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Short communication

Development and validation of a LC–MS/MS method for the determination of the novel oral 1,14 substituted taxane derivatives, IDN 5738 and IDN 5839, in mouse plasma and its application to the pharmacokinetic study

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ABSTRACT

Two LC-ESI–MS and CID-MS/MS methods were developed and validated for pharmacokinetic studies of the novel oral taxane derivatives IDN 5738 and IDN 5839, used for preclinical evaluation in mice. The analysis requires 100 μL of plasma sample, involves the addition of an internal standard and protein precipitation with 0.1% HCOOH in acetonitrile. The HPLC separation was obtained on Sunfire C18 column and Selected Reaction Monitoring technique was used to quantify the taxanes. The recoveries were more than 90%; the methods were linear over the validated concentrations range of 25–1500 ng/mL for IDN 5738 and 25–5000 ng/mL for IDN 5839 and had a limit of detection of 0.14 and 0.25 ng/mL, respectively. The inter-day coefficient of variation (CV%) of the calibration standards ranged between 1.3 and 7.2% for IDN 5738 and between 0.0 and 9.0% for IDN 5839 and the mean accuracy was in the range 85.3–112.0% for IDN 5738 and between 80.0 and 111.0% for IDN 5839. Moreover, analysing quality control plasma samples on three different days, the methods resulted precise and accurate showing intra- and inter-day CV within 12% for both analytes, and accuracy of 92.0–113.3% and 85.9–105.7% for IDN 5738 and IDN 5839, respectively. With these methods, we studied for the first time, the pharmacokinetics of the two taxanes showing for both, good oral bioavailability (>50%).

1. Introduction

Paclitaxel (PTX) and docetaxel (DTX) are among the leading anti-cancer drugs in clinical use today [1,2] for the therapy of several human neoplasms including breast, ovarian and lung cancer. Both drugs are substrates of P-glycoprotein (P-gp) and this explains why P-gp over-expressing tumors are resistant to them [3,4]. The very low bioavailability of PTX and DTX is also related to the expression of P-gp in the gastro-intestinal epithelial cells [5] as demonstrated by the data that inhibitors of P-gp, block the efflux of taxanes from intestinal cells, thus increasing the absorption of these compounds [6]. These considerations explain why it is of great interest to develop new taxanes with a low binding affinity for P-gp and therefore possibly not cross resistant to PTX and DTX and that would possess good bioavailability. The oral route for a taxane is of clinical interest, not only because convenient for patients as well as reduced costs, but also because prolonged administration of low doses of taxanes may be clinically very effective and may possibly involve mechanisms other than anti-mitotic, e.g. anti-angiogenesis [7]. In this perspective, several taxane derivatives have been screened and the research conducted to ortataxel (Fig. 1A) [8]. This compound displayed an excellent cytotoxicity and a wider therapeutic window, along with a much higher potency than PTX against drug-resistant tumors [9,10]. Moreover, ortataxel was found to be highly active when given orally with good bioavailability [9,10]. For these reasons, ortataxel entered phase I–II clinical trials showing activity in heavily pre-treated metastatic breast and non-small cell lung cancer patients [11,12]. With the aim of further improving the pharmacological properties of ortataxel, a new family of taxanes was synthesized generating a series of compounds modified in the C14 position [13]. Two of these, IDN 5738 and IDN 5839 (Fig. 1B and C, respectively), based on their cytotoxic activity, in particular on MDR-cell lines, were selected for the subsequent preclinical evaluation [14].

The aim of this study was to develop and validate an assay to determine the two selected taxanes in mouse plasma and to apply it to characterize the pharmacological profile and the bioavailability of the compounds in mice. For this purpose, the developed methods were based on high-performance liquid chromatography coupled

