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Determination of Aplidin®, a marine-derived anticancer drug, in human plasma, whole blood and urine by liquid chromatography with electrospray ionisation tandem mass spectrometric detection

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Abstract

A sensitive and highly specific liquid chromatographic method with electrospray ionisation tandem mass spectrometric detection (LC–ESI–MS/MS) is reported for the determination in human plasma, whole blood and urine of Aplidin® (APL), a novel depsipeptide derived from the tunicate *Aplidium albicans* with a potent cytotoxic activity under investigation in clinical studies. Didemnin B was used as internal standard and, after protein precipitation with acetonitrile and liquid–liquid extraction with chloroform, APL was separated by liquid chromatography using a reversed-phase C_{18} column and a linear gradient of acetonitrile in water (both containing 0.5% formic acid). Detection was performed using a turboionspray source operated in positive ion mode and by multiple reaction monitoring (MRM; m/z 1111 $\rightarrow$ 295 for APL and m/z 1113 $\rightarrow$ 297 for didemnin B). The method was linear ($r \geq 0.9933$) over the range 1–250 ng/ml, with intra- and inter-batch precision and accuracy below 12.2% (except at LLOQ, $\leq$15.4%) for both plasma and urine. Recoveries were moderate, ranging from 54 to 70% in plasma and blood, and from 46 to 60% in urine, for both APL and didemnin B. The LOD was 0.25 ng/ml for both matrices. APL resulted stable in the different matrices at least for 6 h (both at room temperature and 37 °C), after freeze and thaw cycles and long term storage at −20 °C. The method allowed demonstrating that APL is in a dynamic equilibrium between plasma and blood cells. Moreover, the method was successfully applied to the pharmacokinetic study of Aplidin® in cancer patients.

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1. Introduction

In the last decades, there has been an increasing interest in pharmaceutical properties of marine

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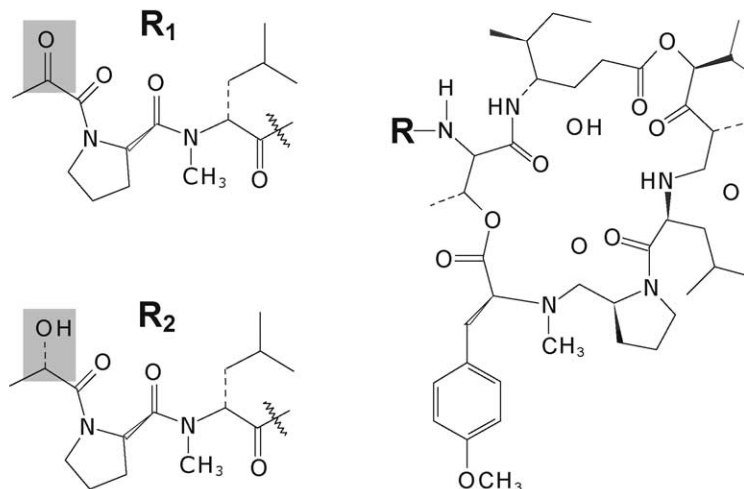

Fig. 1. Chemical structures of APL and didemnin B. R_1 and R_2 are the side chains of APL and didemnin B, respectively.

natural molecules [1–3], especially in those derived from stationary organisms in which natural selection may have developed powerful toxic defences. Among the most studied of these bioactive substances are the members of the didemnins family [4], which are cyclic depsipeptides first isolated from the Caribbean tunicate *Trididemnum* genus [5,6]. All didemnins have an identical macrocyclic structure containing hydroxyisovaleryl propionate and a stereoisomer of the unusual amino acid statine (isostatine). Didemnins mainly differ in the side chain attached to the macrocycle backbone through the amino acidic group of threonine (Fig. 1). This side chain seems to be responsible for their characteristic biological activities [7].

Didemnin B, the first and most studied member of this family, has shown a potent antiviral [8–10], immunosuppressive [11,12] and cytotoxic activity [13,14], as well as the ability to induce a very rapid apoptosis in leukaemia cells [15,16].

Following its antitumoral activity, didemnin B was the first marine natural product entering clinical trials [17].

Aplidin® (APL), which is currently under clinical investigation, differs from didemnin B only in the oxidation of a hydroxylic group of the side chain to ketone (Fig. 1). APL was isolated from the Mediterranean tunicate *Aplidium albicans* in 1990 [18] and recently described as one of the most cytotoxic members of didemnins [7], with a lower toxic effect and better therapeutic indexes in preclinical systems than didemnin B [19,20]. During phase I development Aplidin® demonstrated a positive therapeutic index in treated patients [21,22] and it is currently in phase II development.

In early studies, didemnins were analysed by RIA [23] and ELISA [24], but these methods do not allow distinguishing APL from the other didemnins and their possible metabolites. High performance liquid chromatography (HPLC) has been predominantly used with UV [25,26] and differential refractometry [18] (but with scarce sensitivity), fluorescence [27] and mass spectrometry [28,29] detection systems.

To support the clinical studies of Aplidin® with the characterization of the pharmacokinetic profile of the drug in human, it was necessary to improve the method for the determination of the drug in rat plasma and urine previously developed [28] by adapting it to human biological fluids.

Here is described a liquid chromatographic method with electrospray ionisation tandem mass spectrometric detection (LC–ESI–MS/MS) method for the quantitation of APL in human plasma, whole blood and urine and its application to clinical study. Furthermore, APL stability in the different matrices and the absorption in/onto blood cells was evaluated.

