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SAFE USE OF FLUOROPYRIMIDINES IN ONCOLOGY: DEVELOPMENT OF A METHOD FOR THE DETERMINATION OF PLASMA URACIL AND DIHYDROURACIL LEVELS BY UHPLC-MS-MS AND SCREENING FOR DIHYDROPYRIMIDINE DEHYDROGENASE DEFICIENCY

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ABSTRACT

5-Fluorouracil (5-FU) is a widely used drug in oncology. It can cause toxicity, especially in the case of Dihydropyrimidine Dehydrogenase (DPD) deficiency. An enzyme responsible for the inactivation of more than 80% of the 5-FU dose and for the transformation of uracil (U) into dihydrouracile (UH_2). A sensitive method is needed to screen for DPD deficiency by determination of U level, as recommended by the European Medicine Agency (EMA). Separation was performed by an UHPLC on a C_{18} column and a tandem mass spectrometer performed the detection of U and UH_2. The method's performance was validated according to ICH M10 recommendations. The validated method was used to screen for DPD deficiency in fifty-seven hospitalized patients. None of the patients showed DPD deficiency based on the U-rate instead of the metabolic ratio UH_2/U (7.08%) which might be a more sensitive tool for this screening.

Keywords: Uracil; Screening; DPD Deficiency; Fluoropyrimidines.

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1. INTRODUCTION

Fluoropyrimidines (FPs), with 5-FU as the lead compound, are cytotoxic molecules widely used in various cancer chemotherapy protocols where they play a central role. However, they can cause toxicities in 10 to 40% of cases [1] and are fatal in nearly 1% of patients [2–4]. More than 80% of the administered dose of 5-FU follows a pathway of inactivation conditioned by the activity of a key hepatic enzyme: Dihydropyrimidine Dehydrogenase (DPD) [5–8]. DPD converts 5-FU to 5-fluoro-5,6-dihydrouracil and converts U, its natural substrate, to UH_2 [9,10]. The activity and toxicity of 5-FU are thus closely related to the activity of DPD [11]. Indeed, this enzyme conditions the fraction of 5-FU available to the activation pathway, and a deficiency in this enzyme will lead to overexposure and increased toxicity to 5-FU, which may result in the patient's death [9,12-16]. Therefore, screening for DPD deficiency before starting any FPs-based chemotherapy is important. Several screening methods have been developed based on two complementary approaches: the genotypic approach, which studies the DPD gene, and the phenotypic approach, based on the quantification of uracilemia or the determination of the metabolic ratio UH_2/U [17]. A third multiparametric approach has been proposed combining genotyping and phenotyping [18,19].

The Clinical Pharmacology Oncology Group (GPCO)-UNICANCER and the French National Network of Hospital Pharmacogenetics (RNPGx) recommend pre-therapeutic screening for DPD deficiency by measuring uracilemia, possibly combined with calculation of the metabolic ratio and genotyping by searching for the *2A, *13, p.D949V and B3 variants of the DPD gene [18]. Since the end of 2018, the French Hight Health Authority (HHA) has recommended screening for DPD deficiency by measuring the U blood level before any administration of FPs chemotherapy [20]. Uracilemia levels below 16 $ng.mL^{-1}$ suggest the absence of DPD deficiency, whereas values above 16 $ng.mL^{-1}$ indicate either partial DPD deficiency when between 16 and 150 $ng.mL^{-1}$ or complete deficiency if above 150 $ng.mL^{-1}$ [17,21-23].

Various methods for the determination of U and UH_2 blood levels by liquid chromatography coupled to an ultraviolet detector (LC-UV) [24-27] or with a mass spectrometer (LC-MS-MS) [28-33] have been described in the literature.

The objective of this study is the development and analytical validation according to the ICH M10 protocol [34] of an ultra-high performance liquid chromatography method with tandem mass spectrometry detection (UHPLC-MS-MS) for the determination of plasma levels of endogenous U and UH_2. To our knowledge, the Toxicology Laboratory of the Central Army Hospital, Dr. Mohamed Seghir Nekkache, is the first Algerian laboratory to use such a method to ensure the safe use of FPs through pre-therapeutic screening for DPD deficiency.

