Impact of the preanalytical practices on the uracil and dihydrouracil stability for the diagnosis of dihydropyrimidine dehydrogenase deficiency.

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Background

- In 2019, EMA's safety committee recommended that patients should be tested for dihydropyrimidine dehydrogenase (DPD) deficiency before starting cancer treatment with 5-FU or related medicines (capecitabine and tegafur) **[1]**.
- Phenotypic activity of DPD relies on the measurement of uracil (U) and its metabolite dihydrouracil (UH2) in plasma. Because of the low stability of U and UH2 in biological samples, compliance with pre-analytical conditions is essential for the reliability of the results.
- According to the preanalytical conditions recommended by the French National Health Authority (HAS) **[2]** **plasma should be immediately separated from whole-blood or up to 1.5h after collection if stored at room temperature (RT) or up to 4h if stored at 4°C.**

▷ ***Objective :*** *this study aimed to gather preexisting unpublished data of U and UH2 stability in biological samples in order to consolidate the pre-existing references or establish new preanalytical recommendations.*

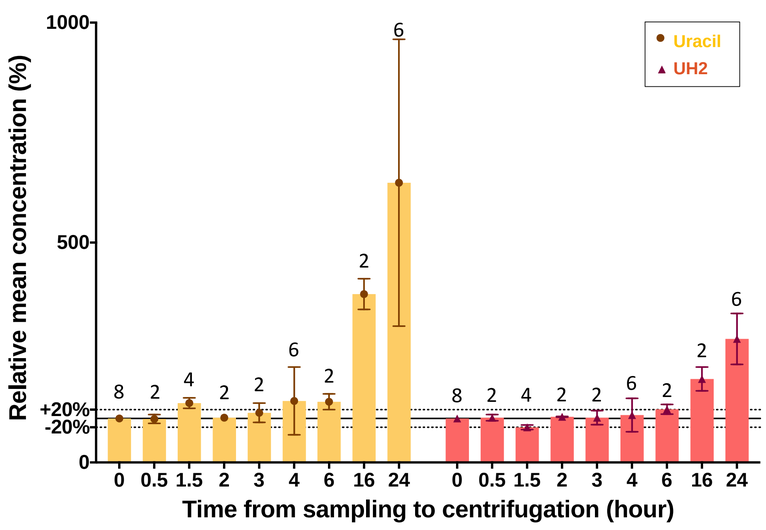
Material and Methods

- **Data collection :** during 8 months plasma concentrations of U and UH2 were collected from ten French and one Belgian laboratories belonging to the French National Pharmacogenetics Network (RNPGx) and the Clinical Oncopharmacology group (GPCO-Unicancer).
- **Evaluated preanalytical parameters :**
  - Stability in whole-blood before plasma separation by centrifugation;
  - Stability in plasma stored during variable periods and at variable temperatures (4°C or RT);
  - Long-term freezing and thawing;
- **The intraindividual variability** of U and UH2 was assessed in routinely analyzed patients that have been drawn at 2 different time points.
- **Graphs** were generated with GraphPad Prism® (v.7.0, La Jolla, CA).

