ELSEVIER

Journal of Chromatography B, 736 (1999) 135–141

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

High-performance liquid chromatographic assay for the determination of the novel taxane derivative IDN5109 in mouse plasma

T. Colombo[a], R. Frapolli[a], E. Bombardelli[b], P. Morazzoni[b], A. Riva[b], M. D'Incalci[a], M. Zucchetti[a,*]

[a]*Laboratory of Cancer Pharmacology, Department of Oncology, Istituto di Ricerche Farmacologiche 'Mario Negri', Via Eritrea 62, 20157 Milan, Italy*

[b]*Indena, Viale Ortles 12, 20139 Milan, Italy*

Received 26 May 1999; received in revised form 22 September 1999; accepted 28 September 1999

Abstract

An HPLC assay was developed to determine the paclitaxel analogue 13-(N-*tert*.-butoxycarbonyl-β-isobutylisoserinyl)-14-hydroxybaccatin-1,14-carbonate (IDN5109) and its epimer in mouse plasma. The method involves solid-phase extraction on cyano cartridges (recovery >75%), HPLC separation on symmetry shield column, a mobile phase of NaH_2PO_4 (10 m*M*) pH 5.2, acetonitrile (47:53) and detection at 227 nm. Retention times of IDN5109, its epiform and internal standard were 15, 24 and 25.5 min, respectively. The assay was linear from 0.10 to 10 μg/ml ($r^2 = 0.999$), with a C.V.<5% and accuracy in the range of 95–107%. LOQ was 50 ng/ml for both compounds.Using this method IDN5109 pharmacokinetic was determined in mice. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Taxane; IDN5109

1. Introduction

IDN5109,13-(*N-tert.*-butoxycarbonyl-β-isobutyl-isoserinyl)-14-hydroxybaccatin-1,14-carbonate (Fig. 1), is a novel paclitaxel analogue synthesized from 14β-hydroxy-10-deacetylbaccatin III, a diterpene present in the needles of Taxus Wallichiana [1,2].

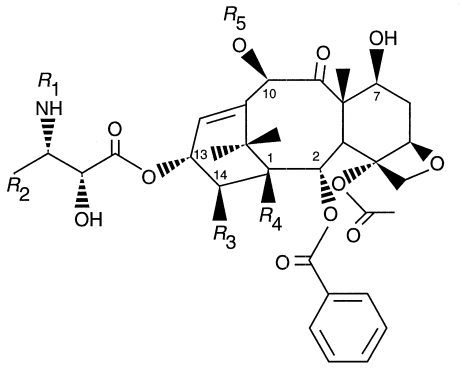
IDN5109 was tested in a panel of different human tumor cell lines and was selected on the basis of the pattern of cytotoxicity superior to that of paclitaxel and docetaxel and the ability to overcome multi drug resistance (MDR) mechanisms [3]. Preclinical investigations on human tumor xenografts in nude mice revealed that IDN5109 has high antitumor activity, also in taxane resistant tumors, and is better tolerated than paclitaxel [4,5]. In addition, IDN5109 is more soluble than the common clinically available taxanes paclitaxel and docetaxel [6].

In order to evaluate the preclinical pharmacokinetic of IDN5109 in mice, we developed an HPLC plasma assay able to determine IDN5109 and its epiform, with good degree of sensitivity, precision

*Corresponding author. Tel.: +39-02-3901-4471; fax: +39-02-354-6277.

E-mail address: zucchetti@irfmn.mnegri.it (M. Zucchetti)

0378-4347/99/$ – see front matter © 1999 Elsevier Science B.V. All rights reserved.
PII: S0378-4347(99)00450-8



	R_1	R_2	R_3 R_4	R_5
	NH	C3'	C14 C1	C10
IDN 5109	t-butoxy-carbonyl	Isobutyl	Carbonate	Acetyl
IDN 5127	t-butoxy-carbonyl	Isobutyl	Thiocarbonate	Acetyl
Paclitaxel	Benzoyl	Phenyl	Hydrogen Hydroxyl	Acetyl

Fig. 1. Chemical structure of IDN5109.

and accuracy. Here we describe the procedure, the validation of the assay and its initial application showing the first pharmacokinetic data of IDN5109 in mice.

