Urinary Calcium for Tracking Bone Loss and Kidney Stone Risk in Space

Junkun Ren; Aleksandra S. Stankovic; Darin A. Knaus; Scott D. Phillips; Dave B. Kynor; Jay C. Buckey

INTRODUCTION: Urinary calcium (Uca) levels in space reflect bone loss and kidney stone risk and could be measured using portable devices. This project evaluated the repeatability of Uca measurements to assess how many repeated measurements would be needed to detect significant urinary calcium elevations in space.

METHODS: A total of six subjects collected 24-h urine samples weekly for 8 wk and took 500 mg of oral calcium carbonate and 400 IU of vitamin D daily in week 7 and 8. Uca concentration was analyzed using a calcein-based system. The effect of the intake of calcium and vitamin D on Uca levels and the correlation between first void concentration and 24-h mass were assessed with linear mixed effect models. The reproducibility coefficient (RPC) for Uca was determined using Bland-Altman analysis on pairs of measurements at different time points.

RESULTS: Oral supplementation did not significantly affect 24-h mass. First void concentration correlated with 24-h mass. The 24-h mass RPCs were 167.0, 116.8, and 108.1 mg for 1-, 2-, and 3-wk average measurements. First void concentration RPCs were 90.6, 76.6, and 72.8 mg $\cdot$ L^{-1}. Skylab astronauts 24-h mass increased by 88.9 $\pm$ 76.0, 123.5 $\pm$ 58.3, 142.2 $\pm$ 56.5, and 159.9 $\pm$ 83.4 mg after 1, 2, 3, and 4 wk in flight.

DISCUSSION: Averaging multiple Uca measurements reduced variability effectively and allowed increases likely to be seen in space to be detected. Consecutive Uca measurements could be tracked over time in space to assess the effectiveness of the countermeasure program. First void concentration could potentially be used rather than 24-h collections.

KEYWORDS: urinary calcium, in-flight bone loss, Bland-Altman analysis, repeatability.

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Astronauts are at risk for bone loss and kidney stone formation during spaceflight as a consequence of low light levels, high ambient CO_2 concentrations, and insufficient skeletal loading.[3] Presently, the effectiveness of in-flight bone loss and kidney stone prevention is established based on postflight measurements.[3,8] A more flexible and effective way of prevention might be to track bone loss in real time during flight so effective countermeasures can be evaluated and adjusted in-flight.

One simple approach is to measure urinary calcium excretion (or perhaps just concentration) at various times throughout the flight.[3] Markers such as deoxypyridinoline and pyridinoline in urine samples demonstrated that increases in urinary calcium excretion levels in spaceflight are associated with an increase in bone resorption.[3,4,15] Urinary calcium excretion has been used as a marker of bone loss in bed rest studies.[21,23] High calcium excretion is associated with decreased trabecular bone mineral density in elderly men, suggesting a relationship between the two.[22] Higher 24-h urine calcium excretion appeared to be a risk factor for increased femoral neck bone mineral density loss over a 3-yr period among families with idiopathic hypercalciuria and stone disease.[1] Many studies have investigated bone loss countermeasures using urinary calcium excretion as one of the measures.[7,16,20] Therefore, employing urinary calcium as a real-time indicator of bone loss in space is promising, but understanding the natural variability of the measures is important to allow astronauts, flight surgeons, and mission planners to know when urinary calcium has increased or decreased significantly (i.e., more than normal variation).

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Urinary calcium has natural intraindividual variability from day to day. On 5 d within a 1-mo period, 24-h urine samples of 22 Neapolitan men with mild blood pressure elevation were collected and showed an intraindividual variance of urinary calcium with a standard deviation of 32% of the overall mean while urinary sodium and potassium were less variable.[14] Kamperis et al. observed a coefficient of variation of approximately 0.2 for intraindividual variability in total 24-h urinary calcium-to-creatinine ratios in over 100 Danish children.[10] One way to reduce the effects of this variability is to take the average of multiple measures rather than just use individual measurements. To implement this approach, it should be verified that a certain number of averaged measurements could bring down the variability to the degree where the desired level of urinary calcium change can be detected. Thus, to use urinary calcium as a bone loss and kidney stone prevention marker in space, more research on the intraindividual variability of urinary calcium is needed. The objective of this study was to assess how many measurements should be averaged to detect the degree of urinary calcium excretion change likely to be seen in space. Another consideration is the effect of changes in oral calcium and vitamin D intake on urinary calcium excretion. Under steady-state conditions, urinary calcium excretion can be affected by calcium load in healthy subjects.[18] This project also studied whether a changing oral calcium and vitamin D intake would result in changes in urinary calcium excretion levels that are indistinguishable from the variation seen during spaceflight.

