Development and validation of a selective SPR aptasensor for the detection of anticancer drug irinotecan in human plasma samples

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Abstract
In this work, a surface plasmon resonance (SPR)-based assay for the quantification of antineoplastic drug irinotecan in human plasma samples has been developed for the first time. The selective binding of irinotecan with an aptamer receptor, operating in human plasma, allowed to set-up a novel analytical methodology to detect the drug in the analytical range of interest by using SPR as detection technique. After hybridizing the aptamer to the sensing platform and optimizing the sample preparation procedure, a quantitative assay was validated according to FDA regulatory guidelines. The analytical working range was found between 100 and 7500 ng mL^{-1} with negligible interferences from plasma components and co-medication associated with the administration of irinotecan. The utility of the new SPR assay was confirmed by analyzing plasma samples in parallel with LC-MS as reference technique, providing a new analytical tool for the therapeutic drug monitoring of irinotecan in patients under chemotherapy regimens.

Keywords Irinotecan · Aptamers · Therapeutic drug monitoring · Anticancer drugs · Surface plasmon resonance · Oncology

Introduction

Antineoplastic drug irinotecan (CPT-11) is a camptothecin derivative, widely indicated in the first-line treatment of patients with metastatic carcinoma of the colon or rectum [1, 2]. Chemotherapeutic regimens involving CPT-11 often include the co-administration of other anticancer drugs such as 5-fluorouracil (5-FU) and leucovorin (i.e., FOLFIRI treatment) [3, 4], or monoclonal antibodies, such as cetuximab and bevacizumab [5, 6].

Antitumor activity of CPT-11 is due to the generation of a 100–1000-fold more cytotoxic metabolite SN-38 from metabolic processes (Scheme 1), which is acting as a topoisomerase I inhibitor [7, 8]. This nuclear enzyme plays a key role in relaxing the supercoils of the DNA double helix during replication, transcription, and repair [9, 10]. Therefore, inhibition of this enzyme causes irreversible DNA damage inducing death of cancer cells by apoptosis.

Other main metabolites known from the metabolic profile of CPT-11 are produced through the cleavage of the (1,4′-bipiperidine) chain from the core structure of CPT-11. In particular, APC and NPC are generated by cytochrome P-450 3A4 (CYP3A4), and SN-38 is further converted into its glucuronide derivative SN-38G by hepatic UDP-glucuronosyltransferase, which is excreted through urine (Scheme 1) [11].

CPT-11 is administered via intravenous infusion for 2 h at a dosage of 350 mg/m^2 every 3 weeks in monotherapy, and 180 mg/m^2 every 2 weeks in combination with folinic acid and 5-FU [3, 4, 12]. The dose should be modified after the first administration according to the degree of adverse effects observed in patients. The side effects of irinotecan are common to most antineoplastic agents and include neutropenia, thrombocytopenia, anemia, acute cholinergic syndrome, alopecia, and gastrointestinal disorders [2, 13, 14].

In recent years, the practice of therapeutic drug monitoring (TDM) has received a growing interest to personalize the drug

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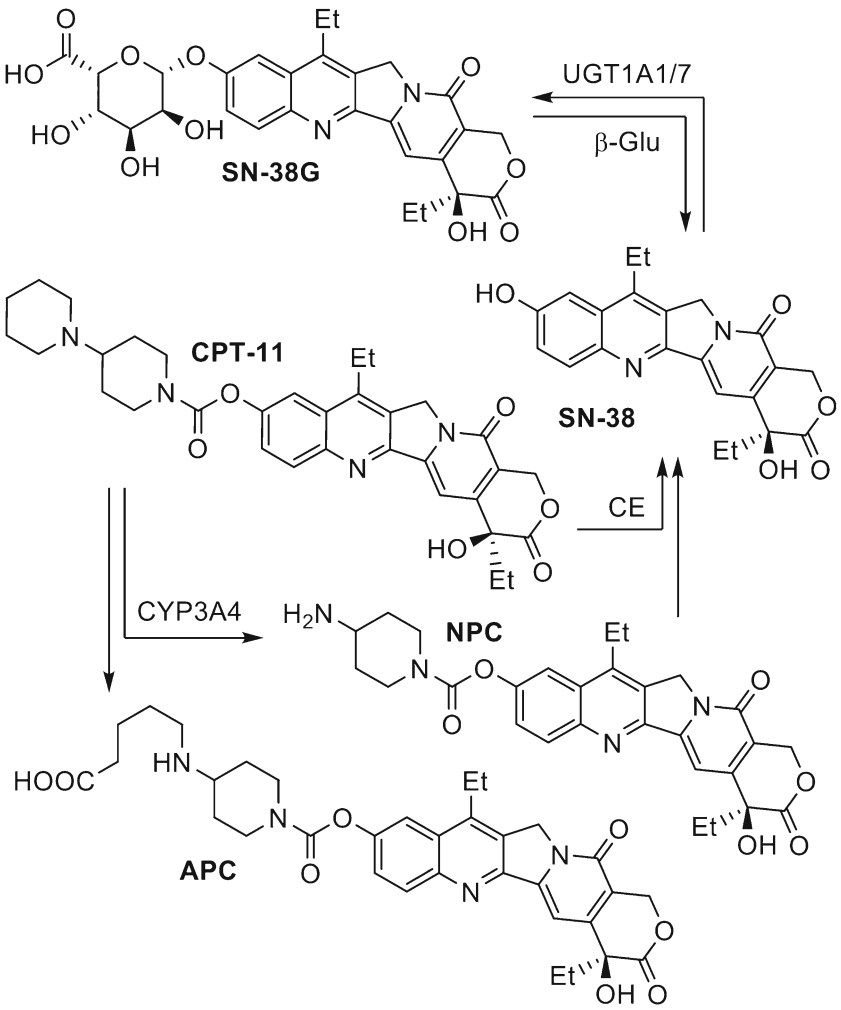

Scheme 1 Metabolic pathway of CPT-11 with its main metabolites. CYP3A4, cytochrome P-450; UGT1A1/7, UDP-glucuronosyltransferase; CE, liver carboxylase

dosage for any individual patient, but its application in oncology is still very limited [15]. In this context, point-of-care testing (PoCT) and biosensing technologies would represent a convenient strategy to extend TDM to a wide number of patients. Indeed, implementing TDM in the clinical practice would ensure that the plasma level of administered drugs is maintained within a therapeutic window of efficacy, preventing therefore most of toxic effects or treatment failures [16].