2. Experimental

2.1. Chemicals

Analytical reference standard of IDN5109 (lot No. 509/18), 7-epi-IDN5109 (lot No. 507/2) and 13-(*N-tert.*-butoxycarbonyl-β-isobutylisoserinyl)-14β-hydroxybaccatin-1,14-thiocarbonate (IDN5127) (lot No. 186/18) were obtained from Indena (Settala, Italy). Control mice plasma was obtained from Charles River Italia (Calco, Italy). Methanol of HPLC grade was obtained from J.T. Baker (Deventer, The Netherlands). Acetonitrile of HPLC grade was obtained from Carlo Erba (Milan, Italy). NaH_2PO_4 of analytical grade was obtained from Merck (Darmstadt, Germany). Tryethylamine was obtained from Fluka (Buchs, Switzerland). Ultrapure water of HPLC grade was obtained from Milli Ro 60 Water System, Millipore (Milford, MA, USA).

2.2. Instrumentation and materials

A Bench Mate Workstation system for solid-phase extraction from Zymark (Hopkinton, MA, USA) was used to extract the analytes from plasma. The HPLC system consisted of a Model 717 WISP autosampler

and a Model 510 pump from Waters (Milford, MA, USA). The detector used was a spectrophotometer model LC290 at variable-wavelength UV–VIS from Perkin-Elmer (Norwalk, CT, USA). The acquisition system was a Millenium 2010 software for chromatography, Waters.

Sep-pak-CN cartridges (100 mg, 1 ml) for solid-phase extraction were obtained from Waters Associates (Milford, MA, USA). Symmetry shield RP8 HPLC column (3.5 μm, 150×4.6 mm) and pre-column (5 μm, 20×4.6 mm) were obtained from Waters. HPLC filter, 0.4 μm Nucleopore PC membrane filter, was obtained from Nucleopore Italia (Milan, Italy). Disposable borosilicate glass tubes, 16×100 mm, were from Corning (New York, NY, USA). Vials and limited volume insert (for WISP 717 autosampler), were obtained from Waters.

2.3. Animals

The experiment was performed with female CDF1 mice (body weight 20±2 g) obtained from Charles River Italia (Calco, Italy). They were housed and handled according to the institutional guidelines.

2.4. Drug

Vials of IDN5109 (5 ml) formulated in ethanol, Tween 80 and citric acid were provided by Indena (batch No. 1711).

2.5. Preparation of plasma standards

An aliquot of 0.4 ml of control mouse plasma was added with different volume amount of 20 μg/ml methanol solutions of IDN5109 and 7-epi-IDN5109, to produce six concentrations (0.1, 0.25, 0.5, 1.0, 2.5 and 5 μg/ml) and four concentrations (0.1, 0.25, 0.5 and 1.0 μg/ml) of IDN5109 and its epiform, respectively. Each point was prepared in duplicate.

2.6. Preparation of quality control samples

Plasma was divided in three fractions of 20 ml (A, B and C) to prepare quality control (QC) samples for IDN5109. A, B and C were added with IDN5109 to obtain a final plasma concentration of 0.375 μg/ml, 1.875 μg/ml and 3.750 μg/ml, respectively. 7-Epi-IDN5109 was added to fraction A to obtain an unique QC at the final plasma concentration of 0.375 μg/ml.

Several aliquots of the three fractions were stored at −20°C, as a control for future assay and to check the stability under storage conditions.

2.7. Extraction procedure

Plasma samples (0.4 ml) were spiked with 50 μl of IDN5127 (20 μg/ml in methanol) as internal standard (IS) and with 0.7 ml of 0.2 *M* ammonium acetate buffer at pH 4.5. The acid buffer had to be added as quickly as possible to avoid the isomerization of IDN5109 into the epiform that can occur at physiologic pH [7].

After vortex mixing for 10 s, the samples were kept at 4°C for 30 min. After centrifugation at 1500 *g* for 10 min, the samples were processed automatically by the use of the Bench Mate workstation, with Sep-pak-CN cartridges for solid-phase extraction (SPE).

