A fast method for the detection of irinotecan in plasma samples by combining solid phase extraction and differential pulse voltammetry

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Abstract

In this paper, a fast method for the detection of irinotecan (CPT-11) in plasma samples was investigated. CPT-11 is widely used in a number of chemotherapeutic treatments of several solid tumors. The method is based on the combination of a solid phase extraction and an electrochemical detection step. The extraction of CPT-11 from plasma was performed using solid phase extraction (SPE) columns and acetonitrile as eluent. The procedure included also a cleaning step to eliminate interference due to plasma endogenous compounds and the co-therapeutics 5-fluoroacil (5-FU) and folinic acid (FA). The latter are administered together with CPT-11 in the FOLFIRI regimen. The detection of CPT-11 was performed by differential pulse voltammetry at a glassy carbon electrode (GCE) in basified acetonitrile media. Under these conditions, a well-defined peak due to the oxidation of the tertiary ammine end of CPT-11, also free from interference due to main metabolites, was obtained. Calibration plots showed a good linear response with limit of detection and quantification of $1.10 \cdot 10^{-7}$ and $3.74 \cdot 10^{-7}$ M, respectively. The suitability of the method proposed here for clinical

applications was verified by determining the concentration of CPT-11 in plasma samples of an oncological patient, collected after 30 and 180 min from the infusion of the drug.

Keywords: Irinotecan, voltammetric detection, solid phase extraction, FOLFIRI regimen

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INTRODUCTION

The antineoplastic agent irinotecan (CPT-11), a semisynthetic derivative of the cytotoxic alkaloid camptothecin [1–2], is widely used in a number of chemotherapeutic treatments, including lung, colorectal, pancreas and breast cancer [3 - 6]. Irinotecan therapy is known for its dose-limiting toxicity and has significant adverse effects, including diarrhea, neutropenia and asthemia [4,7]. Actually, CPT-11 acts as a pro-drug, because it is converted *in vivo* by liver-carboxylesterase enzymes to 7-ethly-10-hydroxy-camptothecin (SN-38), which is a potent inhibitor of Topoisomerase I [4,7]. SN-38 is 100- to 1000-fold more active than CPT-11 itself. However, only 2-5% of irinotecan is converted into SN-38 [4,7]. Therefore, the therapeutic index [8] (i.e., the range of doses at which the drug is effective without unacceptable adverse effects) of CPT-11 is narrow [4,7-9]. Moreover, the drug effectiveness strongly depends on patients, because of their different clinical conditions and genetic background, which play a relevant role in drug pharmacokinetics and metabolism [4-10]. Thus, personalized drug treatments could avoid either over- or under-drug dosages, leading to a more selective chemotherapeutic use of CPT-11. In this respect, therapeutic drug monitoring (TDM) has become essential to establish the best drug dosage for individual patient [10 - 13], although in oncology it has not become yet a fully developed clinical practice [13]. At present, most TDM clinical protocols developed for CPT-11 detection are based on direct fluorometric [14–16], HPLC-fluorescence [17–21], HPLC-UV detection [22], high performance liquid chromatography- mass spectrometry (HPLC-MS) [23-25], and occasionally by ELISA [26] approaches. These methods, however, suffer from problems related to costs, portability of the instruments, long time analysis, while measurements require centralized and well-equipped laboratories. Most of these drawbacks can be overcome using electrochemical techniques [27]. In fact, cost effective, simplicity, portability and possibility of miniaturization represent the main advantages of electroanalytical methods over those based on chromatography, mass spectrometry and spectroscopy. However, only a few papers reported on electroanalytical investigations of irinotecan. Some of them concerned with the voltammetric

behaviour of the drug to establish the functional groups involved in the electrode processes [28–31], or to study the interaction of irinotecan with DNA [32]. Some others dealt with CPT-11 detection using different electrode systems or methodologies [28, 31, 33–37]. In particular, measurements were performed using: static mercury drop electrodes (SDME), coupled with adsorptive square wave voltammetry [31]; polymethylene-blue multiwalled carbon nanotubes modified glassy carbon electrodes [28]; pencil graphite electrodes [34, 36]; graphene quantum dots-polyaniline/zinc oxide nanocomposites [37]; a flowing system coupled with fast Fourier transform continuous cyclic voltammetry at gold microelectrodes [33]. Most of these investigations were carried out in synthetic aqueous media, exploiting electrode processes that occur in the negative potential region, where oxygen could interfere, unless the sample is de-aerated. Some of the proposed methods were also applied to real samples (i.e., urine and blood serum), spiked with irinotecan alone or in mixture with other drugs, and specifically with emodin [34], flutamide [36] and 5-fluoroacil [37].

In plasma samples, the detection of irinotecan can be hampered by several factors that were either partially or completely disregarded in all previous papers. In fact, CPT-11, shortly after its administration [4,7,23], is subject to extensive enzymatic conversion providing several metabolites that share with CPT-11 a similar chemical structure (Scheme S1A). Apart from SN-38, other metabolites, namely, 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]-carbonyloxycamptothecin (APC) and 7-ethyl-10-[4-amino-1-piperidino]-carbonyloxycamptothecin (NPC) are inactive [4,7], but NPC can be further hydrolyzed by carboxylesterase into SN-38 [4,7]. Quantification of irinotecan and its main metabolites is required in pharmacokinetic studies, investigation that are beyond the scope of the present work .

We have reported earlier that CPT-11' metabolites yield voltammetric responses, which overlap with those of irinotecan, especially in the negative potential region [30]. It is also known that irinotecan in plasma is strongly bound to proteins [4,7, 38] and this poses the problem of adequate recovery of the drug, in order to define an accurate analytical protocol for its detection. Other interferences may also arise from co-drugs (often administered concomitantly with CPT-11 in several therapeutic regimens),

as well as from endogenous electroactive compounds of plasma. Also, worth to be mentioned is the circumstance that in clinical regimens, CPT-11 is administered as irinotecan hydrochloride (CPT-11HCl) (Scheme S1B), and chloride ions can represent a further source of interference. To the best of our knowledge, no paper dealt comprehensively with all the above-mentioned aspects.

