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Supplementary information

pH-mediated molecular differentiation for fluorimetric quantification of chemotherapeutic drugs in human plasma

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Abbreviations

PSB: potassium chloride and sodium hydroxide buffer (98 mg NaOH, 372 mg KCl per 100 ml MeOH)

SPE: solid phase extraction

TFAH: trifluoroacetic acid in H_2O (0.05 vol%)

TFAM: trifluoroacetic acid in MeOH (0.05 vol%)

Experimental

Reagents and materials: All chemicals were used without any further purification. Trifluoroacetic acid (99 %), sodium methoxide (95 %), Irinotecan hydrochloride, SN-38 (> 98 %) and Epirubicin hydrochloride (> 90 %) were purchased from Sigma Aldrich. Potassium chloride (reagent grade) and methanol (HPLC grade) were sourced from VWR and Fisher Scientific, respectively. Methotrexate (> 98 %) was purchased from Cambridge Bioscience. Water was purified to ultrapure Type 1 quality using the Milli-Q filtration system from Merck/Millipore.

Optical measurements: The absorbance spectroscopy set-up included a laser-driven light source (Energetiq Technology, EQ-99XFC LDLS), a temperature-controlled cuvette holder (QNW, Q-Pod) and a high sensitivity spectrometer (Ocean Optics, QEPro) as the detector. Fibre optic cables with a core diameter of 1000 µm (Ocean Optics) were used to transmit the light through the system. Disposable 4.5 ml cuvettes made of polystyrene served for absorbance measurements (Fisher Scientific).

Fluorescence measurements were carried out with a spectrofluorimeter (Shimadzu, RF-6000) using a slit width of 5nm and 10 x 10 mm quartz cuvettes (Hellma, Suprasil).

Solid phase extraction: Solid phase extraction experiments were carried out using disposable extraction columns (Bakerbond, SPE octadecyl (C18)) with a size of 3 ml and a sorbent weight of 200 mg, purchased from VWR. The SPE protocol was carried out as follows. First, three volumes of 1.5 ml (1st step TFAM; 2nd step TFAM/TFAH (1/1), 3rd step TFAH) were passed through to condition the column. Then, 0.5 ml of spiked plasma was loaded into the column. The drugs would be previously dissolved in MeOH and spiked in such a way that the amount of MeOH in plasma did not exceed 5% by volume to avoid denaturisation of the plasma content. Subsequently, three volumes of 1.5 ml (1st step TFAH, 2nd step TFAM/TFAH (1/9), 3rd step TFAH) were run to wash the column from its plasma content. Finally, 1 ml of TFAM was run through the column to elute the drugs. The conditioning step included an initial wash with MeOH in order to wet the surface of the sorbent (C18, endcapped) and to penetrate bonded alkyl phases, which then allowed the water to wet the silica surface efficiently. After the conditioning step, the plasma with the spiked drugs of desired concentration was loaded into the cartridge. The three aqueous washing steps were introduced to remove the vast majority of the plasma content and thus avoid parasitic fluorescence when eluting the drugs. The second wash contained 10 % of MeOH by volume in order to promote the elution of plasma content that was not soluble in pure water. This percentage was found to be optimal as a smaller content of MeOH resulted in a stronger parasitic signal from the plasma, and a higher content of MeOH led to unreliable drug recoveries, most likely due to partial elution of the drugs.

pH control: The pH of the media was controlled by using TFAM for the acidic pH (1.4), and PSB for the basic one (12.1). After solid phase extraction, 1 ml of TFAM is added to ensure that the pH stays at pH = 1.4. This dilution is needed since a small fraction of the plasma, a natural buffer, is eluted with the drugs. Thus, the lack of dilution results in unreliable pH values. Once the fluorescence has been measured in acidic conditions, an equal volume of PSB is added to basify the media. The combination of a strong base and a salt result very effective to buffer the solution into the desired solution's basicity (pH = 12.1).