The most common methodologies utilized in centralized laboratories for the quantification of drugs in biological fluids, including CPT-11 and its main metabolites, are currently based on reverse-phase high-performance liquid chromatography (RP-HPLC) coupled with mass spectrometry [12, 17, 18].

However, the development of novel analytical tools at a PoCT level presents several technical challenges related to the analysis of therapeutic drugs, which are often low-molecular weight organic molecules, in biological fluids (blood, plasma, serum, or urine). As general considerations, new analytical tools should minimize sampling procedures and avoid or simplify pre-analytical treatments of biological samples with respect to standard chromatographic methods. Moreover, any analytical method, utilized in the clinical practice, should be validated according to regulatory guidelines, in order to provide clinicians and medical staff with reliable quantitative information to take critical decisions. Finally, portable analyzers could be used to monitor patients in clinical settings, where well-equipped laboratories are not available, such as at patients' bedside, in medical practices or, eventually, at home. This would allow testing to carry out without the need for personnel to receive special training.

Examples of sensing approaches for the detection of CPT-11 and its active metabolite SN-38 are already described in the literature, evidencing the need for the development of new analytical tools applied to anticancer drugs for TDM studies.

In particular, we previously reported an electrochemical biosensor to detect CPT-11, which was based on measuring the inhibition of acetylcholine esterase enzyme in the presence of CPT-11, with excellent selectivity toward its metabolites in simple and complex matrices [19]. An electrochemical biosensor employing aptamer receptors was also developed for in vivo detection of CPT-11 and applied to animal models [20]. Instead, direct detection of CPT-11 in human samples was described by using electrochemical methods, such as

adsorptive stripping square wave voltammetry and differential pulse voltammetry. These techniques were successfully applied to the detection of CPT-11 in biological samples [21–23]. A simple and efficient fluorimetric assay to detect the active metabolite SN-38 was also validated in plasma [24].

Herein, we describe a protocol for the analysis of CPT-11 at clinically relevant concentrations by using SPR as the detection technique, and an ss-DNA aptamer as a selective receptor operating in human plasma.

Aptamers are single-stranded oligonucleotides (RNA or DNA), capable of recognizing and binding different targets such as proteins, peptides, drugs, and small molecules with high affinity and specificity. They can fold into a large variety of secondary structures with specific structural motifs that form along the nucleotide chain (hairpin, pseudo-knot, stem-loop/bulge, and G-quadruplex), providing a three-dimensional structure similar to those observed in proteins. Aptamers undergo adaptive conformational changes and their three-dimensional folding creates a specific binding site. In the presence of the target, the aptamer structure can be stabilized by the establishment of weak interactions [25].

Our protocol was developed using SPR, which allowed the characterization of the steady-state affinity of the aptamer-CPT11 interaction and to develop an assay for the quantification of CPT-11 in plasma samples. The working principle of SPR is typically based on the real-time monitoring of the interaction between the receptor, immobilized on the sensor surface, and analyte molecules in the samples. When a mass change (deriving from such interaction) is occurring at the sensor surface, a change of refractive index at the interface is originated. This SPR signal is recorded in response units (RU). Large biomolecular systems such as antigen-antibody are commonly investigated with SPR [26], while the interaction of low-molecular weight targets (< 1000 Da) with large biomolecular receptors (typically > 50,000 Da) results in the generation of a significantly weaker SPR signals, due to the small local variation of refractive index at the surface [27, 28]. This therefore presents a challenge for monitoring small molecule interactions with high molecular weight affinity ligands (such as antibodies). A typical SPR measurement is based on the sequential injection of the sample across a "blank" reference flow cell (FC1) and a working "receptor immobilized" flow cell (FC2). Information relating to the analyte-receptor binding can be therefore obtained through a differential measurement (FC2-FC1), enabling subtraction of any interference generated by non-specific surface interactions.

Materials and methods

Chemicals

Analytical standards of CPT-11 (7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin, purity 97%) and SN-38 (7-ethyl-10-hydroxycamptothecin, purity 98%) were purchased from Sigma-Aldrich Co. (Milan, Italy). APC (7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]-carbonyloxycamptothecin, purity 95%), NPC (7-ethyl-10-(4-amino-1-piperidino) carbonyloxycamptothecin, purity 98%), and SN-38G (7-ethyl-10-[3,4,5-trihydroxy-pyran-2-carboxylic acid]-camptothecin, purity 95%) were purchased from Toronto Research Chemicals, Inc. (North York, Ontario, Canada). PBS buffer, magnesium chloride ($MgCl_2$), and acetic acid were purchased from Merck Sigma-Aldrich (Milan, Italy). Tween 20 was purchased from VWR (Milan Italy). CPT-11 aptamer (100-mer), 5′biotin CPT-11 aptamer, and 5′biotin immobilization oligomer (15-mer) (see details in Supplementary Information (ESM) and in the recently reported patent [29]) were purchased from Aptamer Group (York, UK). Lyophilized oligomers were solubilized with Milli-Q water upon arrival, aliquoted, and stored at − 20 °C. Co-medications for selectivity tests, in particular dexamethasone, atropine, chlorphenamine, 5-fluorouracil, ondansetron, loperamide, and capecitabine, were provided by the pharmacy of the National Cancer Institute (Aviano, Italy) and folinic acid was purchased from Merck Sigma-Aldrich. LC-MS grade methanol, acetonitrile, and camptothecin were supplied by Merck Sigma-Aldrich. Control human plasma stabilized with K_2EDTA for the preparation of daily standard calibration curves and quality control (QC) samples was obtained from healthy volunteers and was provided by the Transfusion Unit of the Centro di Riferimento Oncologico di Aviano (CRO), Italy.

SPR measurements

SPR measurements were performed using a Biacore X100 workstation (GE Healthcare Life Sciences), at 25 °C, with a flow rate of 5, 10, and 30 μL min^{-1}. Streptavidin-coated sensor chips (SA) were purchased from GE Healthcare Life Sciences.

