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A simple and fast LC-MS/MS method for the routine measurement of cabozantinib, olaparib, palbociclib, pazopanib, sorafenib, sunitinib and its main active metabolite in human plasma

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Highlights:

- Targeted therapies display large pharmacokinetics variability impacting their efficacy or toxicity
- A simple method may help to routinely monitor the concentrations of these drugs
- We present here a simple and fast LC/MS/MS validated method for the routine measurement of these drugs
- We also performed stability study in whole blood and plasma to help the management of the samples in routine situation

Keywords: LC–MS/MS, anticancer targeted therapies, whole blood stability, routine analysis

Declaration of interest: none

The authors do not want the figures to be printed in color

ABSTRACT

Targeted therapies such as cabozantinib (CABO), pazopanib (PAZO), sorafenib (SORA), sunitinib (SUNI) and its main active metabolite N-desethyl-sunitinib (DST-SUNI), olaparib (OLA) and palbociclib (PALBO) display large pharmacokinetics variability impacting their responses in terms of efficacy or toxicity. For the monitoring of these drugs, an analytical method allowing to routinely measure their concentrations in human plasma is needed. Such a method has been developed and validated and is presented here.

The chromatographic separation is achieved on a Zorbax Bonus-RP analytical column using an isocratic elution of 92% V/V of acetonitrile and 8% of water in 0.1% formic acid at a flow rate of 500 $\mu\text{l}/\text{min}$ for 0.5 min and then 300 $\mu\text{l}/\text{min}$ for 2 min. After a liquid-liquid extraction of plasma samples, a step of filtration is performed. This method was validated based on the EMA and French committee of accreditation guidelines.

The analysis time is 2.5 minutes per run, and all analytes eluted within 0.53-1.61 minutes. The standard curves are linear over the range from 1 to 380 ng/ml for SUNI; from 4.3 to 450 ng/ml for DST-SUNI; from 6 to 1000 ng/ml for PALBO; from 75 to 5000 ng/ml for CABO, from 0.17 to 20 $\mu\text{g}/\text{ml}$ for OLA; from 0.35 to 40 $\mu\text{g}/\text{ml}$ for SORA and from 1.7 to 200 $\mu\text{g}/\text{ml}$ for PAZO. The method also showed satisfactory results in terms precision (below 9.5% for within-run and below 13% for between-run) and accuracy (below 13.5% for within-run and below 14% for between-run). After sampling, all the compounds are stable in whole blood at ambient temperature at least for 6h and plasma are stable for 48h at ambient temperature or 4°C.

The method presented here allows to measure the concentrations of 7 targeted therapies in a routine setting. We moreover present here a method that is, to our knowledge, one of the first detailed method aimed at the measurement of human palbociclib in plasma in a routine

setting, together with data useful for the management of samples in routine hospital practice.

1. INTRODUCTION

Cabozantinib (CABO), pazopanib (PAZO), sorafenib (SORA), sunitinib (SUNI) and its main active metabolite, N-desethyl-sunitinib (DST-SUNI) are tyrosine kinase inhibitors (TKIs) which specifically target molecular aberrations of cancer cells by blocking the intracellular signals driving proliferation in malignant cells [1]. They have an important activity in many types of tyrosine kinases involved in angiogenesis, tumor growth and metastatic progression of cancer [2].

These molecules display a large pharmacokinetics (PK) variability mainly due to their absorption *via* P-gp [3] or metabolism *via* P450 cytochromes - 3A4 especially [2] - or food intake [4]. Each TKI generates an important number of metabolites that are often inactive, but SUNI have an active metabolite whose concentration in plasma representing between 23% and 37% of the SUNI plasma concentration [5]. Although they are less toxic than conventional chemotherapy, these variability lead to high interpatient PK variability [6] resulting in exposure levels that may be related to potential toxicity or therapeutic inefficacy [7].

Indeed, in parallel with this PK variability, many of these drugs display an exposure/response relationship [6]. More precisely, SUNI, DST-SUNI and SORA show an exposure – toxicity relationship [8,9] assessed by C_{max} or AUC. Similarly, CABO and PAZO show an exposure – efficacy relationship [10,11] evaluated by C_{max}, AUC, progression-free survival, objective response rate or overall survival.