Quantification of Irinotecan and SN-38:

Irinotecan + SN-38: After dilution of the SPE extract with 1 ml of TFAM, the fluorescence was measured at 432 nm with an excitation wavelength of 370 nm.

SN-38: Subsequent to measuring the fluorescence of the drugs in acidic conditions, the sample was basified by adding an equal volume of PSB. The fluorescence was then measured at 559nm with an excitation wavelength of 430nm. This measurement was compared with the calibration curve made for SN-38 taking in consideration the dilution factor of 8 x to derive the concentration of SN-38.

Irinotecan: First, the established amount of SN-38 was translated into a corresponding emission in acidic conditions based on a previously built calibration curve and subtracted from the overall intensity. The corrected value was then compared with the calibration curve made for Irinotecan taking in consideration the dilution factor of 6.9 x to obtain the concentration of Irinotecan.

NMR Spectroscopy: NMR Spectroscopy was carried out at 297.2K on a Bruker Avance III 500 spectrometer at frequencies of 500MHz for 1H nuclei.

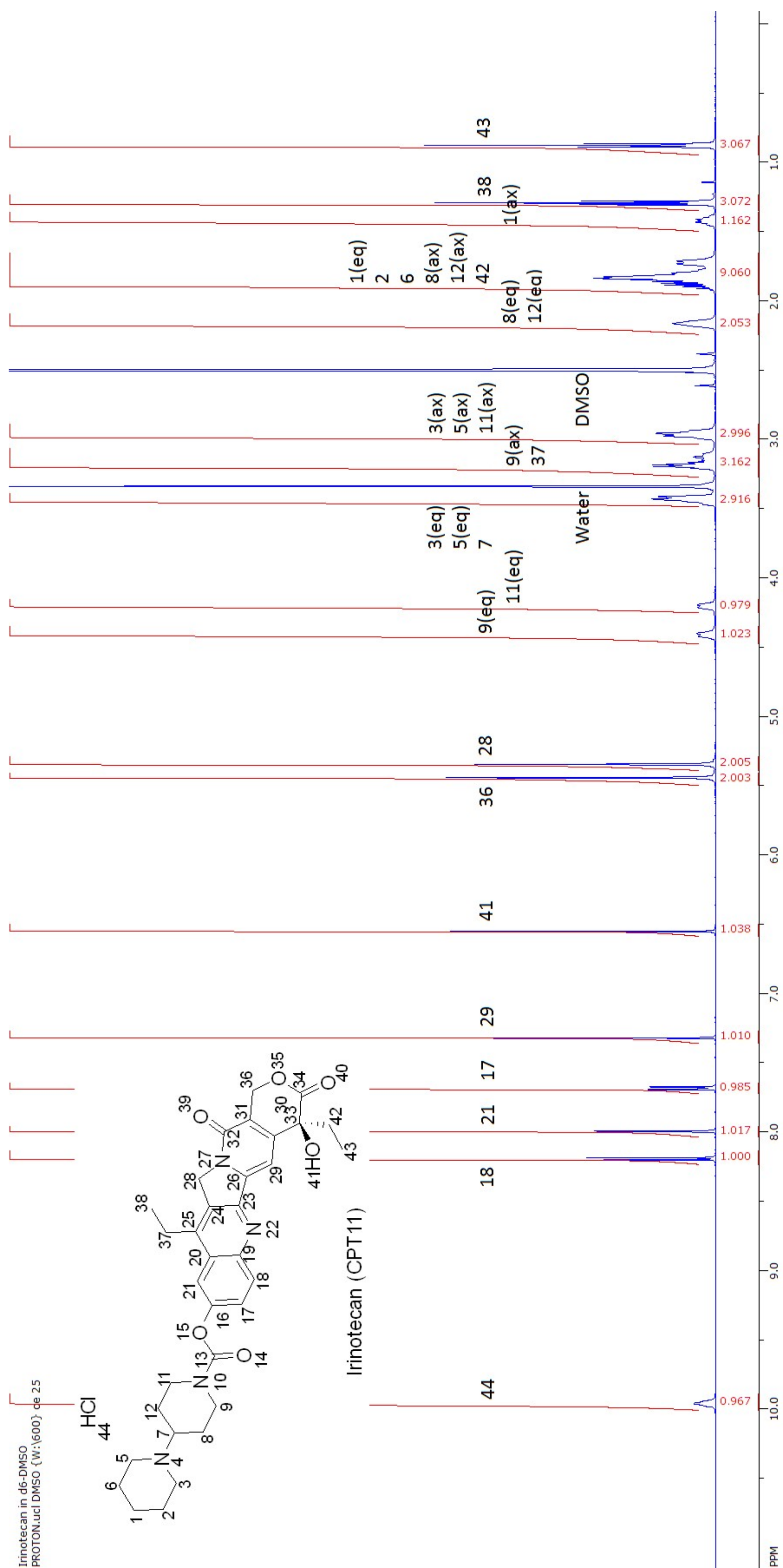


Figure S1. 1H NMR spectra of Irinotecan (CPT11) in d6-DMSO.