2. Experimental

2.1. Chemicals and materials

Acetonitrile, chloroform (both HPLC grade), 98–100% formic acid and ethanol (both analytical grade) were purchased from Carlo Erba Reagenti (Milan, Italy). Deionised water was purchased from Laboratori Diaco Biomedicali (Trieste, Italy). APL standard and the internal standard didemnin B were kindly provided by Pharma Mar S.A. (Tres Cantos, Madrid, Spain). Sodium heparin (Liquemin® 5000 IU/ml) was purchased from Roche (Milan, Italy). Drug-free heparinised blood and plasma, and drug-free urine were obtained in our laboratory from healthy donors.

2.2. Standard solutions

Standard stock solutions of APL and the internal standard (IS) didemnin B were prepared by dissolving the standards in ethanol, both giving a final concentration of 1 mg/ml.

APL stock solution was diluted in acetonitrile–water (50:50, v/v) obtaining a working solution at the concentration of 10 μg/ml.

Two IS working standard solutions at the concentration 400 ng/ml were prepared by diluting the IS stock solution with human blank plasma and urine, respectively. All standard solutions were stored at −20 °C until use.

2.3. Instrumentation and operating conditions

2.3.1. Liquid chromatography

A Perkin-Elmer 200 micro LC pump system (Norwalk, CT, USA) was used. Analyses were performed at room temperature using a Hypersil-5 ODS column (100 mm × 3.0 mm, 5 μm, 120 Å) equipped with a Chromsep guard column (10 mm × 2.0 mm; 5 μm, 120 Å), both purchased from Varian-Chrompack (Middelburg, The Netherlands). Samples were automatically injected using a Perkin-Elmer 200 autosampler (thermostated at 4 °C) equipped with a 20 μl sample loop. Separations were carried out using a linear gradient of acetonitrile (A) in water (B), both with 0.5% formic acid (50% A for 1 min; then to 82% A in 9 min) at the flow rate of 500 μl/min.

2.3.2. Mass spectrometry

A Sciex API 365 triple-quadrupole mass spectrometer (Toronto, Canada) was used. Instrument control and data acquisition were performed with a Power Macintosh G4 (Apple, Cupertino, CA, USA) using Masschrom 1.1.1 software (PE Sciex, Foster City, CA, USA). The mass spectrometer was calibrated with polypropylene glycol (PE Sciex) and the resolution was set at 0.7 ± 0.1 u (as peak width at half height).

The HPLC system was connected to the mass spectrometer through a Sciex turboionspray source. Mass spectrometric parameters were optimised by infusion of APL and didemnin B standard solutions (1 μg/ml in water–acetonitrile, 50:50 (v/v), at the flow rate of 5 μl/min) in the mobile phase (at the initial gradient flow and composition) using a “make-up” system and a model 11 syringe pump (Harvard Apparatus, South Natick, MA, USA).

Analyses were performed in positive ion mode by multiple reaction monitoring (MRM), selecting precursor ion *m/z* 1111 and product ion *m/z* 295 for APL, and precursor ion *m/z* 1113 and product ion *m/z* 297 for didemnin B. The nebulizer gas flow (air) and the curtain gas flow (nitrogen) were both set at 2.7 l/min. The turboprobe was heated at 450 °C with the auxiliary gas flow (nitrogen) set at 8 l/min. The turboionspray, orifice and ring potentials were set at +5000, +75 and +320 V, respectively. For both APL and didemnin B, the collisionally activated dissociation gas pressure (nitrogen) and energy were maintained at 0.36 Pa and 53 eV, respectively, whereas the dwell time was set at 600 ms.

2.4. Extraction procedure

Five hundred microlitres of sample (plasma, blood or urine) were transferred to a 15 ml polypropylene conical tube with the addition of 50 μl of the IS working standard solution (prepared in plasma for plasma and blood samples, and in urine for urine samples) and 2 ml acetonitrile, containing 1% formic acid. After 5 min vortexing at 1500 rpm and then 10 min centrifugation at 2500 × *g* in a benchtop centrifuge, the supernatant was transferred to a new 15 ml polypropylene conical tube. 2 ml of chloroform were added, and after 5 min vortexing at 1500 rpm and then 10 min centrifugation at 2500 × *g*, the upper aqueous phase was discarded. The organic phase was

evaporated to dryness (at 40 °C) under nitrogen flow and the dried residue was stored at −20 °C. Samples were reconstituted just before analysis with 200 μl of water–acetonitrile–formic acid (49.5:49.5:1, v/v/v), centrifuged at 2500 × *g* to remove any particulate material, and transferred to autosampler microvials with pre-cut caps to be injected and analysed.

2.5. Linearity, precision, accuracy and recovery

APL working solution was used to spike plasma and urine in order to obtain standard curves ranging from 250 to 1 ng/ml. To evaluate the linearity, precision and accuracy of the method, three different and independent batches were processed in three separate working sessions. Each batch consisted of five different and independent calibration curves for both plasma and urine.

Peak integration of extracted ion chromatograms (*m/z* 1111 → 295 for APL peaks and *m/z* 1113 → 297 for didemnin B) and all calculations of concentrations and regression parameters were performed using PE Sciex TurboQuan 1.0 software. Other statistical calculations were performed using Excel software (Microsoft Corp., Redmond, WA, USA).

Calibration curves were constructed using weighted (*1/y*) linear regression of internal ratios (APL peak areas/IS peak areas) versus concentration ratios (APL concentrations/IS concentrations). After back calculation of concentrations from the regression curve, precision was expressed as relative standard deviation (R.S.D.%) of the re-calculated concentrations, and accuracy was calculated as:

$$\frac{\text{mean calculated concentration} - \text{nominal concentration}}{\text{nominal concentration}} \times 100$$

The limit of detection (LOD) was considered as the lowest concentration that can be discriminated from the baseline level, with signal intensity at least two times greater than the background level.