In this work, we propose a method based on solid phase extraction (SPE) coupled with differential pulse voltammetry (DPV) as a robust and simple approach enabling a relatively fast detection of irinotecan in plasma samples of patients affected by colorectal cancer, treated with the FOLFIRI regimen [39–42]. The method proposed here is aimed at quantifying CPT-11 in plasma of patients administered with irinotecan within 0.5-3 h upon drug infusion, when monitoring and, in case of necessity, adjusting the concentration of CPT-11 is of particular importance because an over-dosage can lead to severe toxicity [7, 39–42]. Even tough Irinotecan can be found in a range of 10 to 10000 ng/mL in plasma, the peak concentration, which is achieved tipically after 2 hrs from the start of intravenous infusion, is often found below 3000-4000 ng/mL. The other metabolites do not exceed usually 10-20 % of the total amount of CPT-11 and therefore are not expected to provide strong interferences in the electrochemical measure. In a reported pharmacokinetic study, CPT-11 was detectable up to 50 h after the infusion and the elimination half-life of all metabolites were 8.0 h for CPT-11, 16.8 h for SN-38, 18.3 h for SN-38G and 8.7 h for APC for the administration at 310 mg/m^2. [23]

We chose acetonitrile as solvent because it offers two advantages: i) it can denature proteins and allows to extract CPT-11 from the matrix in the solid phase extraction step; ii) it is an ideal medium to quantify CPT-11 by voltammetry [29, 30]. In addition, to avoid interference from irinotecan metabolites, the voltammetric detection was carried out in the anodic potential region, at a glassy carbon electrode (GCE), by exploiting the oxidation process of the tertiary ammine of irinotecan (i.e., the deprotonated form, Scheme S1A), which can be formed in the acetonitrile-basified medium [29, 30]. This was actually proven in our previous work [30], where, in preliminary experiments, DPV was employed to detect micromolar levels of CPT-11 in synthetic acetonitrile solutions. From this

starting point, here we address other issues, not considered before, related to interference due to the plasma matrix (i.e., CPT-11 bound to proteins and endogenous electroactive compounds of plasma samples) and co-drugs. The suitability of the here proposed SPE-DPV procedure for therapeutic drug monitoring of CPT-11 is verified, firstly, in a series of human plasma samples collected from healthy volunteers and spiked with controlled amounts of CPT-11HCl, and, at last, in two samples of a patient administered with CPT-11. To the best of our knowledge, this is the first example of quantification of CPT-11 in plasma samples, also coming from patients undergoing chemotherapeutic treatments by using voltammetric methods coupled with a solid phase extraction purification procedure.

Materials and methods

Chemicals

Acetonitrile (anhydrous, 99.9%), tetrabutylammonium hexafluorophosphate ($TBAPF_6$), tetraethylammonium hexafluorophosphate ($TEAPF_6$), phosphate buffered saline (PBS, tablets, pH 7.4), 7-ethyl-10[4-(piperidino)-1piperidino]-carbonyloxycamptothecin hydrochloride, (irinotecan hydrochloride, CPT-11HCl), 7-ethyl-10-hydroxycamptothecin (SN-38), at purity ≥ 98 %, were purchased from Sigma Aldrich. 7-Ethyl-10-hydroxy-camptothecin glucoronide (SN-38G), 7-ethyl-10-[4-N-(5-aminopentanoicacid)-1-piperidino]carbonyloxycamptothecin (APC), 7-ethyl-10-[4-(1-piperidino)-1-amino]-carbonyloxycamptothecin (NPC), N-(4-{[(2-Amino-5-formyl-4-oxo-1,4,5,6,7,8-hexahydro-6pteridinyl)methyl]amino}benzoyl)glutamic acid (folinic acid, FA) were from Toronto research chemicals, Canada. 5-Fluoro-2,4(1H,3H)-pyrimidinedione **(**5- Fluoruracil, or 5-FU) was from Tocris Bioscience.Sodium tetraborate decahydrate ($Na_2B_4O_7{\cdot}10H_2O$) was from Carlo Erba Reagenti. Methanol (MeOH), LC-MS Grade, was from VWR International. Stock solutions of CPT-11HCl, APC, NPC, SN-38, SN-38G, 5-FU, FA over the concentration range 0.5-5 mM, were prepared

in pure acetonitrile; they were stored at 4 °C and used within ten days after their preparation. Pure N_2 (from SIAD, 99.99% pure) was employed to purge the solutions when required.

Real samples employed and processing

Plasma samples, collected from either healthy volunteers or a patient undergoing chemotherapeutic treatments, were provided by the Centro di Riferimento Oncologico di Aviano (CRO), Italy. They were stored at -20 °C. Once received, the samples were de-frosted by keeping them in ice bath for 1 h. When required, the samples (typically, 500 µL) were spiked with known amounts of CPT-11HCl, and vortexed for about 10 s. Spiked or un-spiked samples were then kept in an ice bath until use. For inter-day and inter-week precision and recovery tests, plasma samples, once spiked with the analyte, were stored at -20 °C and de-frosted the day of the measurement and then kept in ice bath until use.

Electrodes and Instrumentation

A three-electrode cell was employed to carry out the voltammetric experiments. A glassy carbon disk (Ø = 3 mm) was employed as the working electrode (GCE), while a silver wire and a platinum spiral served as quasi-reference electrode (AgQRE) and a counter electrode, respectively. The AgQRE was employed to avoid contamination of the acetonitrile solutions with chloride ions leaching from the classical Ag/AgCl (KCl saturated) reference electrode. The GCE electrode was mechanically polished with diamond suspensions (0.1 µm diameter) placed over a Buehler microcloth, and then they were rinsed with milli-Q water and acetonitrile. All voltammetric experiments were performed using a CHI 920 C bipotentiostat (CH Instruments).

