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ARTICLE in JOURNAL OF CHROMATOGRAPHY. B, ANALYTICAL TECHNOLOGIES IN THE BIOMEDICAL AND LIFE

SCIENCES · OCTOBER 2014; 969:213-8.

DOI: 10.1016/j.jchromb.2014.08.024

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Title:

Development and validation of an ultra-high performance LC-MS/MS assay for intracellular SN-38 in human solid tumour cell lines: comparison with a validated HPLC-fluorescence method

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Abstract

A simple and rapid ultra-high performance liquid chromatography-mass spectrometry/mass spectrometry (UPLC-MS/MS) method has been developed for measuring intracellular concentrations of the anticancer agent 7-ethyl-10-hydroxycamptothecin (SN-38) in tumour cells using camptothecin (CPT) as internal standard. SN-38 extraction was carried out using acidified acetonitrile. SN-38 and CPT were separated on a PFP column using gradient elution with acidified water and acetonitrile. SN-38 and CPT were quantified using a triple quadrupole mass spectrometry system. Least square regression calibration lines were obtained with average correlation coefficients of $R^2 = 0.9993 \pm 0.0016$. The lower limit of detection (LOD) and lower limit of quantification (LOQ) for SN-38 were 0.1 and 0.3 ng/ml, respectively. CPT recovery was 98.5 ± 13% and SN-38 recoveries at low quality control (LQC, 5ng/ml) and high quality control (HQC, 500ng/ml) were 89 ± 6% and 95 ± 8%, respectively. The intra- and inter-day imprecision for LQC was 6.0 and 9.2%, and for HQC was 4.7 and 7.1%., respectively. The method was compared to a validated high performance liquid chromatography-fluorescent method. In addition, the method has been successfully applied to determine the intracellular accumulation of SN-38 investigating the transport through ABCB1 (P-gp) and ABCG2 (BCRP) efflux pumps in colorectal cancer cell lines.

Keywords

SN-38; ultra-high performance LC-MS/MS; HPLC-fluorescence; method validation; method comparison.

1. Introduction

Irinotecan is mainly used in the treatment of colorectal cancer either alone or in combination with other drugs. SN-38 is the active metabolite of irinotecan (**Figure 1**). Irinotecan is converted to 7-ethyl-10-hydroxycamptothecin (SN-38) by human carboxylesterase (hCE) [1]. Irinotecan belongs to a class of anti-cancer drugs known as topoisomerase I poisons. Topoisomerase I enzymes allow DNA to relax by causing single stranded breaks, unwinding of DNA for replication to occur, and then re-annealing of the DNA double helix. If single stranded breaks remain due to the binding of SN-38, it ultimately leads to apoptosis of the affected cell.

SN-38 can undergo glucuronic acid conjugation and thus detoxification to form SN-38 glucoronide inside the body. The extent to which this occurs can greatly alter the side effects of irinotecan therapy such as diarrhoea [2]. Polymorphisms of UDP-glucuronosyltransferase (UGT) 1A1 enzyme can be associated with accumulation of SN-38 with increased risk of toxicity [3]. Therefore, plasma concentrations of SN-38 could serve as a useful marker predicting toxicity when irinotecan is given to cancer patients. In addition, intracellular concentrations of SN-38 are also thought to vary due to efflux of SN-38 via drug efflux pumps such as breast cancer–related protein (ABCG2). This efflux system may vary between tumour types and confers resistance to the drug [4]. So, measuring intracellular SN-38 could serve as an additional marker for drug activity/toxicity in patients.

Previous HPLC quantitative methods described for SN-38 were mainly for plasma or liposomes as opposed to intracellular measurements [5-14]. Some of the plasma methods were adapted to quantify intracellular SN-38 but without full validation of the optimised methods [15,16]. Other groups [17,18] have successfully developed specific methods for intracellular measurements. However, these methods were aimed primarily at separating the lactone and hydroxy forms of SN-38 and involved complex chromatography leading to long run times. In addition, SN-38 peak tailing was notable despite the use of ion pairing. Therefore, we have developed and validated this novel ultra-high performance liquid chromatography-mass spectrometry/mass spectrometry (UPLC-MS/MS) method specifically for the determination of intracellular total SN-38 (hydoxy + lactone); with the aims of shortening the run time and improving SN-38 peak resolution. The method was developed using a pentafluoryl phenyl (PFP) UPLC column which allowed fast analysis and good peak shape without the need for ion-pairing agents. Moreover, the method applicability was tested in a panel of colorectal cancer cell lines and the results were compared to a validated high performance liquid chromatography-fluorescent (HPLC-Flu) method which have been used in a number of previous studies [4,19,20].