To assess the variability of repeated urinary calcium measurements, one effective approach is to use Bland-Altman analysis. Bland-Altman analysis is a graphical method that has long been employed to quantify the agreement between two measurement methods for a given parameter. It calculates the bias between the two methods and the agreement interval within which 95% of the mean differences would fall.[6,13] It can also be used to assess the agreement between multiple measurements using the same measurement method (i.e., repeated urinary calcium measures). In this study, we adapted Bland-Altman analysis to evaluate the average intraindividual variability of urinary calcium excretion levels based on 24-h total mass and first void concentration on six subjects under normal conditions. The measurements on two different time points were compared. While the limits of agreements are defined by the Bland-Altman method, the acceptable limits relative to the changes seen during spaceflight and the frequency of measurements required to meet the acceptable limits can be determined by comparing to the urinary calcium changes in the NASA Skylab data.[9] The Skylab program collected comprehensive urinary calcium data on all crewmembers.

Lastly, the adopted urinary calcium measurement method needs to be practical for use in space. The authors are developing an automated low-power optical system based on the fluorescent indicator reagent calcein, which could be incorporated into a low-power, small device that is well suited for making urinary calcium measurements repeatedly during spaceflight. Also, since collecting void-by-void 24-h urine could be inconvenient in space missions, this study further investigated first void urinary calcium concentration for approximating 24-h urinary calcium mass to evaluate the viability of detecting operationally significant changes in first void concentration to monitor bone loss.

METHODS

Subjects

The study protocol was reviewed and approved by the NASA IRB and the Dartmouth College Committee for the Protection of Human Subjects. Informed consent was obtained from each subject. Six subjects, three women and three men, ages 30–56, were enrolled in this study. Subjects were excluded if they had any history of a disorder of calcium metabolism, or if they were taking any drugs that might affect calcium excretion.

Procedure

This study was run over 8 wk during which urinary calcium excretion levels (24-h mass and first void concentration) were monitored in the six subjects. The subjects were supplemented with calcium carbonate 500 mg and vitamin D 400 IU daily during weeks 7–8. The objective of this study was to assess the variability of urinary calcium excretion without any dietary or other control, so the subjects were instructed to eat their normal diet and do their usual routines. Urine was collected from the subjects once each week over a 24-h period, beginning with the second void of the first day and concluding with the first void the following morning. The urine was typically collected on Thursday and finished on Friday morning, with analysis being carried out on the same day. Sunday/Monday was reserved as a make-up day if a subject missed the usual collection day. Each void from the subjects was collected into a separate 1-L Nalgene container, the date and time were recorded using preprinted labels, and the samples were refrigerated until they were delivered to Dartmouth-Hitchcock Medical Center (DHMC) for analysis. A previous pilot study had shown that overnight refrigeration of the samples does not significantly impact measurements made using the calcein approach. For the clinical calcium analyzer measurements, the samples were acidified to resolubilize the calcium.

Data were collected with a calcein-based prototype urinary analysis system (pUAS) developed by Creare LLC. The device measures urinary calcium concentration using a fluorescent indicator method where calcein is mixed with urine in a predetermined concentration ratio and then passed through an optical excitation and sampling cell. Optical measurements are made by exciting the mixture of urine and calcein at a wavelength of 480 nm and measuring the strength of the resulting fluorescent signal produced at a wavelength of approximately 525 nm. The strength of the signal is proportional to the concentration of dissolved calcium in the urine. For a subset of the samples, to establish comparability between the devices, each void's concentration was analyzed both by the pUAS and the clinical chemistry analyzer at DHMC. Calculating 24-h urinary calcium mass was done by summarizing the products of each void's concentration and volume (measured with a graduated cylinder) within the 24 h. Creatinine was also measured on the samples with the DHMC clinical analyzer. The calcein-based device suitable for

spaceflight, however, does not have the ability to measure creatinine. Thus all data were expressed as a concentration ($mg \cdot L^{-1}$) rather than normalizing for grams of creatinine because the creatinine information would not be available for spaceflight use.

To assess whether a given level of variability in urinary calcium excretion would be acceptable to detect spaceflight changes, we analyzed spaceflight data on urinary calcium 24-h mass. These data came from the Skylab program. The Skylab data used in this study were obtained from the NASA Experiment M071 carried out on the nine astronauts who participated in the three Skylab flights of 28, 59, and 84 d in 1973–1974, which aimed to determine major changes in the chemical state of the muscular and skeletal systems in space.[9] For the experiment, environmental factors were kept as constant as possible from phase-to-phase, that is preflight to in flight to postflight, and from day-to-day. Astronauts were under stringent dietary control. For example, the Commander of the 59-d flight consumed 725 ± 31, 729 ± 72, and 742 ± 40 mg of calcium daily during the three phases, respectively, which was representative of the group. The 24-h total urinary calcium mass for the 15 d prior to launch, the first 14 d, 1 d each in weeks 3 and 4 in flight, and the first 17–19 d postflight were available for this study.

