ELSEVIER

Journal of Chromatography B, 663 (1995) 119–126

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

High-performance liquid chromatographic analysis of the lactone and carboxylate forms of a topoisomerase I inhibitor (the antitumor drug GI147211) in plasma

C.G. Stafford*, R.L. St. Claire III

Drug Metabolism Department, Glaxo Research Institute, P.O. Box 13358, Research Triangle Park, NC 27709, USA

First received 10 June 1994; revised manuscript received 15 September 1994

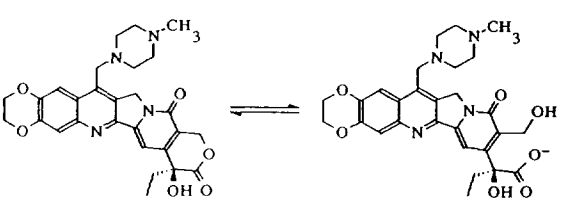
Abstract

A sensitive reversed-phase high-performance liquid chromatographic method has been developed and validated for the quantitation of the lactone form (GI147211) of a topoisomerase I inhibitor and its carboxylate form (GI190421) from dog plasma. The method consists of two solid-phase extraction methods. The first method, referred to as the lactone-only method, utilizes a diol Bond Elut cartridge to separate the two forms and measures the lactone directly. The second method, referred to as the total method, converts the carboxylate form to the lactone through acidification of the plasma sample. Total lactone is then extracted from the sample using a C_{18} Bond Elut cartridge. The carboxylate is quantitated indirectly from the difference of the total and lactone-only. The range of the standard curve for the lactone-only method is 0.096–38.5 n*M* (0.05–20 ng/ml) and for the total method is 0.193–19.3 n*M* (0.1–10 ng/ml). This is the most sensitive method reported to date for a camptothecin analogue.

1. Introduction

GI147211 (I), a novel topoisomerase I inhibitor, is being developed for its antitumor properties. The drug is an analogue of camptothecin, a plant alkaloid obtained from *Camptotheca acuminata* [1]. Camptothecin showed promising antitumor properties in vitro and in animal models but clinical evaluation of camptothecin was discontinued due to its toxicity profile [2]. The poor water solubility of camptothecin was also problematic.

Compound I contains a lactone ring which is stable in acidic media but hydrolyses in neutral and alkaline media to form the carboxylate GI190421 (II) (Fig. 1). Both forms are present at physiological pH and are monitored to support pharmacokinetic studies in the dog. Historically



GI147211 GI190421

Fig. 1. Structures and chemical equilibrium reaction between GI147211 (I) and GI190421 (II).

* Corresponding author.

0378-4347/95/$09.50
SSDI 0378-4347(94)00430-7

in rat and human studies with this class of compounds, the circulating levels of the carboxylate have been higher then the lactone shortly after dosing due to the rapid conversion of the lactone to the carboxylate at physiological pH [3–5]. Because of this stability issue, proper sample handling throughout processing is important in order to obtain a sensitive as well as selective determination of each analyte.

Reported methods for the determination of the lactone and carboxylate forms of camptothecin and its derivatives have utilized liquid–liquid extraction techniques [6,7], protein precipitation [3], and solid-phase extraction [5,8,9]. However, a method with greater sensitivity and attention to specificity was needed. The method described here utilizes two solid-phase extraction methods. One method is specific for the lactone only. The other method measures total lactone and carboxylate collectively as the lactone. This study is unique in that the accuracy of both methods are evaluated with both the lactone and carboxylate forms present in the sample. The two methods described are then used in combination to indirectly calculate the concentration of the carboxylate from the difference of total I and lactone-only I.

2. Experimental

2.1. Instrumental

The liquid chromatographic instrument consisted of Shimadzu components: LC-10AD pumps, RF-10A fluorescence detector, SIL-9A autoinjector, CTO-10A column oven, and the SCL-10A system controller. The chromatographic system employed a BDS Hypersil C_8 (250 × 4.6 mm I.D., Keystone Scientific, Bellefonte, PA, USA) analytical column thermostated at 30°C. The mobile phase for the lactone-only method was acetonitrile–100 m*M* ammonium acetate (pH 4) (25:75, v/v) with a flow-rate of 1 ml/min. The retention time of I was 6.3 min. The mobile phase for the total method was acetonitrile–100 m*M* ammonium acetate (pH 5.5) (24:76, v/v) with a flow-rate of 1 ml/min. The retention time of I was 6.1 min. Detection was by fluorescence with an excitation wavelength of 378 nm and an emission wavelength of 420 nm. Data was acquired on the PE Nelson system (Perkin-Elmer, Cupertino, CA, USA) using a PE Nelson 970 intelligent interface and Turbochrom 3 data acquisition software (version 3.2).