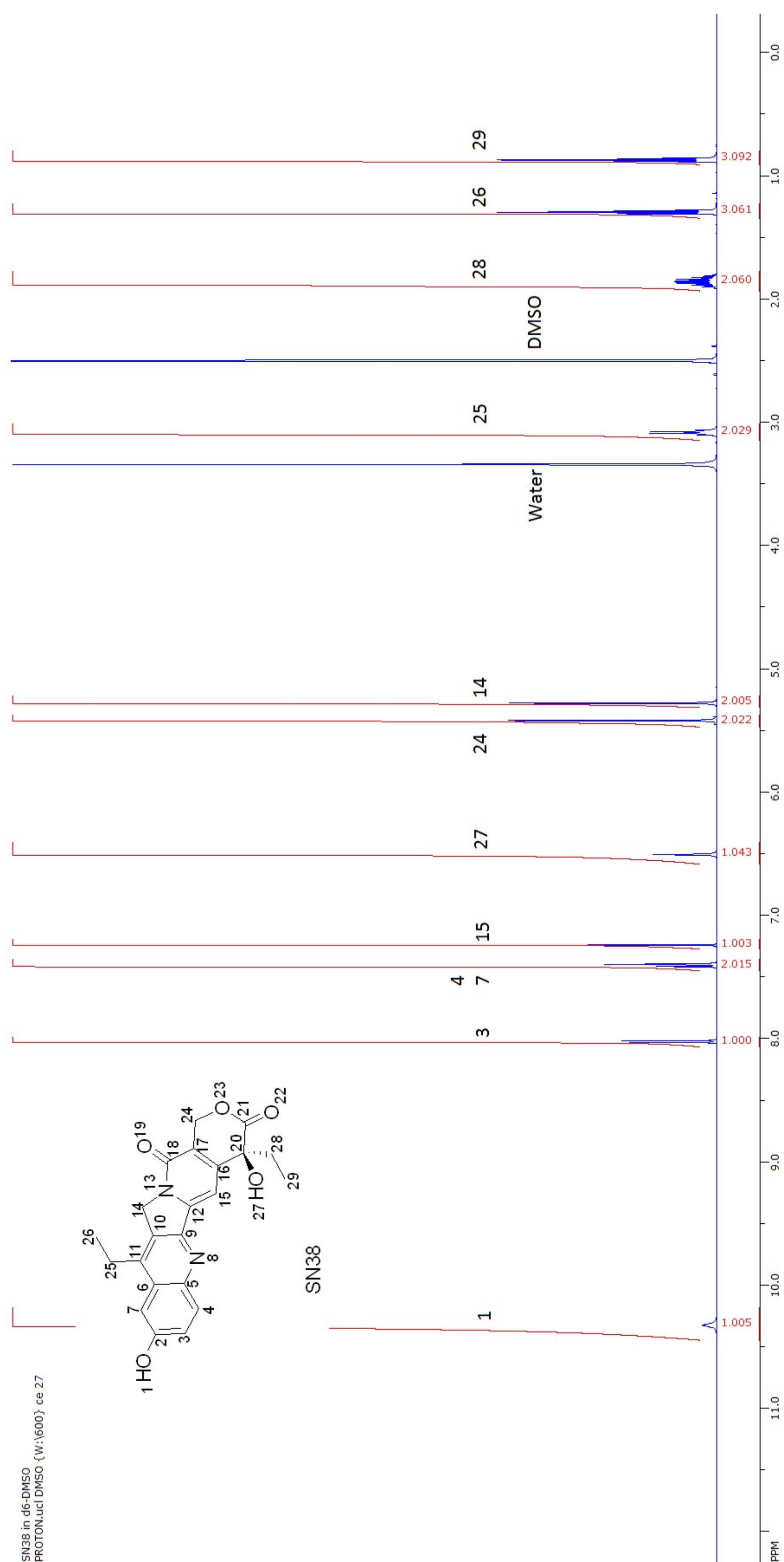


Figure S2. 1H NMR spectra of SN-38 in d6-DMSO.