2. EXPERIMENTAL

2.1. Chemical reagents

The certified reference standards required for determining U and UH_2 were purchased from Analytical Standards and Solutions laboratories(A2S®), namely Standard Uracil ($C_4H_4N_2O_2$ - purity 99±1%) batch U003A180709. Standard Dihydrouracil ($C_4H_6N_2O_2$ - purity 98±1%) batch D837A180709. Standard Uracil-13C, $15N_2$ ($C_4H_4N_2O_2$ - purity 96±1%) batch U025B200403 as an internal U standard (U_{IS}). Standard 5-6 Dihydrouracil-13C, $C_4H_6N_2O_2$ - purity 98.8±1%) batch D809B200407 as an internal standard of UH_2 ($UH_{2\ IS}$). Other chemical reagents used in the assay included: Formic acid (HCOOH - 98% purity) Panreac®, Methanol (CH_3OH - 98% purity) LOBA CHEMIE®, Ethyl acetate ($CH_3COOC_2H_5$- 99.9%) Panreac®, Isopropanol ($CH_3CHOHCH_3$ - purity 99.9%) Panreac®, Ammonium sulfate ($(NH_4)_2SO_4$ - purity 99%) Panreac®, Bisdistilled water on Elga Medica treatment station®.

2.2. Biological reagents

Bovine serum albumin (BSA) was used as a surrogate matrix knowing that U and UH_2 are naturally present in human plasma. Two sources were used: Panreac® (Purity ≥ 96%) batch 9Y011231 and ROTH® (Purity ≥ 98%) batch 169283077. The patient's plasma was obtained by venous blood sampling on EDTA tubes.

2.3. Chromatographic conditions (UHPLC-MS-MS)

The chromatographic system consists of an Agilent Technology UHPLC chain® 1290 Infinity II. The chromatographic separation was performed on an UHPLC Mediterranea sea C_{18} column (1.8 µm, 150x2.1 mm) Teknokroma® equipped with a C_{18} pre-column (1.8 µm, 10x2.1 mm). The mobile phase comprises 0.1% formic acid/Methanol (97/3%). The elution is in isocratic mode with a flow rate of 0.15 $mL.min^{-1}$. The analysis was performed via a triple quadrupole

mass spectrometer (Agilent Technologies® 6420 Triple Quad LC/MS) using the Electrospray Ionization method (ESI) in positive mode with the following source parameters: capillary voltage at 1700 V, nebulization performed at a pressure of 45 psi (3.1 bar), gas temperature 350 °C, gas flow rate 10 $L.min^{-1}$. The compounds were quantified in Multiple Reaction Monitoring (MRM) mode. The chromatographic data collection and processing software used is Masshunter Acquisition Data Qualitative Analysis B.06.00.

2.4. Sample processing

A liquid-liquid extraction (LLE) was used for sample processing as described by ***Neto et al.2018*** [33]. Briefly, in a 5.5 mL glass tube, a quantity of 50 µL of U_{IS} (200 $ng.mL^{-1}$) and 50 µL of $UH_{2\ IS}$ (1000 $ng.mL^{-1}$) are added to 500 µL of the sample (patient plasma, calibration standards and quality controls) to which 500 mg of ammonium sulfate is added (deproteinization). An LLE is then performed by adding to the previous mixture 3.5 mL of ethyl acetate/Isopropanol (85/15) v/v. It is vortexed for 2 min at 3000 rpm^{-1}, then centrifuged at 4000 rpm^{-1} for 10 min. A quantity of 3 mL of the supernatant is evaporated under vacuum at 45°C for 45 min. The dry residue is reconstituted with 100 µL of 0.1% formic acid; 15 µL are injected.

2.5. Validation procedure

The performance of the developed assay method was validated according to the guidelines defined by the International Council for Harmonisation ICH M10 and recommended by the EMA [34].

We examined the following criteria:

2.5.1. Selectivity and Specificity: Selectivity or the ability of an analytical method to differentiate the analyte in the presence of potentially interfering substances in the blank matrix. Its application is impossible for U and UH_2 because they are naturally present in human plasma. We evaluated the selectivity of U_{IS} and $UH_{2\ IS}$ using six plasma sources. The retention time responses should not exceed 5% of the IS response in the sample.

Specificity is the ability of the method to detect and differentiate the analyte from other substances, including related substances (similar structure, metabolites, concomitant drug combination). U and UH_2 are compounds in the plasma, making the specificity evaluation impossible.