Statistical Analysis

To evaluate the effect of oral calcium supplementation on urinary calcium excretion and to investigate the relationship between first void concentration and 24-h total mass, linear mixed-effect models were used to account for the nonindependence resulting from repeated measurements of the same subjects. These models included the interaction between the oral calcium intake indicator (i.e., whether oral calcium supplement was taken) and the time variable (week). The repeatability of urinary calcium 24-h mass and first void concentration measurements were quantified using the reproducibility coefficient (RPC), 1.96 times the standard deviation of the overall urinary calcium differences between measurements at two different time points, and the coefficient of variation (CV), the standard deviation of the differences divided by the overall mean of all data expressed as a percentage.[11] The RPC, also known as the smallest real difference, retains the units of urinary calcium level measurements and indicates the limits of agreement or the 95% confidence interval of the absolute difference between two measurements.[2,12,19]

For each subject, each time point was paired with the next one (the last time point was spontaneously paired with the first one). Measurement pairs from all six subjects were used to calculate overall RPC and CV. The time points were first obtained by randomly permutating the original 8 wk one, two, or three times, giving rise to 8-, 16-, or 24-wk points with new orders and then either directly using the generated 8-wk points (i.e., to assess the repeatability of a single measurement) or averaging every two or three subsequent weeks when two times or three times the number of permuted week points were generated. This normal urinary calcium variability was compared with the expected urinary calcium level changes in space, which were calculated from the Skylab dataset. The changes in 24-h urinary calcium mass were the nine-astronaut averages of individual differences between the measurements at week 1, 2, 3, and 4 in flight and the preflight measurement means. The ratio of the standard deviation of the differences to the overall mean of preflight measurements gave a measure of difference dispersion. In addition to the overall repeatability of urinary calcium excretion among the six subjects in the current study, the repeatability of measurements within an individual subject were assessed using the same method, except the pairs of measurements were confined to a single subject. The agreement between the pUAS and the clinical chemistry analyzer was determined using Bland-Altman analysis based on pairs of measurements from the two instruments. Bland-Altman and correlation plots were used to visualize the results. All analyses were conducted using Matlab R2019a.

RESULTS

The six subjects' averaged urinary calcium excretion obtained using pUAS over 8 wk of the study was 282.0 ± 89.2, 144.8 ± 47.0, 237.6 ± 54.4, 206.1 ± 65.9, 181.6 ± 42.9, and 249.9 ± 80.8 mg 24-h mass and 183.6 ± 68.9, 40.8 ± 20.2, 90.5 ± 21.8, 37.0 ± 21.0, 57.4 ± 24.8, and 152.2 ± 68.4 $mg \cdot L^{-1}$ first void concentration (see **Appendix A, Table AI**, which presents the raw data of the current study).

The interaction term between the oral intake indicator and time was statistically insignificant in the linear mixed effect regression of urinary calcium excretion measurements, showing that the oral supplement of calcium and vitamin D did not significantly influence urinary calcium excretion levels, either 24-h mass or first void concentration. This was true even if the two subjects with the highest urinary calcium excretion were excluded from the analysis.

Accounting for the subject-specific random effects, the first void concentration was highly correlated with 24-h total calcium ($P < 0.001$) with an R-squared of 0.42 as shown in **Fig. 1**.

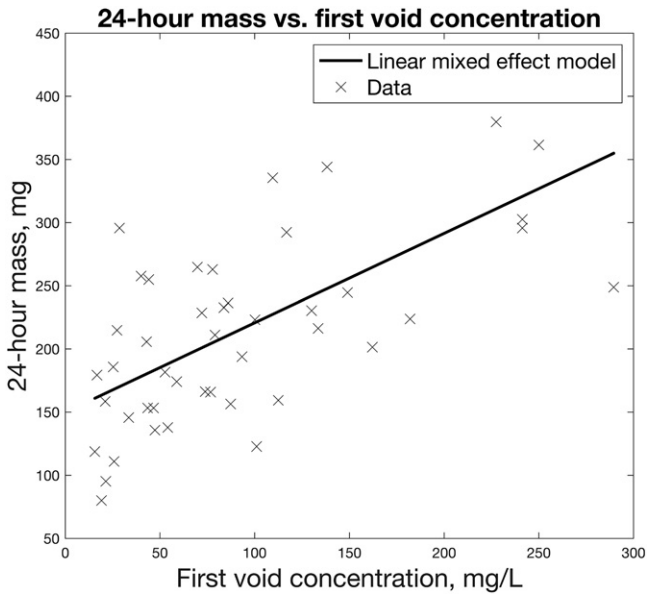

Fig. 1. Approximating 24-h urinary calcium mass using first void urinary calcium concentration.