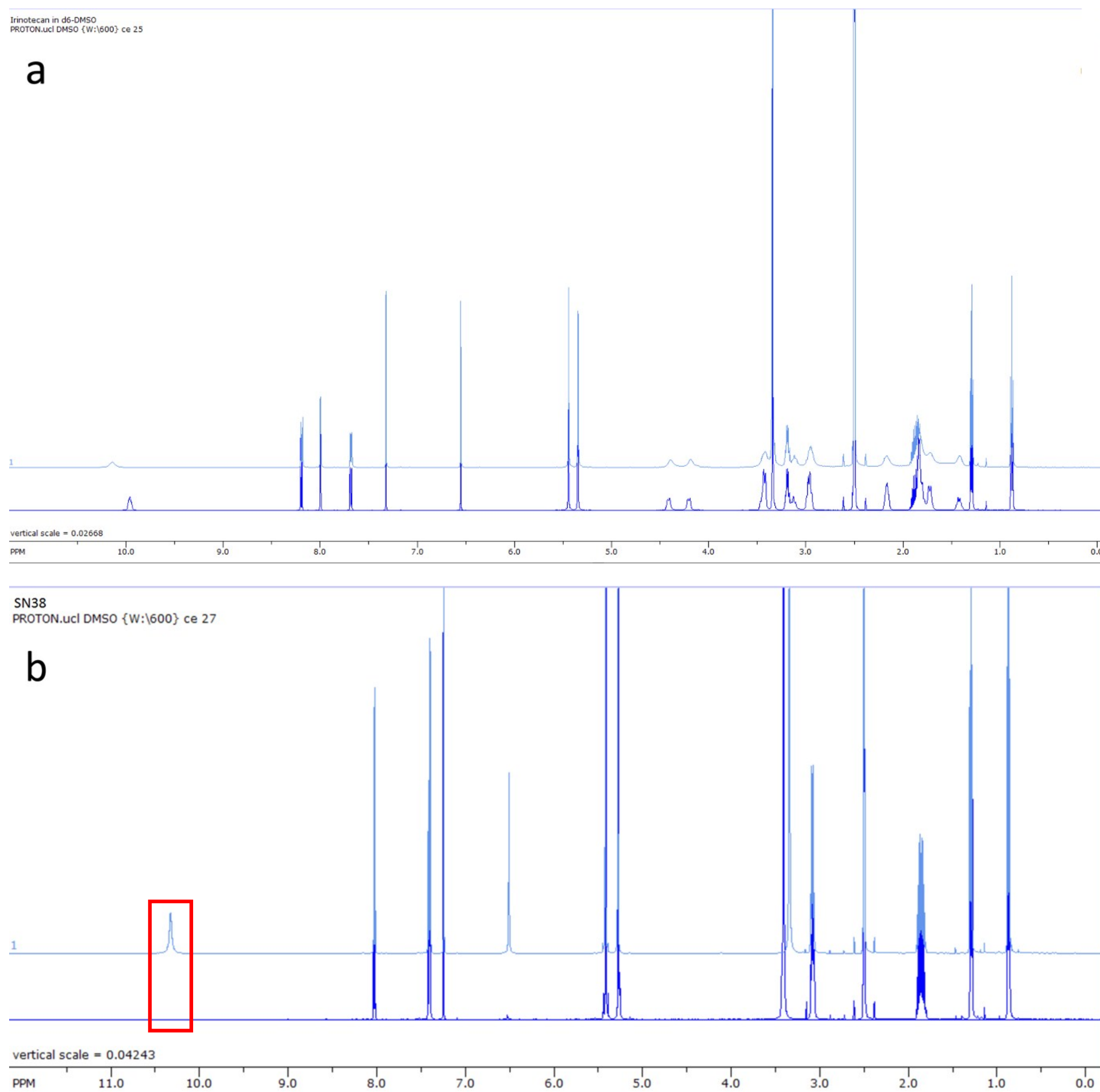


Figure S3. 1H NMR of Irinotecan (a) and SN-38 (b) in d6-DMSO before (top) and after (bottom) the addition of 1mg of NaOH and 2.2mg of KCl to simulate the effect of basification. The signal from the aromatic alcohol group in SN38 is highlighted.

