# Fully automated LC-MS/MS method to assess DPD deficiency in Cancer treatment with 5-FU

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## 1. Overview

- This work describes a fully automated method to quantify uracil (U) and dihydrouracil ($UH_2$) drugs in plasma.
- Uracilemia or $UH_2$/U ratio are used to characterize dihydropyrimidine deshydrogenase (DPD) deficiency in order to prevent toxicity under fluoropyrimidines treatment.

## 2. Introduction

Fluoropyrimidines (5-fluorouracil or capecitabine) are anticancer drugs used in nearly 60% of chemotherapy treatment. It is known that they can lead to severe or lethal toxicities in case of dihydropyrimidine dehydrogenase (DPD) deficiency, therefore it is highly recommended to check for that DPD efficiency status. In France, health authorities recommend the determination of uracil concentration to guide dosing of fluoropyrimidines. Numerous LC-MS/MS methods have been proposed but they include complex liquid-liquid or solid-phase extraction procedures. To answer to the need of high throughput and robust analysis, our objective was to develop a method where the extraction was carried out by a programable liquid handler robot directly coupled to a LC-MS/MS system.

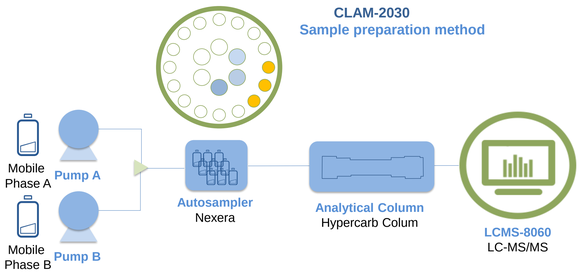


**Figure 1**. Schematic representation of the CLAM-2030 LC-MS/MS method for uracil and dihydrouracil in plasma

## 3. Methods and Materials

Uracilemia is an indirect phenotyping method measuring DPD deficiency by checking ratio uracil/dihydrouracil in plasma. That method, in comparison to genotyping ones, covers all risks of deficiency and thus is preferred for safety. The extraction procedure was performed on a CLAM-2030 coupled to a LCMS-8060 tandem MS (Shimadzu Corporation, Marne-la-Vallée, France) used in positive electrospray ionization mode. The acquisition method targeted MRM transitions for uracil, dihydrouracil, uracil-$^{13}C,^{15}N_2$ and dihydrouracil-$^{13}C,^{15}N_2$.

**Sample preparation for CLAM-2030 :**

1. Add 20 µL isopropanol
2. Add 20 µL sample
3. Add 10 µL internal standard
4. Add 40 µL acetonitrile + 1% formic acid
5. Shake for 2 min at 1 900 rpm
6. Filtrate for 2 min
7. Autosampler dilute 10 µL of the extract with 90 µL of water

Sample preparation and LC/MS/MS analysis can be performed in parallel to accelerate throughput using CLAM-2000.

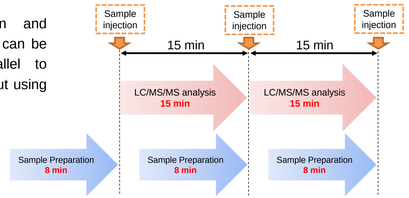


**Figure 2.** Analytical flow with parallel processing

**HPLC Conditions**

| | |
|---|---|
| Analytical column | : Hypercarb Column 2,1x150 mm, 3 µm |
| Mobile Phase A | : Water + 0.5% acetic acid |
| Mobile Phase B | : Acetonitrile + 0.5% acetic acid |
| Rinse solution | : (R0) water |
| Flow rate | : 0.25 mL/min |
| Oven temperature | : 25 °C |
| Injection volume | : 50 µL |
| Time program | : |

| Time (min) | Event | (%) |
|---|---|---|
| 3 | Pump B Conc. | 5 |
| 5 | Pump B Conc. | 25 |
| 9.1 | Pump B Conc. | 25 |
| 9.2 | Pump B Conc. | 100 |
| 10.5 | Pump B Conc. | 100 |
| 10.6 | Pump B Conc. | 5 |
| 15 | Stop | 5 |

**MS Conditions**

| | |
|---|---|
| Ionization | : ESI Positive |
| Interface voltage | : 1 kV |
| DL temp. | : 300 °C |
| Heat Block temp. | : 500 °C |
| Interface temp. | : 380 °C |
| Nebulizer gas flow | : 3 L/min |
| Drying gas flow | : 3 L/min |
| Heating gas flow | : 14 L/min |

| Compounds | Transition MRM (1) | Transition MRM (2) |
|---|---|---|
| Uracil | 113.15>70.1 | 113.15>96.05 |
| Uracil-$^{13}C^{15}N_2$ | 116.2>71.1 | 116.2>98.0 |
| Dihydrouracil | 114.95>30.1 | 114.95>55.0 |
| Dihydrouracil-$^{13}C^{15}N_2$ | 118.3>55.05 | 118.3>76.15 |

## 4. Results

The calibration range was 5 to 320 ng/mL for uracil concentration and 10 to 640 ng/ml for dihydrouracil. The calibration curves that were generated had linear regression values of $R^2$ >0.99 for each curve.