The intra-batch assay was performed using the first batch, whereas the inter-batch assay was performed using the three batches all together.

The applicability of the method to whole blood samples was evaluated by spiking drug-free blood samples ($n = 6$) with 1, 2, 80 and 200 ng/ml of APL, and re-calculating the concentrations using regression curves obtained in plasma. Precision and accuracy were calculated as described above.

Recoveries were estimated at three different concentrations (2, 80 and 200 ng/ml) for APL and at the amount used in the extraction procedure (20 ng) for didemnin B. Peak areas of extracted samples were compared with the peak areas of not extracted standards in water–acetonitrile–formic acid (49.5:49.5:1, v/v/v) at the same concentrations. The repeatability of the extraction procedure was assessed as R.S.D.% of the peak areas.

2.6. Stability

The stability of APL was evaluated by spiking plasma, blood and urine at the concentrations of 10 and 100 ng/ml at different times (from 20 min to 24 h; $n = 4$) either at room temperature (20–22 °C) and 37 °C. Stability after three freeze and thaw cycles and after long term (6 months) storage at −20 °C were also determined ($n = 4$). Stability was expressed as recovery (%) of APL by comparing the obtained concentrations of stored samples with those of freshly prepared samples (expected concentrations). Furthermore, autosampler stability was assessed by re-analysing the processed standard samples 24, 48 and 72 h after the first injection ($n = 4$). The repeatability was assessed by R.S.D.% of the re-calculated concentrations.

2.7. In-vitro kinetics of APL absorption into blood cells

Fresh collected drug-free heparinised blood was spiked with APL to obtain the final concentration of 10 ng/ml, gently mixed and then incubated at 37 °C. 2 ml of the spiked whole blood ($n = 4$) were transferred at different times (from 1 min to 6 h) into 2 ml polypropylene conical tubes. 500 μl were immediately extracted as whole blood, whereas the remaining amount was centrifuged for 5 min at 20, 000 × *g*, in order to separate plasma from blood cells, then 500 μl of plasma were withdrawn and extracted.

2.8. Pharmacokinetic studies

As an example of the applicability of the method, samples from five patients treated with Aplidin® participating the phase I study APL-A-001-98 [2] were

analysed. Aplidin® in the previously described formulation [26] and at the dose of 3.75 mg/m^2, was administered during a 24 h intravenous infusion. Blood samples were collected in heparinised tubes at 3, 6, 8, 12 h during the infusion, at 24 h (end of the infusion) and at 15, 30, 45 min, 1, 2, 4, 8, 12 and 24 h from the end of the infusion. Plasma samples were obtained by centrifugation within 20 min from the collection. Whole blood samples were collected at the end of the infusion and at 30 min and 24 h from the end of the infusion and immediately frozen. Urine were collected in the 0–24 h and 24–48 h ranges starting from the beginning of the administration and then frozen. Control samples (before treatment) for all matrices were also analysed.

3. Results and discussion

3.1. Liquid chromatography–tandem mass spectrometry

The MS conditions were optimised using standard compounds as described in Section 2.3.2 in order

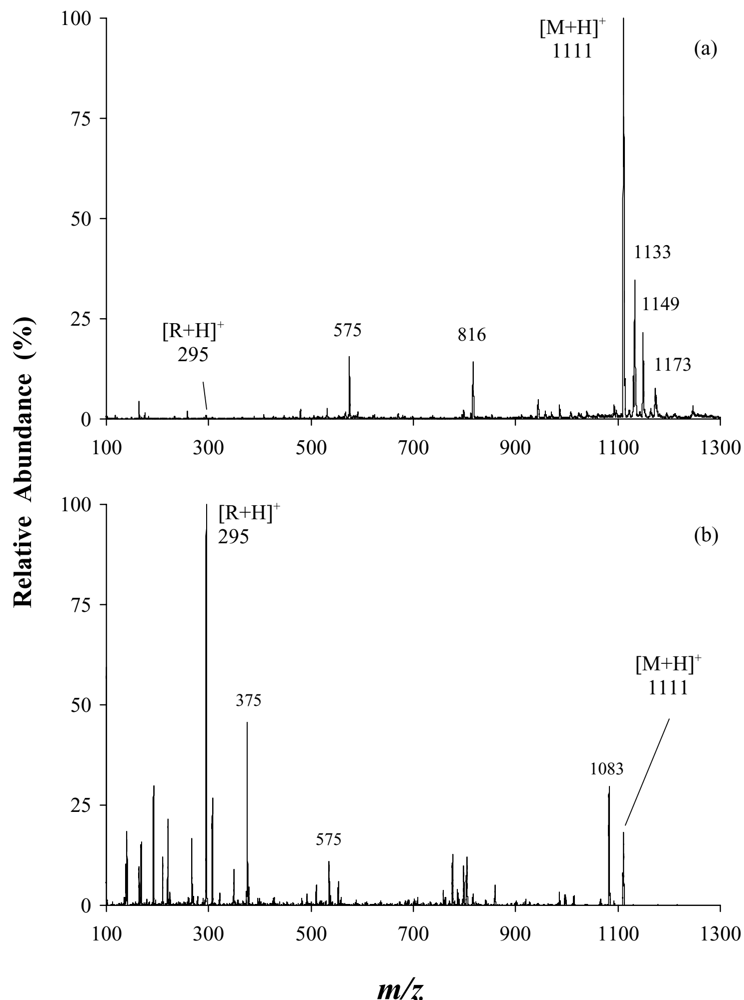

Fig. 2. (a) Full scan mass spectrum and (b) product ion mass spectrum of APL obtained by infusion of the standard (see Section 2.3.2 for details). Ten scans have been summed up during acquisition. Mass spectra of didemnin B showed the same profile with a mass increase of 2 u.