The Bland-Altman plots (**Fig. 2** and **Fig. 3**) demonstrate the distribution of differences and the limits of agreement between urinary calcium measurements at different times. As summarized in **Table I**, the reproducibility coefficients for 24-h total urinary calcium were 167.0, 116.8, and 108.1 mg and the coefficients of variation were 39.2%, 27.6%, and 24.6% using 1-, 2-, and 3-wk time point measurement averages, respectively (Fig. 2). As more time points were averaged for assessment, the agreement interval between 24-h mass measurements narrowed and the dispersion of measurement difference distribution was reduced. In the Skylab study, astronauts had an average increase in 24-h urinary calcium mass of 88.9 ± 76.0, 123.5 ± 58.3, 142.2 ± 56.5, and 159.9 ± 83.4 mg after being in flight for 1, 2, 3, and 4 wk, respectively, compared to the average baseline levels before launch. This corresponded to a 50.2%, 72.6%, 83.5%, and 90.9% increase, respectively, over the preflight baseline. As is shown in **Fig. 4**, by averaging two or three urinary calcium excretion measurements the changes that were seen in the Skylab program could be detected. As shown in Fig. 3, the reproducibility coefficients and coefficients of variation of first void urinary calcium concentration had a similarly decreasing pattern as 24-h total mass: 90.6, 76.6, and 72.8 mg · L^{-1} and 49.4%, 41.5%, and 39.5%, respectively.