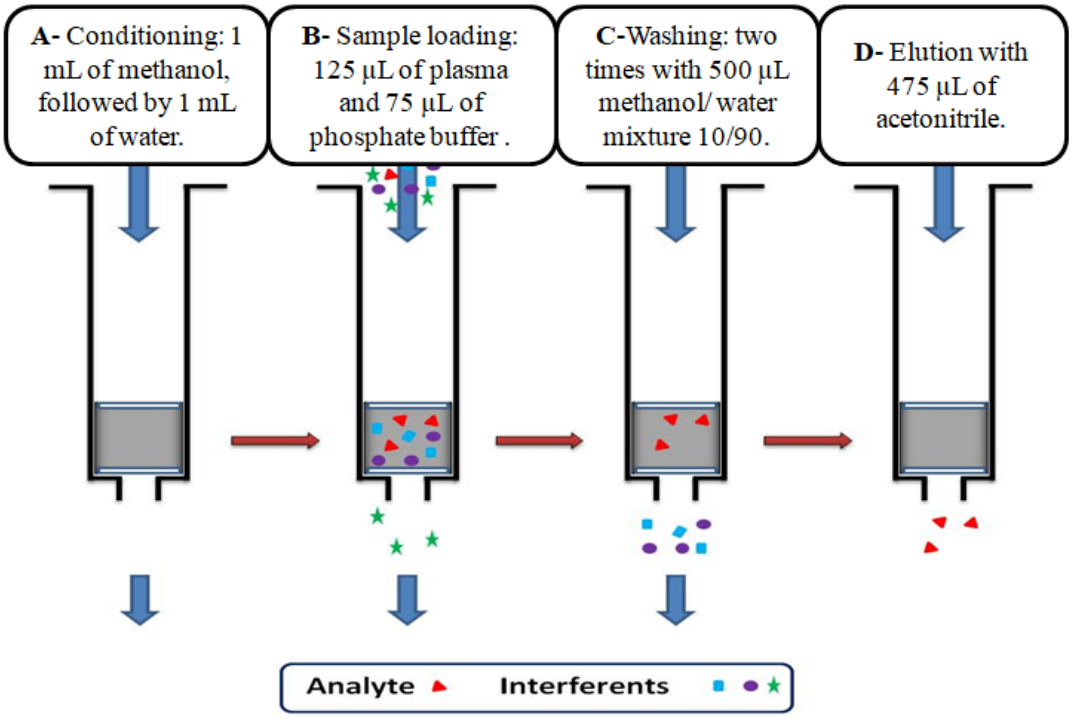
Voltammetric measurements

Unless otherwise stated, the voltammetric measurements, namely cyclic voltammetry (CV) and differential pulse voltammetry, were performed in acetonitrile solutions containing 0.05 M $TBAPF_6$ as supporting electrolyte or in acetonitrile containing 0.05 M $TBAPF_6$ and 0.73 mM $Na_2B_4O_7$. The latter was prepared by adding to anhydrous CH_3CN (generally 0.5 mL) controlled amounts of a $Na_2B_4O_7 \cdot 10H_2O$-saturated aqueous solutions (generally 2 µL). All measurements were performed at room temperature (23 ± 1 °C).

DPV parameters were preliminarily optimised in a 4 µM CPT-11HCl borate-basified acetonitrile solution. The best sensitivity was found using: pulse height 0.05 V; potential increment 4 mV; pulse width 0.2 s; pulse period 0.5 s. Unless otherwise stated, the latter parameters were employed in the DPV measurements reported herein.

Purification and extraction of CPT-11 from plasma samples

The SPE experiments were performed by using Strata-XL cartridges from Phenomenex, USA. They contain a reversed phase functionalized polymeric sorbent, particle size 300 µm, surface area 520 $m^2 g^{-1}$, and sorbent mass of 30 mg in 1 mL polypropylene tubes. The various steps involved in the cleaning and extraction procedure are summarised in Scheme 1.



Scheme 1. Scheme of the procedure used to extract CPT-11 from plasma samples.

The cartridges were activated before use and conditioned by drawing 1 mL of MeOH through, followed by 1 mL of milli-Q water (step A). The column was loaded afterward with 125 µL of plasma sample/calibrator and 75 µL of PBS (pH 7.4) (step B); the latter buffer was used to keep the sample at its physiological value. Two aliquots (600 µL each) of a 90% Milli-Q water and 10% MeOH mixture were forced (by a N_2 stream) to pass through the column to eliminate polar/hydrophilic substances of the sample (step C). CPT-11 from the column was then eluted using 475 µL of pure CH_3CN, which was forced to pass through the column by a pure N_2 stream (step D). The above procedure required overall about 4 minutes.

Prior to electrochemical analysis, the extract (on average 475 (±15) µL) was treated with 23 µL of a CH_3CN solution, containing 1.06 M $TBAPF_6$ and 2 µL of a $Na_2B_4O_7$ saturated aqueous solution (0.18 M), to achieve a final volume of solution of 500 (± 15) µL, having the suitable composition for the voltammetric analysis (i.e., 0.05 M $TBAPF_6$ and 0.73 mM of $Na_2B_4O_7$).

Results and Discussion

General anodic behavior of CPT-11HCl in acetonitrile without and with $Na_2B_4O_7$

As reported in [30], CV of CPT-11HCl at the GCE in CH_3CN solution containing 0.1 M $TBAPF_6$, over the potential range from 0 to +1.5, displayed a single peak at +1.33 V **(Fig S1A**), due to the oxidation of Cl^-. In the presence of $Na_2B_4O_7$, an additional oxidation process appeared at +0.88 V **(Fig S1B**), due to the oxidation of the deprotonated tertiary ammine end of CPT-11. The characteristics of the two processes (i.e., due to CPT-11 and Cl^- in CH_3CN-borated buffered and CH_3CN solution, respectively) were here further investigated by performing CVs at different scan rates (v), over the range 10 - 200 mVs^{-1}. For both processes, no return peak was obtained on the reverse scan **(Fig S1**). Both peak currents depended linearly on the square root of the scan rate **(Fig S1, A'-B'**), thus suggesting the occurrence of diffusion-controlled processes. In addition, peak width at half height [43] was essentially constant at 130 (± 4) mV for CPT-11 and 120 (± 2) mV for Cl^-, regardless of scan rate. These results are congruent with a fairly irreversible electrode process with coupled a fast following chemical reaction [43].