2. Material and methods

2.1 Materials

SN-38, CPT-(s)-(+)-Camptothecin, Ciclosporin A, Fumitremorgin C, Sodium dodecyl sulphate (SDS) GC grade, Hanks balanced salt solution and Dulbecco's modified Eagles

Medium were all obtained from Sigma Aldrich, (Dorset, UK). AnalaR grade HCl and NaH_2PO_4 were obtained from VWR, (West Sussex, UK). Trypsin was purchased from Invitrogen, (Paisley, UK). LC-MS grade formic acid (FA), water, methanol and acetonitrile (ACN) were all obtained from Fisher Scientific, (Loughborough, UK) and SN-38 from Pfizer, (Cambridge, UK). HT29, and SW620 cell lines were obtained from the American Type Culture Collection (Teddington, Middlesex, UK). HCT116 was obtained from the European Collection of Cell Cultures (Wiltshire, UK)

2.2 Instrumentation, chromatographic and mass spectrometry conditions

SN-38 and CPT were separated on an Accela UPLC system, Thermo Scientific, (Hemel Hempstead, UK) equipped with Kinetex 1.7 µm PFP, LC Column 100 × 2.1 mm obtained from Phenomenex, (Macclesfield, Cheshire, UK). Gradient elution was employed using a mobile phase of 0.1% FA in water (buffer A) and 0.1% FA in ACN (buffer B) as follows: Buffer A = 70% at 0 min, from 70 to 10% over 2 minutes, held at 10% for 1 minute, from 10 to 70% over 0.1 minutes, ending with 70% for 3 minutes, all at a flow rate of 250 µl/min.

Triple-stage-quadrupole mass spectrometry (TSQ) Vantage system, Thermo Scientific, (Hemel Hempstead, UK) equipped with an electrospray ion source was used for mass detection. Samples were analyzed in the Multiple Reaction Monitoring (MRM), positive mode at a spray voltage of 3500 V. Nitrogen was used as sheath and auxiliary gases at a flow rate of 30 and 10 arbitrary units, respectively. Argon was used as collision gas with pressure of 1.5 mTorr. The optimum transitional daughter ion mass and collision energy

for SN-38 was: m/z 393.1 → 349.4 (collision energy 25V) and for CPT: m/z 349.1 → 305.4 (collision energy 23V). Data acquisition and chromatography analysis were carried out using Xcalibur software version 2.2 from Thermo Scientific, UK.

The HPLC-Flu system consisted of Perkin Elmer series 200 HPLC system (Perkin Elmer, Cambridge, UK) equipped with Luna ODS, C18(2), 3 µm, 150 × 4.6mm ID, from Phenomenex (Macclesfield, Cheshire, UK), fitted with a C18 security guard cartridge also from Phenomenex (Macclesfield, Cheshire, UK). Analytes were eluted using an isocratic mobile phase consisting of 50 mM NaH_2PO_4 buffer (pH 3.1) containing 10 mM SDS 65%: ACN 35%. At a flow rate of 1 ml/min. Analytes were detected using Perkin Elmer series 200 HPLC fluorescence detector equipped with a xenon lamp operating at wavelengths of 380 and 540 nm for excitation and emission, respectively. Data acquisition and chromatography analysis was carried out using Turbochrom software version 6.2.1 from Perkin Elmer (Cambridge, UK).

2.3 Cell extraction procedure

Cell samples were pelleted by centrifugation at 600 g, each containing 6 million cells. After removal of supernatant, 200µl of 50% ACN containing 0.5% FA and 1 µg/ml CPT was added to each sample, vortexed for 60 seconds and placed on ice for 30 min to allow protein precipitation to occur. Samples were then spun at 10,000 g, 4 ^{o}C, for 10 min to pellet the cell precipitate. 150µl supernatant was then transferred into LC-MS vials and 10 µl injected directly into the UPLC-MS system.

2.4 Preparation of standards and controls

SN-38 and CPT stock solutions were prepared at 1 mg/ml by dissolving in DMSO and chloroform/methanol (4:1 vol:vol), respectively.