2.5.2. Lower limit of quantification (LLOQ): represents the lowest concentration of the analyte for which the analytical method can give a quantified value with good precision. According to lCH M10, it represents the standard with the lowest concentration in the calibration curve, having a coefficient of variation (CV) of less than 20% and an accuracy of ± 20% of the nominal concentration. It is equal to 5 $ng.mL^{-1}$ for U and UH_2.

2.5.3. Calibration curve (calibration range): was calculated between the LLOQ and the upper limit of quantification (ULOQ). Six calibration standards were prepared for the 3 rounds (days) of validation. We used BSA at 40 $g.L^{-1}$ as a substitute matrix for plasma. Stock solutions (SS) of U and UH_2 and their respective IS at 1 $mg.mL^{-1}$ were prepared in 50% methanol. From the U and UH_2 SS, daughter solutions (DS) were prepared at concentrations of 50, 100, 200, 500, 1000 and 2000 $ng.mL^{-1}$ for U and UH_2 plus 4000 $ng.mL^{-1}$ for UH_2. We prepared the calibration standards (CS) by making a one-tenth dilution of each U and UH_2 DS in the BSA solution[35,36]. We added 50 µL of U_{IS} (200 ng.mL-1), and $UH_{2\,IS}$ (1 $\mu g.mL^{-1}$). The accuracy, expressed as relative bias (RB%) of each concentration of the CS, should be ± 20% for LLOQ and ± 15% for the rest of the points. At least 75% of the points on the curve must meet these limits.

2.5.4. Accuracy: RB was assessed using four levels of quality control or QC (LLOQ, Low QC = LQC, Medium = MQC and Hight QC = HQC), and each level was analyzed in 5 replicates per day for 3 days.

The concentrations of the four QC levels were chosen according to ICH M10 criteria; 5, 15, 100 and 150 $ng.mL^{-1}$ for U, 5, 15, 150 and 300 $ng.mL^{-1}$ for UH_2. It was evaluated within and between series (inter-series). The concentrations obtained must be within ± 15% of the nominal concentrations except for the LLOQ, which must be within ± 20%.

2.5.5. Precision: intra-run repeatability (same operating conditions, same technician, and short time interval between replicates) and inter-run intermediate precision were evaluated. Precision was evaluated for all QC levels. It represents the CV of the concentrations determined at each level and should not exceed 15%, except at LLOQ ≤ 20%.

2.5.6. Matrix effect: is an alteration of the analyte response that interferences can cause from undetermined compounds in the matrix. It was determined by analyzing three replicates of LQC

and HQC prepared from two sources of BSA (matrix sparsity). The accuracy should be within ± 15% of the nominal concentration and the CV ≤ 15% in all matrix sources tested.

2.5.7. Carry-over (inter-sample contamination): is a measurement alteration due to analyte residue from a previous sample that remained in the analytical platform. Inter-sample contamination was determined by injecting a blank sample after injecting the ULOQ standard during the 3 validation runs. The retention time response of the analyte must not exceed 20% of the LLOQ response and 5% of the IS response.

2.5.8. Stability study: of the stock solutions (SS) concentrated to 1000 $ng.mL^{-1}$, and that of the samples was carried out. The stability duration explored for SS is 3 months at -45 °C and 1 month for LQC and HQC at -45 °C. We also explored the stability of extracts for 24 h at +4 °C (in the auto-sampler). For this, aliquots of LQC and HQC were analyzed at time zero (reference) and after applying the storage conditions to be evaluated. Three preparations were made for each point, condition and storage time. The average concentration of each QC level must be within ± 15% of the nominal concentration with a CV ≤ 15%.

2.6. Application of the validated method on patient samples

Fifty-seven patients hospitalized in the Oncology and Radiotherapy Department of the Central Army Hospital in Algiers, Algeria, underwent pre-therapeutic screening for DPD deficiency by measuring U and UH_2 levels using the proposed and previously verified method.

Blood samples were collected between 8:30 am and 10 am using EDTA tubes, immediately sent to the Toxicology laboratory of the Central Army Hospital, centrifuged and stored at -45°C while waiting for the analysis (7-day maximum delay).

The decision to initiate FP-based chemotherapy was based on the plasma U level recommended by the EMA and the HHA.