Binding analysis

Binding analysis was performed by using 13 different concentrations of CPT-11 from 0 to 2.0 μM in PBS buffer (10 mM, pH 6.0) containing NaCl (100 mM), KCl (2 mM), $MgCl_2$ (7.5 mM), and Tween 20 (0.05%) on a SA chip functionalized with 5′biotin CPT-11 aptamer (see ESM, Fig. S1 and S2). Experiments were performed in triplicate. Association and dissociation time were 120 and 180 s respectively, with the flow rate set to 30 μL/min. Regeneration of the sensor surface was performed with H_2O for 20 s after each sample injection. Data processing was performed by subtracting the signal recorded on FC1 (reference cell) from the signal recorded on FC2 (functionalized cell). FC2–FC1 data were further referenced by blank subtraction. Association curves were thus

evaluated by non-linear analysis with SPR kinetic evaluation software (BIAevaluation Software, version 2.0.2 Plus Package, GE Healthcare Life Sciences) and the affinity constant was obtained from a 1:1 steady-state model.

Concentration analysis in buffered media

CPT-11 aptamer (1.2 μM) in PBS buffer (50 mM, pH 7.2) containing NaCl (685 mM), KCl (13.5 mM), and $MgCl_2$ (5 mM), defined as hybridization buffer, was injected on FC2 of the SA chip functionalized with 5′biotin immobilization oligomer (see ESM, Fig. S1 and S3) over 600 s at 5 μL min^{-1}. After a stabilization time of 300 s, standard solutions of CPT-11 were sequentially injected on FC1 and FC2 over 420 s at a flow rate of 10 μL/min. Calibration standards of CPT-11 from 50 to 800 ng mL^{-1} in PBS buffer (10 mM, pH 6.0) containing NaCl (100 mM), KCl (2 mM), and $MgCl_2$ (2.5 mM) were used. The running buffer was PBS (25 mM, pH 7.2) containing NaCl (342 mM), KCl (6.75 mM), and Tween 20 (0.05% v/v). Surface regeneration was performed at the end of each run with a mixture of 8 mM NaOH and 160 mM NaCl for 20 s. A blank sample (0 ng/mL) was also included.

Concentration analysis in human plasma

Standards and quality control working solutions

A 1000 μg mL^{-1} stock solution of CPT-11 was firstly prepared in DMSO, aliquoted, and stored at − 20 °C. A series of working solutions (A to G) were obtained by diluting the stock solution with DMSO to the concentrations reported in Table S1 (ESM) and used to prepare calibration standards and quality control (QC) samples at low (QCL), medium (QCM), and high (QCH) concentrations. Aliquots of these solutions were stored at − 20 °C in Eppendorf polypropylene tubes and used for a maximum of three freeze-thaw cycles.

Preparation of standards and quality control samples

A newly prepared seven-point calibration curve was recorded every day during this study. Each calibration and QC sample was prepared by adding 2 μL of working solution from A to G in 38 μL of pooled human plasma in a 0.5-mL Eppendorf polypropylene tube to obtain the final concentrations reported in Table S1 (ESM). Each calibration curve included a blank and three QC samples, which were analyzed in triplicate. Calibration standards and QCs were diluted with 360 μL of PBS buffer (12 mM, pH 5.0) containing NaCl (103 mM), KCl (2 mM), $MgCl_2$ (2.5 mM), and Tween 20 (0.05%), named as incubation buffer, and thoroughly vortexed for 10 s three times. The content of each tube was transferred on a centrifugal filter (Millipore Amicon® Ultra 0.5 mL, 30 kDa cutoff) and centrifuged at 6200 rcf for 18 min at 25 °C. Then, 350 μL of the filtrate was transferred on 11-mm plastic vials (ESM, Scheme S1) and analyzed as described below. CPT-11 standards and samples were kept in ice bath until use.

Concentration analysis

Concentration analysis of plasma samples were performed with the SA chip functionalized with 5′biotin immobilization oligomer (ESM, Fig. S1 and S3), using PBS (25 mM, pH 7.2) containing NaCl (342 mM), KCl (6.75 mM), and Tween 20 (0.05% v/v) as the running buffer. For each run, CPT-11 aptamer (1.2 μM) in the hybridization buffer was firstly injected in FC2 for 600 s at 5 μL/min. After 300 s of stabilization time, CPT-11 standards were injected in both flow cells at 10 μL/min for 480 s in the following order: blank, calibration standards, QCs in triplicate and unknown samples. After each run, the chip surface was regenerated with a mixture of 8 mM NaOH and 160 mM NaCl for 20 s.

Validation study

The validation of the method was carried out by following the guidelines of the Food and Drug Administration (FDA) applied to ligand-binding assays (LBA) [30]. The parameters evaluated for the validation included recovery, intra- and inter-day precision and accuracy, reproducibility, lower limit of quantification (LLOQ), selectivity, and matrix effects (see ESM for full details).

Mass spectrometry

Quantification of CPT-11 in plasma samples, collected from patients on therapy, was performed by using tandem mass spectrometry, as the reference method to assess the quality of the SPR assay. The analysis was conducted with a Shimadzu LC system coupled with an API 4000 QT (Sciex) working in multiple reaction monitoring (MRM) mode. Irinotecan was quantified in plasma samples applying a LC-MS/MS method previously developed and validated, according to FDA/EMA guidelines, by our group [12]. In brief, 100 μL of the actual sample, calibrator, or QC sample was transferred to a 1.5-mL polypropylene tube and mixed with 5 μL of the internal standard solution (camptothecin, 0.5 μg/mL), and successively with 300 μL of 0.1% CH_3COOH/CH_3OH for protein precipitation. The solution was vortexed and centrifuged at 16000*g* for 10 min at nominally 4 °C. Then, 150 μL of the obtained supernatant was transferred to an autosampler glass vial for the analysis. Analyte separation was obtained with gradient elution on a Gemini C18 column (3 μM 110A, 100 × 2.0 mm) coupled with a Security Guard Cartridge (Gemini-NX C18 4.0 × 2.0 mm) (column temperature set at 25 °C). The mobile phases (MP) were 0.1%

CH_3COOH/double distilled water (MP A) and 0.1% CH_3COOH/acetonitrile (MP B). The ESI source operated in positive ion mode at 650 °C and with ion spray voltage of 5500 V. Quantification was performed using the following transitions: *m/z* 587 > 124 for CPT-11 and *m/z* 349 > 305 for the internal standard.

