Electronic Supplementary Information

A Simple, Rapid and Low-Cost Spectrophotometric Method for Irinotecan Quantification in Human Plasma and in Pharmaceutical Dosage Forms

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Supplementary Results

LC/UV Method Verification

The HPLC separation method applied was based on closely-related reversed-phase literature HPLC methods proposed for the analysis of pharmaceutical preparations of irinotecan or the analysis of irinotecan in plasma, as its lactone form (pH around 3.0)[1]. We verified the adopted HPLC method, before using it for comparison purposes with the here-in developed spectrophotometric method. Validation was performed using QC samples of irinotecan dosage form in water matrix: A very good linear relationship was observed between peak-area and drug concentration ($R^2 \geq 0.997$, n=5) upon LC/UV analysis. Linearity was found to be in the range of 0.78 to at least 40.0 μg/ml. Limit of Quantitation was 0.78 μg/ml. The applied LC/UV method was shown to be precise (CV ≤ 3.4 %, for repeatability and ≤5.5 % for intermediate precision for 10 irinotecan aqueous QC samples covering all the 0.78 to 40.00 μg/ml concentration range). It was also demonstrated as accurate (recovery of irinotecan was 96.65% and 99.70%, for two QC samples containing irinotecan at 16.1 and 19.3 μg/ml, respectively). All analytical parameters defined were acceptable and appropriate for the analysis purposes. The LC/UV profile of a solution of irinotecan and UV /Vis spectrum of the peak at 6.85 min are displayed in Figure 1.

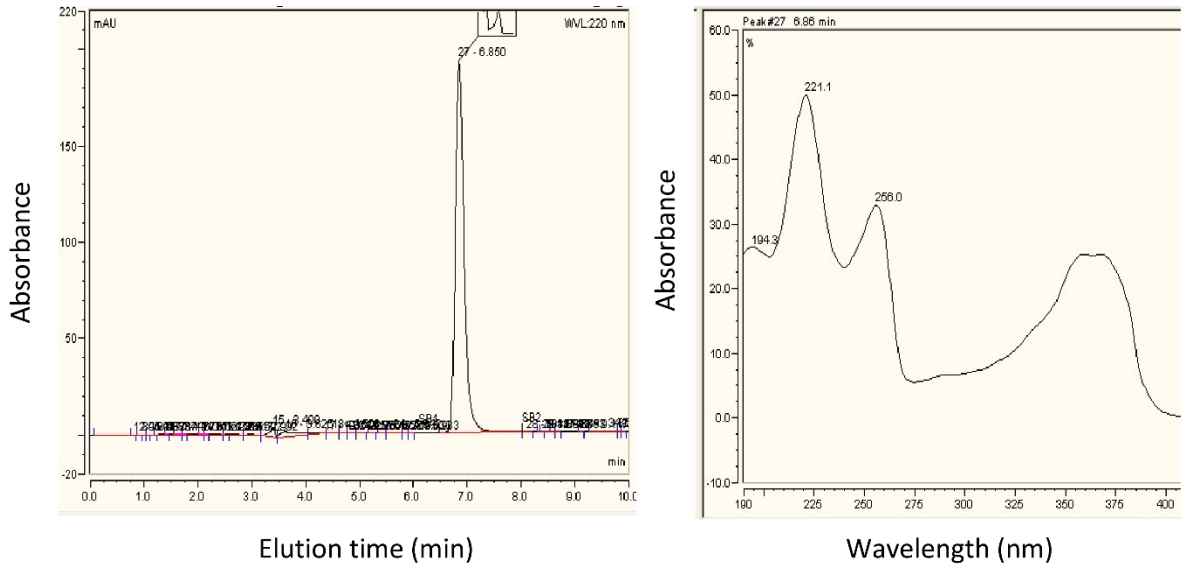

Figure 1. LC/UV analysis profile of a solution of irinotecan prepared in 20 mM disodium phosphate buffer pH 3.2 (left). The UV /Vis spectrum of the peak at 6.85 min (right) verified its identity as being solely irinotecan, displaying the characteristic peaks at 194 nm, 221 nm, 256 nm and a double peak at 358 and 368 nm

Supplementary Materials and Methods

Linearity

Calibration curves used to quantify irinotecan concentration in aqueous dilutions of irinotecan solution for injection or in plasma were prepared using eight standards in duplicates. Standards were prepared in the appropriate matrix (water or plasma). Calibration curves were generated by plotting A400nm, in water matrix, or (A400nm-A55nm), in plasma matrix, versus nominal concentration of irinotecan in each standard. Linear calibration equation, correlation coefficient (R^2) and residual sum of squares (RSS) were determined.

Precision

Repeatability was evaluated by analyzing Quality Control (QC) samples of irinotecan (in water or in plasma matrix) at five concentration levels for each matrix, at least seven times on the same day. The concentrations analysed spread across the specified useful range of the analytical procedure (water matrix), or across the upper region of irinotecan therapeutic window (plasma matrix).There was at least a 15 min difference between each measurement of the same QC sample. Analyses to establish Intermediate Precision was carried out at at least four concentration levels, for at least six days. The concentration of each QC sample was evaluated using a newly measured calibration curve. Precision was evaluated by calculating coefficient of variation (CV%) for the analysis of QC samples. Confidence interval was also calculated for each type of precision investigated.