3. RESULTS AND DISCUSSION

Screening for DPD deficiency before initiating any FP-based therapy is a concern of oncologists in Algeria and worldwide. This pre-therapeutic screening allows the safe use of these anticancer agents. It is now mandatory in France and recommended in other countries. The interest lies in having an accurate, precise and sufficiently sensitive assay method to quantify plasma U and UH_2 levels. This study presents a method for determining plasma U and UH_2 by UHPLC-MS-

MS that meets the requirements of ICH M10. The transitions from precursor ions to product ions for U, UH_2 and the corresponding internal standards were m/z **113.1**→95.8 at 10V (retention time: rt = 5.21 min), m/z **115.1**→73.3 at 10V (rt = 4.63 min), m/z **116.1**→71.2 at 20V (rt = 5.10 min), m/z **118.1**→76.1 at 10V (rt = 4.57 min) respectively. The dwell time was 60 ms for each transition.

Compared to UV spectrometry, mass spectrometric detection is the method of choice for determining U and UH_2 and the metabolic ratio UH_2/U. This ratio is a biomarker for detecting DPD deficiency. The major drawback to its routine use is the lack of consensus values, mainly due to compounds interfering with the retention time of UH_2 during the LC-UV assay.

3.1. Validation procedure

3.1.1. LLOQ: Our assay method describes an LLOQ (ICH M10) at 5 $ng.mL^{-1}$ for our two analytes. Regarding U, the LLOQ is similar to what has been described in the literature by several authors [33,37]. This LLOQ is sufficient for screening for DPD deficiency by detecting U levels > 16 $ng.mL^{-1}$.

3.1.2. Selectivity: U and UH_2 are endogenous compounds found in human plasma, making it difficult to evaluate the method's selectivity. For this purpose, we used the IS. No interference (<5%) was detected at their respective retention times in the 6 different batches of plasma.

3.1.3. Calibration curve (calibration range): The calibration range covers the range of concentrations from 5 to 200 $ng.mL^{-1}$ for U, which covers the range of values that can be used to decide on the status of DPD (from 16 to 150 $ng.mL^{-1}$) [22]. For UH_2, the range of determination extended from 5 to 400 $ng.mL^{-1}$, sufficient to cover the physiological values that this metabolite could take (45.6 to 193.8 $ng.mL^{-1}$ [38]).

The results were obtained as chromatograms representing the intensity of the detector response to the analytes and their respective ISs as a function of time. **Fig.1.** represents the chromatograms related to LLOQ and ULOQ of U and UH_2 and their respective ISs. The U/U_{IS} and $UH_2/UH_{2\,IS}$ area ratios were determined to generate the calibration curve for each of the 3 days of the validation.

The RBs (%) of the calibration curve points on the three validation days fluctuated between -14.26% and +14.16% regarding U and between -8.94% and +9.51% for UH_2. All points'

accuracy (RB) was within ≤ 15% and ≥- 15%. The calibration curves for the 3 validation days correspond to the specifications set by ICH M10.