To quantify CPT-11, current intensity of the peak at 0.88 V was employed, as it was free from interference due to main metabolites [30]. This is shown in **Fig. S2**, which refers to CVs performed at the GCE in a CH_3CN-borated buffered solution containing the main metabolites. In addition, CVs recorded at 0.05 V s^{-1} in basified-acetonitrile solutions containing different CPT-11HCl concentrations ($C_{CPT\text{-}11HCl}$), over the range 0.02 – 0.2 mM (**Fig. S3A**), provided a straight line between I *vs.* $C_{CPT\text{-}11HCl}$ (Fig. S3B); the linear regression analysis of experimental data yielded: $I\ (\mu A) = 27\ C_{CPT\text{-}11HCl}\ (mM) + 0.08$ with a correlation coefficient of 0.994. The reproducibility was within 3% from three replicates.

Series of measurements, performed by DPV at lower concentrations of CPT-11HCl in CH_3CN solutions without and with sodium borate, provided similar results. In fact, in the absence of borate, only the peak at +1.26 V, due to the oxidation of chloride ions was evident, while in the presence of borate buffer, two peaks due to chloride ions and tertiary amine (at + 1.26 V and + 0.88 V,

respectively) appeared (**Fig. 1**) and their peak currents were proportional to CPT-11HCl concentration (see insets in **Fig. 1**).

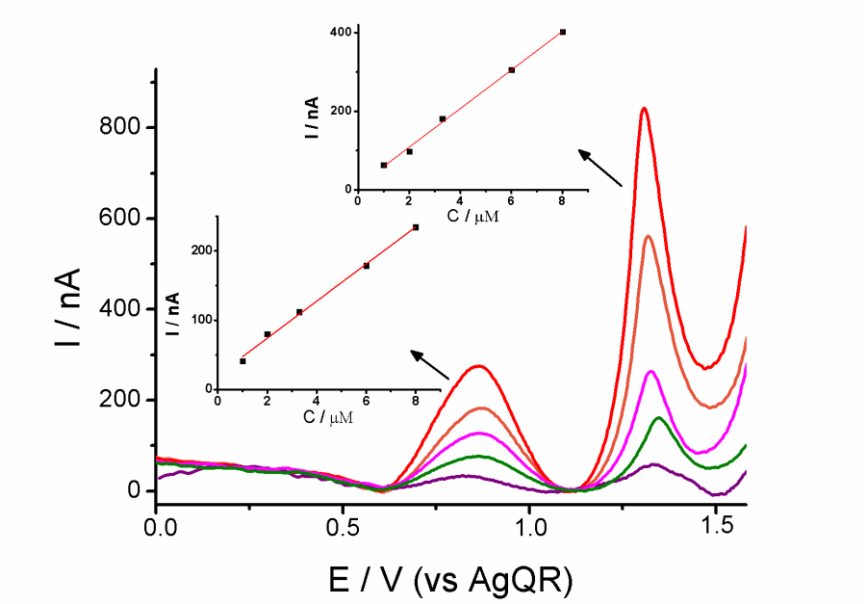


Fig. 1. DPVs of CPT-11HCl at 1, 2, 3 6, 8 µM in CH_3CN, 0.1 M $TBAPF_6$ solutions with 0.73 mM of $Na_2B_4O_7$. Voltammograms are baseline subtracted. Inset calibration plots for the DPV responses as indicated with arrows.

DPV measurements, performed in basified acetonitrile solutions, containing a fixed amount of CPT-11HCl and different amounts of the various metabolites, confirmed that the process at +0.88 V was suitable for the detection of CPT-11 with no interference. As an example, **Fig. S4** shows the CV obtained in a basified acetonitrile solution containing 6 µM CPT-11HCl (black line) and that recorded after it was spiked with 0.5 µM SN38 and 2 µM APC (red line). The intensity of peak at +0.88 V did not change (within 3% RDS), while that at +1.26 V increased, due to the overlapping of the processes due to chloride ions and metabolites. It must be considered that APC is the most abundant metabolite [4,7,23] and its electrode process, in the positive-going scan, is the closest to that of the tertiary ammine of CPT-11 (see also **Fig. S2**).

The analytical characteristics of the calibration plot obtained by DPV in the synthetic $Na_2B_4O_7$-buffered CH_3CN solutions spiked with increasing amounts of CPT-11HCl are shown in Table 1,

second row. Dynamic range, sensitivity, limit of detection (LOD) and quantification (LOQ) (determined as reported in ref [44]) were found suitable for the quantification of CPT-11HCl during intravenous infusion, which typically lasts about 2 h [4, 7, 23, 45–48].

Table 1. Analytical parameters obtained from the experimental DPVs recorded in various media (synthetic CH_3CN solutions, and CH_3CN extracts from plasma samples), spiked with different amounts of CPT-11HCl. Prior to the DPV measurements, the media were amended with 0.05 M $TBAPF_6$ and 0.073 mM $Na_2B_4O_7$.

Medium	Dynamic Range (mol L^{-1})	Linear regression equation $I\ (\mu A) = m\ C\ (\mu M) + b$	R^2	LOD[(1)] (mol L^{-1})	LOQ[(1)] (mol L^{-1})
Synthetic CH_3CN solutions	2.5 x 10^{-7} – 9 x10^{-6}	*I = 0.0326C +0.006*	0.994	1.10 10^{-7}	3.74 10^{-7}
CH_3CN extracts from plasma samples enriched with 5-FU and FA	5 x 10^{-7} – 9 x10^{-6}	*I = 0.0334C +0.008*	0.992	1.28 10^{-7}	4.26 10^{-7}
Plasma samples spiked with CPT-11HCl at different concentrations and examined in the extracts	5 x 10^{-7} – 9 x10^{-6}	*I = 0.0332C +0.003*	0.987	1.28 10^{-7}	4.26 10^{-7}

(1) *LOD* = $\frac{3\ SD_{background}}{m}$ *and LOQ* = $\frac{10\ SD_b}{m}$*, respectively;* SD_b *is the current background* [43].