Working standards of SN-38 at the following concentrations 0.1, 0.3, 1, 3, 10, 30, 100, 300 and 1000 ng/ml were prepared by diluting SN-38 stock in pooled drug free cell pellets. Quality control samples (QC) of 5, 50 and 500 ng/ml were prepared by dilution of SN-38 stock in pooled drug free cell pellets.

Linearity of the assay was determined by injecting the working standards into the UPLC system. The peak area of each analyte concentration was divided by the peak area of CPT internal standard. The resultant ratio was then plotted against the concentration using a weighted ($1/x^2$) quadratic least square regression model, where x is the concentration and y is the peak area ratio.

2.5 Sensitivity

The lower limited of detection (LOD) was determined as the concentration of extracted SN-38 that had a peak area ratio 3 times higher than the baseline noise. The lower limit of quantitation (LOQ) was determined as the lowest conc. of extracted SN-38 with imprecision < 10% and inaccuracy <15%.

2.6 Imprecision and inaccuracy

Interday imprecision was determined by extraction and analysis of three QC samples of known concentration 5 ng/ml (Low Quality Control; LQC), 50 (Middle Quality Control; MQC) and 500 ng/ml (High Quality Control; HQC) over 6 different days.

Intraday imprecision was determined by extraction and analysis of 6 replicates of each of the two QC samples within the same run.

Inaccuracy was determined by calculating the percentage deviation of the measured concentration from the nominal values of each QC sample.

2.7 Extraction recovery

Extraction efficiency (%) was determined at two QC levels (LQC and HQC) by dividing the peak areas from extracted samples with those from spiked post-extracted blank cell extract samples at the same QC concentration. Recovery of the CPT was determined in the same way at a concentration of 1 µg/ml.

2.8 Specificity for SN-38 and CPT

Specificity was evaluated by injecting blank mobile phase to conform that no peaks were co-eluting at the positions of SN-38, CPT. This was also repeated with drug free (blank) cell extract.

2.9 Stability

Stability of SN-38 and CPT were evaluated by repeated injection of standards after freeze thaw cycles over a 3 month period of time.

2.10 Application of method

This method was used to determine intracellular SN-38 concentrations in human cancer cell lines derived from colon cancer (HT29, HCT116 and SW620) in the presence and absence of fumitremorgin C (a known ABCG2 efflux inhibitor) and Ciclosporin A (P-gp efflux inhibitor).

Cells were grown in appropriate media in 90 ml flasks, trypsinized and counted prior to the experiment. Samples of each cell line were then distributed to Falcon tubes at a concentration of 2 million cells /ml (3 ml in each falcon tube). Each sample was then treated with 0.1µM SN-38, with and without 10µM ciclosporin or 5 µM Fumitremorgin C. Incubated at 37^{o}C for 2hrs to allow uptake/efflux to occur, after which, samples were spun at 600 g, 4^{o}C, for 6min to pellet cells. After removal of supernatant the cells were washed once in ice cold hanks and transferred into 1.7ml Eppendorf tubes. Samples were

then re-spun at 600 g, 4^{o}C, for 6 min to pellet cells. Cell extraction procedure was then carried out as previously described in section 2.3.

3. Results

3.1 Method development and optimisation

Two different UPLC columns (PFP and HILIC) were investigated. SN-38 and CPT could not be chromatographically separated on the HILIC column (Kinetex HILIC, 2.1 × 100mm) using 80% acetonitrile isocratic elution at a flow rate of 250µl. SN-38 peak full width at half maximum height (FWHM) was 0.1 minutes and retention time was 1.15 minutes (data not shown). These results suggested that SN-38 was not retained enough on the HILIC column as the void time on this system was expected to be 0.96 minutes. However, the PFP column produced efficient retention and sharp peaks using a simple mobile phase gradient of water and ACN (Figure 2). Finally, the gradient was optimized to achieve high throughput analysis.

3.2 Extraction recovery

Extraction recoveries were reproducible and consistent across 6 independent experiments (total of 203 samples) with mean values of 98.5 ± 13% for CPT. SN-38 recoveries at LQC and HQC (total of 24 samples) was 89 ± 6% and 95 ± 8%, respectively.