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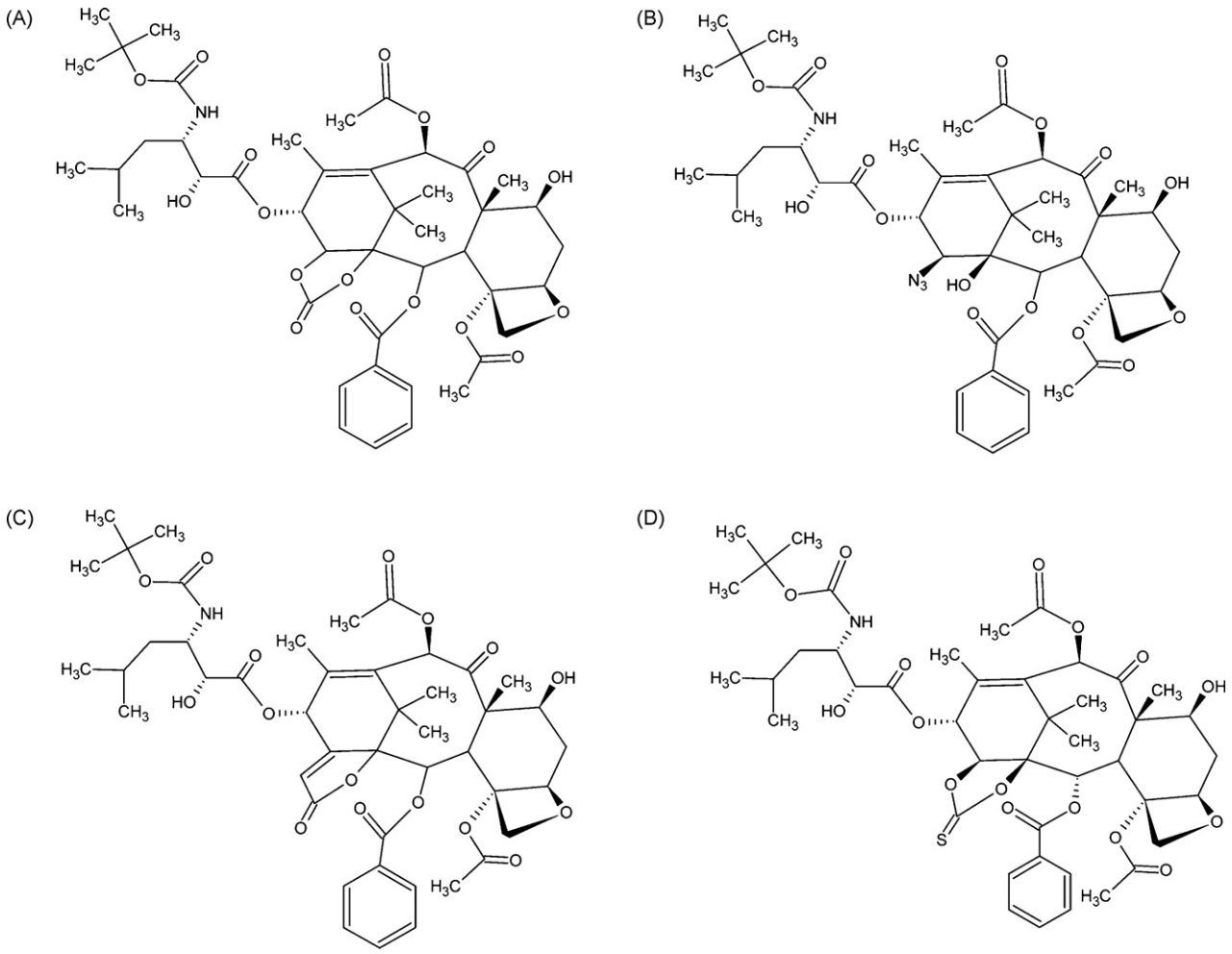

Fig. 1. Chemical structure of ortataxel (A), IDN 5738 (B), IDN 5839 (C) and IDN 5127 (D).

to electrospray ionization low-energy collision tandem mass spectrometry (LC-ESI–CID-MS/MS) technique, because of its success in pharmacokinetic studies with taxanes [15–23] and due to its high sensitivity and specificity. The methods require 100 μL of plasma sample, a simple treatment with acetonitrile and a reasonable time of analysis. Moreover, high selectivity and sensitivity are guaranteed by working in Selected Reaction Monitoring (SRM) mode.

2. Experimental

2.1. Standards and chemicals

IDN 5738 (lot # 624/12, ba 02/957/LR), IDN 5839 (lot # 638/41) and IDN 5127 (internal standard, IS), Fig. 1D (ba 186/18-CoA n.91/061/LR) were obtained from Indena S.p.A., Settala, Milan, Italy.

Control mouse plasma (lot 09-02/03/05) was obtained from Charles River Italia.

Methanol, acetonitrile and formic acid of HPLC grade were obtained from Carlo Erba, Milan, Italy.

Water of HPLC grade was obtained from Milli-Ro 60 Water System, Millipore, Milford, MA, USA.

2.2. Standard and QC solutions

For standards, a stock solution for each analyte was prepared at the concentration of 101.2 μg/mL for IDN 5738 and at 102.4 μg/mL for IDN 5839. For QCs, a stock solution of each analyte was prepared at 100.0 μg/mL. The IS stock solution was prepared at 116.2 μg/mL. All stock solutions were prepared in methanol and stored at −20 °C.

Working solutions to obtain the standard points of the calibration curve and the working solutions to prepare the plasma QC samples (L, M and H), for both the analytes, were obtained by combining different amounts of the stock solutions and further diluted with methanol to obtain IDN 5738 and IDN 5839 at the final concentrations of 0.25, 0.5, 1, 5, 15 μg/mL for IDN 5738 and 0.25, 0.5, 1, 5, 10, 20, 50 μg/mL for IDN 5839.