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Voltammetric behavior of co-therapeutics 5-FU and FA

To establish whether the co-therapeutics could interfere in the detection of CPT-11, the voltammetric behavior of 5-FU and FA was investigated in the basified acetonitrile solutions (i.e., the same medium, where no interference due to CPT-11' metabolites occurred). Typical CVs recorded in the presence of 80 µM of 5-FU and 40 µM of FA are shown in **Fig. 2.**

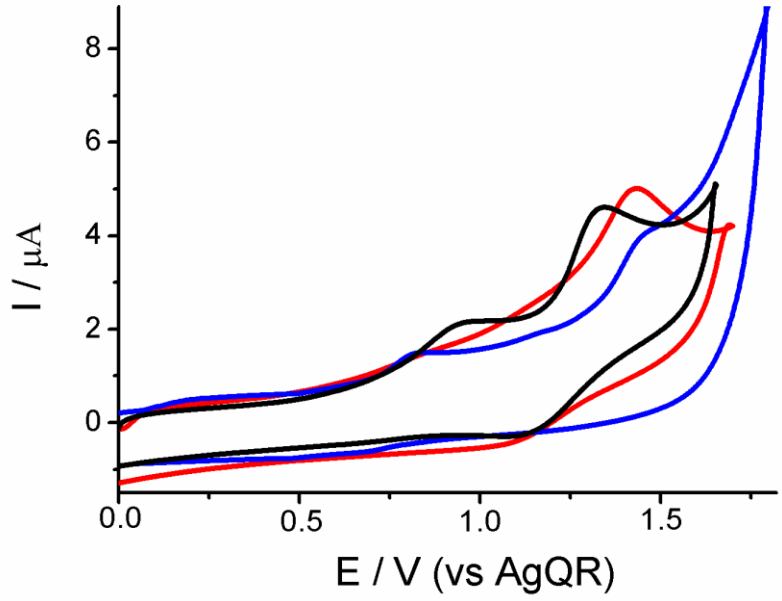

Fig. 2. CVs of 80 µM 5-FU (red line) and 40 µM of FA (blue line), respectively, in CH_3CN/0.05 M $TBAPF_6$ + 0.73 mM $Na_2B_4O_7$. The black line refers to 50 µM CPT-11HCl in the same experimental conditions. Working electrode: GCE. Scan rate 200 mVs^{-1}.

Both 5-FU and FA show irreversible peaks; the first peak of FA falls over the same potential region of that of the tertiary amine of CPT-11 (**Fig. 2** black line). DPVs, performed at lower concentrations

for both co-drugs, alone (**Fig. S5**) or in mixture with CPT-11HCl (CPT-11HCl + 5-FU, **Fig. S6A**, and CPT-11HCl + FA, **Fig. S6B**), provided similar responses. In particular, it was found that major interference arose from the presence of FA (**Fig. S6B**), while 5-FU (**Fig. S6A**) provided mainly an increment of the background current over the potential range from +0.5 to +0.9 V. This, however, could affect the detection of CPT-11 at low concentrations. Therefore, to eliminate interferences due to these co-drugs a cleaning step of plasma samples is needed.

Interference due to endogenous compounds of the plasma.

Plasma samples contain a variety of small and electroactive organic molecules (such as antioxidants [48]), which could also interfere in the detection of CPT-11. This aspect was investigated by performing a series of CVs in 200 µL of plasma, without and with known amounts of CPT-11HCl, mixed with 800 µL of CH_3CN, containing 0.05 M $TEAPF_6$ and 0.73 mM of $Na_2B_4O_7$. $TEAPF_6$ was employed as supporting electrolyte, because the relatively high water content of plasma made the solubility of $TEAPF_6$ in the mixture scarce. **Fig. 3** contrasts typical DPVs recorded either in the absence (black line) or in the presence of 2 and 5 µM of CPT-11HCl (blue and red lines, respectively). As it appears evident, in the CPT-11HCl-free plasma sample, there are several peaks, which fall over the same potential region of that due to the tertiary amine of CPT-11. This result clearly indicates that, even in the absence of co-drugs or metabolites, no reliable measurement of CPT-11 could be performed directly in plasma samples. The lack of a clear peak pertaining to CPT-11 can also be due to plasma proteins, which can interact with the target analyte [4,7, 38], thus masking its electrode process.

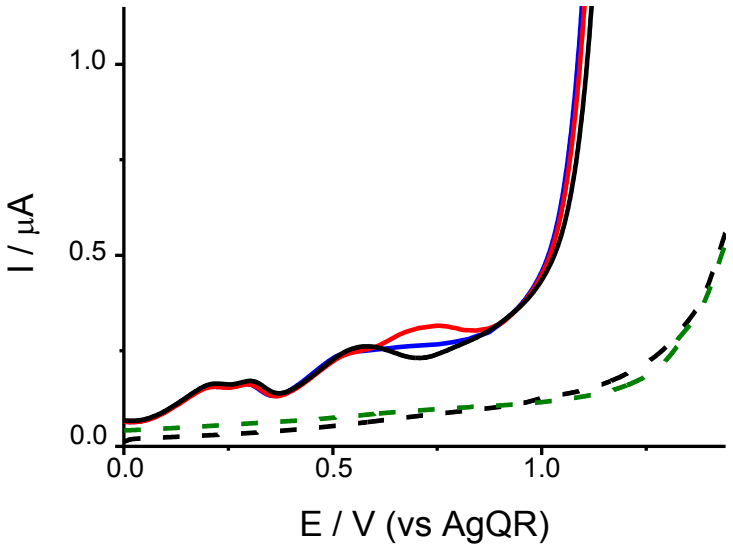

Fig. 3. DPVs recorded in plasma/acetonitrile mixture with 0 (black full line), 2 µM (blue full line) and 5 µM (red line) of CPT-11HCl. Dashed lines contrast DPVs recorded in a CH_3CN extract coming from a plasma sample spiked with 12 µM 5-FU and 10 µM FA (black dashed line) and that recorded in synthetic basified CH_3CN (green dashed line).

Cleaning of the plasma sample using SPE columns

Based on the above results, an effective sample pretreatment procedure was set up in order to remove interfering compounds present in the sample, and to selectively extract CPT-11. SPE, or similar extraction procedures, proved effective for the pretreatment of food and biological matrices [49–52]. In this study, we used strata-XL SPE columns (Phenomenex), which contain a functionalized polymeric sorbent reversed phase, based on copolymer styrene-divinylbenzene backbone modified with pyrrolydone functional groups. Considering the presence of many polar groups (e.g. carbamate, lactone, tertiary amine) and the aromatic polycyclic structure of CPT-11, we attributed the retention mechanism to a combination of several weak interactions (i.e., π-π, dipole-dipole, hydrophobic interactions, as well as hydrogen bonding) between the sorbent material and the molecule of irinotecan. Therefore, a careful optimization of the washing and elution steps allowed an efficient recovery of desired analyte from/in the column, as shown below. Thus, as illustrated in Scheme 2, after a pre-equilibration step to wet the sorbent material, using water and methanol (step A) and

loading of the plasma sample (step B), a washing step was performed with two aliquots of a MeOH(10%)/H_2O(90%) mixture (step C) to eliminate the most hydrophilic compounds present in plasma and, conceivably, 5-FU and FA. In the latter step, it was hypothesized that either free CPT-11 and the fraction still bound to plasma proteins were retained within the sorbent material. Extraction and fully recovery of CPT-11 from the column was performed afterwards using CH_3CN (step D), which was able to elute both the free drug and the fraction still bound to proteins.

