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ORIGINAL ARTICLE

Stability of Urinary Thromboxane A_2 Metabolites and Adaptation of the Extraction Method to Small Urine Volume

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SUMMARY

Background: **Thromboxane (TX) A_2 is a pro-thrombotic prostanoid synthesized by activated platelets, biotransformed into 11-dehydro-TXB_2, measurable in urines. Eleven-dehydro-TXB_2 excretion is increased in high risk cardiovascular diseases; however, this cardiovascular biomarker awaits validation in large trials. The need of large urine volume (8 - 10 mL) and the unknown stability of 11-dehydro-TXB_2 in urine after collection might limit its implementation.**
Methods: **We scaled the original method for urine extraction and 11-dehydro-TXB_2 measurement down to 1 mL, and assessed its stability at 4°C or 25°C up to 6 days after collection. The sensitivity of the 1 mL procedure was also tested in aspirin-treated patients with low 11-dehydro-TXB_2 excretion.**
Results: **The 1 mL adapted method was highly correlated with the original assay (rho = 0.98, $p < 0.001$, n = 33). Both methods showed similar recoveries in samples spiked with exogenous 11-dehydro-TXB_2. Urinary 11-dehydro-TXB_2 values in samples immediately frozen were comparable and highly correlated to values in samples at 4°C (day 6: rho = 0.99, $p > 0.001$, n = 8) or 25°C (day 6: rho = 0.98, $p < 0.001$, n = 23) up to 6 days in controls and patients.**
Conclusions: **Eleven-dehydro-TXB_2 can be measured in small urine volumes and is relatively stable for a few days after collection, even at 25°C. These data allow the validation of this non-invasive cardiovascular biomarker in large studies.**
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KEY WORDS

thromboxane metabolite, stability, measurement, platelet, biomarker

INTRODUCTION

Thromboxane (TX) A_2 is a short-lived (32 seconds), unstable, biologically active derivative of arachidonic acid (AA) via cyclooxygenase (COX)-1 or -2 and TX synthase activities [1], released from activated platelets, promoting vasoconstriction and platelet aggregation [2]. Due to chemical instability, TXA_2 is rapidly and non-enzymatically converted to the stable, inactive hydration product TXB_2 [1], which in humans undergoes hepatic bio-transformation mainly into 11-dehydro-TXB_2 through dehydrogenase and reductase activities [3,4]. Eleven-dehydro-TXB_2 is excreted and measurable in urines, largely inhibited (70 - 80%) by low dose aspirin and its recovery after aspirin withdrawal reflects platelet lifespan [3-5].
Thus, 11-dehydro-TXB_2 reflects *in vivo* platelet activation status. Consistently, 11-dehydro-TXB_2 is increased in diseases at high cardiovascular risk such as unstable angina or stroke [2]. Furthermore, residual

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11-dehydro-TXB_2 levels predicted cardiovascular events in a subgroup of aspirin-treated high-risk patients from a large trial [6]. Thus, 11-dehydro-TXB_2 appears a promising, non-invasive, surrogate biomarker of cardiovascular risk, and/or antiplatelet drug response, but awaits further validation.

The need for small sample volume and stability are crucial for the development and clinical implementation of a biomarker [7]. The main methodological difficulty in validating 11-dehydro-TXB_2 as a non-invasive, cardiovascular biomarker is the need for a relatively large urine volume (~8 - 10 mL), according to the original method [8,9], while its stability before sample freezing is unknown. These features might lower the feasibility of its measurement in multicentric studies, especially if samples need to be shipped to a central lab and storage space is critical.

We adapted the original extraction method to 1 mL urine and assessed the stability of 11-dehydro-TXB_2 up to 6 days after urine collection by comparing measurements of samples immediately frozen, kept at room temperature (20 - 25°C) or 4°C.

MATERIALS AND METHODS

Urines were collected from controls, diabetic patients, or non-diabetics on aspirin as indicated, upon informed consent. Urine was collected from diabetics because, due to possible presence of proteins (microalbuminuria) or glucose, they are more likely to have contaminants possibly affecting 11-dehydro-TXB_2 stability.