Palbociclib (PALBO), a cyclin-dependent kinases (CDK) 4 and 6 inhibitor, displays a quite similar pharmacokinetics profile with mainly hepatic metabolism *via* the P450 cytochromes - 3A4 especially (CYP3A4) - and the sulfotransferase SULT-2A1 enzymes. But there also is an effect of food on variability (it tends to decrease variability) and potential drug – drug

interaction with molecules metabolized through CYP3A [12]. Interestingly, an association between higher PALBO exposure and toxicity (lower longitudinal neutrophil counts) is suggested [13]. Currently, there is few methods in the literature for the human plasmatic measurement of this drug. With respect to its PK/Pharmacodynamics (PD) profile and to the request of the clinicians, the development of an analytical method for the measurement of this drug in the plasma appeared us of interest.

Olaparib (OLA), a poly (ADP-ribose) polymerase (PARP) inhibitor, is also metabolized mainly through CYP3A4 and is subject to drug-drug interaction similarly to the others drugs [14] while the effects of food seem less significant [15,16]. Interestingly, in a surgery context, the authors observed that, in addition to a large inter-patient variability of drug concentrations, the exposures of OLA were around 50% lower than those observed with similar doses in advanced diseases studies [17]. No obvious reasons could be proposed to explain such difference, underlying the interest of additional PK studies of this drug.

The similarity between the metabolic profile and the PK/PD relationship of these molecules suggests the potential interest of their therapeutic drug monitoring (TDM) [18] to improve treatment benefit by reducing toxicity and increasing efficacy. This however needs an assay method that allows to routinely measure the concentrations of these drugs [19].

To meet the request of clinicians and to make the technique convenient for the laboratory, we tried to develop a single method for all these drugs, instead of one for TKIs and one or two for the other drugs. We hereby develop and validate an assay method using Liquid chromatography – Tandem Mass spectrometry (LC-MS/MS) in which CABO, OLA, PALBO, PAZO, SORA, SUNI and DST-SUNI can be simultaneously quantified. With this method, we are able to easily assess the plasmatic concentrations of the molecules that display a PK/PD

relationship to address the TDM practice. It also supports clinical trials for molecule for which PK/PD relationship needs to be studied.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

CABO, OLA, PALBO hydrochloride, PAZO hydrochloride, SORA, SUNI-L-malate, DST-SUNI hydrochloride, and [$^2\text{H}_4$]-CABO, [$^2\text{H}_8$]-OLA, [$^2\text{H}_8$]-PALBO, [^{13}C , $^2\text{H}_3$]-PAZO, [^{13}C , $^2\text{H}_3$]-SORA, [$^2\text{H}_{10}$]-SUNI, the six isotopic internal standards (IS) were purchased with certified weighing from Alsachim (Illkirch, France). As DST-SUNI and SUNI display very close chemical structures and a similar chromatographic behavior, [$^2\text{H}_{10}$]-SUNI was used as IS for both compound. Acetonitrile (CH_3CN), methanol (MeOH), water LC-MS grade were purchased from Carlo Erba reagents (Val-de-Reuil, France). Acid formic and dimethylsulfoxide (DMSO) were purchased from Thermo Fisher scientific (Illkirch, France). Ethyl acetate and sodium hydroxide (NaOH) were purchased from VWR chemicals (Fontenay-sous-bois, France). Ammonium acetate was purchased from Euromedex (Souffelweyersheim, France). Human plasma was purchased from a blood transfusion center (Besançon, France). All plasma and whole blood samples were taken using heparin as anticoagulant.

2.2. Stock solutions, calibration standards and quality controls

For each drug, two stock solutions - for the calibrators and the quality controls (QCs) - were separately prepared with two different certified amounts of drugs. Six stock solutions of IS were prepared as described in table 1. The same volume of each IS stock solutions was mixed to obtain the IS working solution. All of these solutions were stored at -40°C , except PALBO at $+4^\circ\text{C}$ and PAZO at room temperature. SUNI and DST-SUNI were stored in a dark

place because light induces intra-molecular transformations [20]. Calibrators were prepared in advance by appropriate dilution of working solution of each molecule in blank plasma at different level according to the drug (Table 2). QCs were prepared in blank plasma to obtain concentrations of 20, 80 and 480 ng/ml for PALBO; 7.63, 30.52 and 183.12 ng/ml for SUNI; 9.10, 36.4 and 218.4 ng/ml for DST-SUNI; 200, 800 and 4800 ng/ml for CABO; 375, 1500 and 9000 ng/ml for OLA; 750, 3000 and 18000 ng/ml for SORA and 3750, 15000 and 90000 ng/ml for PAZO. Calibrators, QCs and patients' plasma samples were stored at – 20°C.