2.2. Materials

I and II were obtained from Glaxo Research Institute (Research Triangle Park, NC, USA). All reagents were of analytical grade and distilled water (Milli-Q Plus system) was used. Plasma was obtained from beagle dogs.

2.3. Standards

Standards of I for the lactone-only method were prepared in 100 m*M* ammonium acetate (pH 4) and serially diluted to various concentrations used to spike the dog plasma calibration standards. Standards of I for the total method were prepared in water and serially diluted to various concentrations used to spike the dog plasma calibration standards. Standards of II were prepared in 100 m*M* phosphate buffer (pH 10) and serially diluted to various concentrations used to spike the dog plasma samples.

2.4. Sample preparation

The lactone-only method utilized a diol (2OH) Bond Elut cartridge (100 mg) (Analytichem International, Harbor City, CA, USA) for extraction of I from a 1-ml dog plasma sample. The cartridge was conditioned with 1 ml of water prior to sample application. The 1-ml dog plasma sample was diluted with 1 ml of cold (4°C) 0.05 *M* phosphate buffer (pH 7.4) and then applied to the conditioned cartridge. The cartridge was washed with 1 ml of 0.05 *M* phosphate buffer (pH 7.4) followed by two 1-ml washes with water–methanol (60:40, v/v). The endogenous carboxylate, II, was removed by this wash step. Compound I was eluted with two 0.75-ml aliquots of methanol–1 *M* ammonium acetate (pH 4) (95:5, v/v). The eluent was evaporated to dryness under nitrogen at 25°C. The sample was

reconstituted with 200 μl of mobile phase and 100 μl was injected onto the chromatographic system.

The total method utilized an octadecyl (C_{18}) Bond Elut cartridge (50 mg) (Analytichem International) for the extraction of I from a 1-ml dog plasma sample. The cartridge was conditioned with 1 ml of methanol followed by 1 ml of 0.01 *M* HCl. The 1-ml dog plasma sample was incubated for 5 min at ambient temperature with 1 ml of 0.01 *M* HCl to allow conversion of II to I and then applied to the conditioned cartridge. The cartridge was washed with 1 ml of 100 m*M* ammonium acetate (pH 4). Compound I was eluted with 1 ml of methanol and evaporated under nitrogen at 25°C. The sample was reconstituted with 200 μl of 1 *M* ammonium acetate (pH 4) and 100 μl was injected onto the chromatographic system.

2.5. Calibration curves

Calibration curves were prepared in dog plasma by the addition of the appropriate I standard in pH 4 buffer for the lactone-only method or water for the total method to produce the standard curve points. The range of the standard curve for the lactone-only method was 0.096–38.5 n*M* (0.05–20 ng/ml). The range of the standard curve for the total method was 0.193–19.3 n*M* (0.1–10 ng/ml). The lactone-only samples were prepared on ice to minimize conversion of I to II in the plasma sample before application to the solid-phase extraction cartridge. Calibration curve samples were processed as described in section 2.4.

2.6. Accuracy, precision and extraction efficiency

Accuracy was determined by calculating the percent difference of the interpolated values for fortified dog plasma samples to the nominal values. Replicate samples ($n = 6$) for accuracy assessment of the lactone-only method were prepared at concentrations of 0.288, 5.76 and 28.8 n*M*. Replicate samples ($n = 6$) for accuracy assessment of the total method were prepared at concentrations of 0.288, 0.672, 2.88 and 5.76 n*M*.

Precision was determined by calculating the coefficient of variation percentages (C.V.%) of the interpolated values for fortified dog plasma samples. Replicate samples ($n = 6$) for precision assessment of the lactone-only method were prepared at concentrations of 0.288, 5.76 and 28.8 n*M*. Replicate samples ($n = 6$) for precision assessment of the total method were prepared at concentrations of 0.288, 0.672, 2.88 and 5.76 n*M*.

Extraction efficiency was determined by comparing the peak areas of extracted I fortified dog plasma samples to the peak areas of non-extracted samples at identical concentrations. Replicate samples ($n = 3$) for determination of the extraction efficiency of the lactone-only method were prepared at concentrations of 0.288, 5.76 and 28.8 n*M*. Replicate samples ($n = 3$) for the determination of the extraction efficiency of the total method were prepared at concentrations of 0.288, 0.672, 2.88 and 5.76 n*M*.