For scaling down experiments, urines from the same donor were divided into 8, 4, 2, or 1 mL aliquots, 8 mL samples were extracted according to the original, validated method [8,9]. In scaling down experiments, we stepwise modified: acidification, volumes of washes and/or elution steps, and amount of ^3H-TXB_2 (7.7 TBq/mmol, GE Healthcare Amersham, Buckinghamshire, UK) used for calculating final recoveries. Protocols were optimized first to 4 mL, then to 2 mL and 1 mL urine. The 1 mL protocol giving the best results was the following: 60 µL acetic acid and 1,500 cpm ^3H-TXB_2 were added to 1 mL urine, then loaded onto a 1 mL/50 mg C18 column (Bakerbond-spe, JT Baker Inc, Phillipsburg, NJ, USA) prewashed with 1 mL absolute methanol and 2.5 mL distilled H_2O. The column was then washed with 1.6 mL distilled H_2O and 2 mL acetonitrile/water (15:85, vol/vol), dried and eluted with 2.5 mL isooctane/ethyl acetate (1:1, vol/vol). The eluate was loaded on a 1 mL/100 mg SiOH column (Bakerbond-spe, JT Baker Inc) prewashed with 2 mL isooctane/ethyl acetate (1:1,vol/vol), eluted with 2 mL ethylacetate/methanol (60:40, vol/vol). After drying, eluate was resuspended in 1 mL PBS/0.1% BSA buffer, 500 µL were counted for recovery. To assess extraction efficiency, 1 and 8 mL samples from the same donor were spiked with exogenous 11-dehydro-TXB_2 (0.5 to 1.5 ng/mL final concentrations) (Cayman Chemicals, Ann Arbor, MI, USA) or with vehicle (ethanol) and the percentage of cold spike recovery was calculated.

In stability experiments, after urine collection, one aliquot was immediately frozen (baseline), and the remaining sample was incubated in capped, sterile tubes at 4°C or in a water bath at 25°C. Aliquots were frozen daily up to day 6, until assayed. Urines were extracted as already described.

To check whether urinary 11-dehydro-TXB_2 stability could be improved, 1 mmol of the antioxidant 4-hydroxy-TEMPO (Sigma-Aldrich Co, St. Louis, MO, USA) was added immediately after collection to some urine samples and experiments were performed as detailed above. We also measured the 8-iso-prostaglandin (PG) $F_{2\alpha}$ formation in urine, to assess whether non-enzymatic, oxidative products of AA could be generated over the 6-day incubation. Eleven-dehydro-TXB_2 and 8-iso-$PGF_{2\alpha}$ were measured by validated enzyme immunoassay (EIA) [8,10,11]. The cross-reactivity between the two antibodies was < 0.01%.

Statistical analysis

Assuming a value of urinary 11-dehydro-TXB_2 of 383 ± 83 pg/mg creatinine in controls [12] and an inter-assay EIA variability of 15% calculated on an internal control (n = 30, unpublished data), we considered as acceptable a relative variation between baseline and day 6 not higher than 20%. Thus at least 8 samples were required to have 95% power (two-sided alpha). Data were reported as mean ± standard deviation (SD) or median and inter-quartile range [IQR] and analyzed according to their distribution. Comparisons were realized by ANOVA for repeated measurements, paired *t*-test, Deming regression or Bland-Altman analyses, as appropriate. Correlations were analyzed by Pearson or Spearman rank tests, as appropriate. The significance was $p < 0.05$.

RESULTS

By making stepwise modifications to the 8 mL method [8,9], we obtained highly-correlated values of urinary 11-dehydro-TXB_2 in samples extracted from 8, 4, 2, and 1 mL urine (Table 1 and Figure 1A, B). By Bland-Altman analysis, the mean percentage difference of 8 mL minus 1 mL extraction methods versus absolute 11-dehydro-TXB_2 8 mL and 1 mL means was -6.6 ± 12% (Figure 1A). By Deming regression there was no proportional error within the tested concentration range of 11-dehydro TXB_2 (min 142 pg/mL-max 2,700 pg/mL, regression slope coefficient = 0.002 ± 0.003, p = 0.41). Moreover, 32 of 33 measurements (96.9%) were comprised within the ± 1.96 SD. Eleven-dehydro-TXB_2 values of 8 and 1 mL extractions were highly correlated (rho = 0.983, p < 0.0001, n = 33, Figure 1B). Experiments with exogenous 11-dehydro-TXB_2 added to 8 and 1 mL samples showed similar recovery of the exogenously-added compound between the methods: 72 ± 18% in 1 mL (n = 21) versus 73 ± 28% in 8 mL extrac-

Table 1. Pearson correlation between different extraction methods.