2.3. Equipment and LC-MS/MS conditions

The mass spectrometer devices consisted of a Dionex HPLC system controlled with Chromeleon® software and with an Endura (Thermo Fisher Scientific, San Jose, California) triple quadrupole mass spectrometer controlled with TSQ Tune Application® Software. The quantitative analysis was performed using the Tracefinder® software. A Heated Electrospray Ionization Source (HESI) in positive mode was used with the following parameters: ionization voltage set at 4000 V, vaporizer temperature at 250°C, capillary temperature at 250°C, sheath gas flow at 35 arbitrary units. The used transitions are reported in Table 3. The liquid chromatography was performed in isocratic condition with the following elution solvent at room temperature and a flow rate of 500 µl/min for 0.5 min and then 300 µl/min for 2 min (92% V/V of acetonitrile and 8% of water in 0.1% formic acid). Separation was performed on Zorbax Bonus-RP analytical column (150 x 2.1 mm, 1.8 µm) placed in a thermostated column heater at 50°C. The run time is 2.5 min. Data acquisition was performed using Tracefinder® software. The ion values used for the method for each compound are described in the Table 3.

2.4. Sample preparation

Sample preparation was performed as follows: 150 µl of human plasma and 100 µl of NaOH were added to 10 µl of the IS working. Samples were vortexed a few seconds, then 700 µl of ethyl acetate was added. After being vortexed during 30 seconds, the samples were centrifuged at 10000 RPM for 5 minutes. 600 µl of supernatant were collected in an extraction tube and evaporated under nitrogen flow. The dried samples were reconstituted with 150 µl of a mixture of 55% of methanol/45% of solvent A (ammonium acetate 1 M in water, pH adjusted to 3.2 with formic acid). The samples were vortexed during 30 seconds and filtrated using Captiva ND Lipids plates. The filtrates were injected into the system. 8 µl were injected if the sample contains PALBO and 2 µl for all others.

2.5. Assay conditions

After extraction and sample measurements as previously described, a calibration curve was plotted with the ratio drug/IS areas as a function of the respective concentrations. A weighting factor (1/x) was applied to the calibration curve. For each run, the linearity of the calibration curve was assessed by the percentage of deviation between nominal and measured concentration of standards which should be below 15% (20% for the lowest calibration standard).

2.6. Method validation

The method has been validated based on the EMA Guideline on bioanalytical method validation and, when appropriate, on the French committee of accreditation's guidelines for biological method [21,22].

2.6.1. LOQ

The limit of quantification (LOQ) is the lowest concentration which presents a maximum inter-run imprecision (CV) of 20%. LOQ was assessed on 15 samples.

2.6.2. Selectivity

To assess the selectivity, potential endogenous interferences were checked using 6 sources of blank samples. The responses observed should not be greater than 20% of LOQ for each drug and not greater than 5% for each IS.

Moreover, the potential interferences between the 7 drugs were assessed.

2.6.3. Carry-cover

Following the EMA guidelines, 3 blank samples were injected after high concentration calibrators (G7). The responses observed should not be greater than 20% of that of LOQ for each molecule and not greater than 5% for each IS. Moreover, we also followed the method recommended by French committee of accreditation. For this purpose, 3 high-level calibrators (G7) were followed by three samples of the lowest-level calibrator (G1). This sequence was repeated 3 times. The carry-over, expressed in %, was calculated as the ratio of the difference between the mean of the first low-level calibrator (mLC1) and the mean of the third low-level calibrator (mLC3) and the difference between the mean of the high-level calibrator (mHC) and the mean of the third low-level calibrator, as follows:

$$\text{Carry over (\%)} = \frac{(mLC1 - mLC3)}{(mHC - mLC3)} \times 100$$

2.6.4. Accuracy and precision

The between-run accuracy was assessed with a total of 22 controls assayed in 3 different runs performed on 3 different days with different manipulators. The within-run accuracy was assessed on 10 samples of each control. The same pattern was used to assess the between-run precision and within-run precision. The precision was calculated as the coefficient of variation (CV %) and the accuracy as the bias of percentage of deviation between nominal and measured concentration. The accuracies and precisions were accepted if the percentage of deviation between theoretical and experimental concentrations for each calibration level and QC samples were less than $\pm 15\%$.

2.6.5. Matrix effect

Following EMA recommendations, the ME should be evaluated with a CV of the IS-normalized matrix factor which is calculated from 6 lots of matrix and should not be greater than 15%.

2.6.6. Stability

For acceptable stability results, ratios between measured concentrations at the beginning of the study and those measured during the study should be within $\pm 15\%$. This parameter was investigated in QCs–high and –low plasma samples stored in polypropylene tubes. Drug concentrations variations were expressed as a percentage of the initial concentration measured at T0. Analyses were performed in triplicate:

→ Stability of plasma and whole blood spiked with drugs, kept at room temperature (RT) and at 4°C after being left at RT and at 4°C 6h, 24h and 48h.