The SPE columns were preconditioned at a flow rate of 0.26 ml/s with 4 ml of methanol and 3 ml of ultrapure water, then with 2 ml of 0.01 *M* ammonium acetate buffer (pH 4.5). A volume of 1 ml of plasma mixture was loaded (flow rate 0.05 ml/s) on the cartridge, then washed at a flow rate of 0.05 ml/s with 2 ml of 0.01 *M* ammonium acetate buffer and with 1.2 ml of ultrapure water.

After drying the cartridge for 120 s with nitrogen, the final elution (flow rate 0.05 ml/s) was performed with 2.5 ml of acetonitrile–triethylamine (1000:1, v/v) into borosilicate tubes.

The eluted solutions were dried under nitrogen and the residue dissolved in 120 μl of mobile phase. The reconstituted samples were vortexed for 1 min, centrifuged at 10 000 *g* for 10 min and a volume of 80 μl of the supernatant was injected using WISP autosampler into HPLC instrumentation for quantitative analysis.

2.8. Chromatographic conditions

HPLC analyses were carried out using a Symmetry Shield RP8, in line with Symmetry shield pre-column using mobile phase of NaH_2PO_4 (10 m*M*) pH 5.2 and acetonitrile 47:53 previously filtered

through 0.45 μm filters and degassed. The flow-rate was 1.2 ml/min and peaks were detected at 227 nm (i.e., λ max). Chromatograms were evaluated from peak heights.

At the end of the daily analyses the HPLC column was washed with acetonitrile–water (1:1) 30 min at the flow-rate of 1.0 ml/min.

2.9. Validation study

Precision and accuracy were evaluated by determining IDN5109 in five replicates of three QC samples at the nominal concentration of 0.375, 1.875 and 3.750 μg/ml (prepared as shown in Section 2.5) on three different days. To assay the QCs, three different standard calibration curves (see Section 2.6) of six plasma concentrations (0.10, 0.25, 0.5, 1, 2.5 and 5 μg/ml) of IDN5109 (four concentrations for 7-epi-IDN5109) were prepared in duplicate and processed as described in Section 2.7.

To check the linearity of the standard curves, the ratio of peak height (expressed as detector response in microvolts) for IDN5109/IS and for 7-epi-IDN5109/IS were plotted versus concentrations. The linearity of the standard curves was determined by a regression model calculating the Pearson's correlation coefficient r^2 and by comparison of the true and back-calculated concentrations of the calibration standards.

The precision of the method at each concentration was expressed as a coefficient of variation (C.V.) by expressing the standard deviation as a percentage of the mean calculated concentration, while the accuracy of the measure was determined by expressing the mean calculated concentration as percentage of the added concentration.

The percentage extraction recovery of IDN5109 was determined at three different plasma concentrations in triplicate 0.20, 1.0 and 5.0 μg/ml (two concentrations for 7-epi-IDN5109: 0.2 and 1.0 μg/ml). Peak-height ratios of analyte/IS of chromatograms obtained from extracted samples, with I.S. added after the extraction at the moment of drying, were compared to those of the external standards prepared in acetonitrile/triethylamine.

The detection limit (LOD) was defined as the concentration at which the signal-to-noise ratio was 3. The quantitation limit (LOQ) was defined as the lowest amount of the analyte which can be determined in a sample with a precision expressed as intra-day C.V.$<10\%$ and an accuracy $>90\%$. An aliquot of 1 ml of control plasma was added with 50 μl of a 1 μg/ml solution of both IDN5109 and 7-epi-IDN5109 to produce a nominal plasma concentration of 50 ng/ml (close to LOD) of the analytes. Five replicates of the obtained samples were processed and analyzed by HPLC according to the previously described procedure together with a freshly prepared standard curve with a blank control in triplicate.

The stability of plasma samples under storage condition (−20°C) was also checked analyzing five replicates of the QCs, prepared on day 1 of the validation study, after one week and after six weeks of permanence under storage conditions. On the day of this last analysis a standard sample at 10 μg/ml was added to increase the upper range of the calibration curve (0.1→10 μg/ml).