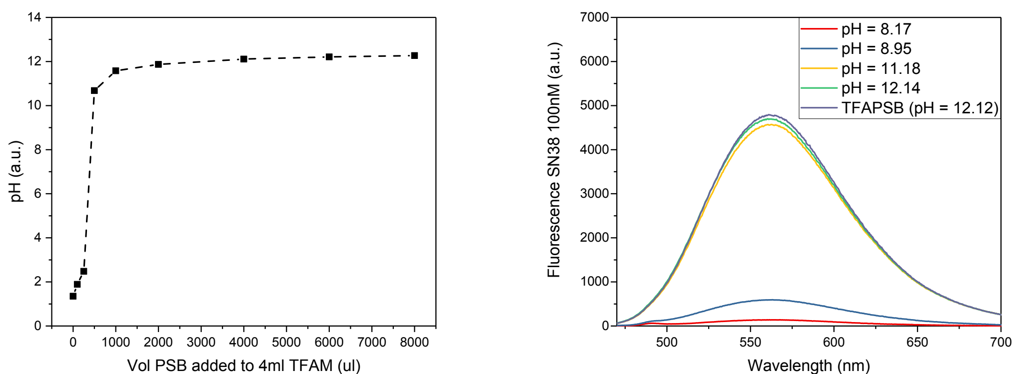


Figure S4. Variations of pH when adding different volumes of PSB to a solution of 0.05 vol% TFA in MeOH (left). Fluorescence spectrum of SN-38 at different pH mediated by TFAPSB with an excitation wavelength of 430 nm (right).