→ Stability of plasma samples kept frozen at -20°C: the response of freshly prepared calibration and QC samples was compared to the same frozen calibration and QC samples stored 3 months at -20°C

→ Stability of drugs in extracts was assessed by re-injection of the extracts after additional storage of 9 days at + 4°C

Moreover, the stability of stock solutions was assessed by comparing their concentrations with newly reconstituted solutions. Stability of extracts kept onboard the autosampler at 10°C was also assessed, as well as the effect of three cycles of freeze and thaw. The mean concentrations should be within $\pm 15\%$ of the nominal values.

2.6.7. Linearity

To assess the upper LOQ, we spiked samples with each drug at the concentration of 750, 1000 and 1500 ng/ml for PALBO; 305.2, 381.5 and 572.25 ng/ml for SUNI; 364, 455 and 682.5 ng/ml for DST-SUNI; 7.5, 10 and 15 $\mu\text{g/ml}$ for CABO; 15, 20 and 30 $\mu\text{g/ml}$ for OLA; 30, 40 and 60 $\mu\text{g/ml}$ for SORA and 150, 200 and 300 $\mu\text{g/ml}$ for PAZO. The concentrations obtained were compared to their theoretical amounts.

2.7 Clinical application

This method aims at the measurement of targeted therapies in plasma of patients. Blood samples were taken as part of the routine clinical care from patients treated with targeted therapies either to perform TDM or to assess the patients' observance. Blood samples were processed and analyzed as previously described.

3. RESULTS

3.1. Chromatograms

Using our isocratic chromatographic conditions, the retention times observed were respectively, around 0.53 min for DST-SUNI, 0.54 min for SUNI, 0.62 min for PAZO, 0.63 min for CABO, 0.78 min for PALBO, 0.89 min for OLA and 1.61 min for SORA as shown in Fig. 1.

3.2. LOQ

The values of obtained and set for routine application LOQ with the corresponding CV are summarized in Table 4.

3.3. Selectivity

No peaks from endogenous compounds were observed at the drugs retention times in any of the 6 blank plasma extracts evaluated. The endogenous responses in blank plasma were always below 2.02% of the signal of the LOQ for each analyte and below 0.75% for the IS. Besides, there were neither interference between the 6 drugs nor between sunitinib and its main active metabolite.

3.4. Carry-over

Following the EMA guidelines, 3 blank samples were injected after high concentration calibrators. The responses (peak area) observed after the injection of blank samples were below 1.72%, 2.81%, 1.20%, 0%, 0%, 0.37% and 0% of that of the LOQ, respectively for PALBO, SUNI, DST-SUNI, CABO, OLA, SORA and PAZO. There was no carry-over for each IS.

As our method is made to simultaneously measure very low and very high concentrations, we also assessed the carry-over effect following the method provided by the French committee of accreditation. When we used this method, the maximal percentage of contamination was 0.08% between the first and the third blank.

3.5. Accuracy and precision

Accuracy and precision results are summarized in Table 5 and fulfill the objectives of EMA.

3.6. Matrix effect

For the higher concentrations, the CV of the IS-normalized matrix factors were 8.09% for PALBO, 1.05% for SUNI, 4.84% for DST-SUNI, 2.11% for CABO, 7.14% for OLA, 3.12% for SORA and 3.77% for PAZO. For lower concentrations, it was 4.02% for PALBO, 6.30% for SUNI, 7.44% for DST-SUNI, 3.98% for CABO, 4.69% for OLA, 2.81% for SORA and 1.88% for PAZO. They therefore fulfill the criteria.

3.7. Stability

Regarding the whole blood stability study; all analytes - except DST-SUNI and PALBO - were found to be stable for 48h with a maximum deviation from initial conditions of 12.3%, 4.6%, -2.3%, 5.6% and 2.6% at ambient temperature and of 7.2%, 1.8%, -7.0%, -6.1% and 5.1% at 4°C for SUNI, CABO, PAZO, OLA and SORA respectively, whatever the level of concentration. In our hands, DST-SUNI was found to be stable in whole blood, at ambient temperature or at 4°C, for 6h with a maximal deviation from initial conditions of 3.3% and 3.2% respectively for the low level of concentration. Regarding PALBO, this analyte was found to be stable in whole blood at ambient temperature for 24h for both levels (maximal deviation: -8.6%). Regarding the stability in plasma, all the analytes were found to be stable for at least 48h for both levels (maximum deviation from initial conditions: -12.3%). When frozen, the analytes were found to be stable for at least 3 months at -20°C. Finally, after extraction, the extracts were stable for at least 9 days when kept at 4°C and at least 24 hours when kept onboard at 10°C.