The IS working solution was prepared at 10 μg/mL by diluting the stock solution with methanol.

2.3. Preparation of standards and QC samples

Control mouse plasma aliquots (90 μL) were spiked with 10 μL of each working solution to obtain a final dilution of 1:10, giving six calibration standards in the range 0.025–1.500 μg/mL for IDN 5738 and seven in the range 0.025–5.000 μg/mL for IDN 5839.

To prepare QC samples, three fractions of plasma were added with an appropriate amount of QC solutions, obtaining QC samples at the final concentrations of 0.075, 0.600 and 1.500 μg/mL for IDN 5738 and 0.075, 1.500 and 4.000 μg/mL for IDN 5839.

Several aliquots of the three fractions were stored at −20 °C as controls for future assays and to check the short term stability under storage conditions. The analytes were considered stable at each concentration when the differences between the freshly pre-

pared samples and the stability testing samples were found to be not exceeding 15% of the nominal concentration.

2.4. Sample preparation

Mouse plasma samples (100 μL) were mixed with 10 μL (100 ng) of IS working solution and 200 μL of 0.1% HCOOH in CH_3CN. After vortexing for 10 s, the mixture was centrifuged at 4 °C for 10 min at 13,000 rpm. The supernatant was recovered and 5 μL were injected into the LC–MS/MS system.

2.5. Chromatographic conditions

The HPLC system consisted of an Alliance separation module 2695 (Waters, Milford, MA, USA).

For both methods, the samples were separated on a SunFire C18 column 3.5 μm, 150 mm × 2.1 mm coupled with a C18 precolumn, 4 mm × 3 mm (Waters) held at 30 °C. The mobile phases for the chromatographic separation were composed of 0.1% HCOOH in water (MP A) and 0.1% HCOOH in acetonitrile (MP B). The HPLC system was set up to operate at a flow of 0.2 mL/min at the following gradient conditions: step 1—from the initial condition of 50% MP B to 95% over 4 min; step 2—maintenance of 95% MP B over 2 min; step 3—from 95% MP B to 100% over 4 min; step 4—maintenance of 100% MP B over 1 min; step 5—from 100% MP B to the initial condition over 2 min; the initial condition was held for 7 min. At the end of the daily analyses, the HPLC column was washed 30 min with CH_3OH/H_2O (1:1) at the flow of 0.2 mL/min.

2.6. Mass spectrometry

The HPLC system was coupled with a Micromass Quattro Ultima Pt triple-quadrupole mass spectrometer (Waters).

The mass spectrometer operated in negative ion mode and was used to obtain both the mass spectra (MS^1) and the product ion spectra (MS^2). The instrument was equipped with an electrospray ionization interface using argon as collision gas. The biological samples were analysed with the ion spray needle operating at −2500 V and the cone voltage at 70 V. The mass spectrometer was programmed to perform low-energy collisions SRM analysis. This was done by selecting the precursor ions $[M + HCOO]^-$ of IDN 5738, IDN 5839 and IDN 5127 at *m/z* 915, 912 and 932 respectively, in the first quadrupole and by monitoring the characteristic CID product ions of these three compounds in the third quadrupole [*m/z* 583 (19.0 eV), 523 (21.0 eV) and 260 (19.0 eV) for IDN 5738; *m/z* 806 (16 eV), 623 (17.0 eV) and 563 (30.0 eV) for IDN 5839; *m/z* 826 (19.0 eV) and 583 (20.0 eV) for IDN 5127]. The fragmentation patterns are reported in Fig. 2.

2.7. Validation study

These methods were set up to quantify two taxane compounds in a preclinical explorative pharmacokinetic study so it was applied a short validation protocol performed over 3 days. Two validation studies were performed, one for each compound. The linearity of the calibration curves was validated over 3 days and calculated by the ratio of the ESI-CID-MS/MS peak areas for IDN 5738/IS and IDN 5839/IS to the nominal concentration of IDN 5738 and IDN 5839 in the sample. The linearity of the standard curves was determined by a regression analysis and the goodness of the regression by calculating the Pearson's determination coefficient R^2 and by comparison of the true and back-calculated concentrations of the calibration standards.

Precision and accuracy were evaluated on three different days by determining the analytes in three replicates of three QC samples at the nominal concentrations of 0.075, 0.600 and 1.500 μg/mL for IDN 5738 and 0.075, 1.500 and 4.000 μg/mL for IDN 5839. A standard calibration curve was prepared and processed each day to analyse the QC samples.