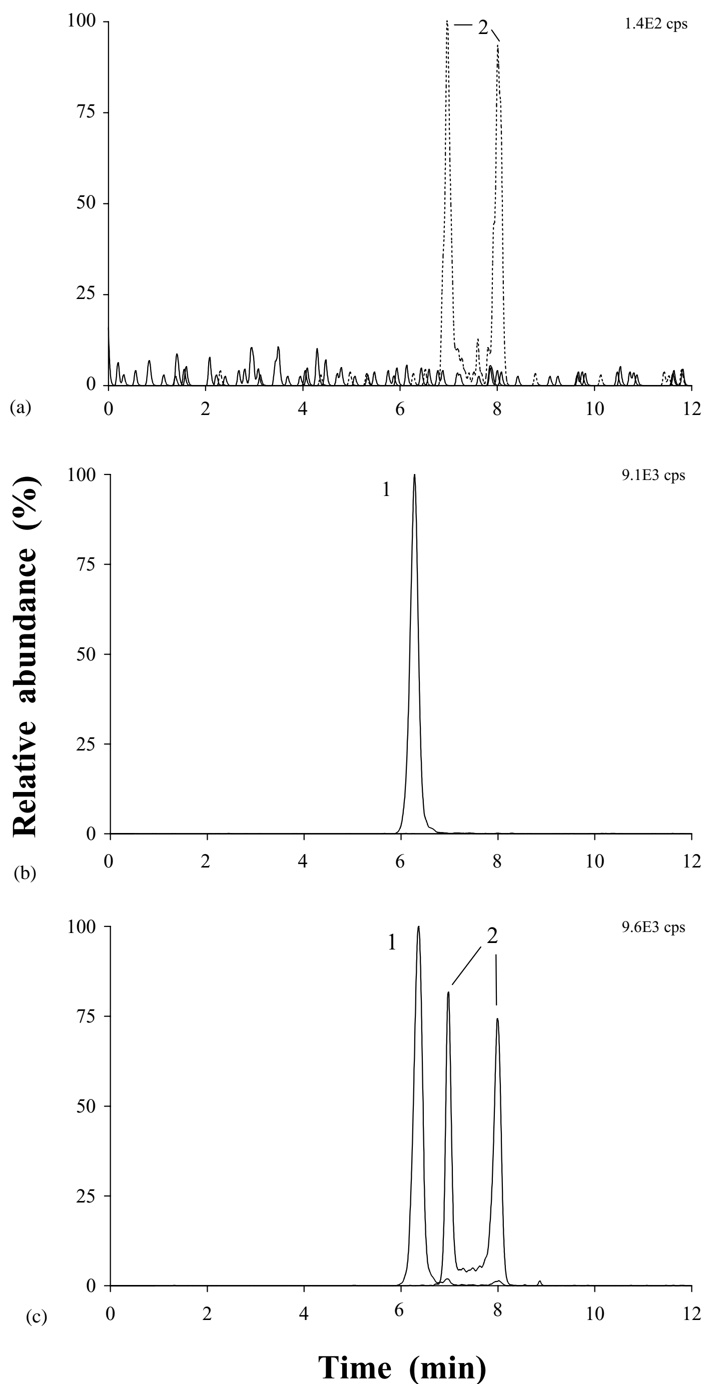

Fig. 3. Representative LC–MRM chromatograms of extracted plasma obtained by superimposition of the extracted ion chromatograms of APL (m/z 1111 → 295) and IS (m/z 1113 → 297). (a) blank plasma, with a further superimposed APL extracted ion chromatogram (broken line) of blank plasma spiked at LLOQ (1 ng/ml); (b) blank plasma spiked with 20 ng IS; (c) blank plasma spiked with APL 50 ng/ml and 20 ng IS. Peak 1, IS; Peaks 2, APL conformers. The chromatographic profiles of APL and IS in whole blood and urine are qualitatively the same as for the plasma samples.

to obtain the best intensity of the molecular ions, and of the most representative fragments (the side chains R_1 and R_2; Fig. 1). Representative APL full scan mass spectrum and the fragmentation pattern obtained from product ion scan are shown in Fig. 2. As reported in our previous paper [28], 0.5% formic acid was used as mobile phase modifier in order to reduce the formation of sodium and potassium adducts and to obtain higher sensitivity, in that trifluoroacetic acid, also used by other authors as ion-pairing agent [26,27,29], could inhibit the ionisation of the analyte because of its competitive ionisation. However, the separation of APL from didemnin B was achieved with satisfying resolution (Fig. 3). As previously reported [18,26–29], APL may show two partially separated peaks corresponding to two conformational isomers at the pyruvoyl proline residue, that are in a temperature-dependent equilibrium. Therefore, APL was quantified as the sum of both peak areas.

3.2. Linearity, precision, accuracy and recovery

The method was linear ($r \geq 0.9933$) over the range 1–250 ng/ml and, as shown in Table 1, with intra- and inter-batch precision and accuracy for both plasma and urine ≤12.2% at all concentrations (except at LLOQ, ≤15.4%). The detection limit was 0.25 ng/ml for both plasma and urine.