In our hands, all the stock solutions were stable for at least 12 months for all drugs at their respective storage temperature.

Finally, there was no effect of freeze and thaw cycles as their concentrations were within 11.2 % of their nominal temperature.

3.8. Linearity

The upper limit of quantification we obtained are summarized in Table 4.

3.9. Clinical application

As described in the figure 2, the compounds were easily detected and measured in all cases.

No interferences could be observed both between the studied targeted therapies and between others drugs than can be administered to patients.

4. DISCUSSION

We present here a method for the simultaneous measurement of 6 drugs and a main metabolite of one of them. The method we developed was made to perform the assessment of these drug concentrations in a routine setting. This implies that our method should be practical, fast and allows to measure the concentrations of these drugs usually observed in patients.

During the development of the method, we evaluated the two mostly used technics for drug extraction, i.e. the protein precipitation (PP) and the liquid-liquid extraction (LLE). In the literature, PP is the most described for all of molecules, [7,23,24] excluding CABO for which only LLE is employed when assessed alone or with other drug [25,26]. Using LLE we observed better signal-to-noise ratio. This is certainly due to a lower matrix effect, as it is known that this method decreases the matrix effect allowing to obtain a better matrix factor than PP [27]. In addition, we performed a step of filtration using a simple device (Captiva ND lipid plates, Agilent) to further improve our results. This filtration aims at removing phospholipids and residual proteins from biofluids as well as reducing ion suppression [28]. The addition of this step clearly enhanced the sensitivity of the method and avoided the overloading of the column.

As the method we propose was made to measure drug concentrations for patients in a routine setting, it was developed so that the LOQ be lower than the minimal plasmatic concentration (C_{min}) for each molecule. In the literature, the LOQ were sometimes lower than those we obtained [7,23–26,29,30]. The lower LOQ may be related to higher volumes of injection volumes (10 to 25 μ L) as compared to those we used (2 μ L, except 8 μ L for PALBO). Nevertheless, we estimated that it was not necessary to improve this parameter since it is already under the trough concentration in routine setting in most cases.

The values of the MQC we chose are 17% of the calibration range, instead 30-50% as recommended by the EMA. In fact, we chose this value with respect of the sampling times in clinical routine. Indeed, blood samples are often taken near through concentration or few hours before. The values of the low QC were chosen to match with concentrations in the first case. The values of the MQC were chosen to match in the latter case.

It could be argue that we don't measure SORA metabolite concentration like SUNI and DST-SUNI. Indeed, SORA has an active metabolite accounting for around 9% to 17% of SORA concentration [31]. However, as the recommendations for the OSRA monitoring are only based on the sole SORA measurement [2,6], we did not include its metabolite in our method. Interestingly, we observed a satisfactory separation of compounds' chromatograms with different chemical structures. We can mention in this regard DST-SUNI and SORA, whose retention times - respectively 0.53 min and 1.61 min - are short and yet the furthest apart. The analysis is fast and only lasts two minutes, so that the results can be delivered in a day.

In the literature, data regarding the stability in whole blood is very rare and even frequently missing. It is however a crucial parameter as a delay between the sampling and the arrival to the laboratory is often observed in routine setting. Moreover, as samples are often performed in another hospital, data about stability are useful in order to manage their shipping, hence the convenience of our method.

More specifically, we found that SUNI should be transferred and centrifuged into the laboratory within 6h due to DST-SUNI instability. However, after centrifugation, the plasma is stable for 48h and can be sent within this length even at ambient temperature. PALBO also displays a lower stability and should be centrifuged within 24h after sampling. In plasma, all the compounds were stable at 4°C or at ambient temperature for 48h, indicating that, after centrifugation, the samples can be sent even at ambient temperature within this length.

Thus, practically, all samples can be transferred to a laboratory that can perform a centrifugation within 48h at ambient temperature except those for PALBO (24h) and both SUNI and DST-SUNI for which the samples should reach this laboratory within 6h. Interestingly, the extracts can be analyzed up to 9 days after the extraction. So, in case of quick emergency analysis needing to use the analyzer previously dedicated to the assay of the present analytes, the measurement of the drugs can be easily delayed facilitating the management of analyzer allocation.

