ELSEVIER

Journal of Chromatography B, 714 (1998) 355–359

JOURNAL OF CHROMATOGRAPHY B

Trace analysis of SN-38 in human plasma by high-performance liquid chromatography

Laurent P. Rivory*, Michael Findlay, Stephen Clarke, James Bishop

Medical Oncology, Sydney Cancer Centre, Royal Prince Alfred Hospital, Missenden Road, Camperdown, NSW 2050, Australia

Received 9 February 1998; received in revised form 17 April 1998; accepted 28 April 1998

Abstract

A reversed-phase high-performance liquid chromatographic method with fluorescence detection was developed and validated for the quantitation of SN-38, the active metabolite of irinotecan (CPT-11), a new anticancer drug. This method uses solid-phase extraction with a C_{18} column for sample clean-up and concentration following acidification of human plasma with two volumes of 0.1 *M* HCl. Using blank plasma spiked with SN-38, we found the assay to be linear over the concentration range of 10–500 p*M* (3.9–195 pg/ml) with acceptable total and within-day imprecision. The recovery of SN-38 ranged from 48.3% (10 p*M*) to 91.5% (500 p*M*) whereas that of the internal standard, 20-(*S*)-camptothecin, was 96.9% (500 p*M*). This method represents a sizeable increase in sensitivity over other published methods and is shown to be suitable for the measurement of 'trough' concentrations of SN-38 during the treatment of patients with a weekly regimen of irinotecan.

Keywords: SN-38; Irinotecan

1. Introduction

Irinotecan or CPT-11 (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin) is a camptothecin analogue currently used for the treatment of advanced colon cancer [1,2]. In vivo, irinotecan is converted to the active metabolite SN-38 (Fig. 1) by carboxylesterases [3,4]. SN-38 is several orders of magnitude more potent in vitro than CPT-11 and is thought to be responsible for most of the cytotoxic activity of the parent drug [5]. The plasma pharmacokinetics of SN-38 have been assessed in several phase I/II studies but the terminal phase is poorly characterized because concentrations often do not decrease in a clear log-linear manner at late time points (up to 24 h) following the administration of CPT-11 [6]. This terminal phase may be of some importance given that the disposition of SN-38 and its β-glucuronide have been implicated in one of the major toxicities associated with CPT-11 — a late onset diarrhoea syndrome [7]. Because camptothecins are more cytotoxic when used in protracted schedules [8], the prolonged retention of SN-38 in plasma even at low concentrations could have a bearing on this toxicity.

The regimen of CPT-11 we currently use is 125 mg/m^2 weekly for 4 weeks followed by a 2-week rest [2]. In order to study a possible link between the late kinetics of SN-38 and toxicity, we aimed to quantitate SN-38 in plasma just prior to the next scheduled dose of CPT-11 (i.e. 'trough' levels). Based on published estimates of the terminal half-life

*Corresponding author.

0378-4347/98/$19.00
PII: S0378-4347(98)00216-3

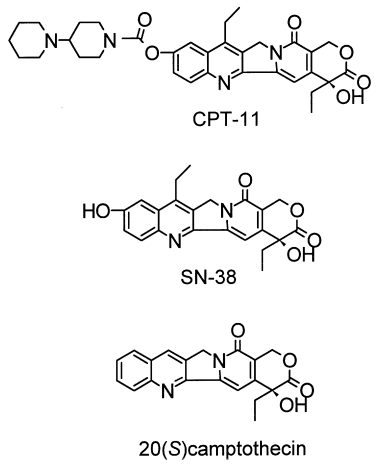

Fig. 1. Chemical structures of the camptothecin analogues mentioned in the text. The internal standard used in this study was 20-(*S*)-camptothecin (CPT).

of SN-38 (~13 h) and typical concentrations 24 h following administration of irinotecan (125 mg/m^2) of ~10 n*M* [9], we estimated that these trough concentrations would be likely to fall in the low picomolar range. This indicated a requirement for a method with two to three orders of magnitude more sensitivity than currently available methods [10–12]. In this paper, we describe the development of assay methodology suitable for the measurement of trace concentrations (as low as 10 p*M*) of SN-38 in human plasma.