Results and interpretation

Stability of U and UH2 in whole-blood samples

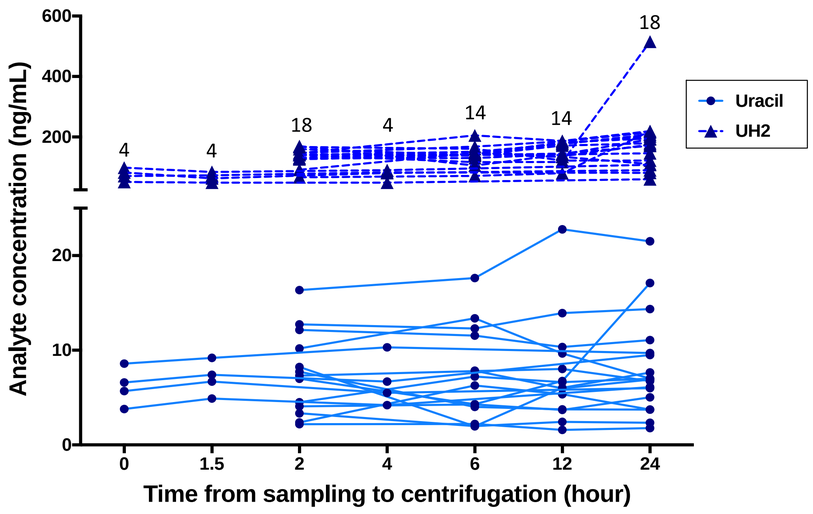
Fig.1: Storage of whole-blood samples at room temperature (RT)



- The aim of this assay was to determine the optimal delay and storage temperature before centrifugation of the whole-blood samples (*sample sizes on graph, data represented as relative means and standard deviations)*.
- At room temperature (**fig.1),** U rapidly increased as soon as 1h30 (+35%), whereas UH2 was stable until 6 hours (+21%). Both U and UH2 were increased after 16h (+283% and +90%, respectively).

▸ **These results at RT showed the important instability of U as early as 1h30 after sampling. However, only a small number of samples at each time point were available.**

Fig.2: Storage of whole-blood samples at 4°C



- **At 4°C** (**fig.2**), U and UH2 concentrations were stable and did not exceed 20% of the reference value until 12 h However at 24 h, UH2 increased significantly in one sample (+267%), whereas U only varied by +13%.

▸ **It appears that storage at 4°C could maintain stable concentrations of U and UH2 until 12 hours. This must be confirmed in a larger number of samples.**

Stability of U and UH2 during freezing

- The objective was to evaluate the stability of U and UH2 after long-term freezing and to assess the impact of thawing plasma samples.
- According to the results, long-term freezing of samples stored either at -20°C or -80°C was not accompanied by any severe variation of U and UH2 concentrations (**table 1**).
- In **table 2**, U and UH2 concentrations were measured before freezing and then after 1, 2, 3 or 19 cycles of 24 hour-freezing and 1 hour-thawing.

▸ **According to the results, freezing and thawing of plasma samples do not seem to impact uracilemia and UH2.**

Analyte	Temperature (°C)	Mean CV% 0-7d (n)	Mean CV% 1w-1m	Mean CV% >1m-1y	Mean CV% >1y
U	-20	-6% (4)	-1% (7)	*N/A*	*N/A*
	-80	10% (1)	-4% (3)	-7% (7)	-5% (3)
UH2	-20	-2% (4)	3% (7)	*N/A*	*N/A*
	-80	-4% (1)	6% (3)	3% (7)	6% (3)

Table 1: Impact of time and freezing temperature of plasma samples on U and UH2. *d : days; w : week; m: month; y: year; N/A : not available*

Freezing/thawing cycles (n samples)	Mean of uracil CV (%) [min-max]	Mean of UH2 CV (%) [min-max]
After 1 cycle (28)	0,49% [-20% – 34%]	5,18% [-26% – 28%]
After 2 cycles (21)	-4,42% [-10% – 3%]	-0,72% [-6% – 12%]
After 3 cycles (17)	1% [-29% – 33%]	8% [0% – 18%]
After 19 cycles (3)	8% [6% – 10%]	8% [7% – 9%]