The effectiveness of the cleaning step was assessed using a series of plasma samples, collected from healthy volunteers, spiked with different amounts of FU and FA. **Fig. 3** (black dashed line) shows a typical DPV obtained in in the final acetonitrile extract (fortified with supporting electrolyte and borate buffer), coming from a plasma sample spiked with 12 µM of 5-Fu and 10 µM of FA. It is worth noticing that, in any case, no peak was recorded up to about +1.5 V. The latter DPV feature was almost identical to that recorded, as background, in the basified CH_3CN solution (**Fig. 3**, green dashed line). Similar results were obtained using CV and extracts from plasma samples enriched with larger amounts of 5-FU and FA (CVs not shown). Furthermore, to demonstrate that no proteins capable of binding CPT-11 was present in the extracts, calibration plots were constructed from the DPV outputs of acetonitrile extracts spiked with CPT-11HCl over the concentration range 0.5-6 µM (**Fig. S7**). The analytical features of the plot thus obtained are included in Table 1, second row. These are almost identical to those obtained previously when considering synthetic acetonitrile solutions.

It must be considered that the analytical characteristics of the method proposed here in terms of sensitivity, LOD and LOQ (shown in Table 1) compare with the electrochemical detection approaches reported in the literature using polymethylene blue-multiwalled carbon nanotubes modified glassy carbon electrode [28] (or Ref 3 in Table S1). They are however less favorable of those reported in the literature when modified negative potential regions [31,34,36] (refs 4,7 in Table S1), mercury [31] or more elaborated electrode systems/methodologies [33,37] were employed (Refs 5,8 in Table S1).Our methodology, however, was developed to exclude possible interferences by metabolites and co-medication of irinotecan. Moreover, the method was tested using plasma, which is the sample matrix

of interest for TDM studies, against other previously reported work, in which simpler real matrices, such as urine or serum, were used [34,36,37].

Recovery of CPT-11 from plasma samples following the extraction step

Extraction/recovery tests of CPT-11 were performed in a series of plasma samples, collected from healthy volunteers, spiked with different amounts of CPT-11HCl, each of them prepared in triplicate. The quantification of CPT-11 in the extracts was achieved by DPV, using the standard addition method (see examples in **Fig. 4** and **Fig. S8**), while Table 2 shows the CPT-11 concentrations expected (C_{ex}) and experimentally found (C_f). The recovery percentage varied between 97 and 104% (Table 2, third entries).

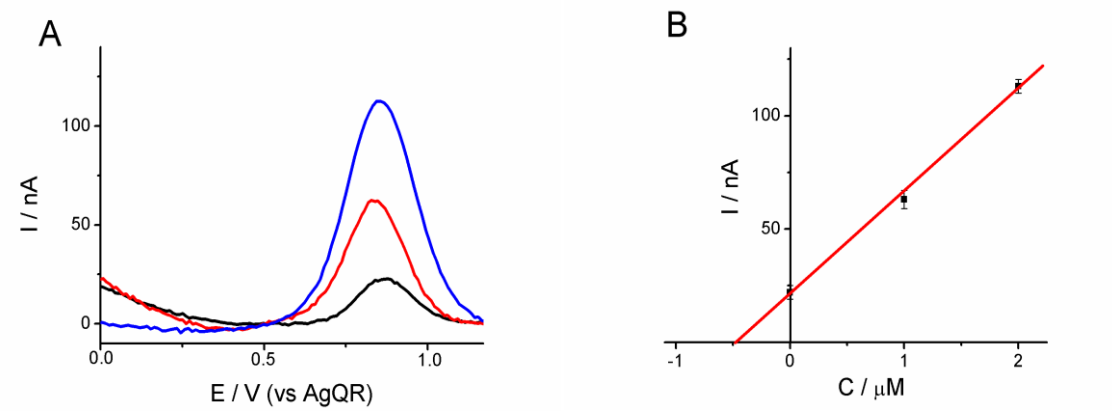

Fig. 4. (A) Typical DPVs obtained in the plasma extract, initially spiked with 0.5 µM CPT-11HCl (black line) and then with standard addition of 1µM CPT-11HCl (red line) and 2 µM CPT-11HCl (blue line). (B) Calibration plot obtained from the standard addition.

Table 2. Recovery tests performed in plasma samples spiked with CPT-11 at different concentrations and subjected to the extraction procedure summarized in Scheme 2.

C_{ex} (µM)	C_f (µM)[1]± SD	Recovery % ± RSD %
2.00	2.08 ± 0.24	104 ± 10
3.00	2.84 ± 0.24	95 ± 8
4.00	4.08 ± 0.31	102 ± 9
6.00	5.80 ± 0.40	97 ± 7

10.00	9.76 ± 0.48	97 ± 5
18.00	18.4 ± 0.40	102 ± 2
24.00	23.4± 0.72	97 ± 3

[1]Value corrected for plasma dilution

Using the current intensities from DPVs and the corresponding C_f (Table 2), it was possible to obtain a calibration plot (**Fig. S9**), whose relevant analytical characteristics are included in Table 1, third row. These features are very close to those obtained in both synthetic acetonitrile solutions and acetonitrile extracts from plasma samples (Table 1). This result further confirms the suitability of the SPE columns and extraction procedure for recovery of total CPT-11, including the fraction bound to proteins. Also, the method proved its efficacy in eliminating interferences.