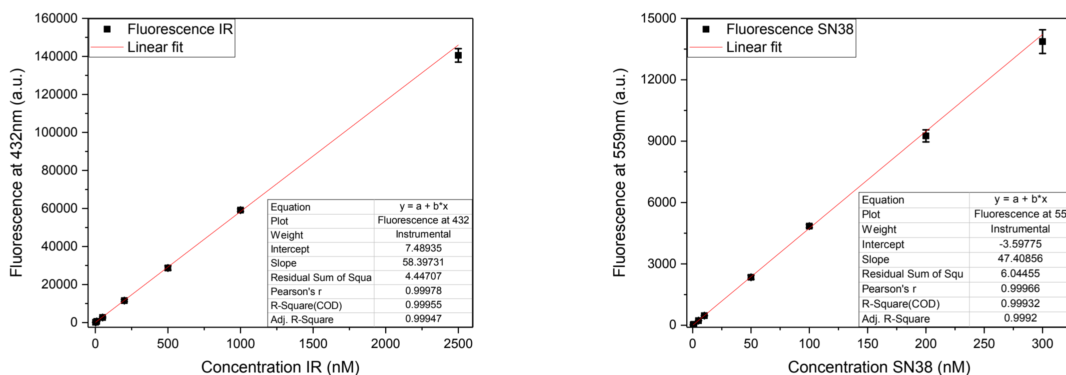


Figure S5. Fluorescence of Irinotecan (Ex = 370 nm, left) and SN-38 (Ex = 430 nm, right) at different concentrations (n = 3).

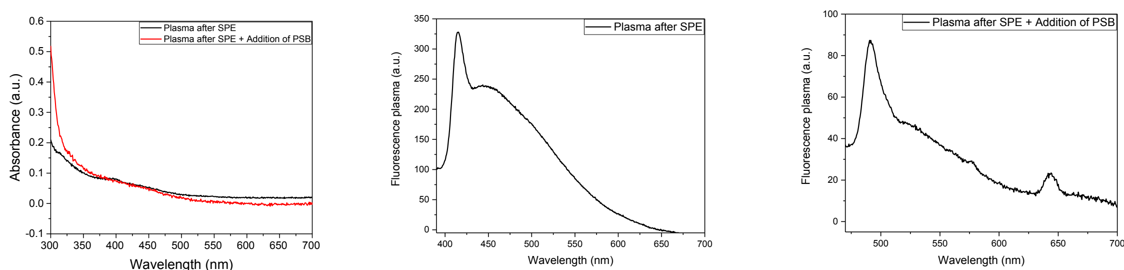


Figure S6. Absorption (left) and fluorescence spectra of pooled plasma after SPE (Ex = 370 nm, middle), and after SPE and basification (Ex 430 nm, right).

<table>
<tr><th></th><th>Conditioning</th><th>Loading</th><th>Washing</th><th>Elution</th></tr>
<tr><td>Step 1</td><td>1500 ul TFAM</td><td rowspan="3">500 ul Human Plasma</td><td>1500 ul TFAH</td><td rowspan="3">1000 ul TFAM</td></tr>
<tr><td>Step 2</td><td>1500 ul TFAM/TFAH (50/50, v/v)</td><td>1500 ul TFAM/TFAH (10/90, v/v)</td></tr>
<tr><td>Step 3</td><td>1500 ul TFAH</td><td>1500 ul TFAH</td></tr>
</table>

Table S1. Optimised conditions for the SPE of Irinotecan and SN-38 from human plasma with a Bakerbond 3 ml SPE column with a bed weight of 200 mg (C18, endcapped). TFAM refers to 0.05 vol% of TFA in MeOH, whereas TFAH refers to 0.05 vol% of TFA in water.

Sample N°	Concentration Irinotecan (nM)	Expected fluorescence (Cal. Curve) (/6.93)	Average fluorescence measured (a.u.)	Concentration Irinotecan calculated (nM)	Error Average (%)	Error (%) (STDEV)
1	2200	18550	21549	2212	9.0	5.0
2	1681	14174	16295	1677	8.2	5.3
3	1136	9579	9898	1086	0.7	8.1
4	756	6375	6744	775	6.0	6.7
5	327	2758	2651	283	-4.4	6.7
6	103	869	896	92.0	9.3	6.3

Table S2. Summary of data for the quantification of Irinotecan.