Finally, the method we developed is one of the first in the literature for the routine measurement of PALBO in human plasma using a mass spectrometry detection. Indeed, the methods described until now either concerned studies on animals, [32–35] or measurement in plasma with UV detection [36]. Only two very recent articles describe methods for the measurement of PALBO in human with mass spectrometry detection [37,38]. The calibration range of the first one is between 50 to 1000 ng/mL [37] while that of the second one is 2 to 200 ng/mL [38]. In our experience, the concentrations we measured in patients for routine follow-up ranged between 13 to 229 ng/mL. Of note, more than 17% of the concentrations were below 50 ng/mL, emphasizing the importance of a LOQ below this value. In our case, the calibration range allowed us to measure all these patients in a single run, without dilution for the highest concentrations. This is interesting insofar as more studies are needed to confirm the potential PK/PD relationship previously described [13].

5. CONCLUSION

We have thus developed and validated a simple and fast method that enables to routinely quantify 6 drugs and one metabolite simultaneously in human plasma using LC-MS/MS. This technique is adapted to TDM and can be used in clinical routine for most of these molecules

and also for the PK studies for PALBO and OLA. The measurement method for PALBO in human is all the more interesting that its detection parameters are suitable to 6 other molecules. In the future, this technique could be easily and quickly adapted to incorporate active metabolites or additional drugs.

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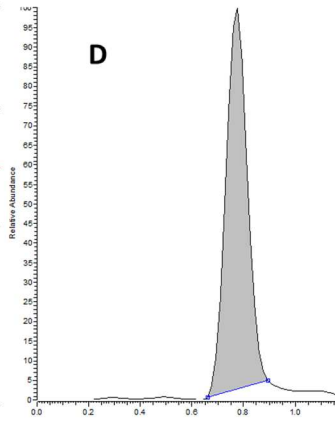
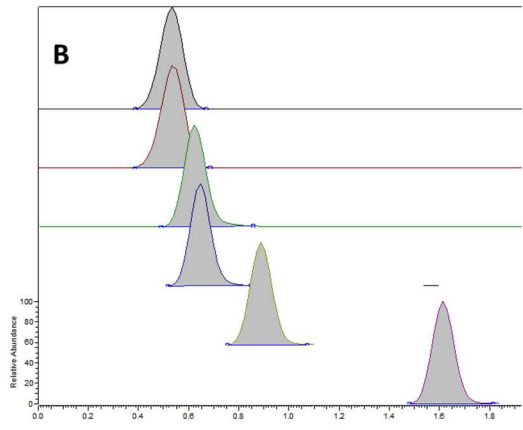
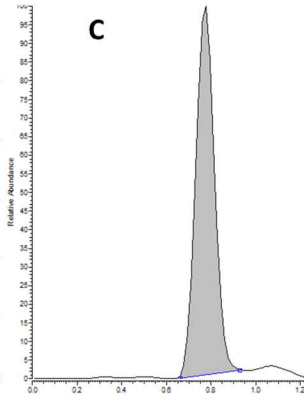
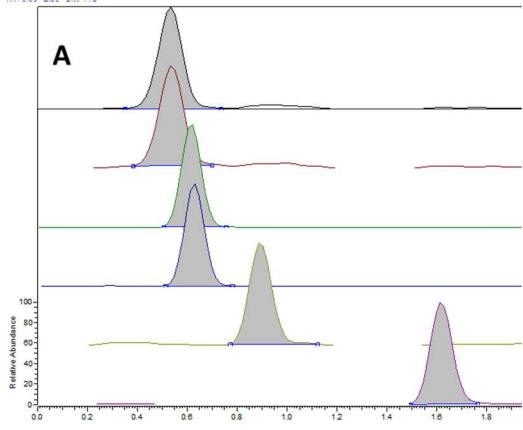
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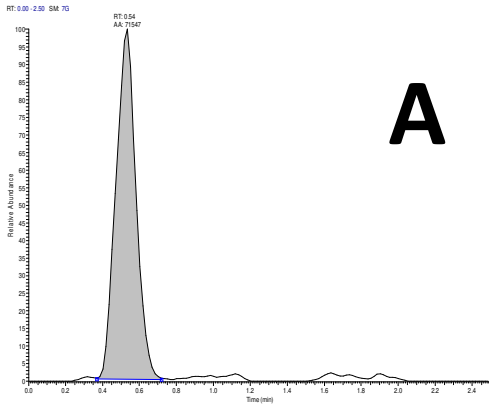
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Figure legends

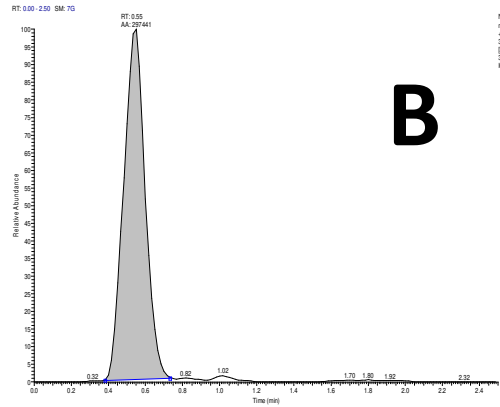
Figure 1: Chromatograms obtained at 2 μL of respectively, from top to bottom: SUNI, DST-SUNI, PAZO, CABO, OLA, SORA, for the lowest calibrator concentration (A) and the highest one (B). PALBO's chromatogram obtained at 8 μL for the lowest calibrator concentration (C) and the highest (D).