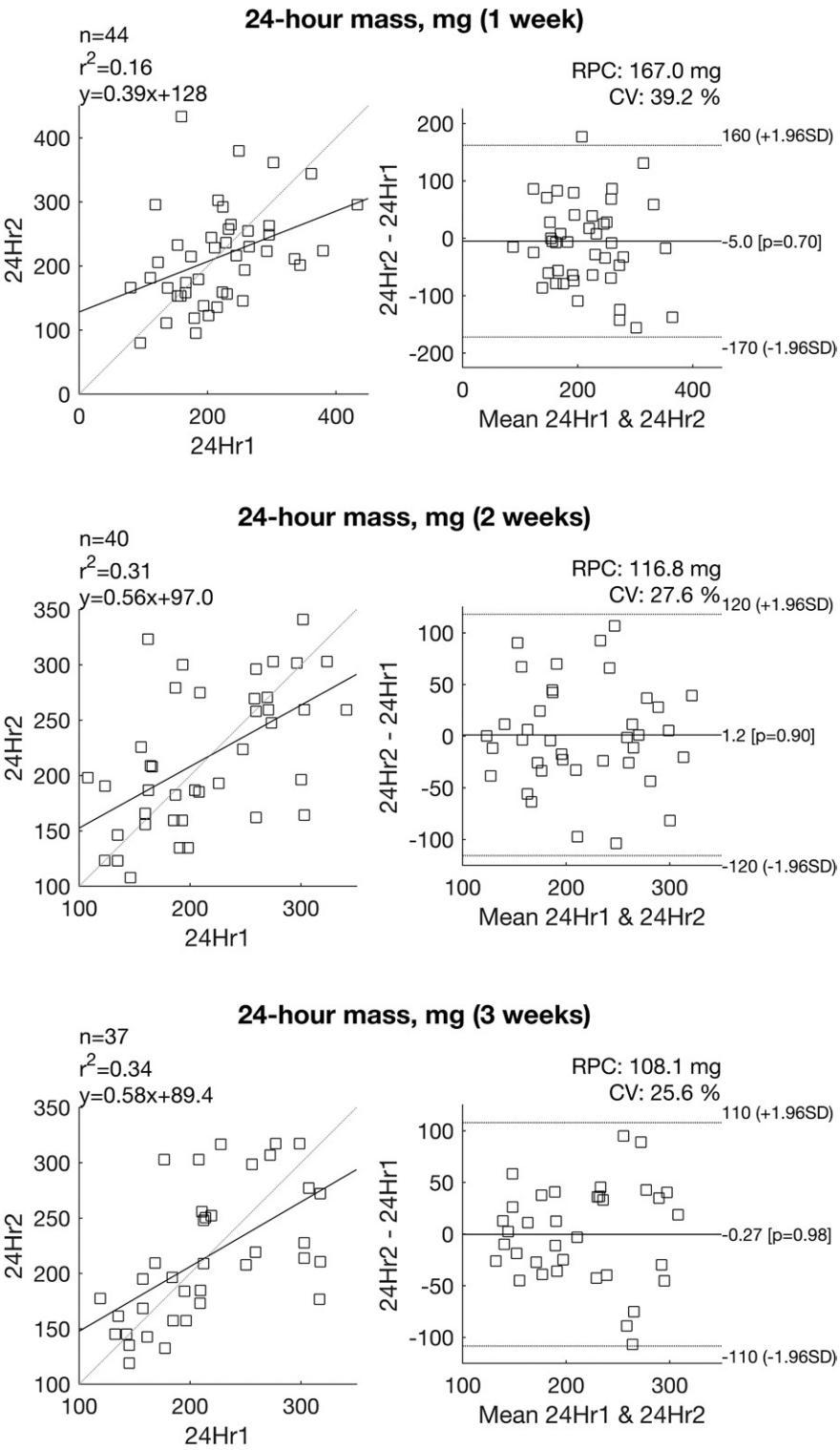

Fig. 2. 24-h mass Bland-Altman plots using 1-wk, 2-wk average, and 3-wk average measurements.

The reproducibility coefficients and coefficients of variance of 24-h urinary calcium mass and first void concentration for each subject are shown in **Table II**. For an individual, averaging measurements at more time points did not necessarily reduce the repeatability.

The results of pUAS and the clinical chemistry analyzer at DHMC were highly correlated and reproducible. The measurements of the two instruments demonstrated a squared Pearson correlation coefficient of 0.98, a reproducibility coefficient of 22.1 mg, and a coefficient of variance of 5.6% for 24-h urinary calcium mass, and 0.99, 12.4 mg · L^{-1}, and 7.4% for first void urinary calcium concentration.

DISCUSSION

To the authors' knowledge, this is the first study that establishes the repeatability of urinary calcium 24-h mass measurements and first void concentrations to assess how frequently the measurements should be taken to detect operationally significant changes in space. This study shows that while a single measurement of 24-h urinary calcium mass would only be useful for detecting very large changes in urinary