2.7. Stability/interconversion assessment

Dog plasma samples were prepared and assayed using the lactone-only method to evaluate the stability of I and II during sample processing and thus assess the specificity of the method for I. Dog plasma samples were prepared that contained only I at a concentration of 0.29 or 28.9 n*M*. Also dog plasma samples were prepared that contained both I and II. One set of samples contained 0.29 n*M* of I and 0.26 n*M* of II. The other set contained 28.9 n*M* of I and 25.7 n*M* of II. All the samples described above were prepared in triplicate. The samples were prepared and then stored at −70°C until analyzed, thus mimicking the storage conditions of unknown samples.

2.8. Assessment of carboxylate conversion to lactone

The conversion of the carboxylate, II, to the lactone, I, was examined to ensure an accurate measurement of total I. Samples were prepared in duplicate in dog plasma. The first sample set

contained 0.674 n*M* of I and 0.597 n*M* of II. The second sample set contained 6.74 n*M* of I and 5.97 n*M* of II. Samples were processed using the total method to assess the conversion of II to I.

2.9. Method applications

Three sets of samples were prepared in triplicate in dog plasma at various concentrations of I and II. The first set of samples contained 1.16 n*M* of I and 0.29 n*M* of II. The second set contained 0.725 n*M* of I and 0.725 n*M* of II. The third set contained 0.29 n*M* of I and 1.16 n*M* of II. Samples were processed using the lactone-only method and the total method. The moles of II were calculated through subtraction of the lactone-only moles from the total moles. For a given volume, these amounts were converted to concentration (n*M*).

3. Results and discussion

3.1. Chromatography

Chromatograms of extracted dog plasma samples at the lowest concentration of the calibration curve processed using the lactone-only and total methods are shown in Figs. 2 and 3, respectively. A 100-m*M* ammonium acetate (pH 4) buffer was initially used as the mobile phase buffer for both methods but an interfering endogenous peak was present in the dog plasma samples processed using the total method. A 100-m*M* ammonium acetate (pH 5.5) buffer was examined and found to resolve the endogenous plasma peak and the peak of interest. The conversion of lactone to carboxylate is slow at pH 5.5. There was insufficient on-column conversion to compromise the measurement of I at pH 5.5. However, on-column conversion of I to II was observed at a pH higher then 5.5.

3.2. Calibration curves

The range of the standard curve for the lactone-only method was 0.096–38.5 n*M* (0.05–20 ng/ml) and 0.193–19.3 n*M* (0.1–10 ng/ml)

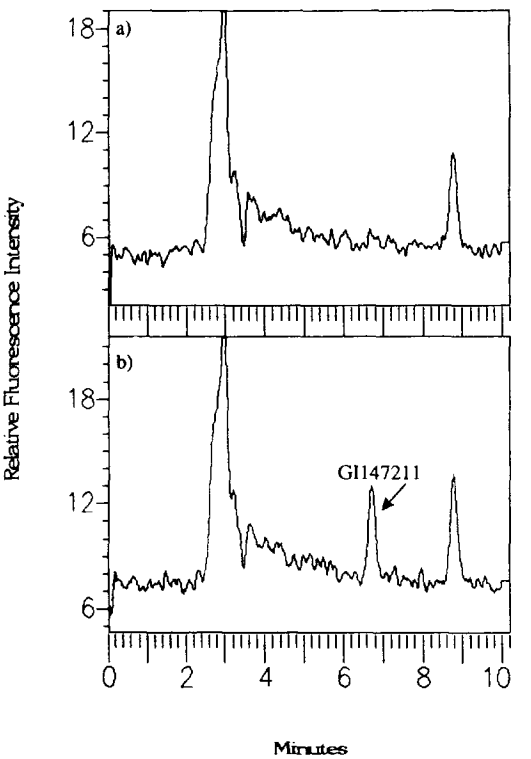


Fig. 2. Chromatograms of extracted dog plasma samples processed using the lactone-only method. (a) Blank, (b) 0.096 n*M* of I. BDS Hypersil C_8 (250 × 4.6 mm I.D.) column at 30°C, acetonitrile–100 m*M* ammonium acetate (pH 4) (25:75, v/v), flow-rate 1 ml/min. Fluorescence detection λ_{ex} = 378 nm, λ_{em} = 420 nm.

for the total method. A $1/x$ weighing scheme was determined to be most suitable for both methods. The standard curves for both methods had acceptable linearity and goodness of fit with a correlation coefficient of 0.99 or greater.

3.3. Accuracy, precision and extraction efficiency

Accuracy and precision data for the lactone-only and the total methods are shown in Table 1. Accuracy was acceptable for the lactone-only and the total methods, being ± 20% of the nominal concentration. Precision for the lactone-only method was 5.7% or better. Precision for the total method was 9.2% or better.