3.3 Standard curves and linearity

The mean response factor across 6 experiments (standards were run in duplicate) was 239.5 ± 18.2 (Stdev). The average r^2 was 0.9993 ± 0.0016. (**Table 1**). The imprecision at the 0.1 ng/ml concentration was not acceptable (34.1%), so this calibrator point was removed from the standard curve used in quantifying intracellular SN-38.

3.4 Imprecision and Inaccuracy

Intra-day imprecision was less than 6.0% over 6 experiments. Highest imprecision was noted with LQC. The mean measured value for the LQC was 5.1 ± 0.3 with inaccuracy of 1.2%. The mean measured concentration for HQC was 492.0 with inaccuracy of -1.6% (**Table 2**).

Inter-day imprecision were less than 9.2% across 6 experiments. The mean measured value for the LQC was 5.1 ± 0.5 with inaccuracy of 1.9%. The mean measured concentration for HQC was 491.5 with inaccuracy of -1.7% (**Table 2**).

3.5 Sensitivity

As shown in **Figure 2 (E) and Table 1**, although SN-38 was detectable at a concentration of 0.1ng/ml, the imprecision at this concentration exceeded 10%. Therefore LOQ was identified as 0.3 ng/ml and LOD identified as 0.1 ng/ml. The impression at LOQ and LOD was 8 and 34%, respectively.

3.6 Specificity

Extracted blank cell extract showed no peaks eluting at the retention times of SN-38 and CPT (1.75 and 1.89 minutes, respectively). **Figure 3 (D & G)** shows example chromatograms for blank cell extracts with no co-eluting compounds at the expected retention time of SN-38 and CPT, respectively.

3.7 Freeze-thaw stability

The results of the stability experiments are shown in **Table 3**. SN-38 stock solution was found to be stable at −40°C for 3 months with a concentration difference of less than 5% after 3 months of storage. The analytical method is therefore proved to be applicable for routine analysis over this period of time.

3.8 Method Application

Figure 3 shows that Ciclosporin A had little effect on the accumulation of SN-38 in the three colorectal cell lines with significant increase of 57% in HT29 cell line ($p<0.05$). These results suggest that ABCB1 has a minor role in SN-38 efflux. However, Fumitremorgin C (a specific ABCG2 inhibitor) resulted in a dramatic increase in SN-38 intracellular concentrations in all colorectal cell lines with 4.3, 2.1 and 3.1 fold increase in HT29, HCT116 and SW620 cell lines, respectively. Combined administration of ciclosporin A and fumitremorgin C did not result in more SN-38 accumulation compared to fumitremorgin C alone treatment. These results suggest that SN-38 efflux in colorectal cell lines is mainly via ABCG2.

3.9 UPLC-MS/MS Vs HPLC-Flu Method Comparison

This novel UPLC-MS/MS method resulted in sharp peak shapes for both SN-38 and CPT. FWHM for SN-38 and CPT was >0.1 minute for UPLC-MS method compared to 0.2 and 0.3 minutes, respectively for the HPLC-Flu method. The retention times obtained for the UPLC-MS method were 1.75 and 1.89 minutes for SN-38 and CPT, respectively compared to 3.7 and 4.6 minutes for the HPLC-Flu. This resulted in a shorter total run time for the UPLC-MS method (6 minutes compared to 7 minutes for HPLC-Flu). In addition, the UPLC-MS method had higher sensitivity with LOQ value of 0.3 ng/ml compared to 1 ng/ml for HPLC-Flu. The fluorescent detection of the HPLC-Flu method was saturated at a SN-38 concentration of 300 ng/ml, while the mass spectrometry detector of the UPLC-MS method detected SN-38 concentrations up to 10,000 ng/ml without saturation. Therefore, this novel UPLC-MS method covered a wider dynamic range (0.3-1000ng/ml compared to 1-200 ng/ml for HPLC-Flu). Imprecision and inaccuracy values were < 10% for both methods. The two methods were used to determine intracellular SN-38 concentrations in a panel of colorectal cancer cell lines. The results were then compared using Bland-Altman analysis in order to determine the degree of agreement between the two methods (**Figure 3D**). The average bias between the two methods was close to zero (0.16 ng/ml). In addition, the 95% limits of agreement were between +8.29 and -7.97 ng/ml.

