive even more information about fatty acid metabolism malfunctions than from plasma analyses.

References

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