2.10. Application of the method

Animals were treated intravenously with 60 mg/kg of IDN5109 diluting the drug vials with saline solution. Blood samples were collected in mice at different times during the first 24 h after IDN5109 administration. Four animals were treated per time point. Blood was obtained from the retro-orbital plexus under diethylether anesthesia and collected in heparinated tubes. The animals were sacrificed by cervical dislocation. The plasma fraction was immediately separated by centrifugation (10 min, 2000 *g*, 4°C) and stored at −20°C until analysis of IDN5109 and its epiform. The IDN5109 and its epiform plasma levels were determined in the samples by using the present methods. The concentration data at each time point represented the mean±standard deviation obtained from four animals. Pharmacokinetic parameters were calculated by using a non-linear fitting program [8]. The experimental 24 h area under the curve of the concentration vs. time points (AUC) of IDN5109 and its epiform were calculated by the trapezoidal rule.

3. Results and discussion

3.1. Chromatography

Fig. 2A shows a typical chromatogram of an extracted mouse plasma sample before drug administration. No interfering substances were present at the retention time of IDN5109 and I.S.. Fig. 2B shows a plasma blank spiked with 1 μg of I.S. Part C shows a chromatogram of a mouse plasma sample taken 2 h after the i.v. treatment with 60 mg/kg of IDN5109 and corresponds to an amount of 7.5 μg/ml of IDN5109. From the chromatogram it appears that the epimerization of IDN5109 to 7-epi-IDN5109 occurs in vivo and amounts to 1.0 μg/ml.

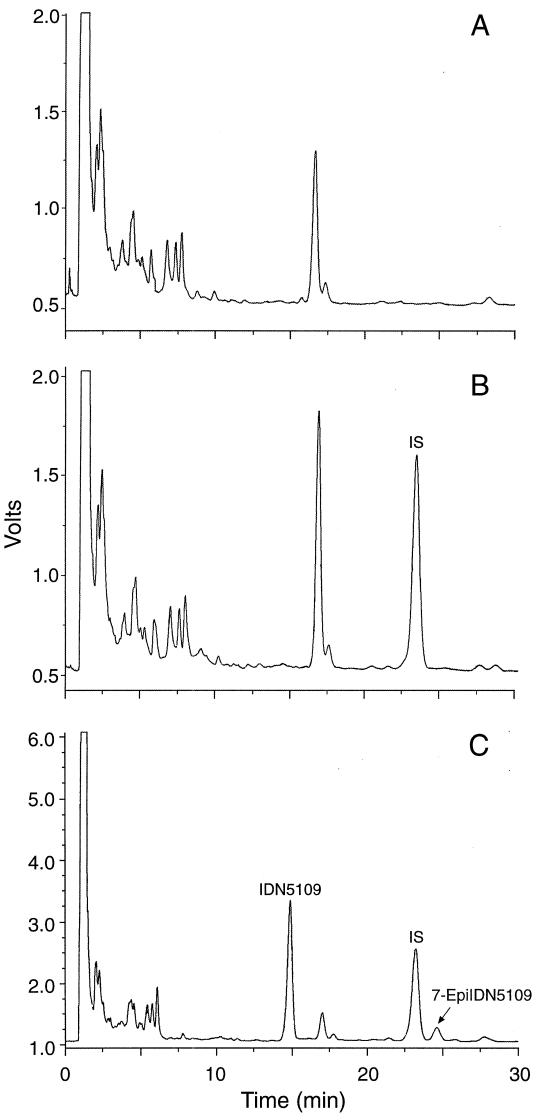


Fig. 2. Chromatograms of a mouse blank plasma sample (part A) and a plasma sample added with I.S., part B. Part C shows a chromatogram of a plasma sample taken 2 h after the i.v. treatment with 60 mg/kg of IDN5109.

The retention time of IDN5109, I.S. and 7-epi-IDN5109 were 15, 24 and 25.5 min, respectively. The chromatographic separation takes a long time, but the conditions described achieve complete separation and good resolution of the three peaks from the plasma matrix.

The identities of the peaks were checked by evaluating the absorbance spectra using a diode-array detector.