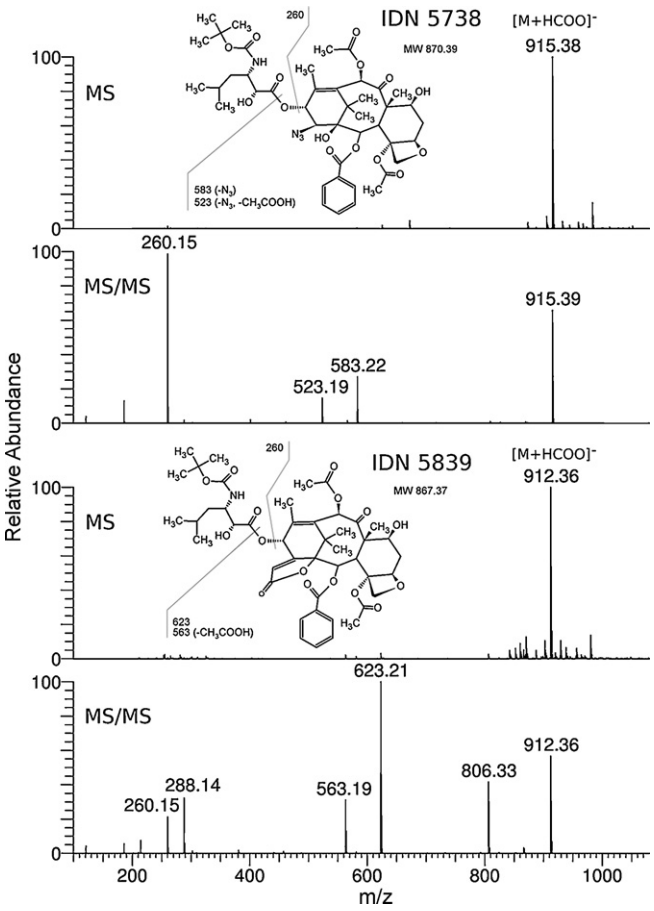

Fig. 2. High resolution MS and MS/MS mass spectra of IDN 5738 and IDN 5839, with chemical structures and identification of the main fragment ions.

The precision of the method at each concentration was reported as a coefficient of variation (CV%), 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 nominal concentration.

The detection limit (LOD) was defined as the concentration at which the signal-to-noise ratio was 3. The limit of quantitation (LOQ) was defined as the smallest amount of the analyte that could be measured in a sample with sufficient precision (±15%) and accuracy (range 80–120%).

The percentage extraction recovery of the two analytes was calculated in triplicate and at two plasma concentrations (0.100 and 1.000 μg/mL) for IDN 5738 and at three (0.100, 1.000 and 5.000 μg/mL) for IDN 5839. The recovery was estimated by comparing peak-area ratios of both analytes from extracted plasma samples to those from external standards prepared in methanol. It was also ensured the absence of the matrix effect that could influence the ionization of IDN 5738 and IDN 5839.

The stability of the taxanes was evaluated analysing QC samples in triplicate at each QC concentration level, immediately after preparation and after approximately 1 month of storage at −20 °C. The analytes were quantified in the stored samples, using a freshly prepared calibration curve.