4. Conclusion

The method described in this work is the first UPLC-MS method to be developed specifically for intracellular quantification of SN-38. The extraction method is simple and reproducible with high extraction yield. The method is highly sensitive, precise and accurate. A good separation was achieved with excellent peak resolution and minimal peak tailing without using any ion pairing agents. Moreover, the chromatography run time was shorter permitting high sample throughput. This method has been successfully applied to the measurement of intracellular SN-38 accumulation in a number of colorectal cell lines studying SN-38 efflux. In all the studied cell lines, SN-38 efflux was found to be mainly regulated by ABCG2 with minimal contribution of ABCB1. These results suggest that the newly developed UPLC-MS method is ideally suited for this type of analysis.

Acknowledgment

The authors would like to thank Professor David Perrett, Bioanalysis, William Harvey Research Institute, Queen Mary University of London, for assistance in editing this manuscript.

Figure Captions

Figure 1. Chemical structure of irinotecan (A) and SN-38 (B)

Figure 2. Examples of mass spectral analysis of SN-38 (A) and CPT (B). Typical standard curve for SN-38 is also shown (C) with quadratic curve fit. Example chromatograms produced using HT29 colon cancer cells, with blank cell extract with no SN-38 (D), lower limit of detection (LOD) at 0.1 ng/ml SN-38 (E) and SN-38 accumulation at 33 ng/ml (F). The internal standard CPT chromatograms are also shown in double blank cell extract (G) with no CPT and CPT concentration of 1 µg/ml added to the extraction solution (H).

Figure 3. Intracellular accumulation of SN-38 before and after treatment with ABCB1 (P-gp) inhibitor, ciclosporin (CA), ABCG2 (BRCP) inhibitor fumtremorgin C (FMC) and a combination of CA + FMC in three colorectal cell lines HT29, HCT116 and SW620 corresponding to Figure A, B and C, respectively (NS = non-significant, * = significant with $p<0.05$ and *** = significant with $p<0.001$). Bland Altman plot showing the agreement in determining the SN-38 intracellular concentrations in the three colorectal cell lines between the UPLC-MS and HPLC-Flu is also shown (D)

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***Highlights (for review)**

- An UPLC-MS/MS method was developed for measuring intracellular concentrations SN-38
- SN-38 was separated using a pentafluoryl phenyl UPLC column
- Fast analysis and good peak shape obtained without the need for ion-pairing agents.
- Method was used to investigate SN-38 efflux in colorectal cancer cell lines.
- The method results were compared to a validated HPLC- fluorescent method.

Figure

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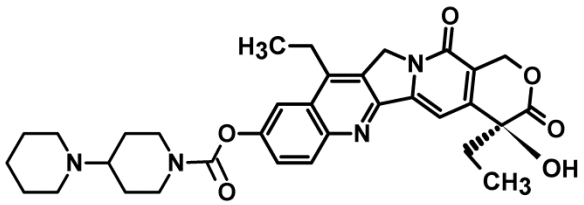
Figure 1A)



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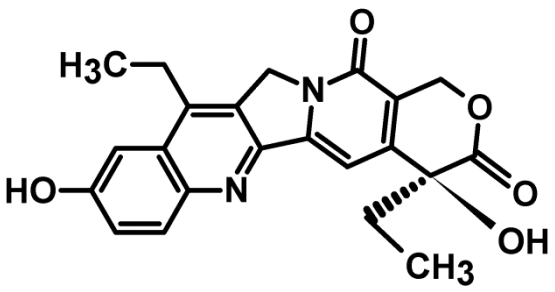

Figure

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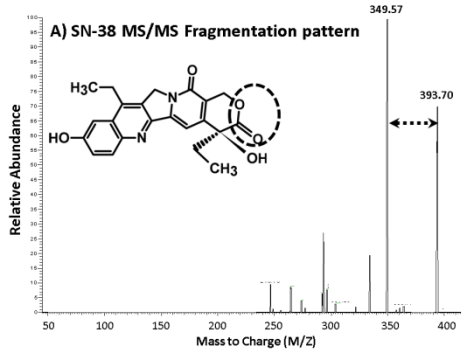
Figure 2A)