2. Experimental

2.1. Chemicals and HPLC reagents

SN-38 was provided by Rhône-Poulenc Rorer (Neuilly, France). 20-(*S*)-camptothecin (CPT), purchased from Sigma (St. Louis, MO, USA), was used as the internal standard (I.S.). Both were dissolved in dimethylsulfoxide (DMSO) to a concentration of 5 mg/ml and stored at −20°C until use. High-grade methanol and acetonitrile (ChromAR-HPLC, Mallinckrodt) were obtained from Biolab Scientific (Melbourne, Australia). Water was of Milli-Q grade (Millipore, Sydney, Australia) and all solutions were filter-degassed using a 0.45-μm filtration system (Millipore). The mobile phase was 0.075 *M* ammonium acetate buffer (pH 5.3)–acetonitrile (77:23, v/v). Date expired fresh-frozen plasma was kindly provided by the Royal Prince Alfred Hospital Blood Bank.

2.2. Apparatus

Separation of SN-38 and CPT was performed at ambient temperature using a Waters Nova-Pak Radial-Pak C_{18} reversed-phase column (100×5 mm I.D., 4 μm) preceded by a matching C_{18} guard column (Guard-Pak, Nova-Pak, Waters). The HPLC system (Shimadzu, Sydney, Australia) consisted of a LC-10AT pump, FCV-10AL gradient valve, DGU-12A in-line solvent degasser, SCL-10A system controller, SIL-10AXL autoinjector and RF-10AXL fluorometric detector. Data were collected and analysed on a personal computer using CLASS VP software (version 4.2, Shimadzu). Solvent flow was maintained at 1.5 ml/min.

2.3. Method optimisation

Standards of SN-38 and CPT were prepared in acetonitrile–0.01 *M* HCl (20:80, v/v) from stock solutions prepared in DMSO. Direct injections of SN-38 and CPT-11 onto the column were made to optimise the excitation and emission wavelengths as described previously [11]. The detector settings which yielded the highest sensitivity were: Gain=1, Sens.=1 and Resp.=3. For SN-38, the wavelength pair of 380 and 532 nm was selected. The emission wavelength was automatically changed to 500 nm for the detection of CPT prior to its elution. Changing the wavelengths to maximise the detector response to CPT (excitation 370 nm, emission 432 nm) caused a baseline jump and an increase in noise. The change of only the emission wavelength for CPT (to 500 nm) avoided this and yielded an approximately four-fold improvement in response relative to that obtained with 532 nm.

2.4. Sample preparation

For sample preparation, several C_{18} solid-phase extraction (SPE) cartridges were trialled with different elution protocols. In addition to analyte recovery, specific attention was paid to the stability of the

baseline of the chromatograph at the elution times of SN-38 and CPT when blank plasma extracts were injected. Bond-Elut SPE columns (*t*C-18, 200 mg, 3cc Varian, Harbor City, CA) were selected. These were prepared by sequential rinsing with 2×2 ml methanol and 2×2 ml water. Standards were prepared by spiking plasma (1 ml) with 100 μl of the appropriate solution of SN-38 and 100 μl of CPT (5 n*M*) in sterile 10-ml polypropylene tubes (Sarstedt, Adelaide, Australia). The plasma was acidified by the addition of 2 ml of 0.1 *M* HCl and vortex-mixed. The mixture was loaded onto the extraction column which was then rinsed with 2×2 ml water, 1×2 ml aqueous methanol (various strengths tested, see Section 3) and dried under full vacuum for 10 min. Compounds were eluted with 1.6 ml methanol into polyurethane tubes and the solvent evaporated overnight at ambient temperature in a Speed-Vac (Savant, Holbrook, NY, USA). The residue was resuspended into 200 μl of acetonitrile–0.01 *M* HCl (20:80, v/v), transferred to a Mini-Eppendorf tube and centrifuged at 8000 *g* for 5 min. The supernatant was transferred to a 100-μl microvolume polypropylene insert (Alltech, Deerfield, IL, USA) and 25 μl injected onto the chromatogram.

The recovery of SN-38 and CPT from plasma was assessed by comparing areas obtained from spiked samples (as described above) and direct injections of 25 μl of the appropriate standard solution in acetonitrile–0.01 *M* HCl (20:80 v/v).