The concentrations of spiked whole blood samples were re-calculated using the plasma standard curves and the results of concentrations, precision and accuracy are reported in Table 2, showing that the method is also applicable to whole blood samples.

As shown in Table 3, recoveries of both APL and didemnin B were moderate, ranging from 54 to 70% in plasma and blood and from 46 to 60% in urine (R.S.D. ≤ 14.9%). Different solvents and solvent volumes were unsuccessfully tested in order to improve the liquid–liquid extraction efficiency (data not shown). A SPE procedure was also described to give similar recoveries with plasma samples [27]. Although low recoveries may be partially attributed to the matrix effects (see Section 3.3), a contribution due to APL-binding to plasma albumin [30] may also be present for plasma and blood matrices. However, a low recovery was obtained also for urine, theoretically having no proteins.

Table 1
Intra- and inter-batch precision and accuracy of APL after extraction from spiked human plasma and urine

Nominal concentration (ng/ml)	Plasma						Urine					
	Intra-batch (n = 5)			Inter-batch (n = 15)			Intra-batch (n = 5)			Inter-batch (n = 15)		
	Observed concentration (ng/ml) (mean ± S.D.)	R.S.D. (%)	Accuracy (%)	Observed concentration (ng/ml) (mean ± S.D.)	R.S.D. (%)	Accuracy (%)	Observed concentration (ng/ml) (mean ± S.D.)	R.S.D. (%)	Accuracy (%)	Observed concentration (ng/ml) (mean ± S.D.)	R.S.D. (%)	Accuracy (%)
1.00	1.12 ± 0.15	13.7	11.6	1.05 ± 0.13	12.8	4.8	1.08 ± 0.10	9.0	7.9	0.963 ± 0.148	15.4	−3.7
2.50	2.38 ± 0.17	7.0	−4.7	2.43 ± 0.14	5.6	−2.9	2.55 ± 0.31	12.2	1.9	2.44 ± 0.28	11.3	−2.2
5.00	4.73 ± 0.17	3.6	−5.5	4.88 ± 0.49	10.1	−2.4	4.40 ± 0.09	2.1	12.1	4.92 ± 0.52	10.7	−1.5
10.0	9.77 ± 0.27	2.7	−2.4	9.83 ± 0.51	5.2	−1.7	9.91 ±0.41	4.1	−0.9	10.4 ± 0.9	8.8	3.8
25.0	25.2 ± 1.9	7.4	0.6	24.9 ± 1.7	6.8	−0.3	25.0 ±1.6	6.6	0.1	24.5 ± 1.4	5.7	−2.1
50.0	51.0 ± 2.6	5.0	2.0	50.2 ± 4.1	8.2	0.4	51.4 ± 1.6	3.2	2.8	51.1 ± 2.2	4.4	2.1
100	99.0 ± 8.8	8.9	1.0	102 ± 7	6.9	1.9	98.7 ± 3.8	3.9	−1.3	102 ± 6	5.9	2.3
250	252 ± 10	3.8	0.7	250 ± 9	3.4	−0.2	251 ± 4	1.1	0.4	248 ± 6	2.4	−0.9

Table 2
Precision and accuracy of spiked blood samples

Nominal concentration (ng/ml)	Observed concentration (ng/ml) (mean ± S.D.)	R.S.D. (%)	Accuracy (%)
1.00[a]	1.10 ± 0.17	15.7	10.2
2.00	2.22 ± 0.20	8.9	10.8
80.0	71.4 ± 1.7	2.4	−10.7
200	202 ± 16	7.8	1.1

The concentrations were re-calculated using calibration curves obtained with spiked plasma ($n = 6$).
[a] LLOQ.

3.3. Selectivity and matrix effects

To evaluate the method selectivity, control plasma, whole blood and urine from six healthy donors (three males and three females) were extracted with and without the addition of the IS. As a result of the highly selective MRM mode and the relatively high molecular weights of APL and didemnin B, no peaks potentially interfering with the quantification of the analyte and the IS areas were observed. Carry-over was totally absent in the linearity range and resulted to be not significant until the concentration of 1 μg/ml.

As shown in Fig. 3c, peaks due to the naturally-occurring isotope *m/z* 1113 of APL, well represented because of its high molecular weight, were observed in the extracted ion chromatogram of the IS transition (*m/z* 1113 → 297). These peaks, representing less than 2% of those of APL transition, are detected above 25 ng/ml APL; however, the different retention times of APL and IS, make these peaks negligible for the quantification purposes.

Matrix effects due to endogenous interferences that could inhibit or enhance the ionisation efficiency of analytes, were evaluated for each matrix by spiking the extracts of six blank samples (deriving from different sources) and the mobile phase solvent (water–acetonitrile–formic acid, 49.5:49.5:1, v/v/v) at three different concentrations of APL (2, 80 and 200 ng/ml) with the IS at the amount used in the extraction procedure (20 ng). The peak areas were then compared and an ionisation suppression was observed for both APL and IS in all the matrices with a consequent average reduction of the peak areas of 14.1% (R.S.D. ≤ 12.2%). However, as shown in Table 1, matrix effects did not compromise the accuracy and precision of the method.

3.4. Stability

As shown in Table 4, APL resulted substantially stable (≥85%) until 6 h, in plasma and blood, and also for 24 h, in urine, both after incubation at room temperature and at 37 °C. Moreover, APL resulted stable in all the tested matrices during three freeze and thaw cycles and after 6 months storage at −20 °C.