Sample N°	Concentration SN-38 (nM)	Expected fluorescence (Cal. Curve) (/8)	Average fluorescence measured (a.u.)	Concentration SN38 calculated (nM)	Error Average (%)	Error (%) (STDEV)
1	188	1113	1096	185	-1.3	4.2
2	126	746	835	141	12.3	4.1
3	57	337	338	57.6	1.1	3.1
4	23	135	147	25.6	11.1	9.8
5	33	195	168	28.9	-12.2	2.1
6	19	112	118	20.5	8.2	4.9

Table S3. Summary of data for the quantification of SN-38

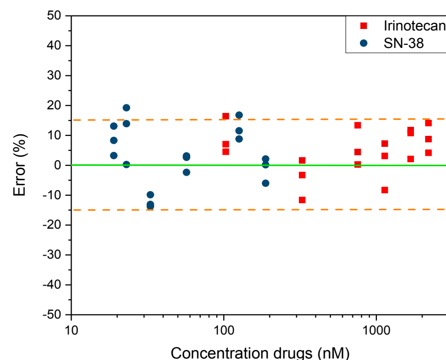


Figure S7. Individual results on the quantification of Irinotecan and SN-38 (n=3). Each experiment was carried out in triplicates.

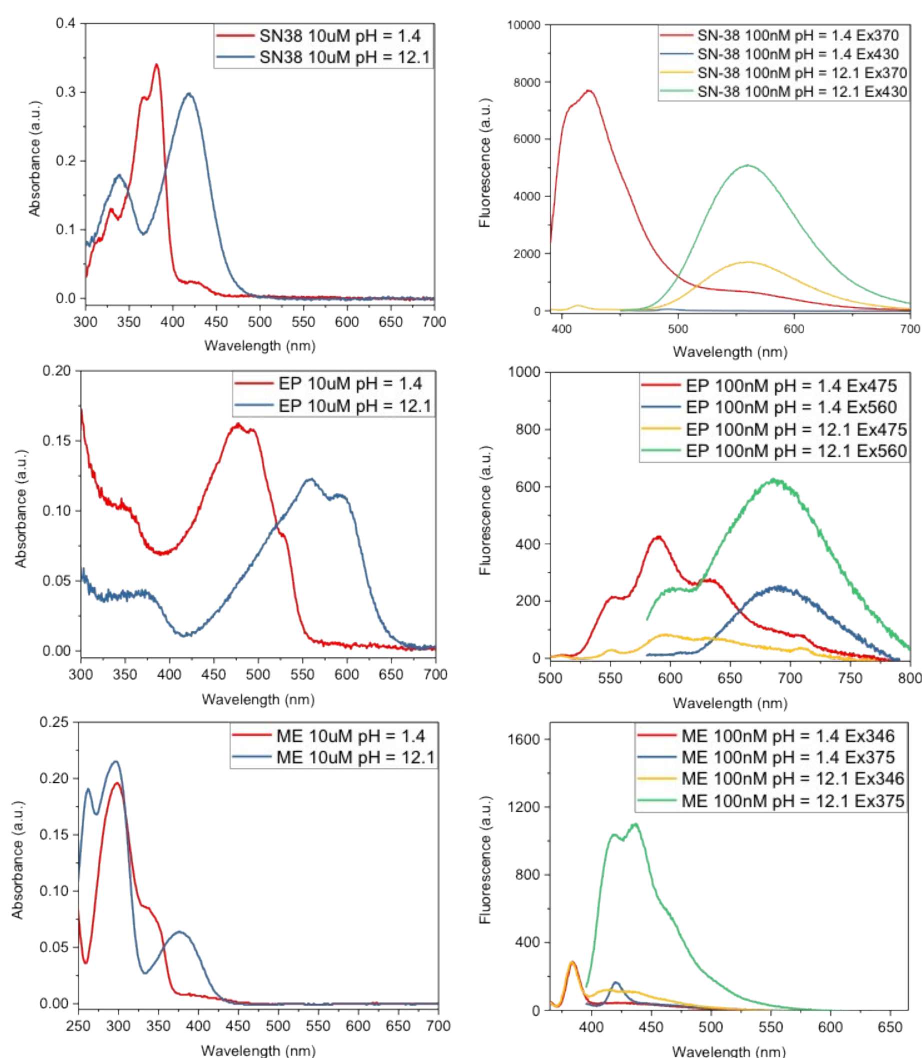
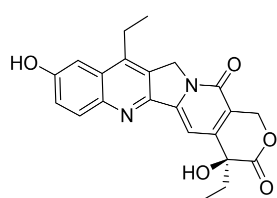
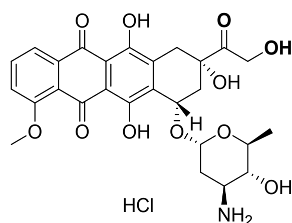
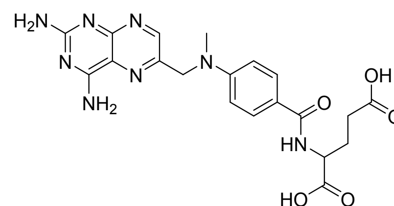