Replicate samples ($n=6$) containing SN-38 at six concentrations (10, 25, 50, 100, 200 and 500 p*M*) were processed on a single day to assess the mean accuracy and the within-day imprecision (C.V.). Standard samples were also processed in triplicate on 5 different days to assess mean accuracy and total imprecision according to the method of Krouwer and Rabinowitz [13].

2.5. 'Trough' pharmacokinetics

The concentrations of SN-38 in the plasma of patients treated with irinotecan (125 mg/m^2 weekly) were determined following the collection of 9 ml venous blood into heparinised tubes on the morning of the scheduled drug infusion. The plasma was collected and stored at −70°C until analysis. SN-38 is stable for at least 6 months under these conditions [11]. The study was approved by the Ethics Committee of the Royal Prince Alfred Hospital (X96-0209).

For the analysis, the plasma samples were thawed and 1 ml transferred to a 10-ml tube to which the I.S. was added (100 μl of 5 n*M* CPT). These samples were processed exactly as for the six standards (10–500 p*M*) according to the procedure detailed above. Concentrations of SN-38 were calculated according to the peak area ratio of SN-38 and CPT. Low and high concentration quality control (QC) samples (40 and 200 p*M*, respectively) were included in duplicate within the run. The run was accepted only if at least three of the four QC samples were within 15% of the expected concentration.

3. Results and discussion

As illustrated in Fig. 2, the HPLC method enabled the detection of SN-38 in human plasma at concentrations ranging from 10 to 500 p*M*. The mean retention times of SN-38 and CPT were 4.0 and 5.1 min, respectively, although these varied slightly from day to day depending on exact mobile phase com-

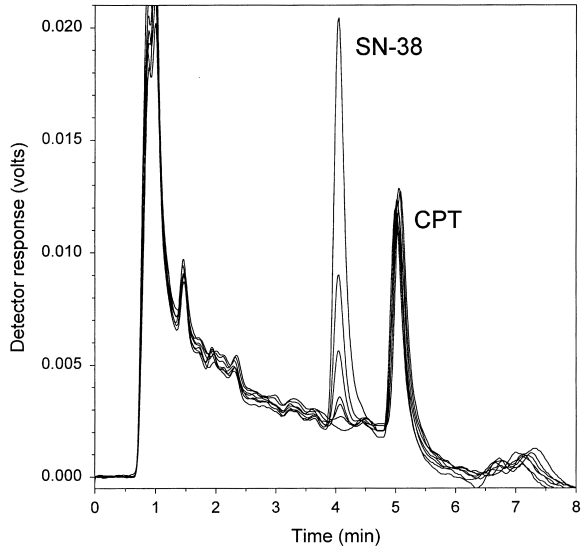

Fig. 2. Overlaid chromatograms of extracts of blank human plasma spiked with increasing amounts of SN-38 to yield final concentrations of: 0 (blank), 10, 25, 50, 100, 200 and 500 p*M*. Concentration of CPT in all plasma samples was 500 p*M*. Note the absence of interfering peaks at the retention time of SN-38.

position and ambient temperature. The retention time of the parent drug, CPT-11, was 3.5 min but CPT-11 was not detected in the clinical samples. Although these compounds were well resolved within 6 min, a run-time of 8 min was adopted. This allowed a more stable baseline for the next injection by ensuring the elution of relatively retained endogenous compounds.

As could be expected at this level of sensitivity, there was significant endogenous fluorescence associated with the sample, particularly at earlier retention times. However, the use of aqueous methanol washes in the SPE procedure reduced this interference (Fig. 3). However, increases in the methanol content of this wash beyond 40% (to 45 and 50%) progressively reduced the recovery of SN-38 (200 p*M*) from 85.0 to 78.5 and 27.1%, respectively. The optimal methanol content of 40% (v/v) in this wash step was therefore used in the method validation and sample analysis.

The recovery of SN-38 from human plasma (Table 1) was concentration dependent and ranged from 48 up to 92%. Because of the consequential effects of this on nonproportionality, we opted for log–log calibration curves with least-squares linear regression (no weighting). The mean (±S.D.) intercept and slope of the log–log calibration curves (10–500 p*M*) were -2.95 ± 0.12 and 1.16 ± 0.05, respectively, over the 5-day validation period. The corresponding r^2 values were 0.996 ± 0.003 ($n=5$).