3.2. Validation study

Extracted standard curves made for IDN5109 at the concentrations of 0.1, 0.25, 0.5, 1.0 and 5.0 μg/ml showed linearity, with Pearson's coefficient of correlation r^2 always more than 0.999 (see Table 1). The r^2 for the 7-epi derivative was more than 0.990 (Table 2). A model also including the quadratic term was evaluated, but the result did not show a significant improvement of the fitting. The tables also report the results of the calibration curves accuracy in the three days of the validation study. Mean accuracy values were always around 100% for

Table 1
Correlation coefficients r^2 and comparison of true and back-calculated standards of the IDN5109 plasma calibration curves in the three days of the validation study

Day	Calibration curve accuracy (%)[a]						r^2
	0.1[b]	0.25	0.5	1.0	2.5	5.0	
1	89.0	93.0	98.8	104.2	99.7	99.8	0.9997
2	110.0	98.9	96.8	103.2	98.5	100.3	0.9997
3	100.1	109.4	106.6	94.6	99.6	100.4	0.9992
Mean	99.7	100.4	100.7	100.7	99.3	100.2	
±SD	10.5	8.3	5.2	5.3	0.7	0.3	
C.V.%	10.5	8.3	5.1	5.2	0.7	0.3	

[a] Accuracy = calculated conc./theoretical conc.) × 100.
[b] Theoretical concentration of standards (μg/ml).

Table 2
Correlation coefficients r^2 and comparison of true and back-calculated standards of the 7-epi-IDN5109 plasma calibration curves in the three days of the validation study

Day	Calibration curve accuracy (%)[a]				r^2
	0.1[b]	0.25	0.5	1.0	
1	91.5	98.0	103.2	99.0	0.9968
2	114.8	97.2	92.9	100.8	0.9979
3	96.4	101.0	100.0	100.0	0.9920
Mean	100.9	98.7	98.7	99.9	
±SD	12.3	2.0	5.3	0.9	
C.V.%	12.2	2.0	5.3	0.9	

[a] Accuracy = calculated conc./theoretical conc.) × 100.
[b] Theoretical concentration of standards (μg/ml).

both the analytes, and C.V. was in the range 0.3–10.5% and 0.9–12.2% for IDN5109 and the epi-derivative, respectively.

The reproducibility of the method was evaluated analyzing five replicates of three QC samples containing IDN5109 at the nominal concentrations of 0.375, 1.875 and 3.750 μg/ml in three different days using extracted standard curves made in the range 0.1– 5 μg/ml. The intra- and inter-day precision and accuracy are reported in Table 3. The method was

Table 3
Summary of intra- and inter-assay precision and accuracy data for IDN5109 and 7-epi-IDN5109 (values in brackets)

Day	*n*	Mean observed	C.V. %	Accuracy % nominal conc.[a]
1	4	0.395	1.9	105.3
	(5)	(0.387)	(6.3)	(103.2)
	5	1.860	3.8	99.2
	5	3.910	1.0	104.3
2	4	0.400	2.2	106.7
	(4)	(0.382)	(2.3)	(102.0)
	5	1.947	1.9	103.8
	5	3.747	1.4	99.9
3	5	0.385	3.6	102.7
	(4)	(0.369)	(6.5)	(98.4)
	4	1.960	2.5	104.5
	5	3.855	2.2	102.8
Overall	13	0.392	3.1	104.7
	14	1.920	3.6	102.4
	15	3.835	2.3	102.3

[a] QC nominal conc. 0.375, 1.875 and 3.750 μg/ml (prepared on day 1).

Table 4
Extraction recovery of IDN5109 from mouse plasma

Added conc. (μg/ml)	Recovery[a] (%)	C.V. (%)
0.2	85.6	2.7
1.0	78.5	2.9
5.0	83.8	1.1

[a] Each value is the mean of three determinations.

found to be highly precise, with a C.V. ≤ 5% and highly accurate >99% and <107% for each of the concentrations tested.

As shown in Table 4, the mean extraction recovery for IDN5109 performed at three representative concentrations over the calibration range: 0.2, 1.0 and 5.0 μg/ml, is around 80% with a reproducibility expressed as C.V. in the range 1–3%. The recovery of the epi-derivative determined at 0.2 and 1.0 μg/ml were of 77.7±1.2% and 76.3±2.3%.

The LOD was defined as the concentration at which the signal-to-noise ratio was 3. The mean of the noise recorded in chromatograms of blank plasma samples in the intervals between 14 and 16 min and between 23 and 26 min (comprising the retention times of both the analytes and I.S.) was equivalent to an amount of 15 ng/ml of analyte, the resulting LOD is 45 ng/ml. The LOQ was fixed at 50 ng/ml, at this concentration (very close to the LOD) the within-day C.V. and accuracy were 6.1% and 108% for IDN5109, 5.4% and 92% for its epiform.