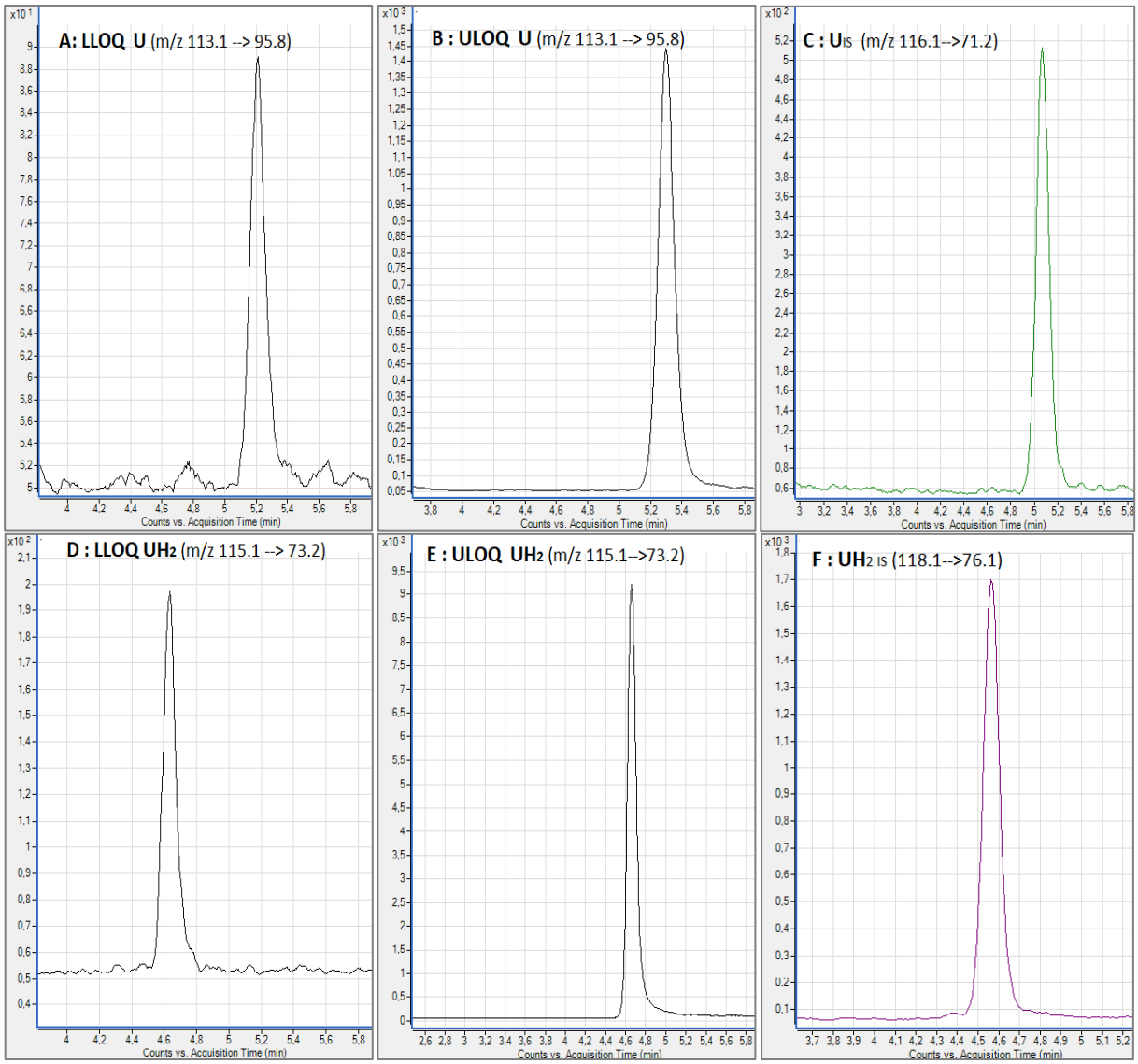

Fig.1. Chromatograms **A**: LLOQ of U (5 ng.L^{-1}). **B**: ULOQ of U (200 ng.L^{-1}). **C**: U_{IS}. **D**: LLOQ of UH_2 (5 ng.L^{-1}). **E**: ULOQ of UH_2 (400 ng.L^{-1}). **F**: $UH_{2\ IS}$

3.1.4. Accuracy and precision: the overall accuracy (intra- and inter-run) at the LLOQ, LCQ, MQC and HCQ fluctuated between -4.02% and +5.46%, which is in line with the requirements of ICH M10 (U). The precision (CV%) ranged from 0.86% to 7.45%, meeting the ICH M10 recommendations. **Table 1.** and **Table 2.** list the main results of the validation for uracil and dihydrouracil respectively.

The latest recommendations for screening for DPD deficiency propose a threshold value of uracilemia at 16 ng.mL^{-1} to characterize a partial deficiency, which implies having a sufficiently

accurate and precise method, especially around this threshold value.

The LQC at the concentration of 15ng.mL^{-1} in uracil presented a precision and an accuracy lower than 4% (RB in absolute value), reducing the risk of interpretation error (Partial DPD deficiency or no deficiency).

3.1.5. Carry-over and matrix effect: No inter-sample contamination was observed, which secures the interpretation of the assays. The matrix used did not interfere with the precision of our assays (**Table 1.** and **Table 2.**).

Table 1. Results of the validation for uracil.

Linear regression (R^2)	QC sample	Nominal concentration (ng.mL^{-1})	Precision (CV %)				Accuracy (RB %)				Matrix effect (CV %)	
			Within-run			Between-run	Within-run			Between-run		
			Run 1	Run 2	Run 3		Run 1	Run 2	Run 3		B1	B2
y = 0.0095x + 0.0162	**LLOQ**	**5**	7.45	5.73	4.49	6.10	3.87	-4.02	-0.81	5.46		
(0.999)	**LQC**	**15**	3.59	1.78	0.86	3.46	0.91	1.41	0.81	1.05	6.40	2.96
	MQC	**100**	5.08	3.90	4.83	4.28	1.17	1.21	0.09	0.82		
	HQC	**150**	3.28	3.30	3.68	3.19	0.79	0.38	-1.49	-0.11	6.06	0.71

QC: Quality control. B: Batch.