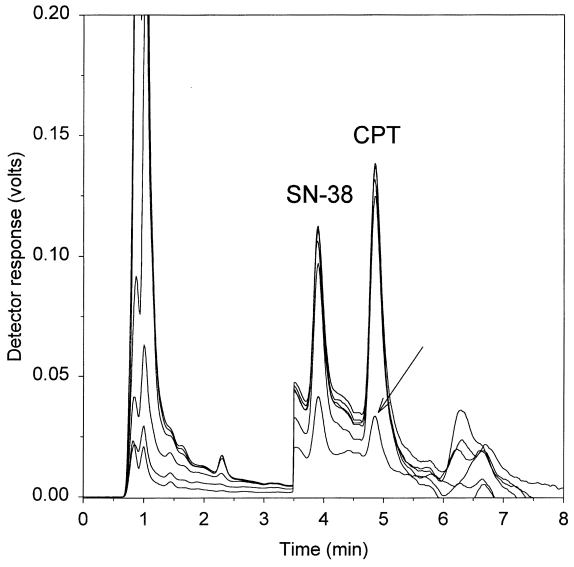

Fig. 3. Overlaid chromatograms from extracts of blank human plasma spiked with SN-38 and CPT (final concentrations of 200 and 500 p*M*, respectively) prepared by SPE with aqueous washes containing an increasing proportion of methanol (0, 10, 20, 30, 40 and 50% v/v). The detector response has been magnified by 10-fold after 3.5 min for illustrative purposes. Note the sharp drop in recovery of SN-38 and CPT when 50% methanol was used (arrow). The 40% wash was adopted for the routine sample preparation protocol.

Table 1
Recovery of SN-38 and CPT from spiked human plasma

Concentration (p*M*)	Recovery (%)	S.D.	*n*
SN-38			
10	48.3	15.8	6
25	55.2	5.9	6
50	63.9	1.6	6
100	73.1	10.1	6
200	80.9	6.1	6
500	91.5	4.5	6
CPT (I.S.)			
500	96.9	6.4	36

The concentration-dependence of the recovery of SN-38 observed is likely to be due to some high-affinity/low-capacity interaction of the analyte with plasma proteins or with the disposable laboratory ware. In any case, the log–log transformation linearised the peak response and, more importantly, enabled accurate and precise determinations of concentrations across the concentration range of 10 to 500 p*M*.

Both the within-day and total imprecision estimates indicate that the method is highly reproducible and suitable for the detection of SN-38 with a lower limit of quantification of 10 p*M* (total C.V. <20%; Tables 2 and 3). This represents a major increase in

Table 2
Within-day accuracy and imprecision (C.V.) of the determination of SN-38 in human plasma ($n=6$)

Concentration (p*M*)	Accuracy (%)	C.V. (%)
10	97.5	16.5
25	108.9	4.4
50	98.5	2.6
100	103.4	3.6
200	102.7	2.3
500	93.8	1.8

Table 3
Average accuracy and total imprecision of the determination of SN-38 in human plasma during a 5-day validation ($n=3$ each day)

Concentration (p*M*)	Accuracy (%)	C.V. (%)
10	99.9	16.2
25	102.3	7.6
50	91.4	5.3
100	99.3	6.6
200	106.4	3.3
500	99.1	4.6

sensitivity (2 to 3 orders of magnitude) over other published methods including a previous one by our group [11]. Although SN-38 exists in two forms in plasma, being in a dynamic equilibrium of lactone and ring-opened forms [14], this new assay is limited to the determination of the total SN-38 following acidification and transformation to the lactone. The large increase in sensitivity was made possible by the concentrative effects of the SPE procedure, the high sensitivity of the detector and the optimisation of chromatography to improve the selectivity of the technique. The parent compound CPT-11 did not interfere with the assay. The extraction method and the detection parameters were not optimised for this compound.