	8 mL	4 mL	2 mL
8 mL		0.987	0.99
		n = 18	n = 22
		p < 0.001	p < 0.001
4 mL	0.987		0.80
	n = 18		n = 20
	p < 0.001		p < 0.001
2 mL	0.99	0.80	
	n = 22	n = 20	
	p < 0.001	p < 0.001	
1 mL	0.983		0.99
	n = 33		n = 14
	p < 0.001		p < 0.001

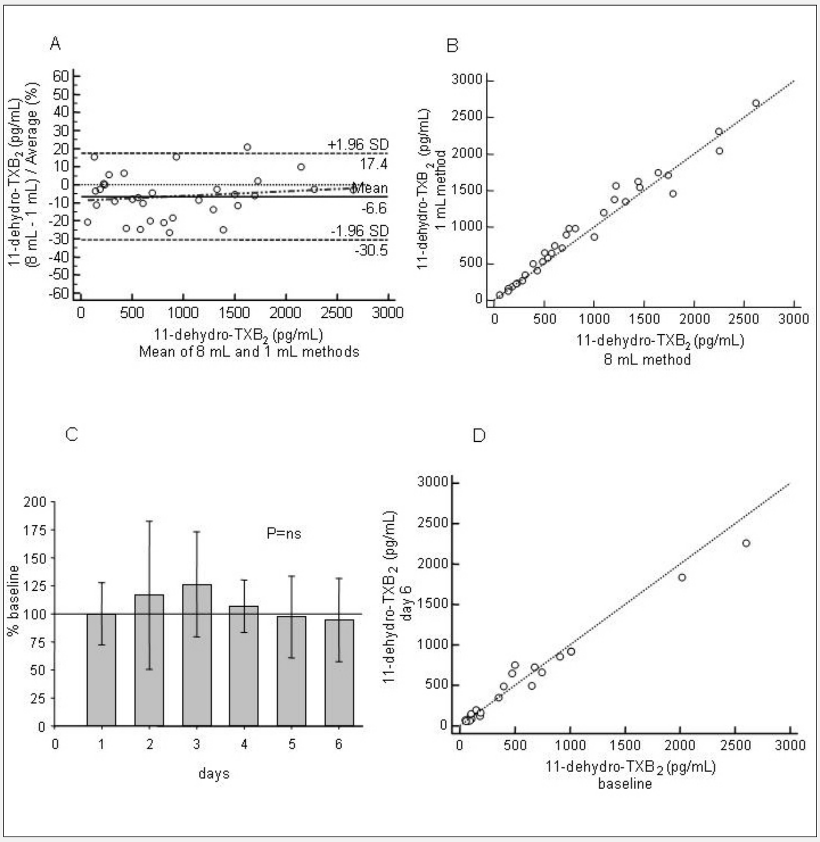

Figure 1. Panel A depicts the Bland-Altman analysis of 11-dehydro-TXB_2 values obtained from the 8 mL and 1 mL extraction methods (n = 33). The dotted line represents the equality line, the dashed lines represent the ± 1.96 SD and the correspondent values, the dotted-dashed line represents the Deming regression. Panel B shows absolute 11-dehydro-TXB_2 values from samples extracted with the 8 mL and 1 mL methods, the line of equality is represented in the plot. Panel C shows data of 11-dehydro-TXB_2 from samples incubated for different intervals at 25°C without 4-hydroxy-TEMPO, values are represented as % of baseline (= 100%, samples frozen within 3 hours from collection), each column represents mean ± SD of 8 - 10 determinations. Panel D represents the absolute values of 11-dehydro-TXB_2 obtained at baseline versus the corresponding values of samples incubated for 6 days; the line of equality is represented in the plot.