Results and discussion

The CPT-11 aptamer was preliminary tested to establish the optimal experimental conditions to detect CPT-11. Our approach included also the utilization of 5′biotin immobilization oligomer, which is a short sequence (15-mer) complementary to a hybridization region within the CPT-11 aptamer (100-mer) [29]. The functionalization of the SPR platform was easily carried out by using 5′biotin CPT-11 aptamer and immobilization oligomer, which were successfully immobilized on a streptavidin-coated (SA) sensor chip (see ESM, Fig. S1). Indeed, the simple injection of a 5 μM solution of biotinylated DNA in 25 mM PBS buffer provided an immobilization yield of 1767 and 2092 RU on FC2 for 5′biotin CPT-11 aptamer and immobilization oligomer respectively (see ESM, Figs. S2 and S3). The surface density for the aptamer and the immobilization oligomer on the chip was estimated in 1.767 and 2.092 ng mm^{-2}, respectively, after conversion of the corresponding RU as reported for Biacore technology, in which a response of 1000 RU corresponds to a change in surface density of about 1 ng mm^{-2} of proteins or DNA [31].

Steady-state affinity study

The evaluation of the affinity constant (K_D) between CPT-11 aptamer and CPT-11 was carried out using an SA chip prepared by immobilizing 5′biotin CPT-11 aptamer on FC2 only, as described in ESM (Fig. S2). After injecting CPT-11 in PBS buffer at a flow rate of 30 μL/min across both FC1 and FC2, the response, obtained from the differential measurement (FC2-FC1), rapidly achieved a plateau. As soon as the injection stopped, the signal decreased immediately, demonstrating that both association and dissociation of CPT-11 from the aptamer were fast processes, as shown in Fig. 1a.

The affinity constant (K_D) was evaluated by analyzing CPT-11 in a concentration range from 0 to 2 μM and fitting the steady-state affinity curve, obtained by plotting the RU at the plateau against the concentration of CPT-11 for each curve, with a 1:1 binding model (Fig. 1b, BIAevaluation Software, version 2.0.2 Plus Package, GE Healthcare Life Sciences). Even though the binding of CPT-11 provided a relatively weak SPR response, the model gave a K_D of 285 ± 17 nM, a typical value for the binding of small molecules [28].

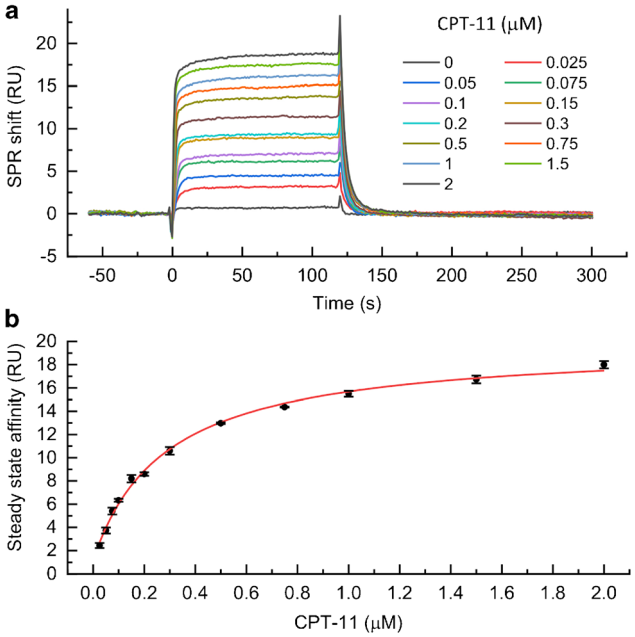

Fig. 1 **a** Reference subtracted sensorgrams (FC2-FC1) and **b** steady-state affinity curve obtained after injecting CPT-11 in the concentration range 0–2.00 μM in PBS buffer (10 mM, pH 6.0) containing $MgCl_2$ (7.5 mM) and Tween 20 (0.05%) on the SA chip functionalized with 5′biotin CPT-11 aptamer. To evaluate K_D, binding values were collected 4 s before injection stop and were further referenced by blank subtraction. Experiments were performed in triplicate

Detection of CPT-11 in buffered media

Although direct binding of CPT-11 with the aptamer immobilized on the sensor surface provided a measurable SPR response, we pursued a different strategy to develop a concentration assay. In fact, the aptamer has a molecular weight (MW) of 31000 Da, which is considerably higher than the CPT-11 molecule (MW of 586.7). Binding of the aptamer has a higher refractive index change at the interface and consequently results a higher SPR response from the binding process. Interferences from non-target molecules (e.g., plasma proteins) would hamper the analysis of real samples. For this reason, we were discouraged from following the direct detection approach. We then moved to a strategy based on functionalizing the SA chip with 5′biotin immobilization oligomer on both flow cells (see ESM, Fig. S3), followed by the hybridization of CPT-11 aptamer to form a duplex structure on FC2. Under this configuration, the conformational change of the aptamer, in the presence of CPT-11, would lead to the release of the aptamer from the duplex structure as shown in Fig. 2 [32]. As a result, the mass decrease from the sensor chip would be significantly greater and more consistent than the direct binding of CPT-11 to the aptamer immobilized at the surface.

During our investigation, we found a satisfactory hybridization level of CPT-11 aptamer on SA sensor chip by using a 1.2 μM solution of the aptamer in 50 mM PBS containing

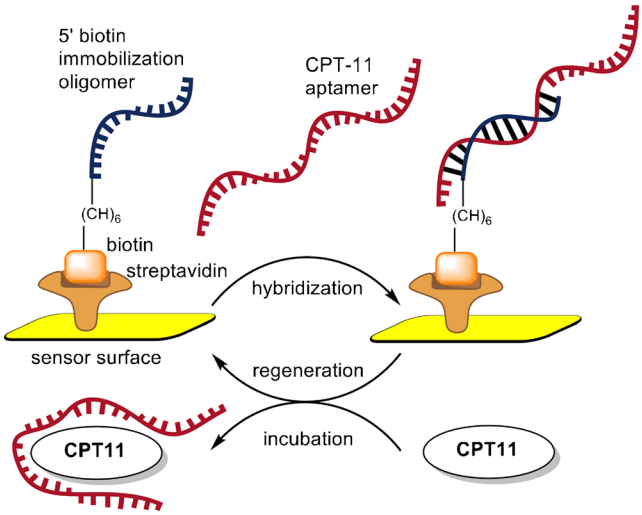

Fig. 2 Schematic representation of the SPR aptasensor to detect CPT-11. After hybridization of the CPT-11 aptamer on the chip surface in FC2, the injection of a sample containing CPT-11 leads to the release of the aptamer from the chip surface with the generation of a SPR response suitable for analytical quantification