After resuspension the extracted samples were stable at least for 24 h in the autosampler, but for longer time storage, concentration values were not reproducible (data not shown). This was not due to degradation of APL and didemnin B, but to the evaporation of acetonitrile from microvials bearing pre-cut caps, even though the autosampler was kept thermostated at 4 °C. Because of the different solubilities of APL and didemnin B in water, the decrease of acetonitrile volume gives rise to a not proportional decrease of the peak areas and, consequently, to an incorrect APL/IS area ratio. This was demonstrated by adding in the

Table 3
Recoveries of APL and didemnin B from spiked plasma, blood and urine samples

Nominal Concentration (ng/ml)	Amount (ng)	Recovery (%) (R.S.D.%)		
		Plasma	Blood	Urine
APL ($n = 6$)				
2.00	1.00	59.9 (11.6)	54.1 (14.9)	46.3 (11.2)
80.0	40.0	66.0 (2.4)	59.0 (2.2)	48.4 (9.5)
200	100	70.2 (3.2)	64.3 (9.8)	54.8 (10.3)
Didemnin B ($n = 18$)				
–	20.0	63.5 (9.5)	60.9 (8.5)	59.5 (11.2)

Table 4
Stability of APL in plasma, blood and urine at room temperature (RT) and 37 °C expressed as mean recovery% (R.S.D.%) ($n = 4$)

Time	10 ng/ml		100 ng/ml	
	RT	37 °C	RT	37 °C
Plasma				
0 (control)	100.0 (4.3)	100.0 (4.3)	100.0 (4.0)	100.0 (4.0)
20 min	93.2 (5.2)	99.8 (16.0)	94.3 (2.1)	99.1 (1.0)
1 h	89.7 (2.1)	89.7 (5.6)	102.9 (6.4)	89.2 (1.0)
6 h	91.9 (9.3)	93.0 (2.6)	99.4 (5.3)	88.9 (1.7)
24 h	72.9 (20.4)	44.1 (2.5)	75.5 (13.4)	47.8 (3.4)
Three freeze and thaw cycles	98.0 (9.1)		91.3 (7.4)	
24 weeks (−20 °C)	107.9 (11.1)		95.5 (3.5)	
Blood				
0 (control)	100.0 (6.6)	100.0 (6.6)	100.0 (5.7)	100.0 (5.7)
20 min	96.9 (3.8)	99.8 (10.1)	98.4 (3.4)	101.7 (6.2)
1 h	93.3 (4.6)	85.9 (1.7)	94.8 (1.4)	90.4 (3.1)
6 h	89.1 (2.5)	90.7 (0.3)	84.7 (7.6)	87.8 (1.4)
24 h	74.4 (12.8)	61.8 (12.8)	72.8 (26.9)	66.9 (10.4)
Three freeze and thaw cycles	90.5 (11.6)		91.3 (11.4)	
24 weeks (−20 °C)	96.9 (3.0)		103.5 (3.3)	
Urine				
0 (control)	100.0 (8.5)	100.0 (8.5)	100.0 (1.3)	100.0 (1.3)
1 h	94.9 (8.5)	101.2 (2.6)	92.7 (9.0)	90.9 (1.0)
6 h	101.4 (4.2)	101.7 (4.2)	97.8 (0.2)	94.9 (6.2)
24 h	102.8 (1.5)	102.2 (3.6)	101.2 (1.9)	88.9 (2.9)
Three freeze and thaw cycles	94.3 (6.4)		96.0 (4.6)	
24 weeks (−20 °C)	92.8 (4.0)		89.5 (2.0)	

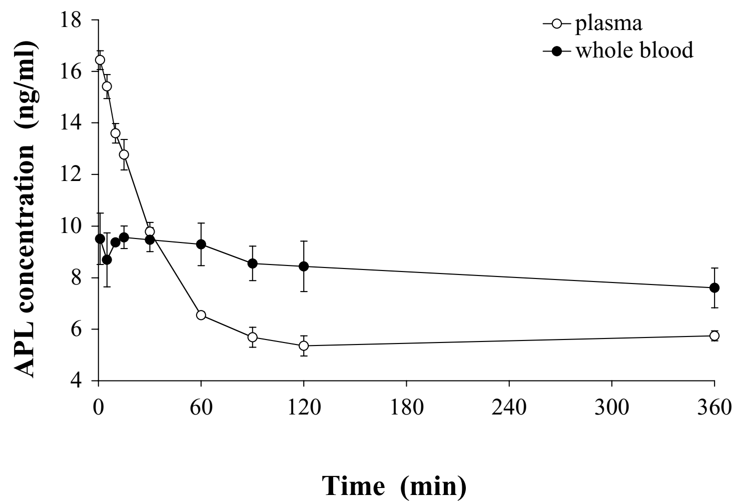

Fig. 4. Concentration–time profile of APL in whole blood and plasma after spiking of whole blood with APL 10 ng/ml ($n = 4$).

Table 5
APL concentrations in whole blood and plasma after spiking whole blood with APL 10 ng/ml

Time (min)	Blood		Plasma		APL in plasma (%)
	Calculated concentration (ng/ml) (mean ± S.D.)	R.S.D.%	Calculated concentration (ng/ml) (mean ± S.D.)	R.S.D.%	
90	11.3 ± 1.2	10.6	4.56 ± 0.12	2.2	40.5
180	9.35 ± 0.60	6.4	3.28 ± 0.22	6.1	35.1

After 90 min, the plasma was removed by centrifugation and replaced with an equal volume of drug-free plasma ($n = 5$).