Extraction efficiencies for I for the lactone-

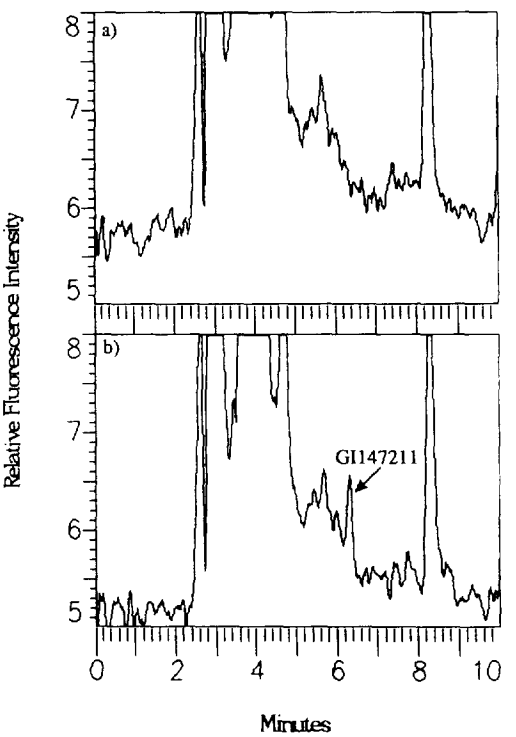


Fig. 3. Chromatograms of extracted dog plasma samples processed using the total method. (a) Blank, (b) 0.193 n*M* of I. BDS Hypersil C_8 (250 × 4.6 mm I.D.) column at 30°C, acetonitrile–100 m*M* ammonium acetate (pH 5.5) (26:74, v/v), flow-rate 1 ml/min. Fluorescence detection λ_{ex} = 378 nm, λ_{em} = 420 nm.

only method were 65, 60 and 57% for the 0.288, 5.76 and 28.8 n*M* samples, respectively. Extraction efficiencies for I for the total method were 72, 73, 87 and 73% for the 0.288, 0.672, 2.88 and 5.76 n*M* samples, respectively.

3.4. Stability/interconversion assessment

The presence of II does not contribute to the calculated concentration of I in the fortified dog plasma samples containing both I and II, as is shown in Table 2. The calculated concentration of I in the samples containing both forms is not significantly different from the concentration calculated for the samples containing only I. During method development it was found that approximately 97–98% of the carboxylate is washed off of the solid-phase extraction cartridge during the sample preparation. The slight amount (2–3%) of carryover of the carboxylate that may occur is insignificant considering the amount of error associated with the method.

3.5. Assessment of carboxylate conversion to lactone

The total method involves the conversion of II to I through the acidification of the plasma sample. Total I is then extracted. The conversion

Table 1
Accuracy and precision for the lactone-only and total method

Theoretical concentration (n*M*)	Measured concentration (mean ± S.D.) (n*M*)	Coefficient of variation (%)	Percentage of nominal
Lactone-only			
0.288	0.316 ± 0.018	5.70	110
5.76	5.79 ± 0.291	5.04	100
28.79	25.59 ± 1.28	4.99	88.9
Total			
0.288	0.323 ± 0.024	7.40	112
0.672	0.658 ± 0.032	4.84	97.9
2.88	2.87 ± 0.265	9.23	100
5.76	6.76 ± 0.113	1.67	117

$n = 6$.

Table 2
Compound I fortified dog plasma samples with and without II processed using the lactone-only method

Theoretical concentration of I (n*M*)	Measured concentration of I (n*M*)	
	Without II	With II (0.26 n*M*)
0.289	0.322	0.339
	0.291	0.322
	0.339	0.326
Average	0.317	0.329
S.D.	0.024	0.009
C.V. (%)	7.67	2.70
Percentage of nominal	110	114
	Without II	With II (25.7 n*M*)
28.9	27.03	25.97
	25.63	24.47
	25.20	25.24
Average	25.95	25.23
S.D.	0.957	0.750
C.V. (%)	3.69	2.97
Percentage of nominal	89.8	87.3

of II to I was examined to ensure an accurate measurement of total I. Table 3 shows the results from samples containing both forms that have been processed with the total method. The calculated values for total I are within ± 15% of the theoretical value of the sample.