5 mM $MgCl_2$ (hybridization buffer) over the sensor surface for 600 s at 5 μL/min. A PBS concentration of 50 mM was beneficial to increase the hybridization level, even though higher salt concentrations were avoided to prevent the possible formation of salt precipitates in the microfluidic cell of the instrument. Moreover, PBS concentration was kept at 25 mM in the running buffer to reduce the non-specific displacement of the aptamer from the surface. On the other hand, the surface regeneration could be efficiently performed with a solution of 8 mM NaOH and 160 mM NaCl for 20 s, thus enabling the possibility to recycle the SA chip after each experiment.

According to our tests, the aptamer hybridization level decreased from 1100 to 1000 RU after 400 runs with an average value of 1053 ± 34 RU and a CV of 3.2% (see ESM, Fig. S4). After such usage, a loss of activity occurred due to an excessive degradation of the surface and the chip was replaced. The aptamer was hybridized only on FC2, while FC1 remained unfunctionalized and was used as reference cell, to exclude non-specific interactions of CPT-11 with the sensor surface during data processing.

Upon hybridization of the aptamer on FC2, solutions of CPT-11 were sequentially injected in both flow cells. Dissociation of aptamer from FC2 occurred with a decrease of SPR signal proportional to the concentration of CPT-11 (Fig. 3a). Several buffer compositions were tested in order to optimize the interaction between the aptamer and CPT-11, providing that 10 mM PBS at pH 6 containing $MgCl_2$ in the range 2.5–7.5 mM could be used for the incubation. After the incubation step, the chip was regenerated for the next run.

Sensorgrams were processed in two steps as follows. (i) SPR data of each run were referenced by subtracting the response of FC1 (reference cell) from FC2 to provide FC2–FC1 data (Fig. 3a), and (ii) sensorgrams were further corrected by subtracting the blank from each non-zero sample and "flipping of data" to plot the aptamer dissociation as a positive response (Fig. 3b).

The correlation of ΔRU values, which were taken 200 s after the stop of each sample injection, against the concentration of CPT-11, provided a response in the concentration range from 50 to 800 ng mL^{-1} that could be fitted by using a non-linear regression (Fig. 3c). A four-parameter logistic (4PL) model was applied following FDA guidelines for LBA validation (see Supporting Information for a brief description of the model) [30]. We put a huge effort to obtain a linear response during this phase of the method development, including the modification of the buffer composition as well as the utilization of different contact times and flow rates. Unfortunately, any variation of experimental conditions had an impact on the relative intensity of the SPR response but not in the shape of the calibration curve. In general, more diluted buffers led to a higher response, whereas media with higher ionic strength reduced the sensitivity of the method, due to an improved stability of the aptamer hybridized to the surface. As an example, the two calibration curves shown in Fig. 3c, obtained diluting CPT-11 with a PBS buffer (10 mM) containing 2.5 and 7.5 mM $MgCl_2$ respectively, clearly showed a higher response when using a more diluted buffer. The detection limit in buffered media was evaluated by analyzing the blank variability (three times the standard deviation of the blank on eight replicates) with the 4PL model, providing a LOD of 9.1 ng mL^{-1}.

Detection of CPT-11 in human plasma

Having found a strategy suitable to detect and calibrate CPT-11, we next moved to the application of our method to human plasma samples. Contrary to a simple buffered media, human plasma is a complex matrix with a high protein content, especially albumin and α-glycoproteins, which are known to bind 60–70% of CPT-11 [33].

The first step in our investigation was therefore the treatment of plasma samples for SPR analysis. Direct injection of plasma samples into the flow cells of Biacore X100 instrument was avoided to reduce the risk of blockages to the instrument microfluidics.

A 1:10 dilution step was applied to match the instrumental response, previously obtained between 50 and 800 ng mL^{-1}, with a concentration range of CPT-11 that can be reasonably found in plasma samples, usually up to 5000 ng mL^{-1} [12].

Unfortunately, the simple dilution of plasma was not sufficient to allow SPR analysis, as plasma proteins still contributed a significant response due to adsorption on the sensor surface, obscuring the aptamer dissociation process. An additional step

of microfiltration with 30 kDa centrifugal filters before SPR analysis was introduced to completely avoid the response of large protein content contained in plasma. In this case, sensorgrams corresponding to the analysis of plasma appeared similar to those previously recorded in simple buffer (Fig. 3a and b). However, the response of CPT-11 was too weak for analytical purposes, due to the binding with plasma proteins, which prevented enough recovery of the drug in the filtrate. The addition of Tween 20 in the buffer successfully reduced non-specific binding (NSB) and allowed a significant fraction of CPT-11 to pass the centrifugal filter. Other surfactants (e.g., sodium dodecyl sulfate or cetyl trimethyl ammonium bromide) or standard dextran NSB reducer [34] (GE Healthcare Life Sciences) did not provide significant recovery from plasma. Tween 80 or PEG were also tested and found to be less effective than Tween 20 (see ESM, Fig. S5). The amount of surfactant in the buffer was 0.05%.