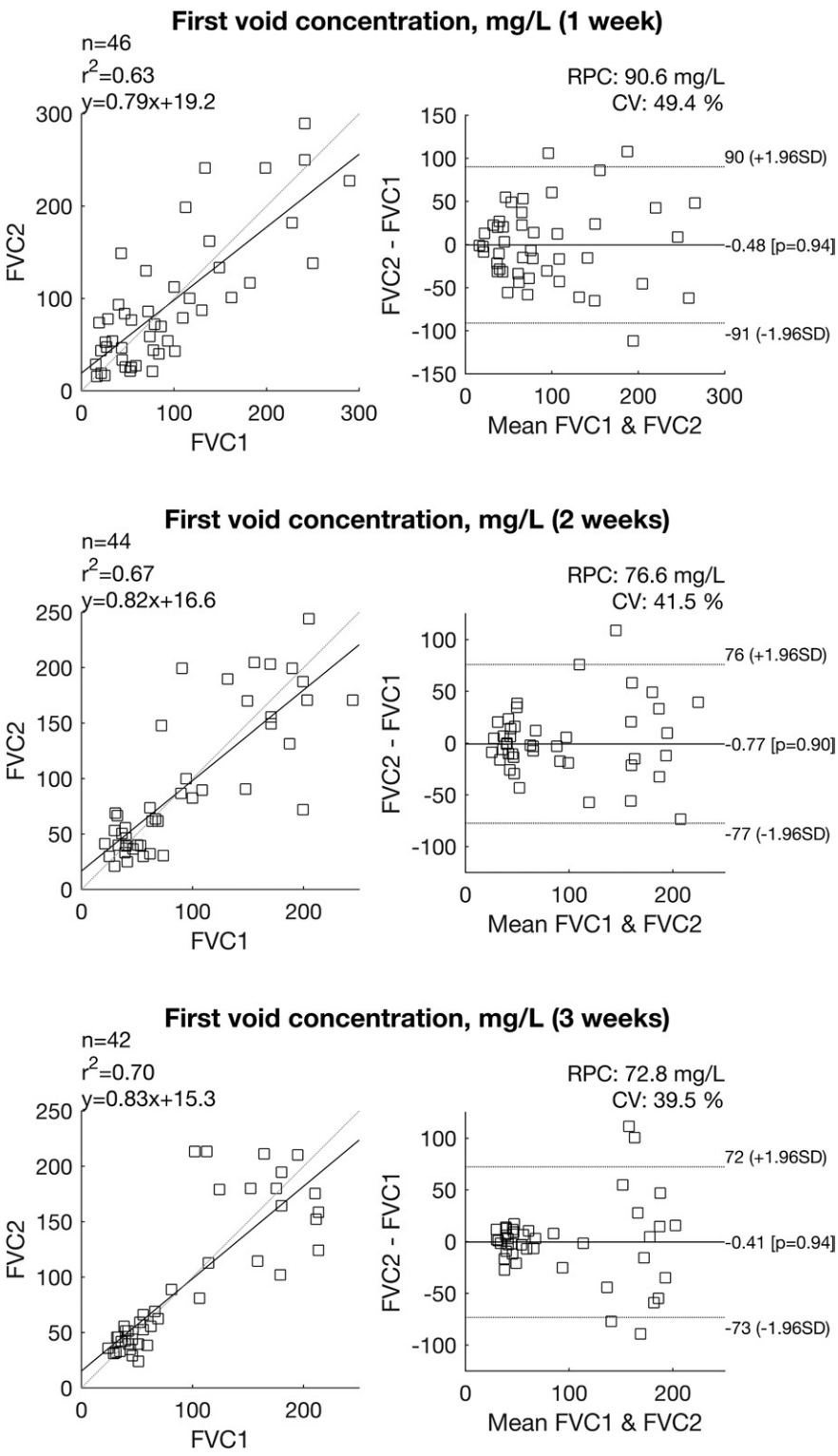

Fig. 3. First void concentration Bland-Altman plots using 1-wk, 2-wk average, and 3-wk average measurements.

calcium excretion compared to a preflight baseline, by averaging repeated measures of 24-h urinary calcium mass, changes similar to those seen in the Skylab program could be detected easily (Fig. 4). The possibility also exists that first void urinary calcium concentration measures could be used. Although first void calcium concentration can be influenced by water intake and urine volume, first void urinary calcium concentration highly correlates with 24-h urinary calcium mass and does not require measuring urine volume and calculating calcium mass over 24 h. Despite first void concentration's limited performance of predicting 24-h mass (it only predicted 42% of the variance), by making multiple measurements over time this limitation could potentially be overcome. Further data are needed on the number of measurements needed to make this approach successful, but it is very attractive for operational use. It is very likely any astronaut who has persistently high first void concentrations would be detected using this approach. One reason for using a calcein-based device in this study is that the calcein approach is well-suited for making a small, portable, calcium measurement system that could be used in space. A calcein-based device that is small enough to fit a hand and is easy to use is currently under development. Such a device could potentially be used to make repeated first void measurements.

The oral calcium and vitamin D supplementation used in this study did not affect urinary calcium excretion levels significantly. After the oral supplementation, urinary calcium excretion levels did not show a statistically significant increase. Previous studies observed a significant elevation in 24-h urinary calcium excretion after a 1-wk oral intake of 3 g of calcium carbonate per day.[5,17] The difference in our results could be due to the much lower oral supplement amount (500 mg calcium carbonate with 400 IU vitamin D) and the small sample size.

One of the goals of this study was to assess the normal variability of human urinary calcium excretion without dietary control (as would be the case before and during spaceflight). This range provides a sense of when an abnormal change caused by events such as spaceflight could be detected. The Bland-Altman analysis was developed to evaluate the agreement between different approaches. In this study we adapted this method to measure urinary calcium levels between different time point pairs to quantify the urinary calcium variability. The Bland-Altman plots and correlation scatter plots presented in Fig. 2 and

Table I. Overall Repeatability of Urinary Calcium Excretion Using One Measurement (1 wk), Two Measurement Average (2 wk), or Three Measurement Average (3 wk) from pUAS.

	24-h TOTAL URINARY CALCIUM, mg			FIRST VOID CONCENTRATION, mg · L^{-1}		
	1-wk	2-wk	3-wk	1-wk	2-wk	3-wk
Reproducibility coefficients	167.0	116.8	108.1	90.6	76.6	72.8
Coefficient of variance, %	39.2	27.6	24.6	49.4	41.5	39.5

Fig. 3, as well as the reproducibility coefficient and coefficient of variance, showed the agreement between urinary calcium excretion levels at different times, which can be further interpreted as its variability. The Pearson correlation coefficient r-squared and the slope that are closer to 1 are associated with tighter agreement intervals, which indicate a better repeatability between different urinary calcium measurements and smaller variability. Since we expect that 95% of the differences between two sequential measurements fall in the agreement interval, the interval can be adopted as the bound where any changes that are beyond the bound are likely to be detected. The natural variability of urinary calcium in space could be smaller in space due to the controlled environment and potentially less varied diet. Thus, the results from this study may be conservative, making averaging repeated measures even more promising than presented here. Also, Subjects 1 and 6 had relatively higher levels of urinary calcium excretion compared to the other subjects and the overall variability was lower when these subjects were excluded from the analysis. This indicates that for some subjects, fewer measurements may be needed.