Table 2. Results of the validation for Dihydrouracil.

Linear regression (R^2)	QC sample	Nominal concentration (ng.mL^{-1})	Precision (CV %)				Accuracy (RB %)				Matrix effect (CV %)	
			Within-run			Between-run	Within-run			Between-run		
			Run 1	Run 2	Run 3		Run 1	Run 2	Run 3		B1	B2
y = 0.0114x + 0.0004	**LLOQ**	**5**	3.80	2.09	4.43	6.91	6.71	6.62	0.84	4.72		
(0.999)	**LQC**	**15**	2.55	0.17	3.91	3.74	4.57	0.58	-2.66	0.83	5.09	2.32
	MQC	**150**	4.38	1.08	6.88	4.13	2.82	3.19	1.00	2.34		
	HQC	**300**	1.46	2.39	3.44	2.58	0.98	0.52	-3.21	-0.57	2.78	2.20

QC: Quality control. B: Batch.

3.1.6. Stability: We demonstrated the stability of our compounds in the SS used to prepare the calibration curve points for 3 months at -45°C, significantly reducing the number of preparation of these solutions over time. ***Jacobs et al. 2016*** proved the stability of these analytes in SS taking 148 days at -20°C [39]. Our analytes were also stable in the matrix for 1 month at -45°C. This interval was more than enough knowing that the assays of U and UH_2 levels of our patients were done weekly (maximum delay of 7 days).

Jacobs et al. 2016 reported the stability of these compounds in the matrix for a longer duration of 93 days but at a temperature of -70 °C [39]. We also proved the stability of these compounds in extract for 24 h at +4°C, which allowed us to shift the assays to the next day when the number of processed samples was too large. Moreover, ***Jacobs et al. 2016*** noted that U and UH_2 remained stable for up to 5 days under the same storage conditions [39].

Jacobs et al. 2016 noted an increase in U in whole blood stored at +4°C for a duration of 4h, as well as the UH_2 in whole blood left at room temperature for the same duration [39]. The same observations were made by ***Coudoré et al. 2012*** [30]. This finding would be due to the enzymatic activity of monocyte uridine phosphorylase and leukocyte and platelet DPD [40,41]. We avoided this phenomenon by centrifuging and storing our samples immediately.

3.2. Clinical application of the validated assay method

The validated method is routinely used to screen for DPD deficiency in patients hospitalized in the Oncology and Radiotherapy Department, with FPs (5-FU or Capecitabine) treatment. The median U level in the 57 patients screened was 7.35 $ng.mL^{-1}$ with extremes at 3.52 and 15.93 $ng.mL^{-1}$. Based on the threshold value above which the patient is considered DPD deficient ($U > 16\ ng.mL^{-1}$), the uracilemia results would suggest that none of our patients were DPD deficient. Simultaneous determination of the UH_2 level allowed us to determine the metabolic ratio, whose median was 12.38 with extremes ranging from 4.33-24.43.

Based on the threshold value of the metabolic ratio in favor of a DPD deficiency described by ***Boisdron-Celle et al. 2007*** (< 6), 7.02% of our patients were in a partial DPD deficiency situation [22].

The U level is currently the only biomarker used consensually for pre-therapeutic screening of

DPD deficiency. It has been correlated with DPD activity, unlike the metabolic ratio. It is feasible to combine the U level with the metabolic ratio. This practice would significantly increase screening sensitivity, as noted by ***Boisdron-Celle et al. 2007*** and ***Capitain et al. 2020*** [22,23].

4. CONCLUSION

In this study, an analytical method was developed to simultaneously determine U and UH_2 plasma levels. The proposed method meets the requirements dictated by the ICH M10 guidelines. We are, to our knowledge, the first Algerian laboratory to perform this analysis, thus ensuring the safe use of fluoropyrimidines (FPs) for our patients via the pre-therapeutic screening of DPD deficiency. It would be interesting to evaluate the capacity of the combined method (U level and/or metabolic ratio) in the detection of DPD deficiency in our patients.

5. CONFLICTS OF INTEREST

Authors do not declare any conflict of interest.

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