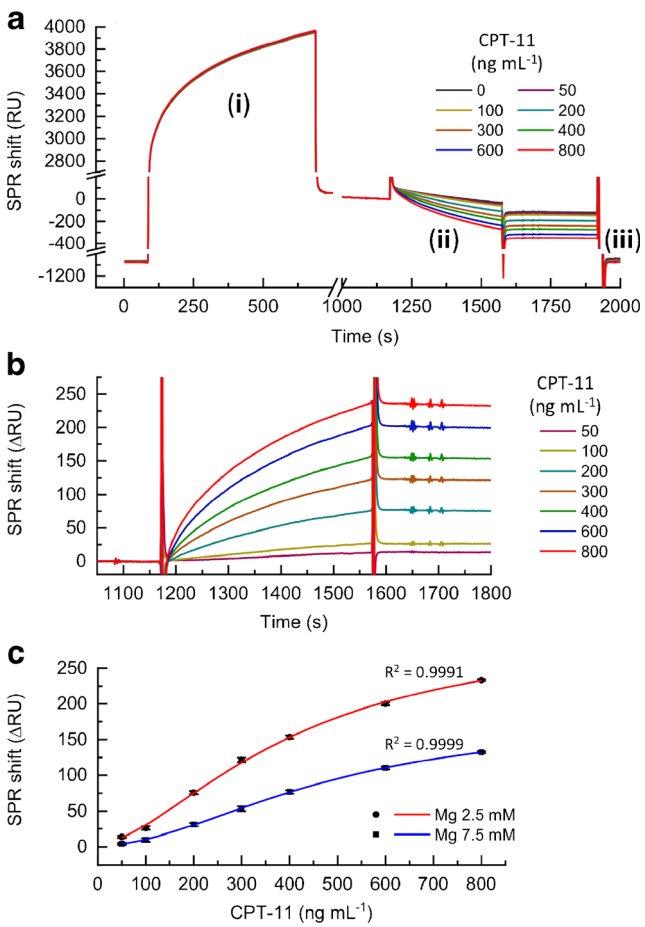

Fig. 3 **a** Significant portions of sensorgrams for the overall calibration experiment (FC2-FC1). The three sections are summarized as follows: (i) injection and hybridization of the aptamer on the sensor chip surface, (ii) sample injection, and (iii) regeneration of the chip surface. **b** Flipped sensorgrams obtained after blank subtraction, to plot the aptamer dissociation as a positive response. **c** Calibration curves obtained by plotting ΔRU values, evaluated 200 s after the end of sample injection, vs CPT-11 concentrations. Mg = $MgCl_2$. Experiments were performed in triplicate

Concentration of $MgCl_2$ in the incubation buffer was kept at 2.5 mM to have a good sensitivity, analogously to the measurement in simple buffered media. As previously observed, the correlation of SPR response against concentration of CPT-11 in plasma required a 4PL model to properly fit the calibration points, as suggested also by LBA validation guidelines (see ESM for a brief description of the model) [30].

Next, once the procedure to prepare plasma samples for SPR analysis was defined, the aptamer-based assay for the quantification of irinotecan assay was validated according to FDA guidelines to assess the suitability of the method.

Calibration curves

Accuracy and precision for each standard point were evaluated on six calibration curves recorded in different working days, as summarized in Table 1. The SPR shift against the nominal concentration of the analyte was used to obtain calibration curves by using a 4PL regression (Fig. 4a). Acceptable back-calculated concentrations in a concentration range from 100 to 7500 ng mL^{-1} were obtained with a Pearson's coefficient of determination (R^2) higher than 0.995 for each set of measurement, while the accuracy was found between 94.5% and 105.2% and precision from 1.6 to 5.8% in terms of CV%.

Recovery

As previously described, the preparation of samples for the SPR analysis was carried out by diluting plasma 1:10 with an incubation buffer followed by centrifugal microfiltration with 30 kDa cutoff centrifugal filters (ESM, Scheme S1). To evaluate the recovery of CPT-11 from the sample preparation procedure, the SPR response of QC samples, prepared by spiking CPT-11 in plasma before the dilution and centrifugal microfiltration, was compared to the response of QCs in which CPT-11 was added after the dilution and microfiltration of plasma. Recovery was evaluated at three concentration levels and was found in the range 64.3–73.7% with CV ≤ 10% (Table 1). The significance of recovery, in our method, is referred to the efficiency of centrifugal microfiltration procedure to recover the analyte from the sample, and indicates the fraction of CPT-11 found in the filtrate, with respect to the theoretical amount present in the sample. Even though the microfiltration step was found to result in a significant loss of the analyte, it must be considered that the same procedure was applied to both calibration standards and samples. In fact, standards and QCs were prepared by spiking working solutions of CPT-11 in plasma before the microfiltration, to simulate the treatment of real samples as much as possible. To confirm this hypothesis, we prepared QCs using independent stock solutions of CPT-11 than those utilized for calibration standards, and determined intra- and inter-day accuracy and precision, as described below.

Table 1 Summary of validation data for the SPR analysis of CPT-11. *N. C.*, nominal concentration; *B. C.*, back-calculated concentration; *SD*, standard deviation; *CV*, coefficient of variation

Entry	N. C. (ng mL^{-1})	Mean B. C. ± SD (ng mL^{-1})	Precision (%)	Accuracy (%)
Calibration curves	100	105 ± 6	5.8	105.2
	250	236 ± 8	3.4	94.5
	500	508 ± 26	5.1	101.5
	1000	1004 ± 26	2.6	100.4
	2500	2504 ± 62	2.5	100.2
	5000	4977 ± 153	3.1	99.5
	7500	7521 ± 118	1.6	100.3
Intra-day	200	200 ± 7	3.5	100.2
	1500	1468 ± 6	0.4	97.8
	6500	6432 ± 45	0.7	98.9
Inter-day	200	199 ± 19	9.5	99.7
	1500	1541 ± 71	4.6	102.7
	6500	6489 ± 492	7.6	99.8
Matrix effect	100	103 ± 10	9.5	102.9
selectivity	200	181 ± 9	5.1	90.3
	1500	1470 ± 26	1.8	98
	6500	6216 ± 138	2.2	95.6
		Recovery (%) ± SD	CV %	
Recovery	200	65.0 ± 6.1	9.4	
	1500	64.3 ± 4.1	6.4	
	6500	73.7 ± 3.0	4.0	
	LOD = 55 ng mL^{-1}		LLOQ = 100 ng mL^{-1}	

Intra-day and inter-day precision and accuracy and reproducibility

To establish the precision and accuracy of the method, we analyzed QC samples in triplicate (200, 1500, 6500 ng mL^{-1}) within a single run for intra-day and over six sets of measurement for the inter-day assessment. The intra- and inter-day accuracy was found in the range 97.8–100.2% and 99.7–102.7% respectively. Moreover, the method demonstrated an excellent intra- and inter-day precision (CV%) in the range 0.4–3.5% and 4.6–9.5% (Table 1). Inter-day precision and accuracy were also evaluated by combining data collected from three different SA chips at the QC concentration levels, in order to assess the reproducibility of the method using different chips. In particular, we found a precision (CV%) of 5.7, 2.9, and 6.2% at 200, 1500, and 6500 ng mL^{-1}, whereas accuracy at the same concentrations was 99.4, 96.6, and 99.4% respectively.