Averaging more measurements lowers the detection bound. In this study we randomly permutated the order of weeks, where on average every week's measurement was used equally. Skylab data were precisely measured and therefore can be used to evaluate the measurement strategy. The resulted repeatability coefficient by averaging 3 wk, 108.1 mg, was smaller than the urinary calcium change after entering space for 2, 3, and 4 wk, 123.5, 142.2, and 159.9 mg (Fig. 4). This implies that consecutive measures in space would enhance the stability of results and in turn increase the probability of detecting relatively minor changes. A similar decreasing pattern in repeatability was observed in the first void concentration. The first void concentration was highly correlated to and might be used to approximate 24-h total urinary calcium mass. Also, the ability to detect high urinary calcium concentrations can be useful for identifying higher risk of kidney stone formation. Therefore, although first void data in space to make a comparison are currently not available, substituting first void concentration for 24-h urinary calcium mass may be feasible. The individual subject results in Table II show that there are interindividual differences as well. This indicates that the measurement frequency could be individualized to the astronaut rather than using an average value. Collecting and analyzing the first void concentration for 3 d each week and then monitoring potential changes could be an effective way to track bone loss in real time during space missions.

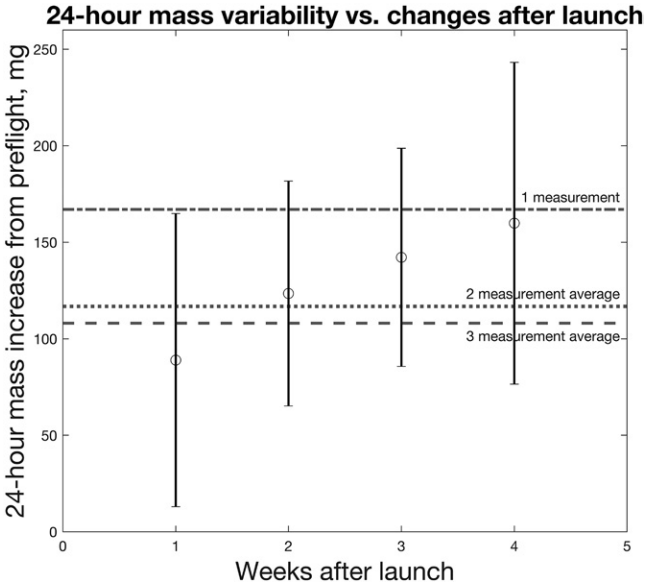

Fig. 4. Comparing 24-h mass single measurement and multiple measurement average variability to 24-h mass changes after 1, 2, 3, and 4 wk in space in the Skylab program.

Since permutation introduces randomness, the authors performed multiple simulations to explore how the results would vary across simulations. The results, including the reproducibility coefficients and coefficients of variance, obtained from the original data, one-time permutation, and averages of 10 times and 20 times of permutation are close to each other (shown in Appendix A, **Table AII**), which validates the single-permutation results presented in Fig. 2 and Fig. 3 and their comparison to the urinary calcium excretion change in Skylab data.

Besides a feasible measurement strategy, this study also employed the calcein-based fluorometric approach in the prototype device (pUAS) developed by Creare LLC. The Bland-Altman analysis on pUAS and the clinical chemistry analyzer demonstrated highly consistent results between the two instruments. The pUAS demonstrated the feasibility of the calcein technique for urinary calcium measurements. This technique is well suited for creating a small (handheld), portable, and low-power device for use in a spacecraft. A new device based on the calcein technique is under development and will use a capillary tube to make rapid urinary calcium measurements.

There were some abnormalities in the week 1 data in this study. There seem to be few measurements with high calcium concentrations in the first week relative to other weeks. The measurement activities in week 1 could have been affected by lack of familiarity with the procedures, which improved over time. An inspection of the original records showed that the

Table II. Repeatability of Urinary Calcium Excretion for Individual Subjects Using One Measurement (1 wk), Two Measurement Average (2 wk), or Three Measurement Average (3 wk).

	24-h MASS, mg			FIRST VOID CONCENTRATION, mg · L^{-1}		
RPC	**1 wk**	**2 wk**	**3 wk**	**1 wk**	**2 wk**	**3 wk**
Subject 1	288.0	73.5	108.3	110.3	83.2	67.9
Subject 2	125.5	108.8	63.7	61.1	17.0	26.7
Subject 3	117.5	171.0	100.9	72.4	23.2	45.8
Subject 4	191.7	96.1	70.6	53.1	36.5	20.0
Subject 5	90.8	74.5	59.1	80.5	55.4	23.4
Subject 6	160.5	187.7	194.9	154.2	155.2	156.0
	24-h MASS			**FIRST VOID CONCENTRATION**		
CV, %	**1 wk**	**2 wk**	**3 wk**	**1 wk**	**2 wk**	**3 wk**
Subject 1	52.1	13.3	19.6	30.7	23.1	18.9
Subject 2	44.2	38.3	22.4	76.4	21.3	33.4
Subject 3	25.4	35.2	21.7	41.4	12.6	26.2
Subject 4	45.9	24.9	17.1	73.4	50.4	27.56
Subject 5	25.5	20.9	16.6	71.5	49.3	20.8
Subject 6	32.8	38.3	39.8	51.7	52.0	52.3