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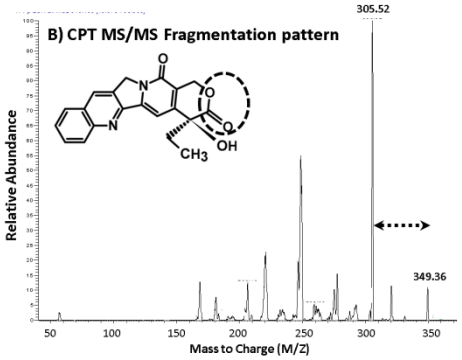

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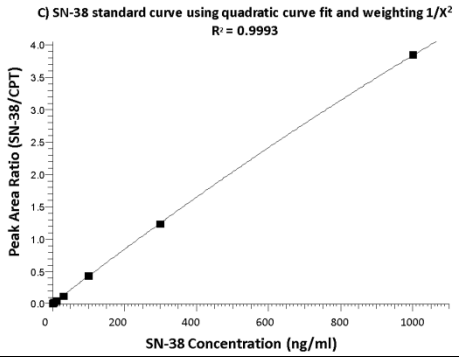

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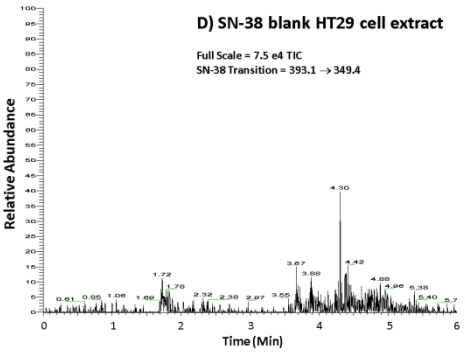

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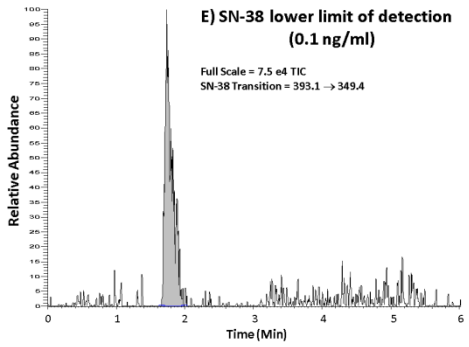

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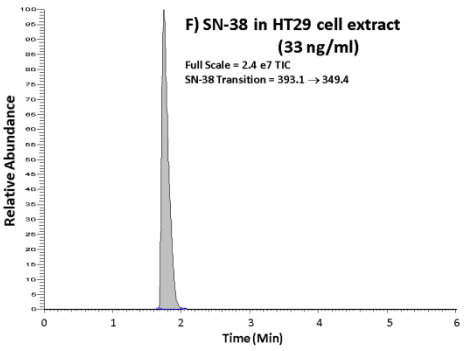

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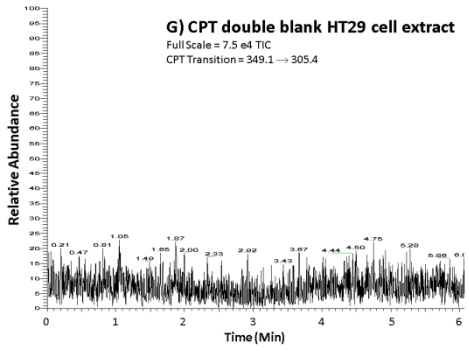

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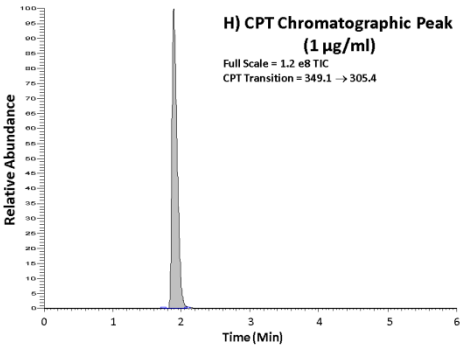

Figure

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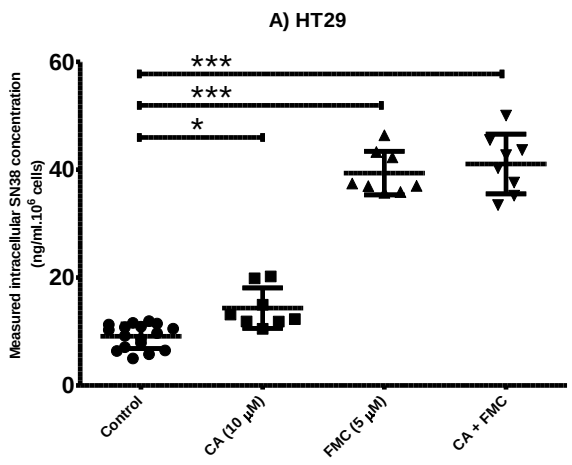
Figure 3A)