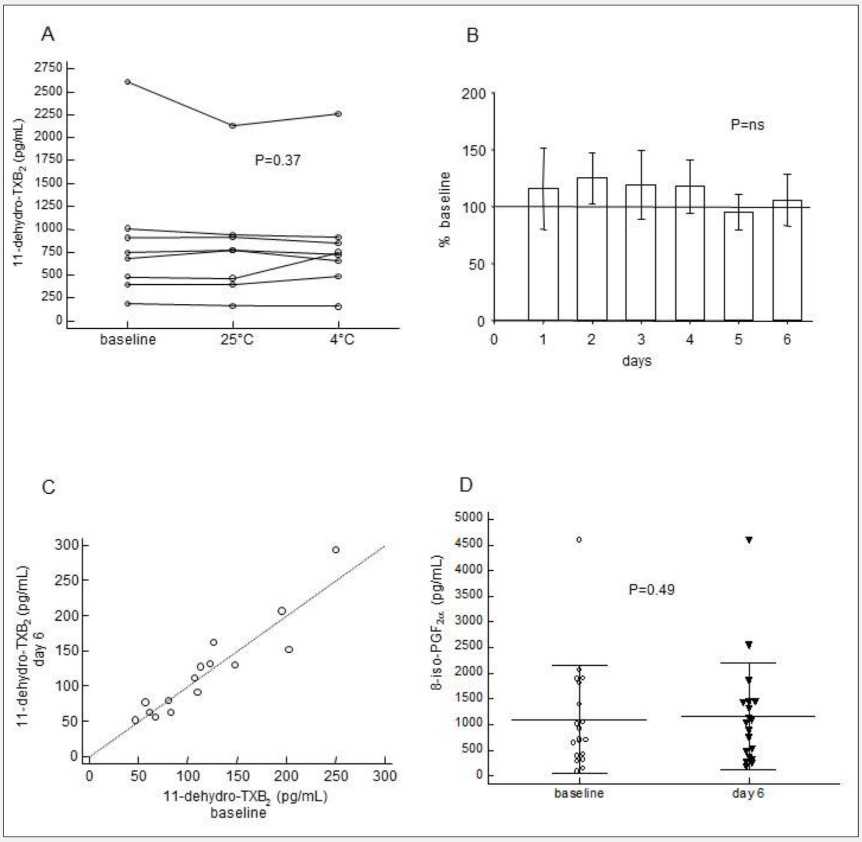

Figure 2. Panel A shows the absolute values of 11-dehydro-TXB_2 obtained at baseline and the corresponding values in samples incubated for 6 days at 25°C or 4°C (n = 8). Each group of dots and line diagram represents an experimental set. Panel B shows 11-dehydro-TXB_2 data obtained from samples incubated for up to 6 days at 25°C with 1 mM 4-hydroxy-TEMPO, values are represented as % of baseline (= 100%), each column represents mean ± SD of 8 - 10 experiments. Panel C shows absolute 11-dehydro-TXB_2 values from samples at baseline and day 6 of incubations at 25°C with 4-hydroxy-TEMPO, the line of equality is represented in the plot. Panel D represents the absolute values of 8-iso-$PGF_{2\alpha}$ at baseline and after 6 days of incubation at 25°C without 4-hydroxy-TEMPO, individual data as well as means ± SD are represented.

tions (n = 10, p = 0.8).

We then checked whether the 1 mL method could be used in patients treated with aspirin, which would be the population with the lowest 11-dehydro-TXB_2 excretion values as compared to aspirin-naïve subjects [2]. In 10 non-diabetic patients, who were fully responsive to aspirin on the basis of serum TXB_2 measurements [2], we could detect 11-dehydro-TXB_2 values of 37 ± 32 pg/mL (min 10 pg/mL - max 110 pg/mL) in the urine extracts. The stability of urinary 11-dehydro-TXB_2 over 6 days at 25°C was assessed with or without 4-hydroxy-TEMPO. Eleven-dehydro-TXB_2 values up to 6 days of incubation without 4-hydroxy-TEMPO did not differ from baseline at any time point (Figure 1C) and highly correlated with baseline (day 6: r = 0.988, p < 0.001, n = 22; Figure 1D). Eleven-dehydro-TXB_2 values at baseline averaged 526 ± 653 pg/mL and on day 6 averaged 505 ± 579 pg/mL (n = 22, p = 0.44), with a similar pattern in patients and controls: (controls: 257 ± 317 pg/mL baseline versus 247 ± 283 pg/mL day 6, p = 0.60, n = 8; patients: 680 ± 751 pg/mL baseline versus 652 ± 659 pg/mL day 6, p = 0.49, n = 14). To further assess stability, we also compared values from urine samples incubated at 4°C and 25°C versus baseline, assuming that 4°C was a better condition to preserve 11-dehydro-TXB_2 stability, and found no significant differences (Figure 2A). Similarly, 11-dehydro-TXB_2 values over 6 days of incubition were observed also by adding 4-hydroxy-TEMPO (Figure 2B), with highly correlated values at day 6 (r = 0.934, p < 0.001, n = 15 and Figure 2C).