Figure 2: Typical chromatograms of the targeted therapies obtained in plasma of patients. The compounds are respectively SUNI (A), DST-SUNI (B), PAZO (C), CABO (D), PALBO (E), OLA (F) and SORA (G).

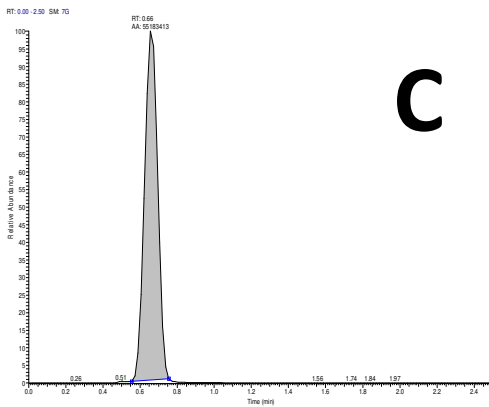




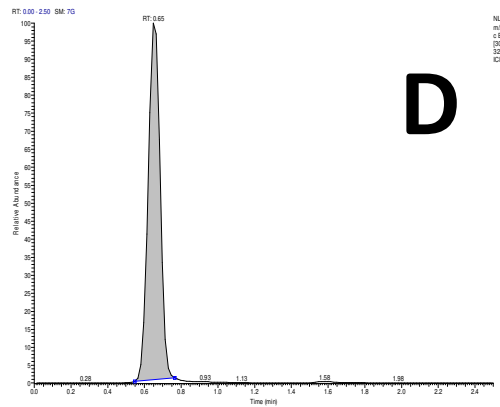
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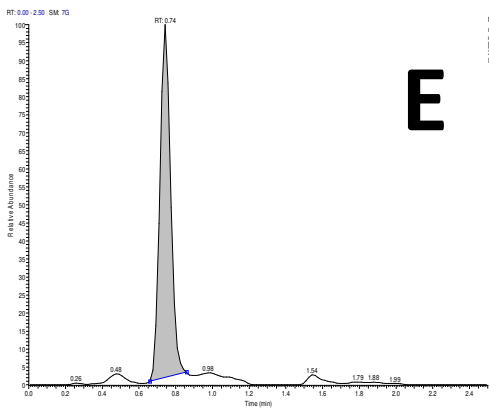
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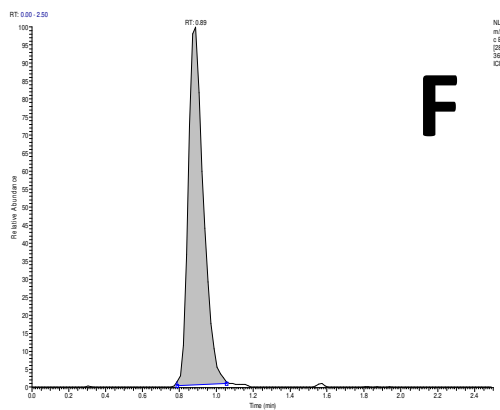
C



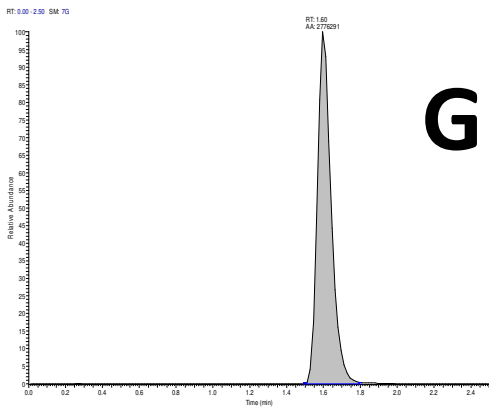
D



E



F



G

Table 1: Preparation of the stock solutions of calibrators, quality controls and internal standards.