Lower limit of quantification, limit of detection, and matrix effect

According to guidelines by FDA, a bias up to 25% is allowed at the lower limit of quantification (LLOQ) level for LBA tests, even though a tighter value was suggested to be beneficial when fitting a model to the standards [35]. Having used a non-linear fitting for the preparation of the calibration curve, we evaluated LLOQ by analyzing the biases of eight independent replicates for each standard, which were plotted against the nominal concentration of CPT-11. Biases were within 15% for all calibrators except at 100 ng mL^{-1}, in which a ± 17% value was found appropriate as the LLOQ and the lowest standard of the calibration curve (see ESM, Fig. S6). Lower concentrations were not considered, as the absolute SPR response at 100 ng mL^{-1} was found between 7.5 and 10.4 RU, which is, reasonably, the lowest response for quantitative detection of CPT-11. Therefore, lower responses would have been inaccurate. The limit of detection (LOD) of the method was evaluated on eight replicates of the blank within a single set analysis. The analysis of the blank variability (three times the standard deviation of the blank), with the 4PL model, resulted a LOD of 55 ng mL^{-1} in plasma. Consequently, this method can be applied to evaluate irinotecan in samples taken from patients up to 20 h after intravenous infusion, in which the concentration of CPT-11 is close to 100 ng mL^{-1} [12]. The method was also designed to detect high concentrations of the drug, which are leading to excessive toxicity, enabling therefore to properly adjust the administered dosage.

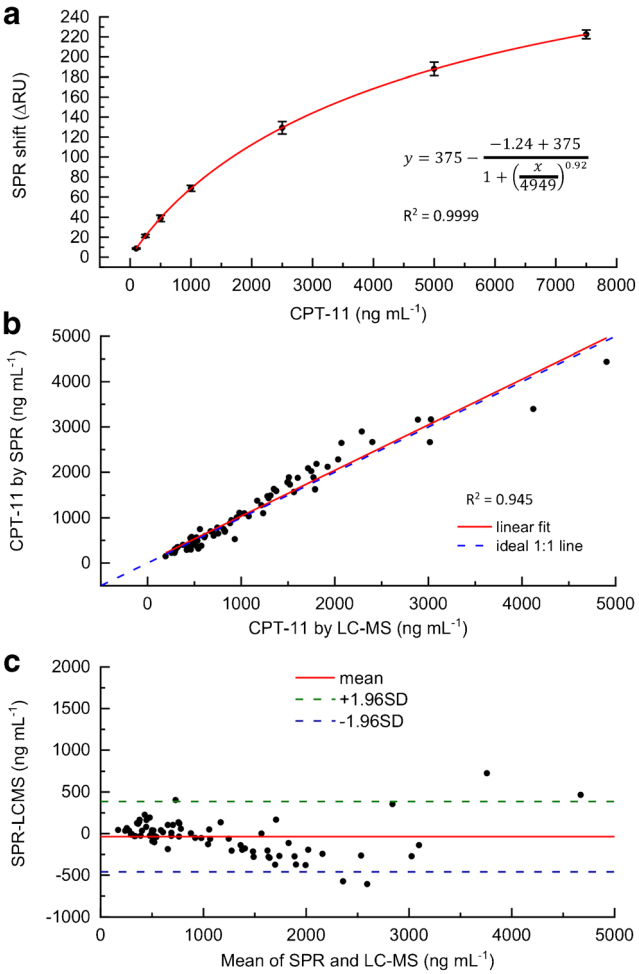

Fig. 4 **a** Calibration curve of CPT-11 in plasma obtained upon blank subtraction and 4PL regression. Concentration of CPT-11 corresponds to the concentration in plasma (C_{fin}) reported in Table S1 (ESM). Experiments were performed in triplicate; **b** correlation of SPR and HPLC-MS data for the quantification of CPT-11 in plasma samples; **c** Bland-Altman plot showing biases vs HPLC-MS

The evaluation of matrix effects and other interferences in the sample was tested at the LLOQ with six sources of human plasma, obtained from different donors. The quantification through a calibration curve, prepared with pooled plasma, provided the same back-calculated concentration at 100 ng mL^{-1} with a precision of 9.5% and accuracy of 103.0%. These values demonstrate that the microfiltration of samples to remove most of the biological interference is effective, whereas the aptamer maintain the selectivity toward the recognition of CPT-11 against those low-molecular weight species remaining in the sample.

Selectivity

Selectivity was evaluated by testing the interaction with other known interfering low-MW compounds found in samples, which included co-medications such as dexamethasone, atropine, chlorphenamine, 5-fluorouracil, ondansetron, loperamide and capecitabine and folinic acid, commonly associated with the administration of CPT-11, and the main metabolites of irinotecan SN-38, SN-38G, APC, and NPC. As expected, co-medications provided a SPR response similar to that of a blank sample, demonstrating that no interaction takes place with the immobilized aptamer (see ESM, Table S2 and Fig. S7). Other camptothecins spiked in plasma in pure form provided an average response of 2% for SN-38G, 11% for SN-38, 97% for NPC, and 29% for APC with respect to CPT-11 at the same concentrations of QCs (see ESM, Table S3). However, when analyzing QC samples containing a mixture of CPT-11 and the other metabolites, as found in real samples, we found a negligible interference with respect to the response of QCs containing CPT-11 alone. This was demonstrated from the quantification of CPT-11 in QCs containing also a mixture of metabolites having a composition of 8% APC, 4% SN38G, 1.5% SN38, and 1.5% NPC with respect to CPT-11, averaging those found in real samples (see ESM, Fig. S8) [12, 36]. The quantification of CPT-11 in the presence of metabolites provided in general a negative bias with accuracies ranging from 90.3 to 98% (Table 1), which is fully acceptable according to guidelines (bias within 20%). Interestingly, the presence of metabolites decreased the efficiency of the aptamer to detect CPT-11 and, in our opinion, this was ascribed to the ability of metabolites to inhibit the aptamer, which is prevented to freely interact with CPT-11. However, we can conclude that validation data confirmed the utility of the biosensing approach to the detection of CPT-11 in human plasma.