The drug appears stable in frozen plasma samples, in fact more than 94% of IDN5109 were found in samples after 6 weeks of permanence at −20°C, as checked by analyzing five replicates of three QC plasma samples containing IDN5109 at the nominal concentrations of 0.375, 1.875 and 3.750 μg/ml. On this day the calibration curve made in the range 0.1 – 10 μg/ml maintained great linearity ($r^2 = 0.9991$) and was superimposable to those obtained in the 3 days of the validation study.

3.3. Application of the method

Fig. 3 shows the pharmacokinetic profile of IDN5109 in plasma of CDF_1 mice after i.v. administration. The drug disappears from plasma according to a two compartment open model with a distribution

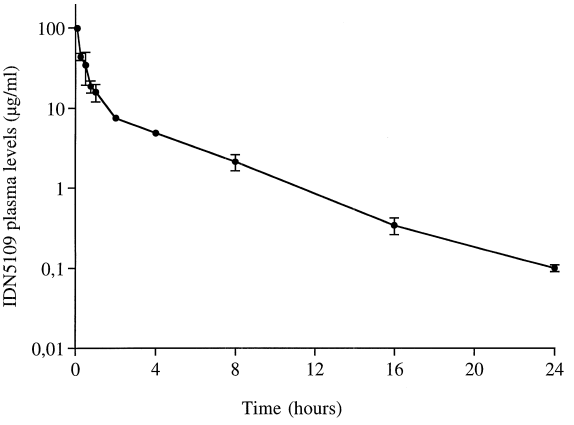


Fig. 3. IDN5109 plasma decay curves obtained in CDF1 mice after an intravenous dose of 60 mg/kg of drug. Each point represented the mean of the concentrations obtained from four animals. Bars are s.d.; not visible when smaller than symbols.

and elimination half-life of 16 min and 3.5 h, respectively.

IDN5109 was still detectable at 24 h at levels higher than the LOQ. In addition, we determined the 7-epiform of IDN5109 that was detectable in plasma during the first 4 h after the treatment. Its AUC is ten times lower than the AUC of the parent drug.

4. Conclusion

This report describes a method to measure IDN5109 and its epiform in mouse plasma. It has a sufficient degree of selectivity and a good sensitivity, precision and accuracy. It should be noted that the method is rapid requiring solid-phase extraction procedure and HPLC analysis. We applied successfully the assay evaluating the pharmacokinetic profile of IDN5109 and its 7-epiform in mice.

References

[1] G. Appendino, P. Gariboldi, B. Gabetta, R. Pace, E. Bombardelli, D. Viterbo, J. Chem. Soc. Perkin. Trans. 1 (1992) 2925.
[2] I. Ojima, J.C. Slater, S.C. Kuduk, C.S. Takeuchi, R.H. Gimi, C. Sum, Y. Park, P. Pera, J.M. Veith, R.J. Bernacky, J. Med. Chem. 40 (1997) 267.
[3] M. Distefano, G. Scambia, C. Ferlini, C. Gaggini, R. De Vincenzo, A. Riva, E. Bombardelli, I. Ojima, A. Fattorossi, P.B. Panici, S. Mancuso, Int. J. Cancer 72 (1997) 844.
[4] C. Rossi, T. Mincione, I. Nicoletti, E. Bombardelli, A. Riva, M.B. Donati, R. Giavazzi, Ann. Oncol. 9 (Suppl. 2) (1998) 92.
[5] D. Polizzi, G. Pratesi, M. Tortoreto, R. Supino, A. Riva, E. Bombardelli, F. Zunino, Cancer Res. 59 (1999) 1036.
[6] G. Pratesi, D. Polizzi, A. Riva, F. Zunino, in: Proceedings of the 89th Annual Meeting of the American Association for Cancer Research, March 1998, 1998, p. 164.
[7] J.L. McLaughlin, R.W. Miller, R.G. Powell, C.R. Smith Jr., J. Nat. Prod. 44 (1981) 312.
[8] G. Sacchi-Landriani, V. Guardabasso, M. Rocchetti, Comput. Programs Biomed. 16 (1983) 35.