Urinary 8-iso-$PGF_{2\alpha}$ levels were also measured in the same samples to assess whether during *in vitro* incubation a non-enzymatic, oxidation-derived transformation of AA occurred in urine. No significant changes were

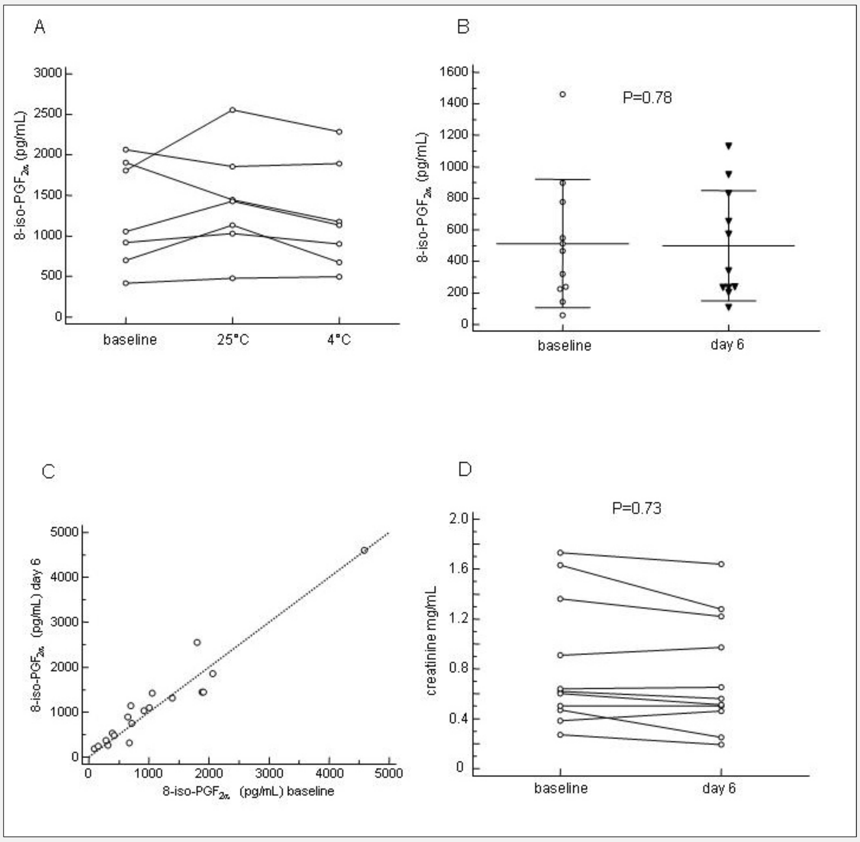

Figure 3. Panel A shows the absolute values of 8-iso-PGF$_{2\alpha}$ obtained at baseline versus the corresponding values from samples incubated for 6 days at 25°C or 4°C (n = 7). Each group of dots and line diagram represents one experimental set. Panel B: the plot depicts the measurements of 8-iso-PGF$_{2\alpha}$ at baseline and after 6 days of incubation at 25°C with 4-hydroxy-TEMPO, individual data as well as means ± SD are represented. Panel C represents the absolute values of 8-iso-PGF$_{2\alpha}$ obtained at baseline versus the corresponding values of samples collected at day 6, the line of equality is reported. Panel D: the figure shows creatinine values at baseline and at day 6 at 25°C (n = 10).

observed at different time points versus baseline without 4-hydroxy-TEMPO at 25°C (Figure 2D) and 4°C (Figure 3A), or with 4-hydroxy-TEMPO (Figure 3B), and values measured at day 6 were highly correlated with baseline (Figure 3C, rho = 0.96, n = 19, $p < 0.001$). A similar pattern was observed in controls or patients. Because 11-dehydro-TXB$_2$ excretion values are corrected for urinary creatinine concentrations, we measured the stability of urinary creatinine as well, and observed no significant changes between baseline values versus day 6 at 25°C, both in patients and controls (Figure 3D).