Figure S8. Absorbance spectra (left) and fluorescence spectra (right) of SN-38 (top), Epirubicin (EP, middle), and Methotrexate (ME, right). The fluorescence spectra have the excitation wavelength used in the legend. Compounds were dissolved at the indicated concentration in TFAM (pH = 1.4) for characterisation under acidic conditions before an equal volume of PSB was added for basification (pH = 12.1).







SN38 Epirubicin Methotrexate

Compound	λ_{max} Abs (pH = 1.35)	λ_{max} Abs (pH = 12.12)	λ_{max} Em (pH = 1.35)	λ_{max} Em (pH = 12.12)
SN-38	380 nm	420 nm	422 nm	559 nm
Epirubicin	475 nm	560 nm	589 nm	686 nm
Methotrexate	346 nm	375 nm	422 nm	437 nm

Table S4. Chemical structure and maximum intensities for the absorbance and fluorescence in acidic and basic conditions of SN-38, Epirubicin and Methotrexate. The maximum in absorbance was used as the excitation wavelength for the fluorescence spectra.

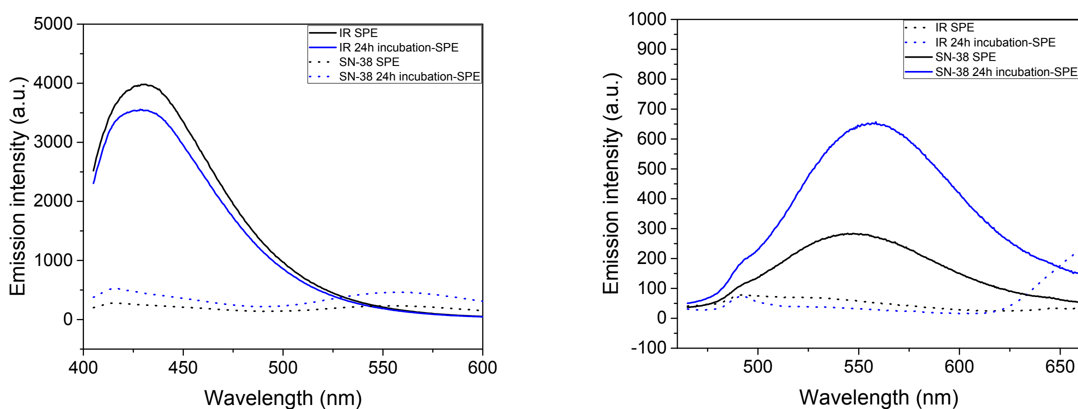


Figure S9. Fluorescence spectra of Irinotecan (1000 nM) and SN-38 (100 nM) spiked plasma samples when solid-phase extracted and analysed instantly (black) and after 24 h storage at 4 °C (blue). Spectra are shown in acidic condition in TFAM (Ex = 370 nm, left), and after basification with an equal volume of PSB (Ex 430 nm, right).