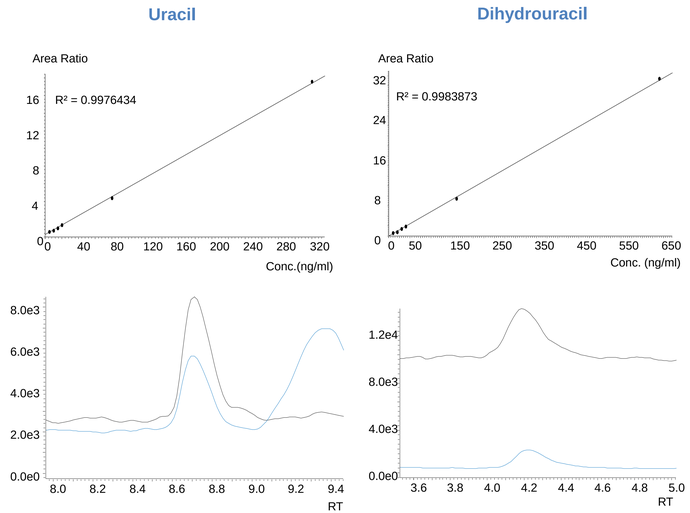


**Figure 3.** MRM chromatograms (at the LOQ) and calibration curves for uracil ad dihydrouracil

The method was fully validated according to the International Standards Organization ISO-15189 standard (repeatability, reproducibility, recovery, matrix effect and selectivity).

Proposed threshold values of 16 and 150 ng/mL for uracilemia characterize a partial or a complete DPD deficiency, respectively. Inaccurate quantification, in particular around these threshold values may totally change patient care and medical decisions. Intra and inter assay test were realized for these threshold values during the validation. Great accuracy and very low CV were obtained.

| | | | Intra-assay (n=6) | | | Inter-assay (n=6) | | |
|---|---|---|---|---|---|---|---|---|
| Compound | Associated IS | Accuracy Repeatability | QC 1: U:13 $UH_2$:26 | QC 2: U:16 $UH_2$:32 | QC 3: U:150 $UH_2$:300 | QC 1: U:13 $UH_2$:26 | QC 2: U:16 $UH_2$:32 | QC 3: U:150 $UH_2$:300 |
| Dihydrouracil | Dihydrouracil $^{13}C^{15}N_2$ | Acc (%) | 85.3 | 86.5 | 101.3 | 89.5 | 93.8 | 100.6 |
| | | RSD (%) | 7.7 | 5.7 | 8.8 | 3 | 2.8 | 4.8 |
| Uracil | Uracil $^{13}C^{15}N_2$ | Acc (%) | 104.6 | 96.6 | 98.4 | 100.8 | 97.1 | 98.3 |
| | | RSD (%) | 4.7 | 4.4 | 3.1 | 6.6 | 3.8 | 6 |

**Table 1.** Accuracy and RSD obtained for intra and inter assay test

The whole procedure was finally applied to 64 real patient samples and its results were compared to a validated LC-MS/MS method used in routine at the Limoges University Hospital (France). A good agreement was obtained between the two methods. In this study, 100% of the Bland Altman plots were within the ±2SD interval for uracilemia.

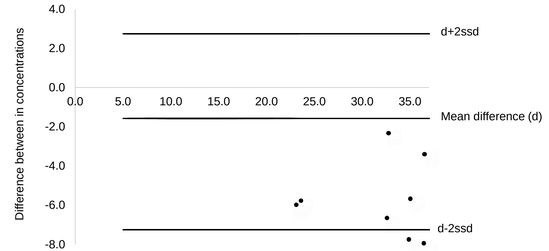


**Figure 4.** : Bland Altman diagram for uracilemia with the new procedure and the method validated in Limoges.

## 5. Conclusions

- Fully automated sample preparation ensures an accurate and robust measurement without requiring precious lab staff time.