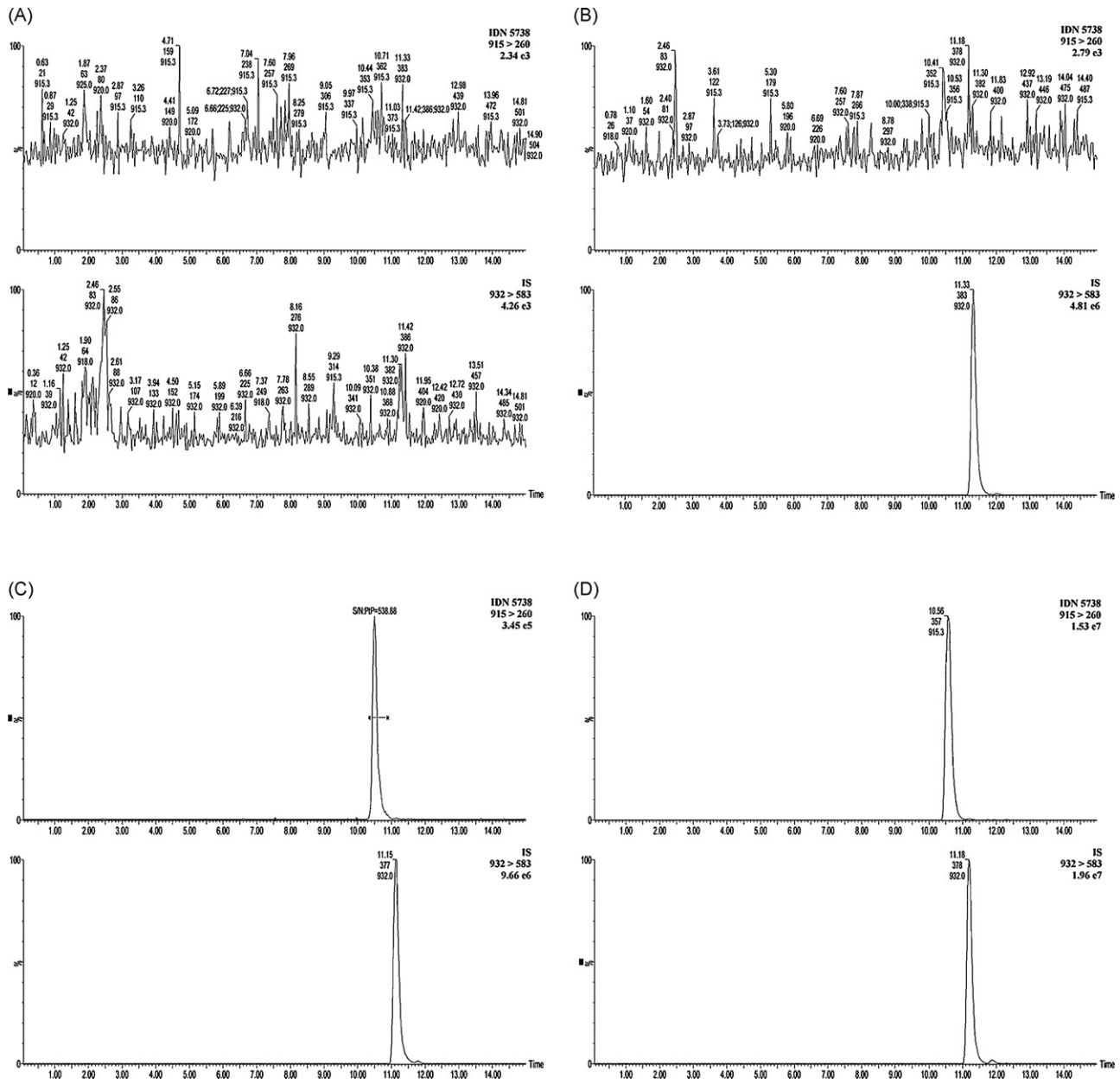

Fig. 3. (A) SRM chromatograms of a mouse blank plasma sample; (B) SRM chromatograms of a mouse blank plasma with IS added; (C) signal-to-noise ratio of IDN 5738 at LOQ concentration (25 ng/mL); (D) SRM chromatograms of an extracted plasma sample of a treated mouse showing IDN 5738 and IS. The measured concentration was 70.7 μg/mL.

2.8. Application of the method: bioavailability study of IDN 5738 and IDN 5839 in mice

The experiments were carried out in 8–10 weeks old female CD1 mice (body weight 25 ± 2 g) purchased from Charles River Italia. They were housed and handled according to the institutional guidelines (Legislative Decree 116 of Jan. 27, 1992. Authorisation n.169/94-A issued Dec. 19, 1994 by Ministry of Health) and international laws and policies (EEC Council Directive 86/609, OJ L 358. 1, Dec. 12, 1987; Standards for the Care and Use of Laboratory Animals, United States National Research Council, Statement of Compliance A5023-01, Nov. 6, 1998).

The compounds were dissolved in Tween 80/ethanol (1:1) and diluted with saline to a concentration of 6 mg/mL.

To determine the pharmacokinetic profile and the bioavailability of IDN 5738 and IDN 5839, each taxane was administered by gavage or injected in the tail vein of the mice at the dose of 60 mg/kg. For IDN 5738 study, blood samples were taken at 5, 15, 30 min and 1, 2, 4, 8, 16 and 24 h after i.v. administration and at 15, 30, 45 min and 1, 1.5, 2, 4, 8, 16 and 24 h after oral treatment while for IDN 5839, the samples were collected at the same times but up to 10 h. Blood was obtained from the retro-orbital plexus under isoflorane anesthesia and collected in heparinized tubes. After centrifugation at 2500 rpm at 4 °C for 10 min, plasma was separated and stored frozen at −20 °C until analysis. Three mice were used for time point and animals were sacrificed by cervical dislocation.

2.9. Pharmacokinetic analysis

The pharmacokinetic parameters were calculated by using the software WinNonLin Pro Node 4.1 (Pharsight, Mountain View, Ca,