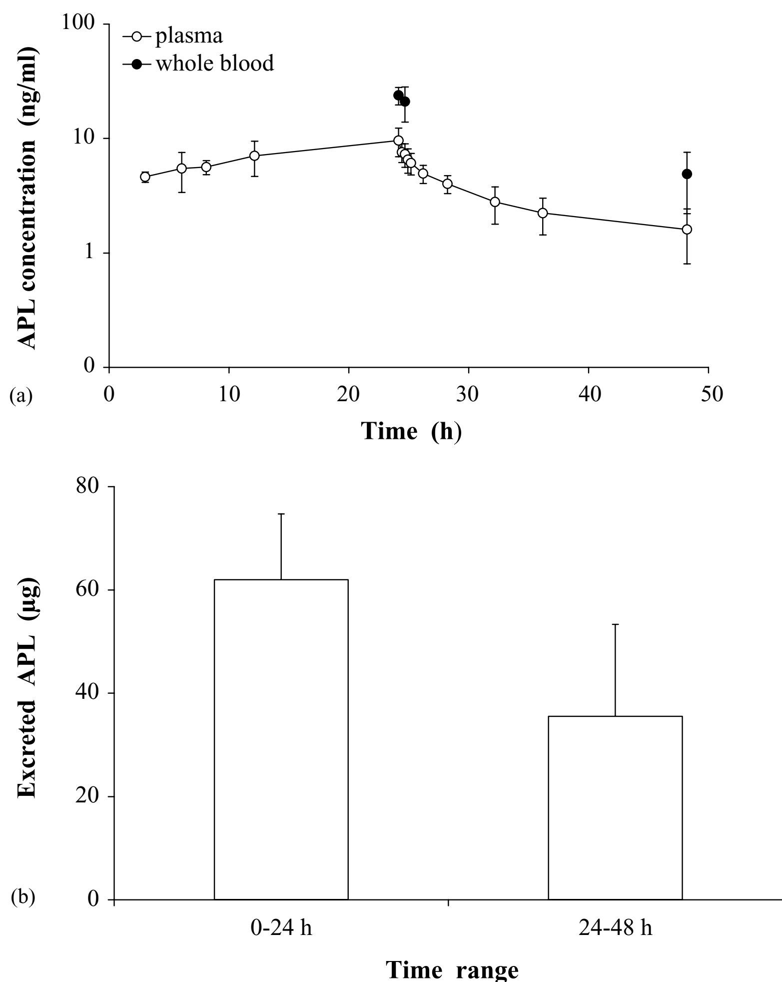

Fig. 5. (a) Concentration–time profile of APL in plasma and whole blood (semi-logarithmic scale) and (b) excretion of unmodified APL in urine of 5 patients treated intravenously for 24 h with 3.75 mg/m^2 of Aplidin®.

microvial a volume of acetonitrile equal to that previously evaporated, obtaining again the initial internal ratio.

3.5. In-vitro kinetics of APL association with blood cells

During preliminary experiments, unexpected high concentrations of APL were observed in plasma of partially haemolysed samples. Hence, we investigated whether a possible absorption/intake of APL on/into blood cells occurred. As shown in Fig. 4, APL concentration in whole blood remained substantially constant during the 0–360 min time-course experiment, whereas the concentration of APL in plasma decreased exponentially reaching the equilibrium within 90 min, in a very similar way as previously described for diacetyl didemnin B [30]. At the equilibrium, APL-containing plasma was removed by centrifugation after 90 min from whole blood, and blood cells were resuspended in an equal volume of blank plasma. Also in this case, the concentration of APL in plasma (35–40% of the total amount found in the whole blood; see Table 5) was newly established when a new equilibrium was reached after further 90 min. This indicating that a dynamic equilibrium between plasma and blood cells may occur.

3.6. Pharmacokinetic findings

Fig. 5 shows the pharmacokinetic profile of APL in plasma, blood and the urinary excretion of the drug. The plot represents the mean plasma decay curves obtained in five patients treated with 3.75 mg/m^2 of Aplidin® given as 24 h continuous intravenous infusion. At the end of administration, APL reached a mean steady state concentration of 10 ng/ml, that was about twice as low than that found in blood according to what found in, in vitro kinetics (Section 3.5). After the end of the infusion, the drug was cleared from plasma according to a two-compartment model with a terminal half-life of 21 h and a clearance of 13.6 l/(h m^2).

Unmodified APL seems to be excreted in small amount in the urine of patients. In the five cases investigated, the percentage of the administered dose recovered up to 48 h was $<1.9\%$ with a mean percentage of 1.5%.

4. Conclusions

A selective, sensitive, precise, accurate and simple LC–ESI–MS/MS method has been developed for the quantification of APL in human plasma, whole blood and urine, using protein precipitation and liquid–liquid extraction for sample preparation. The method was successfully applied to the study of APL pharmacokinetic profile in cancer patients treated with Aplidin® during the phase I clinical studies. The method also allowed confirming that APL is in a dynamic equilibrium between plasma and blood cells.