Analyte	Solvent	Final concentration
Calibrators and QCs		
PALBO	Water	1 mg/ml
SUNI	MeOH / 2% HCOOH	0.763 mg/ml
DST-SUNI	MeOH	0.91 mg/ml
CABO	MeOH	1 mg/ml
OLA	MeOH	200 µg/ml
SORA	MeOH	1 mg/ml
PAZO	DMSO	10 mg/ml
IS		
[² H ₈]-PALBO	CH ₃ CN	20 µg/ml
[² H ₁₀]-SUNI	CH ₃ CN	20 µg/ml
[² H ₄]-CABO	CH ₃ CN	20 µg/ml
[² H ₈]-OLA	CH ₃ CN	20 µg/ml
[¹³ C, ² H ₃]-SORA	CH ₃ CN / 2% DMSO	200 µg/ml
[¹³ C, ² H ₃]-PAZO	MeOH / 4% DMSO	200 µg/ml

Table 2: Target concentrations of the calibrators (ng/ml).

Analyte	G1	G2	G3	G4	G5	G6	G7
PALBO	10	25	50	100	150	250	500
SUNI	3.8	9.5	19.1	38.2	57.2	95.4	190.8
DTS-SUNI	4.6	11.4	22.8	45.5	68.3	113.8	227.5
CABO	100	250	500	1000	1500	2500	5000
OLA	200	500	1000	2000	3000	5000	10000
SORA	400	1000	2000	4000	6000	10000	20000
PAZO	2000	5000	10000	20000	30000	50000	100000

Table 3: Precursor molecular ion/product for quantification or confirmation ; and detection parameters (tube lens voltage (TL)/collision energy (CE)) for each analyte.

Analyte	Precursor ion	Product ion quantification	TL (V)	CE (V)	Product ion confirmation	CE (V)
PALBO	448.22	380.10	298.48	28.00	362.17	38.31
SUNI	399.12	326.11	153.47	20.82	283.07	26.18
DST-SUNI	371.15	283.07	132.24	20.21	326.11	16.27
CABO	502.13	323.04	251.46	38.92	307.00	55.00
OLA	435.03	367.11	232.65	21.33	281.00	33.56
SORA	465.02	252.04	273.30	33.71	270.11	24.06
PAZO	438.12	357.11	298.48	30.38	341.11	46.30
[²H₈]-PALBO	456.32	388.22	260.86	30.22	370.22	41.35
[²H₁₀]-SUNI	409.29	326.11	205.04	22.69	283.04	27.44
[²H₄]-CABO	506.23	323.11	298.48	39.38	307.07	55.00
[²H₈]-CABO	443.26	375.16	190.18	22.29	281.07	34.37
[¹³C, ²H₃]-SORA	469.15	256.11	298.48	35.63	274.11	25.52
[¹³C, ²H₃]-PAZO	442.22	361.16	298.48	31.08	341.18	47.92

Table 4 : LOQs obtained and selected (ng/mL) with their corresponding CV (%) and the range of linearity (ng/mL).

Analyte	CV (%)	Obtained LOQ (ng/ml)	Set LOQ (ng/ml)	Linear range tested (ng/ml)
PALBO	15.37	5.37	6.0	6 – 1000
SUNI	12.44	0.97	1.0	1 – 380
DST-SUNI	3.29	4.24	4.3	4.3 – 450
CABO	6.00	72.92	75	75 – 5000
OLA	3.92	169.19	170	170 – 20000
SORA	4.65	347.44	350	350 – 40000
PAZO	2.05	1683.23	1700	1700 – 200000

Table 5: Accuracy and precision results obtained with the 3 levels of control.

Concentration (ng/mL)	Within-run precision (%) (n = 10)	Between-run precision (%) (n = 22)	Within-run accuracy (%) (n = 10)	Between-run accuracy (%) (n = 22)
PALBO				
20	8.81	12.91	9.53	13.32
80	9.36	9.95	0.45	7.48
480	4.61	5.02	3.09	2.84
SUNI				
7.63	3.65	5.03	-1.45	-3.18
30.52	3.66	4.37	-11.38	-12.65
183.12	2.68	2.12	-13.18	-13.57
DST-SUNI				
9.10	4.57	5.42	1.73	2.64
36.4	3.38	3.59	-10.5	-9.35
218.4	3.87	6.68	-6.8	-11.8
CABO				
200	2.03	3.75	2.49	1.84
800	2.98	3.06	2.17	2.59
4800	4.17	4.05	0.49	1.80
OLA				
375	4.53	4.58	4.28	4.48
1500	2.92	7.26	-6.36	-4.91
9000	3.64	6.69	-3.77	1.46
SORA				
750	4.57	7.10	5.40	4.13
3000	3.48	4.83	-4.35	-1.60
18000	3.92	4.55	2.74	-0.21
PAZO				
3750	1.96	3.10	8.50	6.82
15000	2.23	3.22	6.10	7.64
90000	1.40	1.99	4.51	4.37