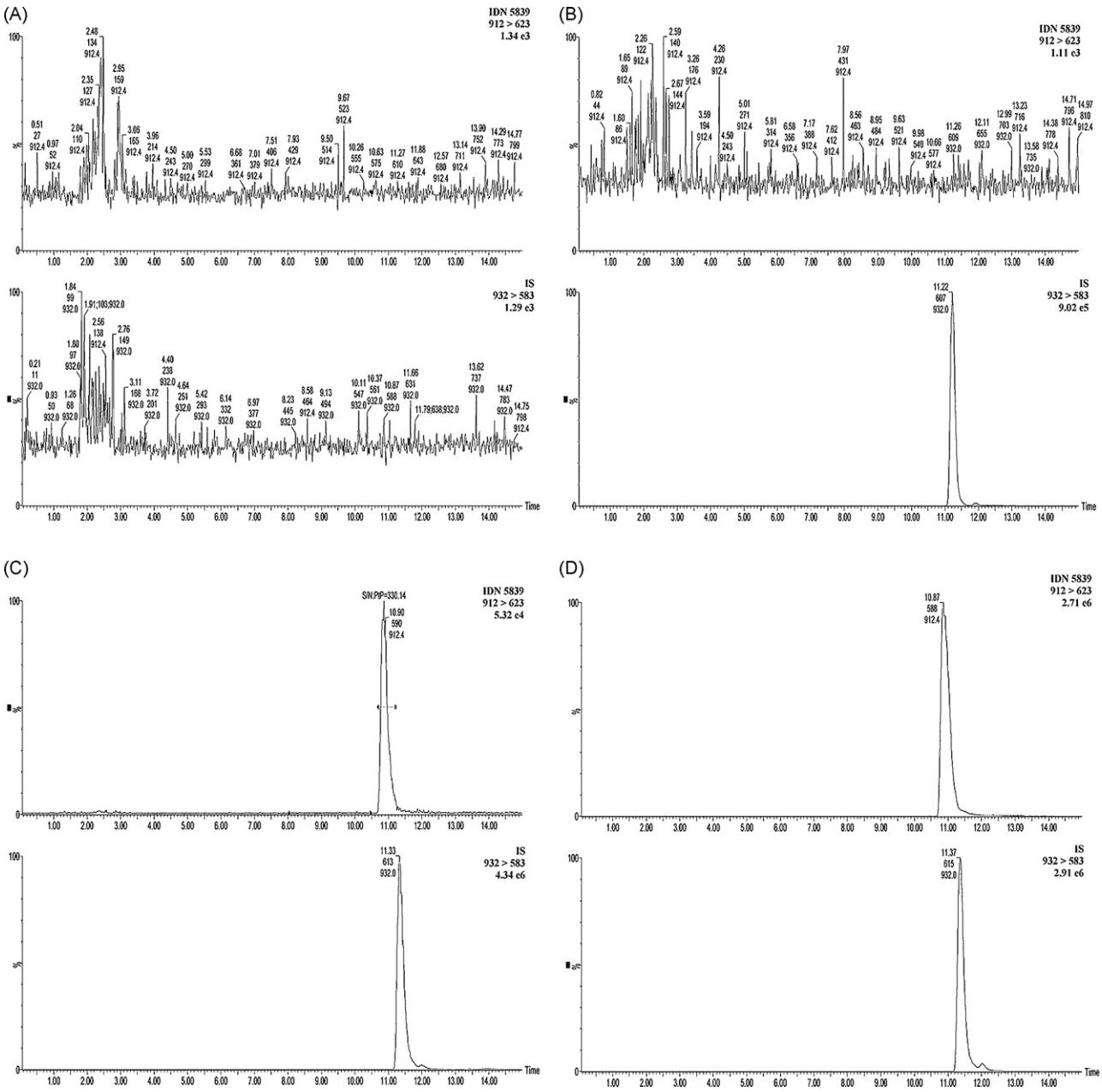

Fig. 4. (A) SRM chromatograms of a mouse blank plasma sample; (B) SRM chromatograms of a mouse blank plasma with IS added; (C) signal-to-noise ratio of IDN 5839 at LOQ concentration (25 ng/mL); (D) SRM chromatograms of an extracted plasma sample of a treated mouse showing IDN 5839 and IS. The measured concentration was 57.8 μg/mL.

USA). A non-compartmental analysis was applied. The parameters considered were: C_{max} (maximum plasma concentration), AUC (area under the concentration–time curve from time 0 to the last detectable sample), $T_{1/2}$ (plasma half-life in the terminal phase), Cl_p (plasma clearance defined as dose/AUC) and the bioavailability after oral administration ($F = AUC_{p.o.}/AUC_{i.v} \times 100$).

3. Results and discussion

3.1. LC-ESI/MS and CID-MS/MS analyses

In Figs. 3 and 4 (panel C) are reported the typical SRM chromatogram for IDN 5738 and IDN 5839 respectively, showing the quantifier transition of IDN 5738 and IDN 5839 for an extracted mouse plasma standard sample at LOQ, containing the compounds at the concentration of 25 ng/mL and IS at 1 μg/mL in comparison with a chromatogram of a blank plasma sample (panel A) and a blank plasma sample with IS added (panel B).

The elution of the analytes was selective with a good separation of peaks in about 11 min (IDN 5738 at 10.6 min, IDN 5839 at 10.9 and IS at 11.2). The relative length of the elution was necessary to avoid the cross talking phenomenon caused by a common fragment of the IS and one of the analytes (*m*/*z* 583), which was due to the particular characteristics of the collision cell of the mass spectrometer. No interfering peaks were present at the retention times and the peaks of all compounds were completely resolved from the plasma matrix.