LOD and LOQ

Limit of Detection (LOD) was calculated as: LOD = 3.3 σ/ S where σ is the standard deviation of the response and S is the mean slope of the calibration curves. The calculation of σ was based on the Standard Deviation of the Blank, after analysing six-eight blank samples and calculating the standard deviation of the responses measured. The limit of Quantitation (LOQ) was calculated as LOQ=3*LOD.

Stabilities Study

Irinotecan stability in pre-treated (acidified (and extracted)) samples was evaluated by analyzing standards of irinotecan (prepared in acidificed to pH 0.2 water, or in HCl/NaCl treated-plasma) at eight different concentrations for each matrix and at two time points: the very beginning of incubation and the end of the incubation interval. The concentrations analysed spread across the specified useful analytical range of the analytical procedure for the aqueous matrix, or across the upper region of irinotecan therapeutic window for the plasma matrix. The standards were stored at RT in the dark for those in the aqueous matrix, or in the fridge or freezer for those in the plasma matrix. Stability was considered sufficient when the standard curves built at both time points (beginning and end of incubation period) had a R^2>0.995 and the final calibration curve slope differed by less than 7.0 % of the initial slope.

Recovery Studies

Recovery studies were carried out by analysing homogenous pools of QC samples, prepared by spiking QC samples with blank matrix, or with a higher concentration QC sample in the same matrix. Recovery studies were performed in water and plasma matrix. QC samples were analysed by the proposed method after applying the entire pre-treatment protocol (i.e. pH lowering to 0.2, (centrifugation) and absorbance measurement). Recovery was assessed using at least four concentration levels. It is reported as percent recovery by the new assay of known added amount of analyte in the QC sample.

Selectivity Studies (Investigation of Interferents Effects)

Selectivity was determined to confirm the influence of endogenous interferents, like hemolysis and icterus, on irinotecan recovery from plasma samples. For this reason, we constructed calibration plots in plasma, in the presence and absence of each interferent whose slopes we compared for significant difference. We used hemoglobin-free, commercial serum (Precipath U Plus, Roche Diagnostics) containing Bilirubin at 3.84 mg/dL plus non-commercial, non-icteric plasma containing hemoglobin at 1.37 mg/dL (mild hemolysis range). Hemoglobin concentration in the hemolysed samples was determined as below: Hemolysis was achieved by fluidic shear when passing whole venous blood through a syringe. Blood was then centrifuged at 2,678 g for 5 min in order to obtain plasma. Icteric serum and lysed plasma were subsequently processed by the general pre-treatment protocol. After pre-treatment, irinotecan was added at 8 different concentrations covering the range 0 to 14.7 μg/ml. The absorbance at 400 nm and 455 nm was recorded and a calibration plot was constructed. In parallel, a second calibration plot was generated in HCl/NaCl-treated non-icteric, non hemolysed plasma, containing irinotecan at the same set of 8 concentrations, as for the sera containing

the interferents, giving rise to 8 baseline standards. The absorbance of each icteric or hemolytic standard was used to calculate each standard concentration based on the baseline calibration curve. The change (bias) in the analyte concentration was determined for each standard concentration using the formula: bias% = 100 × (concentration in hemolytic (or icteric) sample – concentration in baseline sample)/(concentration in baseline sample).

Hemoglobin Determination

To measure iron bound by hemoglobin, we applied a technique entailing acid-permanganate-mediated digestion of blood proteins, which releases all protein-bound iron and converts it to Fe(lll), as modified by Panter[2]. A 0.284 M aqueous KMnO4 solution was prepared which was mixed in a 1:1 volume ratio with 1.2 N HCl solution. 500 µl of the mixture was added to 50 µl of hemolysate (2 replicates) and the resulting sample was incubated at RT for 10min. Protein digestion in a water bath (2h at 60 ℃) followed. The sample was subsequently centrifuged at 10,000 rpm for 10 min, the supernatant was discarded, and the residue was collected. It was then completely solubilised in 500 µl of 0.1 M pH 4.5 acetate buffer in the presence of 1.05% w/v, SLES. 0.4 ml of the solubilised pellet was subsequently treated with 2ml acetate buffer 0.1 M pH 4.5, containing 0.04 mM ascorbic acid and 1.88 mM ferrozine. An appropriate standard and blank sample were also prepared. All samples were incubated for 10 minutes at room temperature, during which a red iron-chromophore complex was formed which was measured at 565 nm. Absorbance at 565 nm was proportional to iron concentration in the sample. After correcting for dilution, for the relative percentage of hemoglobin in blood (75%), while also taking into account that each molecule of hemoglobin (tetramer) contains four iron atoms, hemoglobin concentration was calculated.

Supplementary References

1 R. Mullangi, P. Ahlawat and N. R. Srinivas, Irinotecan and its active metabolite, SN-38: review of bioanalytical methods and recent update from clinical pharmacology perspectives, *Biomed. Chromatogr*., 2010, **24**, 104.

2 S. S. Panter. Release of Iron from Hemoglobin. San Francisco: Army Medical Research and Development, Division of Blood Research, 1986.