Recovery of CPT-11 from plasma samples spiked with mixtures of CPT-11, 5-FU and FA

Recovery tests of CPT-11 were also performed in plasma samples spiked with CPT-11HCl, 5-FU and FA. **Fig. 5** compares two series of typical DPVs obtained upon analysis of the extracts of plasma samples spiked with only CPT-11HCl (2.5 µM, black solid line, and 6 µM, red solid line) and those spiked with same amounts of CPT-11HCl and with 12 µM of 5-FU and 10 µM of FA (black and red dashed lines for 2.5 and 6 µM of CPT-11HCl, respectively). Evidently, in all cases, the DPVs of the extracts of plasma samples without and with the co-drugs almost overlap (RSD 3%), which further confirms the effectiveness of the cleaning/extraction procedure. The above measurements were performed in triplicate. The recovery of CPT-11, which was also carried out under the latter conditions, was within the same range shown in Table 2.

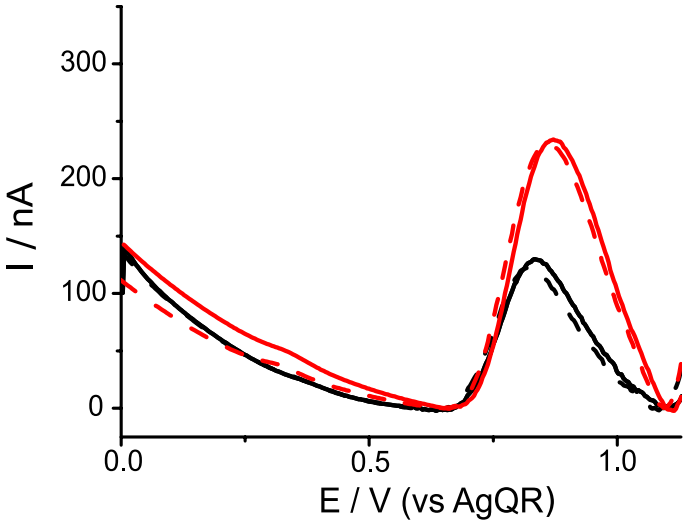

Fig. 5. DPVs obtained in the CH_3CN extracts of plasma samples spiked with 2.5 (black lines) and 6 µM (red lines) CPT-11 HCl and with same amounts of CPT-11HCl as above described plus 12 µM of 5-FU and 10 µM of FA (dashed black and red lines, respectively).

Stability during sample handling

Stability of CPT-11 in the extracts, kept on the bench-top at room temperature, was assessed using plasma samples, initially spiked in triplicate with 6 µM of CPT-11HCl and then subjected to the extraction procedure. A series of DPVs, recorded at different times over 2 h, are shown in **Fig. S10**. The results indicated that the peak currents were almost constant (RSD within 2.6%) up to about 0.5 h; then they decreased with respect to the initial value of about 7% and 23% after 1 h and 2 h, respectively. Therefore, good reproducibility can be obtained in replicate measurements, as soon as they are performed within 30-40 min. This is the case for the proposed voltammetric approach, as each DPV required no more than about 30-40 s to be completed.

Intra-day and, inter-day and inter-week precision and recovery

Intra-day, inter-day and inter-week precision and recovery were evaluated in individually spiked plasma samples (125 µL each) in triplicate at three different CPT-11HCl concentration levels (i.e., 0.5, 2, 4 µM). The DPV measurements were performed in acetonitrile solutions, obtained from the above reported extraction procedure, and after the addition of the supporting electrolyte and borate

buffer. Inter-day and inter-week measurements were performed during three consecutive days (24 h from one another) and three consecutive weeks, respectively. The concentration of CPT-11 in the extracts were determined by using the standard addition method. Typical DPVs thus obtained are shown in **Fig. S11**, while Table 3 summarizes the concentration found (C_f), RDS% and recovery. RDSs% and recoveries found here for intra- and inter-day measurements compare satisfactorily with those obtained in similar tests employing HPLC-MS (i.e., about 9% and 10%, respectively) [23].

Table 3. Intra-day, inter-day, inter-week recovery and precision of the method: C_{add} is the concentration of CPT-1HC1 spiked in the plasma sample, C_f is the concentrations found experimentally.

C_{add} (μM)	intra-day			inter-day			inter-week		
	C_f (μM)	RSD%	Recovery(%)	C_f (μM)	RSD%	Recovery(%)	C_f (μM)	RSD%	Recovery(%)
0.50	0.52	10.1	104	0.47	12.3	94	0.56	11.9	112
2.00	1.96	7.9	98	2.07	8.2	103	1.88	8.5	94
4.00	4.11	5.1	103	3.99	6.3	100	4.14	5.7	104

RDS% from three replicates

Detection of CPT-11 in plasma samples from a cancer patient

The entire analytical protocol described above (i.e., extraction and DPV analysis) was applied to two available plasma samples collected from a patient affected by colorectal cancer, undergoing the FOLFIRI chemotherapeutic treatment. The samples were collected at 30 min and 180 min from administration. DPVs, recorded in plasma extracts, are shown in **Fig. 7A-B** (red lines), along with those obtained after standard addition (green and blue lines). **Fig. 7A'-B'** shows relevant calibration plots. Average CPT-11 concentration found (from three replicates) are shown in Table 4. Reproducibility was within 8% (RSD). The values were compared with those obtained in the same stock of samples by using a HPLC-MS method [23]. Differences in the values were -11.8% and 20.7 % for the two samples. It must be considered that the HPLC-MS method, apart from the instrumental

analytical techniques employed, involved a different processing procedure of the sample (i.e., a mixture of 0.1% of CH_3COOH in CH_3OH was employed to extract CPT-11 from plasma), which led to a lower recovery of the target analyte (i.e, 67-70%). This might explain the relatively high difference found, which, however, can be considered satisfactorily, according to regulatory guidelines for the cross-validation of bioanalytical methods [53].