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References

[1] F. Flam, Science 266 (1994) 1324–1325.
[2] K.L. Rinehart, Med. Res. Rev. 20 (2000) 1–27.
[3] A.M.S. Mayer, V.K.B. Lehmann, Anticancer Res. 21 (2001) 2489–2500.
[4] G. Schwartsmann, A. Brondani da Rocha, R.G.S. Berlinck, J. Jimeno, Lancet Oncol. 2 (2001) 221–225.
[5] K.L. Rinehart Jr., J.B. Gloer, J.C. Cook Jr., S.A. Mizsak, T.A. Scahill, J. Am. Chem. Soc. 103 (1981) 1857–1859.
[6] K.L. Rinehart, J.B. Gloer, R.G. Hughes Jr., H.E. Renis, J.P. McGovren, E.B. Swynenberg, D.A. Stringfellow, S.L. Kuentzel, L.H. Li, Science 212 (1981) 933–935.
[7] R. Sakai, K.L. Rinehart, V. Kishore, B. Kundu, G. Faircloth, J.B. Gloer, J.R. Carney, M. Namikoshi, F. Sun, R.G. Hughes Jr., D. García Grávalos, T. García de Quesada, G.R. Wilson, R.M. Heid, J. Med. Chem. 39 (1996) 2819–2834.
[8] K.L. Rinehart Jr., J.C. Cook Jr., R.C. Pandey, L.A. Gaudioso, H. Meng, M.L. Moore, J.B. Gloer, G.R. Wilson, R.E. Gutowsky, P.D. Zierath, L.S. Shield, L.H. Li, H.E. Renis, J.P. McGovren, P.G. Canonico, Pure Appl. Chem. 54 (1982) 2409–2424.
[9] P.G. Canonico, W.L. Pannier, J.W. Huggins, K.L. Rienehart, Antimicrob. Agents Chemother. 22 (1982) 696–697.
[10] S.D. Weed, D.A. Stringfellow, Antiviral Res. 3 (1983) 269–274.
[11] D.W. Montgomery, C.F. Zukoski, Transplantation 40 (1985) 49–56.
[12] D.W. Montgomery, G.K. Shen, E.D. Ulrich, C.F. Zukoski, Ann. N. Y. Acad. Sci. 712 (1994) 301–314.

[13] T.L. Jiang, R.H. Liu, S.E. Salmon, Cancer Chemother. Pharmacol. 11 (1983) 1–4.

[14] S.L. Crampton, E.G. Adams, S.L. Kuentzel, L.H. Li, G. Badiner, B.K. Bhuyan, Cancer Res. 44 (1984) 1796–1801.

[15] D.R. Grubb, E.J. Wolvetang, A. Lawen, Biochem. Biophys. Res. Commun. 215 (1995) 1130–1136.

[16] K.L. Johnson, F. Vaillant, A. Lawen, FEBS Lett. 383 (1996) 1–5.

[17] H.G. Chun, B. Davies, D. Hoth, M. Suffness, J. Plowman, K. Flora, C. Grieshaber, B. Leyland-Jones, Invest. New Drugs 4 (1986) 279–284.

[18] K.L. Rinehart, A. Lithgow-Bertelloni, International Application PCT/GB90/01495, International Publication number WO91/04985 (1991).

[19] J.L. Uridiales, P. Morata, I. Núñez De Castro, F. Sanchez-Jimenez, Cancer Lett. 102 (1996) 31–37.

[20] H. Depenbrock, R. Peter, G.T. Faircloth, I. Manzanares, J. Jimeno, A.R. Hanauske, Br. J. Cancer 78 (1998) 739–744.

[21] E. Raymond, L. Paz-Ares, M. Izquierdo, K. Belanger, J. Maroun, A. Bowman, A. Anthoney, D. Jodrell, J.P. Armand, H. Cortes-Funes, J. Germa-Lluch, C. Twelves, N. Celli, C. Guzman, J. Jimeno, Eur. J. Cancer 37 (Suppl. 6) (2001) 107a.

[22] E.M. Ciruelos Gil, C. Twelves, M.J. Dominguez, H. McKay, A. Anthony, D. Castellanos, S. Bezares, A. Ruiz, L. Lòpez-Lazaro, J. Jimeno, N. Celli, H. Cortes-Funes, L. Paz-Ares, Proc. Am. Soc. Clin. Oncol. (2002) 422a.

[23] F.A. Dorr, J.G. Kuhn, J. Phillips, D.D. Von Hoff, Eur. J. Cancer Clin. Oncol. 24 (1988) 1699–1706.

[24] T.J.G. Raybould, P.G. Grothaus, S.B. Simpson, G.S. Bignami, C.B. Lazo, R.A. Newman, J. Clin. Lab. Anal. 6 (1992) 136–142.

[25] J.N. Hartshorn, W.P. Tong, J.A. Stewart, J.J. McCormack, J. Liquid Chromatogr. 9 (1986) 1489–1501.

[26] B. Nuijen, M. Bouma, R.E.C. Henrar, P. Floriano, J.M. Jimeno, H. Talsma, J.J. Kettenes-van den Bosch, A.J.R. Heck, A. Bult, J.H. Beijen, PDA J. Pharm. Sci. Technol. 54 (2000) 193–208.

[27] R.W. Sparidans, J.J. Kettenes-van den Bosch, O. van Tellingen, B. Nuyen, R.E.C. Henrar, J.M. Jimeno, G. Faircloth, P. Floriano, K.L. Rinehart, J.H. Beijnen, J. Chromatogr. B 729 (1999) 43–53.

[28] N. Celli, A. Motos Gallardo, C. Rossi, M. Zucchetti, M. D'Incalci, D. Rotilio, J. Chromatogr. B 731 (1999) 335–343.

[29] J.C.M. Waterval, J.C. Bloks, R.W. Sparidans, J.H. Beijnen, I.M. Rodriguez-Campos, A. Bult, H. Lingeman, W.J.M. Underberg, J. Chromatogr. B 754 (2001) 161–168.

[30] J.L. Phillips, R. Schwartz, D.D. Von Hoff, Cancer Invest. 7 (1989) 123–128.