RPC: reproducibility coefficient; CV: coefficient of variation.

number and volume of voids were well recorded and reasonable. Therefore, there was no legitimate reason for the authors to exclude the first week data. Participating in the study may have subconsciously made the subjects change their behavior too, although it is not clear why this would lead to greater urinary calcium excretion.

In summary, averaging multiple urinary calcium measurements reduces data variability and allows smaller changes in urinary calcium excretion levels to be detected. The calcein-based approach is suitable for creating a low-power, small, and valid device for use in space. These data provide the basis for monitoring consecutive urinary calcium measurements over time to assess the effectiveness of the countermeasure programs and to determine trends in urinary calcium excretion. Since the first void concentration is highly correlated to the 24-h total mass, it is reasonable to expect that first void concentrations could be used rather than complete 24-h collections and further research is needed in that area. By repeatedly measuring first void concentrations, real-time in-flight direct monitoring for bone loss and accompanying kidney stone risks may become practicable, which in turn enables individualized countermeasure programs for crewmembers.

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APPENDIX A.

Table AI. Urinary Calcium Measures Using pUAS.

		WEEK							
MEASURES	**SUBJECT**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
24-h mass, mg	1	223.1	292.3	223.8	159.3	295.7	433.3	248.8	379.7
	2	95.2	181.6	110.9	80.0	174.1	166.2	214.8	135.7
	3	NaN	156.4	230.3	335.5	228.5	211.0	236.4	264.7
	4	118.7	179.3	185.8	295.7	254.9	262.9	145.6	NaN
	5	165.8	137.8	193.9	158.4	153.2	153.3	232.7	257.7
	6	244.6	205.8	122.8	216.3	361.4	302.6	344.0	201.4
24-h total volume, L	1	2.4	4.1	2.1	1.8	2.6	2.5	2.7	1.6
	2	4.2	3.7	3.7	4.3	3.4	4.1	4.5	3.3
	3	3.6	2.3	3.6	2.7	3.0	3.2	3.8	2.8
	4	3.6	4.1	3.3	2.6	1.9	2.5	1.7	2.0
	5	2.4	2.5	2.9	2.1	2.3	1.9	2.6	2.1
	6	2.1	1.8	1.1	2.0	1.4	1.4	2.3	1.4
First void concentration, mg · L^{-1}	1	100.2	116.8	182.0	112.5	241.3	198.7	289.6	227.5
	2	21.4	52.7	25.7	19.1	58.8	73.9	27.3	47.3
	3	NaN	87.3	130.1	109.5	72.1	79.1	86.0	69.8
	4	15.6	16.7	25.3	28.6	44.1	77.8	33.5	53.9
	5	76.8	54.1	93.3	21.1	46.6	43.5	83.8	40.0
	6	149.0	42.9	101.0	133.5	250.0	241.3	138.2	162.1

RPC	**24-h MASS, mg**			**FIRST VOID CONCENTRATION, mg · L^{-1}**		
	1 wk	**2 wk**	**3 wk**	**1 wk**	**2 wk**	**3 wk**
No permutation	165.4	NA	NA	103.6	NA	NA
10 simulations average	183.2	119.8	105.6	124.1	85.8	72.7
10 simulations standard deviation	10.49	14.9	10.2	14.6	11.1	14.1
20 simulations average	184.5	126.3	106.8	123.18	88.58	69.0
20 simulations standard deviation	10.8	17.2	12.8	12.68	12.4	12.8
Presented in Figures	167.0	116.8	108.1	90.6	76.6	72.8

RPC: reproducibility coefficient.

Table AII. Result Fluctuation Across Simulations.

	24-h MASS			**FIRST VOID CONCENTRATION**		
CV, %	**1 wk**	**2 wk**	**3 wk**	**1 wk**	**2 wk**	**3 wk**
No permutation	38.6	NA	NA	56.2	NA	NA
10 simulations average	43.1	28.4	25.0	67.5	46.7	39.5
10 simulations standard deviation	2.4	3.6	2.4	7.9	6.1	7.6
20 simulations average	43.4	29.8	25.3	67.0	48.1	37.4
20 simulations standard deviation	2.5	4.1	3.0	6.8	6.8	7.0
Presented in Figures	39.2	27.6	24.6	49.4	41.5	39.5

CV: coefficient of variation.