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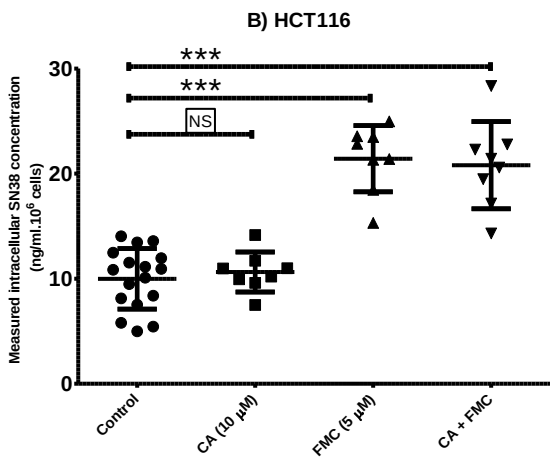


Figure 3C)

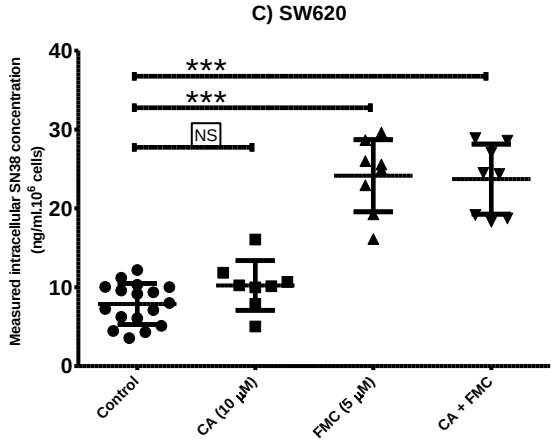
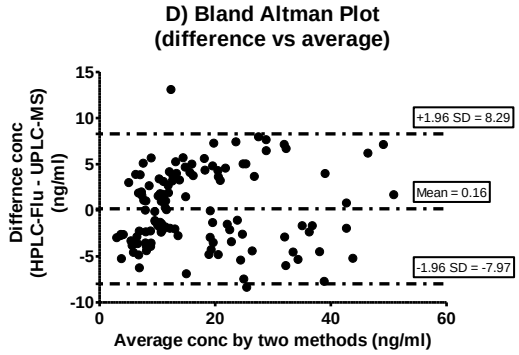


Figure 3D)



Tables

Table 1, Measured Vs Theoretical concentrations of SN-38 standards along with Imprecision (CV) and Inaccuracy (percentage error).

Theoretical Standards	Mean of the Measured standards	Imprecision (CV)	Inaccuracy (Error %)
0.1	0.11	34.1	6.2
0.3	0.33	8.2	8.9
1	0.98	8.9	-2.2
3	3.06	7.3	1.9
10	9.34	7.8	-6.6
30	30.41	8.1	1.4
100	95.58	7.4	-4.4
300	306.21	7.0	2.1
1000	965.74	3.0	-3.4

Table 2. Intra- and inter-day imprecision of SN-38.

	Intraday (n=6)			Interday (n=6)		
	LQC	MQC	HQC	LQC	MQC	HQC
Theoretical value (ng/ml)	5.0	50.0	500.0	5.0	50.0	500.0
Mean measured value (ng/ml)	5.1	48.5	492.0	5.1	49.4	491.5
SD	0.3	0.9	23.5	0.5	1.5	35.1
Imprecision (CV)	6.0	1.9	4.7	9.2	2.9	7.1
Inaccuracy (Error %)	1.2	3.0	-1.6	1.9	-1.2	-1.7

Table 3. SN-38 stability experiments carried out on 3 cycles over a period of 90 days.

	Std 30	Std 3
Cycle 1	32.715	3.097
Cycle 2	30.93	3.16
Cycle 3	32.40	3.14
Mean	32.01	3.13
Std	0.95	0.03
Imprecision (CV)	2.98	0.95
Inaccuracy (Error %)	-2.01	-0.13