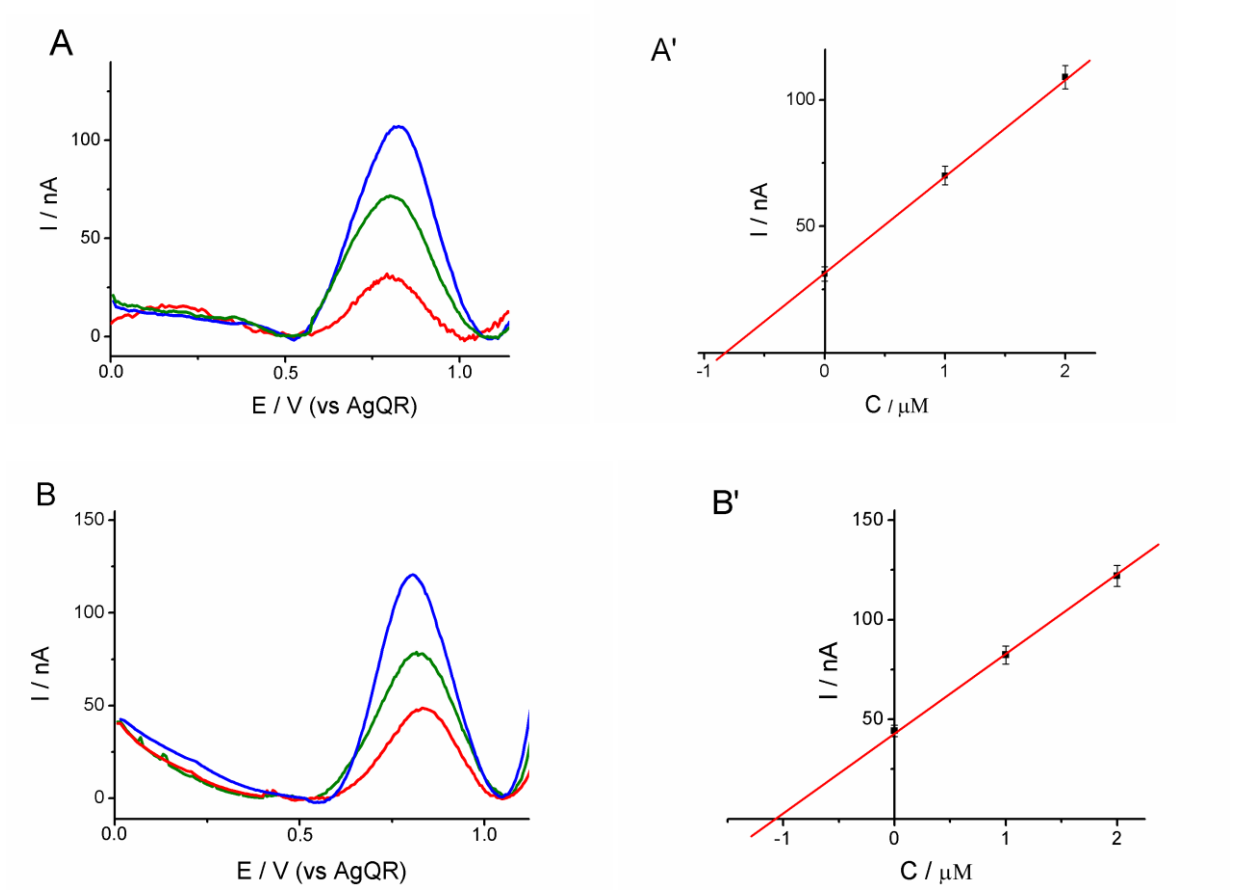

Fig. 7. DPVs recorded in plasma extracts collected from an oncological patient undergoing chemotherapeutic treatment (red lines) and after addition of 1 µM (green lines) and 2 µM (blue lines) of CPT-11HCl. A and B refer to plasma sample collected after 30 and 180 min from infusion, respectively. A' and B' refer to calibration plots obtained from the corresponding current peaks of the DPVs in A and B.

Table 4. Comparison of CPT-11concentrations found with the here proposed method and by HPLC-MS

Sampling time min	$C_{HPLC-Ms}$ (µM) (RSD%) [(1)]	C_{DPV} (µM) (RSD %) [(2)]	E (%)
30	3.59 (≤ 10%)	3.21 (3)	-11.8
180 .	4.06 (≤ 10%)	4.70 (8)	+20.7

[(1)] Obtained by HPLC-MS. [(2)] Obtained by DPV from 3 replicates. Relative error assuming the HPLC-MS as the reference value

Conclusions

In this work a fast method, involving voltammetry and solid phase extraction, has been proposed for the detection of CPT-11 in plasma samples. The method exploits the oxidation process of the tertiary amine of CPT11 in basified acetonitrile media, which falls at a potential where no interference due to main metabolites occurs. To avoid interference, which might be caused by the co-therapeutics 5-FU and FA, as well as from endogenous electroactive compounds of plasma, an extraction procedure, relying on commercially available SPE columns, has been set-up. Acetonitrile, used to elute CPT-11 from the SPE columns, allowed also to denature proteins and freed the bound CPT-11 fraction. To enhance sensitivity, the detection step has been performed using differential pulse voltammetry. The optimised analytical procedure is adequate, in terms of LOD, LOQ, reproducibility and accuracy, for monitoring CPT-11 in the upper region of the therapeutic window (i.e., between 0.5-9 µM) [4,7,23, altri Ref recenti?]. This CPT-11 level is typically achieved in body fluids within the time frame 0.5-3 h from infusion or during infusion. In fact, the hepatic enzyme carboxylesterase starts immediately the conversion of CPT-11 in its metabolites (i.e., during drug infusion). The conversion rate, during the infusion, could be an important diagnostic tool linked to drug tolerance and therefore to drug dosing decision [47]. The suitability of the method proposed here for real world applications has been verified by determining the concentration of CPT-11 in plasma samples of an oncological patient, collected after 30 and 180 min from irinotecan infusion. Worth to be considered is also that the overall

procedure requires, for a single measurement (including plasma treatment and the CPT-11 quantification step), about 5 min.

We are aware that the sensitivity, LODs and LOQ of the proposed method do not allow achieving better values than those reported in the literature for more complex electrode systems/methodologies. However, this work stands out for the interference study and real sample pretreatment for their elimination. Better analytical performance could be achieved by a suitable modification of the electrode surface to enhance the signal to noise ratio of the voltammetric responses. We are currently working in this direction, by using novel engineered nanomaterials, able to catalyze the CPT-11 electrode process.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

For all experiments conducted with patient plasma, informed consent was obtained and approval was granted by the Medical Ethics Committee of the Centro di Riferimento Oncologico di Aviano (CRO), Italy