IDN 5738, IDN 5839 and IS were quantified using the transition *m*/*z* 915 > 260, *m*/*z* 912 > 623 and *m*/*z* 932 > 583, respectively.

As final example, panel D of Figs. 3 and 4 shows the SRM chromatograms of two mouse plasma sample taken 5 min after the intravenous treatment with the compounds. They correspond to

Table 1
Intra- and inter-day validation of the method for quantitative determination of IDN 5738 and IDN 5839.

	IDN 5738 theoretical concentration (μg/mL)			IDN 5839 theoretical concentration (μg/mL)		
	0.075	0.600	1.500	0.075	1.500	4.000
Intra-day						
Day 1						
Mean ($N=3$)	0.076	0.553	1.117	0.068	1.528	3.505
CV%	5.7	2.3	1.0	3.7	4.0	2.5
Accuracy%	101.3	92.2	93.1	91.1	101.9	87.6
Day 2						
Mean ($N=3$)	0.085	0.582	1.183	0.076	1.586	3.527
CV%	2.0	1.8	11.2	3.3	4.6	2.9
Accuracy%	113.3	97.1	98.6	100.9	105.7	88.2
Day 3						
Mean ($N=3$)	0.084	0.552	1.252	0.074	1.429	3.436
CV%	2.2	8.2	2.8	3.4	1.8	2.0
Accuracy%	112.0	92.0	104.3	99.1	95.2	85.9
Inter-day						
Mean ($N=9$)	0.082	0.562	1.184	0.073	1.514	3.489
CV%	5.8	5.5	7.4	5.5	5.6	2.4
Accuracy%	109.2	93.6	99.2	97.0	100.9	87.2

a concentration of 70.7 μg/mL of IDN 5738 and 57.8 μg/mL of IDN 5839.

3.2. Calibration curves

The accuracy and precision for each analyte were determined at each day of the two validation studies.

The peak-area ratios of analyte/IS versus the nominal concentrations were plotted and a least-squares linear regression weighted by the reciprocal of the concentrations was applied to generate the calibration curves. The calibration curves, prepared on three different days, showed good linearity and acceptable data over a wide range of concentrations (0.025–1.500 μg/mL for IDN 5738 and 0.025–5.000 μg/mL for IDN 5839), with R^2 equal to or better than 0.996. Mean accuracy was in the range 85.3–112.0% for IDN 5738 and between 80.0 (at LOQ) and 111.0% for IDN 5839. The precision expressed as CV% was in the range 1.3–7.2% for IDN 5738 and 0.0–9.0% for IDN 5839.

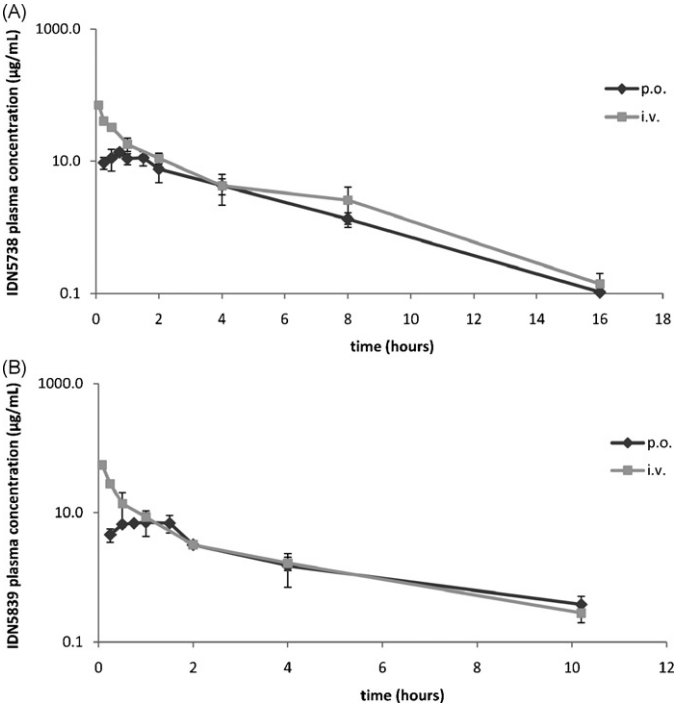

Fig. 5. IDN 5738 (panel A) and IDN 5839 (panel B) plasma decay curves after intravenous or p.o. administrations of a dose of 60 mg/kg of the compounds in CD1 female mice.

3.3. Precision, accuracy and LOQ

The precision and accuracy of the two methods were evaluated analysing three replicates of the QCs at 0.075, 0.600 and 1.500 μg/mL for IDN 5738 and 0.075, 1.500 and 4.000 μg/mL for IDN 5839, within a single-run analysis, for intra-day study and over three consecutive runs for inter-day study. The accuracy and precision for the analytes are shown in Table 1. The methods were precise and accurate, with intra- and inter-day CV ≤12% and accuracy in the range of 92.0–113.3% for IDN 5738 and 85.9–105.7% for IDN 5839.