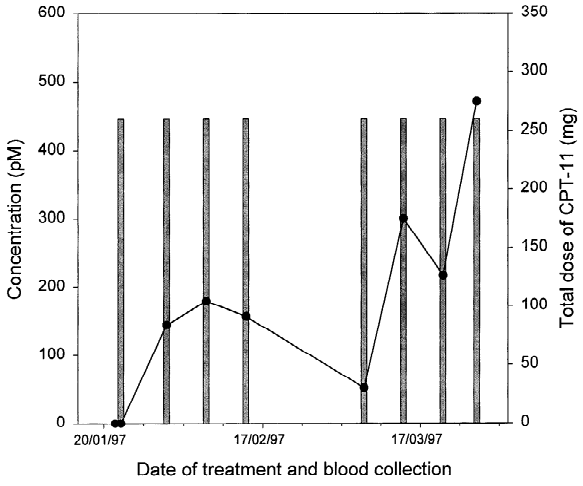

Fig. 4. Concentrations of SN-38 in plasma prior to each administration of irinotecan at the doses shown for a single patient. The concentration in plasma prior to commencement of the second cycle of weekly infusions remained detectable (52.0 p*M*) despite the sample being taken 3 weeks following the last dose of CPT-11.

The method was applied to blood samples collected prior to scheduled doses of irinotecan and found to be suitable. An example of such a study is shown in Fig. 4. Some retention of SN-38 at the time of commencement of the second cycle was noted as was the apparent accumulation of SN-38 during this second cycle. These measurable concentrations indicate that the terminal elimination of SN-38 is likely to be extremely slow. With this technique, we are currently investigating whether retention of SN-38 is related to the severity of toxicity observed during therapy with CPT-11.

References

[1] P. Rougier, R. Bugat, Semin. Oncol. 23 (1996) 34.
[2] M.L. Rothenberg, J.R. Eckardt, J.G. Kuhn, H.A. Burris 3rd, J. Nelson, S.G. Hilsenbeck, G.I. Rodriguez, A.M. Thurman, L.S. Smith, S.G. Eckhardt, G.R. Weiss, G.L. Elfring, D.A. Rinaldi, L.J. Schaaf, D.D. Von Hoff, J. Clin. Oncol. 14 (1996) 1128.
[3] T. Satoh, M. Hosokawa, R. Atsumi, W. Suzuki, H. Hakusui, E. Nagai, Biol. Pharm. Bull. 17 (1994) 662.
[4] L.P. Rivory, M.R. Bowles, J. Robert, S.M. Pond, Biochem. Pharmacol. 52 (1996) 1103.
[5] Y. Kawato, M. Aonuma, Y. Hirota, H. Kuga, K. Sato, Cancer Res. 51 (1991) 4187.
[6] L. Rivory, M. Haaz, P. Canal, F. Lokiec, J. Armand, J. Robert, Clin. Cancer Res. 3 (1997) 1261.
[7] E. Gupta, T.M. Lestingi, R. Mick, J. Ramirez, E.E. Vokes, M.J. Ratain, Cancer Res. 54 (1994) 3723.
[8] P.J. Houghton, P.J. Cheshire, J.D. Hallman 2nd, L. Lutz, H.S. Friedman, M. Danks, J.A. Houghton, Cancer Chemother. Pharmacol. 36 (1995) 393.
[9] M. Rothenberg, J.G. Kuhn, H.A. Burris 3rd, J. Nelson, J.R. Eckardt, M. Tristan-Morales, S.G. Hilsenbeck, G.R. Weiss, L.S. Smith, G.I. Rodriguez, M.K. Rock, D.D. Von Hoff, J. Clin. Oncol. 11 (1993) 2194.
[10] I. Barilero, D. Gandia, J.P. Armand, A. Mathieu Boue, M. Re, A. Gouyette, G.G. Chabot, J. Chromatogr. 575 (1992) 275.
[11] L.P. Rivory, J. Robert, J. Chromatogr. B. 661 (1994) 133.
[12] H. Sumiyoshi, Y. Fujiwara, T. Ohune, N. Yamaoka, K. Tamura, M. Yamakido, J. Chromatogr. 670 (1995) 309.
[13] J.S. Krouwer, R. Robinowitz, Clin. Chem. 30 (1984) 290.
[14] J. Fassberg, V.J. Stella, J. Pharm. Sci. 81 (1992) 676.