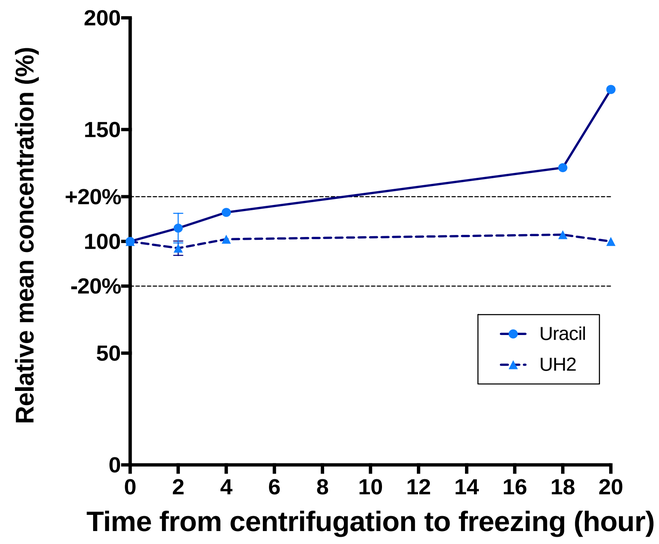
Table 2: Impact of freezing and thawing plasma samples on U and UH2 concentrations.

Stability of U and UH2 in plasma samples

- According to recommendations plasma should be frozen immediately after centrifugation. However, in some situations (as long-lasting transport) plasma may be stored at 4°C. The objective was to determine the maximum duration of plasma storage at 4°C.
- In **fig.3**, uracilemia increased at 18 h (+33%) whereas UH2 was stable up to 20 h.

▸ **These preliminary results are limited by the low number of data available and in particular, the lack of timepoints between 4h and 18h. Additional experiments are currently ongoing to investigate the stability of U and UH2 in plasma.**

Fig.3: Storage of plasma samples at 4°C (n = 5)



Intraindividual variability

- Many biological and environmental factors could influence U and UH2 concentration, such as the circadian DPD activity.
- In this evaluation, 102 patients analyzed in routine lab were included. Phenotypic activity of DPD was assessed at 2 different timepoints for every patient.
- The mean CV% was 29% for U [min: 0% – max: 12%, n = 98] and 21% for UH2 [0% – 96%, n = 79].
- Of these patients, 59 had their samples processed in accordance with pre-analytical recommendations while 43 had a least one non-compliant sample. The latter had a higher CV% for U (25% and 34%, respectively) but UH2 was consistent between the two groups (20% and 22%, respectively).
- Of note, among the 59 patients with 2 compliant samples, the diagnosis of deficiency according to the 16 ng/mL threshold was different between the two samples for 9 patients (15%) (**table 3, results shown for 6 patients**): 4 patients were misdiagnosed at the first sampling and 5 patients were diagnosed with a deficiency at the second sampling.

	Sample 1 (ref)			Sample 2			Time between sample 1 and sample 2 (days)	Bias (%)	
Patient (P)	U (ng/mL)	UH2/U	Deficiency?	U (ng/mL)	UH2/U	Deficiency?		U (ng/mL)	UH2/U
P1	12.4	8.2	No	23.1	6.1	Yes	43	+86,3	-25,6
P2	11.5	10.5	No	16	9.8	Yes	80	+39,1	-6,7
P3	15.4	7	No	16.5	10.2	Yes	53	+7,1	+45,7
P4	32.7	2.1	Yes	12.3	6.7	No	21	-62,4	+219
P5	20.8	4	Yes	8.6	10.6	No	112	-58,7	+165
P6	14.7	7.3	No	16.2	4.8	Yes	8	+10,2	-34,2

Table 3: Intraindividual variability of U and UH2 for 6 of the 9 patients with different diagnosis.

▸ **For patients #3 and #6, the difference may be explained by the fact that their U values are close to the threshold and by the variability of the analytical method (15-20%).**

▸ **This intra-individual variability analysis is currently being extended to more patients.**

Conclusion

These experimental results together with a review of the literature will allow giving recommendations concerning samples handling for DPD phenotyping by uracilemia measurement in plasma. The collection of additional data will help validate those results.

References

[1] European Medicines Agency – Recommendations, April, 2020 – Available here (English version).
[2] Inca/HAS : Haute Autorité de Santé – Recommendations, December, 2018 – Available here (French version).

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