The LOQ was defined as the lowest concentration that could be measured with a precision within 15% and accuracy between 80 and 120%. Consistent with our aims, we set the LOQ at 25.0 ng/mL, validated through three replicates. The intra-day CV% and accuracy were respectively 7.9 and 96.8% for IDN 5738 and 9.6 and 103.2% for IDN 5839. It is to note that, as shown in panel C of Figs. 3 and 4, because of the high signal-to-noise ratio (>500 for IDN 5738 and >300 for IDN 5839), the possibility to find a lower LOQ could have been verified for both the analytes being LODs of 0.14 and 0.25 ng/mL for IDN 5738 and IDN 5839, respectively.

3.4. Recovery

The recovery was evaluated in triplicate and over 2 or 3 concentrations for IDN 5738 and IDN 5839, respectively. The matrix effect, at each concentration level, was determined by comparing the mean area of the analytes prepared in solvent with the same quantity of the taxanes added to biological matrix, after the entire procedure of recovery. We verified the absence of significant variations (<15%) for the areas of both the analytes, so it was possible to exclude any matrix effect of ion suppression or enhancement. As result, the mean extraction recovery for IDN 5738 at 0.100 and 1.000 μg/mL was >96%, with good reproducibility indicated by the CV <3%. The recovery of IDN 5839 at 0.100, 1.000 and 4.000 μg/mL was higher than 90% with reproducibility expressed as CV ≤14%.

3.5. Stability in frozen matrix

The stability of the two taxanes was assessed by analysing QC samples at concentrations of 0.075, 0.600 and 1.500 μg/mL for IDN 5738 and 0.075, 1.500 and 4.000 μg/mL for IDN 5839. The results obtained for the short term stability in frozen matrix let us conclude that they are stable over 1 month in mouse plasma at −20 °C. For IDN 5738, the accuracy was in the range 99.1–109.4% and the CV% between 6.1 and 12.2% while for IDN 5839, the accuracy and the CV% were in the range 85.1–100.9% and 0.0–12.8%, respectively.

3.6. Application of the methods: pharmacokinetic results

Fig. 5 (panels A and B) reports the plasma concentration–time profiles of IDN 5738 and IDN 5839 in CD1 mice after i.v. and p.o. administration of 60 mg/kg of the novel taxanes. Plasma samples from 5 min to 1 h after i.v. treatment with IDN 5738 and at 5 min after administration of IDN 5839, in which the concentrations were superior to the highest standard point of the calibration curve, were suitably diluted with control plasma and re-analysed. Following the intravenous bolus of IDN 5738, a C_{max} of 71.2 μg/mL was achieved and after a rapid distribution phase the compound was eliminated with $T_{1/2}$ of 2.2 h and Cl_p of 0.6 L/h/kg. After oral treatment, the compound was rapidly absorbed achieving at 45 min a C_{max} of 13.7 μg/mL and eliminated with the same $T_{1/2}$ observed after the intravenous treatment. A comparison between the AUCs obtained after oral and intravenous treatment, revealed good absorption being the bioavailability of 53.1%. Similar results were obtained for IDN 5839, that after intravenous bolus achieved a C_{max} of 55.2 μg/mL and it was eliminated with $T_{1/2}$ of 2.4 h and Cl_p of 1.5 L/h/Kg. After oral administration, it achieved within 1 h a C_{max} of 7.1 μg/mL, then plasma concentration declined with $T_{1/2}$ of 2.8 h; the bioavailability was 56.1%.

Our data defined for these taxanes an interesting pharmacokinetic profile superimposable with that of ortataxel particularly after oral route [9]. They were rapidly absorbed with bioavailability higher than 50% and plasma concentrations that are consistent with those indicated active in cytotoxic studies on mammary and lung cancer cell lines sensitive and resistant to paclitaxel [14]. These concentrations were maintained in plasma of mice up to at least 16 and 10 h after the administrations of IDN 5738 and IDN 5839, respectively.

4. Conclusion

The analytical procedure here described, is based on a simple protein precipitation and LC separation followed by ESI-CID-MS/MS analysis (SMR technique), and allows the determination of the novel taxane derivatives, IDN 5738 and IDN 5839, in mouse plasma. Both methods require 100 μL of sample and they are selective, sensitive, precise and accurate. The methods were successfully employed to measure plasma concentrations of the two compounds in mice and to study their preclinical pharmacokinetics. The studies showed that the novel taxanes possess favorable pharmacokinetics and good oral bioavailability (>50%).

Based on these findings, we believe that IDN 5738 and IDN 5839 are good candidates for further preclinical and clinical development.

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