DISCUSSION

Atherosclerosis and cardiovascular diseases are the first cause of death worldwide. Clinical or biochemical surrogate biomarkers enabling the identification of patients at cardiovascular risk are crucial and may help the therapeutic decision. Cholesterol and blood pressure are well established cardiovascular biomarkers, used for risk stratification, and targets of pharmacological interventions [13]. Inflammation and platelet activation contribute to atherosclerosis [2]. C-reactive protein, an inflammatory biomarker, has been validated in a phase III trial as surrogate endpoint predicting cardiovascular complications [14]. *In vivo* platelet activation has no validated biomarker so far. Optical platelet aggregation has high intra- and inter-individual variability, is not standardized, needs fresh blood, is scarcely sensitive to aspirin, and consistently failed to predict thrombosis in cardiovascular diseases [15,16]. TXA_2 is a major product of activated platelets, rapidly and fully converted by a non-enzymatic reaction to TXB_2 which is enzymatically transformed in the liver to the stable, urinary-excreted 11-dehydro-TXB_2 [3,4]. Low-dose aspirin largely inhibits 11-dehydro-TXB_2, further suggesting its platelet origin *in vivo* [3-5]. Thus, urinary 11-dehydro-TXB_2 reflects the degree of *in vivo* platelet activation and is sensitive to aspirin. Small, cross-sectional, observational studies showed that 11-dehydro-TXB_2 is elevated in myocardial infarction, stroke, hypercholesterolemia, hypertension, diabetes, and atherosclerosis [17-24] and might predict poor cardiovascular outcome in high risk, aspirin-treated patients, possibly being an index of poor drug response [6,18]. However, before its clinical implementation, it should be validated on large, prospective studies vis-à-vis hard clinical endpoints. A major advantage of this biomarker is the non-invasiveness, possibility of storing frozen samples before analysis, and a simple interpretation of the standard immunometric assay. Disadvantages are a large sample volume required and its unknown stability, which would affect feasibility in multicentric studies where samples might be shipped under different conditions to a central laboratory and storage space might be limited. We addressed both issues.

The 8 mL extraction method was adapted to 1 mL urine for 11-dehydro-TXB_2 measurement. As shown in Figure 1A, the 1 mL extraction method did not give lower recoveries and measurements of endogenous 11-dehydro-TXB_2 as compared to the original method, and the line of equality (e.g., between-method difference = 0) was included within the 1.96 SD limits of the percentage difference mean. Moreover, the two methods were highly correlated and the 1 mL extraction method showed the same efficiency as 8 mL extraction, as shown by similar recoveries of exogenously added 11-dehydro-TXB_2. Finally, the 1 mL method could be efficiently used also to study patients while on aspirin, where 11-dehydro-TXB_2 is expected to be inhibited by ~80% as compared to non-aspirinated patients.

We further showed that 11-dehydro-TXB_2 is quite stable in urines stored for up to 6 days at room temperature, in normal and in pathological conditions, such as diabetes, where contaminants (glucose, proteins) might facilitate *in vitro* oxidation and/or degradation processes [25]. The addition of 4-hydroxy-TEMPO as antioxidant seemed unnecessary. Eight-iso-$PGF_{2\alpha}$ was not significantly generated *in vitro* over the 6 day incubation, with or without 4-hydroxy-TEMPO. Creatinine was also stable in urines within the same interval. In conclusion, urinary 11-dehydro-TXB_2 stored in capped, sterile tubes up to 6 days at 25°C appears relatively stable both in normal and pathological conditions with or without antioxidants and can be measured in small urine samples. These data might facilitate its implementation and validation as cardiovascular biomarker in large, multicentric studies.

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Declaration of Interest:
The Authors have no conflicts of interest to disclose.

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