Comparison of the SPR assay with HPLC-MS

The final test to assess the potential of our SPR assay for TDM application was the re-quantification of patient's samples previously analyzed by standard HPLC-MS reference technique. Our protocol was applied to 72 available samples, taken from patients administered with irinotecan at the Centro di Riferimento Oncologico di Aviano (CRO), Italy. The samples were processed following the same procedure for standards and QCs (ESM, Scheme S1). As shown in Fig. 4b, the correlation of the SPR assay with respect to HPLC-MS provided a slope of 1.09 and a Pearson coefficient of 0.945. The mean difference of the values obtained from the two techniques was 0.035%, with 54 samples out of 72 (75%) analyzed with the SPR aptasensor which displayed a deviation from the reference method lower than 20% (see ESM Table S4). Moreover, an acceptable degree of correlation between the two techniques was obtained by analyzing data with the Bland-Altman plot (Fig. 4c).

Analytical performances of our methodology, in terms of analytical range and LOD, were compared to the recently reported (in the last 5 years) electrochemical and biosensing strategies for detecting CPT-11 in biological samples (Table 2).

Table 2 Comparison of the analytical performances of recently reported methods for detecting CPT-11 in biological samples

Method	Analytical range (ng mL^{-1})	LOD (ng mL^{-1})	Sample type	Pre-treatment procedure (sample volume, μL)	Reference
HPLC-MS	10–10000	0.116	Plasma (human)	1:4 dilution with 0.1% CH_3COOH/CH_3OH followed by centrifugation (100)	[12]
Pencil graphite electrodes coupled with square wave cathodic adsorptive stripping voltammetry	495–3639	13.1	Serum (human)	1:3 dilution with CH_3OH followed by centrifugation (450)	[21]
Solid-phase extraction coupled with differential pulse voltammetry	312–5608	79.8	Plasma (human)	Loading on SPE columns followed by elution with CH_3CN (125)	[23]
Amperometric enzyme-based sensor	10–10000	1.5	Fetal bovine serum	No sample treatment (n/a)	[19]
Graphene quantum dots-polyaniline/zinc oxide nanocomposites electrochemical sensor	62–15579	8.7	Serum (human)	1:50 dilution with Britton-Robinson buffer (n/a)	[22]
Electrochemical aptamer-based sensor	60–10000	n/a	Whole blood (rat)	In vivo measurement after surgery (n/a)	[20]
SPR aptamer-based sensor	100–7500	55	Plasma (human)	1:10 dilution with incubation buffer followed by centrifugal microfiltration (40)	This work

Although LOD is still far from the reference HPLC-MS method (0.116 ng mL^{-1}) [12], our SPR aptasensor displayed a working range (100–7500 ng mL^{-1}) and a LOD (55 ng mL^{-1}) of the same order of magnitude as reported for other detection approaches. The main advantage of the SPR aptasensor with respect to chromatographic [12] and electrochemical [21, 23] methodologies is that no organic solvents are needed for the analysis, making our approach more suitable to be transferred at a PoCT level. Moreover, our protocol for CPT-11 detection was applied to human plasma, instead of simpler matrix, such as fetal bovine serum utilized to test our previously reported amperometric enzyme-based sensor [19]. Although the analytical range and the LOD of our method do not achieve better values than direct electrochemical [22] and electrochemical aptamer-based [20] sensors, we developed our methodology to exclude interferences, especially by metabolites and co-medications of CPT-11, and finally performed a cross-validation with HPLC-MS, to verify the utility of our method. On the other hand, the two main limitations are represented by the sample pre-treatment and the time to analysis. In particular, the centrifugal microfiltration of plasma limited the recovery of the drug from the sample to 64.3–73.7% and was found a time-consuming step. Overall time to process and analyze samples, including the run time for SPR analysis, was around 1 h. Ideally, this is needing to be further shortened, in order to respond to the timely need for drug administration or reduction.

Considering the excellent validation and cross-validation data of the method, obtained from this pilot study, we conclude that a biosensing approach employing aptamers would be beneficial to the development of novel analytical tools for the quantification of irinotecan in clinical samples. This is a further example showing that advances in biosensor technology would extend the practice of TDM in oncology to optimize the drug dosage during administration and prevent those toxicity events occurring many hours or days after the administration of CPT-11, especially leuco-neutropenia and diarrhea [11].

Conclusions

In conclusion, we described the development of an SPR assay to detect the anticancer drug irinotecan in human plasma samples employing an aptamer as a selective biomolecular receptor. Although direct detection of small molecules with SPR provides typically a low response, we successfully developed a quantitative assay for irinotecan with consistent validation data covering the plasmatic concentrations found in clinical samples. In our opinion, a significant technical result of this study is that a simple procedure with inexpensive and non-toxic reagents can be used to prepare samples for the analysis. Furthermore, this work demonstrates that biosensor-based strategies can be implemented to the detection of small drug molecules in biological fluids with reliable validation data required for clinical analysis by regulatory

guidelines. We therefore prospect that aptamer-based assays will find more space in the development of novel analytical tools aimed at improving the practice of TDM in clinical oncology.

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Compliance with ethical standards

Conflict of interest D. Bunka is a Co-founder and Director of Aptamer Group and therefore has financial interest in Aptamer Group Ltd. A. Puscasu, M. Zanchetta, B. Posocco, S. Tartaggia, and G. Toffoli have no conflicts to declare regarding the publication of this manuscript.

Ethics approval Plasma samples were collected from patients enrolled in a phase Ib clinical study (CRO-2009-25, Prot. n. 0041793(09)-PRE.21-984) approved by the ethics committee of CRO-National Cancer Institute of Aviano (Italy) and by Istituto Superiore di Sanità (ISS, Italy), which was conducted according to the principles expressed in the Declaration of Helsinki. Informed consent was obtained from all participants involved in the study.

Source of biological material Control human plasma stabilized with K_2EDTA for the preparation of daily standard calibration curves and quality control (QC) samples was obtained from healthy volunteers and was provided by the Transfusion Unit of the Centro di Riferimento Oncologico di Aviano (CRO), Italy.

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