Table 3
Conversion of II to I in dog plasma

Theoretical concentration of I (n*M*)	Total concentration of I calculated (n*M*)
Total I: 1.27 n*M*	1.36
(0.674 n*M* of I + 0.597 n*M* of II)	1.41
Average	1.39
S.D.	0.04
C.V. (%)	2.9
Percentage of nominal	109
Total I: 12.71 n*M*	14.26
(6.74 n*M* of I + 5.97 n*M* of II)	14.43
Average	14.35
S.D.	0.12
C.V. (%)	0.8
Percentage of nominal	113

Table 4
Calculated concentrations of II from I and II fortified dog plasma samples

Concentration of I only (n*M*)		Concentration of II (n*M*)		Total concentration of I (n*M*)	
Theoretical	Calculated	Theoretical	Calculated	Theoretical	Calculated
1.16	1.23	0.29	0.25	1.45	1.48
	1.25		0.17		1.42
	1.17		0.30		1.47
Mean	1.22		0.24		1.46
% Nominal	105		82.8		101
0.725	0.751	0.725	0.579	1.45	1.33
	0.700		0.700		1.40
	0.674		0.486		1.16
Mean	0.708		0.588		1.30
% Nominal	97.7		81.1		89.7
0.29	0.365	1.16	0.905	1.45	1.27
	0.383		0.887		1.27
	0.284		1.11		1.39
Mean	0.344		0.967		1.31
% Nominal	119		83.4		90.3

3.6. Method applications

The application of the lactone-only and total methods to determine the concentration of II indirectly through the difference of total I and lactone-only I is exhibited in Table 4. The calculated lactone-only and total I in samples containing both analytes and processed using the two methods was within ± 20% of the theoretical concentration. The indirect determination of II was also within ± 20% of the theoretical concentration. These results are quite acceptable since the approximation of the carboxylate concentration is defined by the combination of the errors of the lactone-only and total methods.

4. Conclusions

One method that measures both I and II in a biological sample would be ideal. However, due to the stability problems associated with the interconversion of I and II and the low concentrations of I in biological samples, this is an extraordinary challenge. Attempts to develop such a method included evaluating traditional protein precipitation and liquid–liquid extraction methods. Both of these methods lacked specificity and sensitivity due to analyte interconversion during evaporative procedures and poor extraction efficiency for the carboxylate. A solid-phase extraction method that separates I and II and then measures I only was ultimately developed. Concurrently, a solid-phase method was developed to determine total I. The two methods were used in combination to indirectly calculate the concentration of II from the difference of total I and lactone-only I.

In addition to being the most sensitive method reported to date for a camptothecin analogue, this approach incorporates a more comprehensive evaluation of the specificity of the lactone-only method. At the time of analysis of the physiological sample, interconversion is ongoing and in favor of the carboxylate form. The analytical process must in effect halt this process without any perturbation of the natural lactone/

carboxylate ratio in order to achieve an accurate lactone-only measurement. The successful application of the two methods described here to samples containing known amounts of both carboxylate and lactone at various concentrations illustrates the soundness of this dual-method approach to directly measure lactone and to indirectly calculate the carboxylate concentration.

References

[1] M.E. Wall, M.C. Wani, C.E. Cook, K.H. Palmer, A.T. McPhail and G.A. Sim, *J. Am. Chem. Soc.*, 88 (1966) 3888–3890.

[2] U. Schaeppi, R.W. Fleischmann and D.A. Cooney, *Cancer Chemother. Rep. (Part Suppl).*, 5 (1974) 25–36.

[3] J.H. Beijnen, B.R. Smith, W.J. Keijer, R. van Gijn, W.W. ten Bokkel Huinink, L. Th. Vlasveld, S. Rodenhuis and W.J.M. Underberg, *J. Pharm. Biomed. Anal.*, 8 (1990) 789–794.

[4] J.H. Beijnen, H. Rosing, W.W. ten Bokkel Huinink and H.M. Pinedo, *J. Chromatogr.*, 617 (1993) 111–117.

[5] C.H. Takimoto, R.W. Klecker, W.L. Dahut, L.K. Yee, J.M. Strong, C.J. Allegra and J.L. Grem, *J. Chromatogr. A*, 665 (1994) 97–104.

[6] J.P. Loh and A.E. Ahmed, *J. Chromatogr.*, 530 (1990) 367–376.

[7] L.G. Hart, J.B. Call and V.T. Olivero, *Cancer Chemother. Rep. (Part 1)*, 53 (1969) 211–214.

[8] S.M. Blaney, F.M. Balis, D.E. Cole, C. Craig, J.M. Reid, M.M. Ames, M. Krailo, G. Reaman, D. Hammond and D.G. Poplack, *Cancer Res.*, 53 (1993) 1032–1036.

[9] I. Barilero, D. Gandia, J.P. Armand, A. Mathieu-Boué, M. Ré, A. Gouyette and G.G. Chabot, *J. Chromatogr.*, 575 (1992) 275–280.