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Journal of Chromatography B, 731 (1999) 335–343

JOURNAL OF CHROMATOGRAPHY B

Analysis of aplidine (dehydrodidemnin B), a new marine-derived depsipeptide, in rat biological fluids by liquid chromatography–tandem mass spectrometry

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Received 19 March 1999; received in revised form 17 May 1999; accepted 28 May 1999

Abstract

Aplidine (dehydrodidemnin B) is a new marine-derived depsipeptide with a powerful cytotoxic activity, which is under early clinical investigation in Europe and in the US. In order to investigate the pharmacokinetic properties of this novel drug, an HPLC–tandem mass spectrometry method was developed for the determination of aplidine in biological samples. Didemnin B, a hydroxy analogue, was used as internal standard. After protein precipitation with acetonitrile and extraction with chloroform, aplidine was chromatographed with a RP octadecylsilica column using a water–acetonitrile linear gradient in the presence of formic acid at the flow-rate of 500 μl/min. The method was linear over a 5–100 ng/ml range (LOD=0.5 ng/ml) in plasma and over a 1.25–125 ng/ml range (LOD=0.2 ng/ml) in urine with precision and accuracy below 14.0%. The intra- and inter-day precision and accuracy were below 12.5%. The extraction procedure recoveries for aplidine and didemnin B were 69% and 68%, respectively in plasma and 91% and 87%, respectively in urine. Differences in linearity, LOQ, LOD and recoveries between plasma and urine samples seem to be matrix-dependent. The applicability of the method was tested by measuring aplidine in rat plasma and urine after intravenous treatment.

Keywords: Aplidine; Didemnin; Depsipeptide

1. Introduction

In the last decades there has been an increasing interest in pharmaceutical properties of marine natural molecules [1,2], especially in those derived from stationary organisms in which natural selection has

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0378-4347/99/$ – see front matter
PII: S0378-4347(99)00262-5

developed elaborate and powerful toxic defences. Among the most studied of these bioactive substances are the members of the didemnins family [3], and didemnin B was the first marine natural product entering clinical trials [4].

Didemnins are cyclic depsipeptides first isolated from the Caribbean tunicate (sea squirt) *Tridemnum solidum* [5,6]. The structure of didemnin B and other members of the didemnins family have been well elucidated by X-ray crystallography [7,8] and NMR studies [5,8] and several didemnins syntheses have been achieved [9,10]. Didemnin B has a macrocyclic structure containing hydroxyisovaleryl propionate and a stereoisomer of the highly unusual aminoacid statine (isostatine) [5] (Fig. 1). It has been shown to have a good antiviral activity against a broad range of DNA and RNA viruses [11–13], as well as potent immunosuppressive effects [14,15]. However, the major interest of didemnins is in their potent cytotoxic activity [16,17] mainly due to protein synthesis inhibition [18,19] at the elongation stage [20].

In 1990, aplidine (dehydrodidemnin B) was isolated from the Mediterranean tunicate *Aplidium albicans*, and recently described as one of the most cytotoxic members of didemnins [21].

Aplidine differs from didemnin B only by two hydrogen atoms in the side chain attached to the macrocycle backbone through the aminoacidic group of threonine (Fig. 1).

In recent studies [22,23], aplidine seems to be less toxic and to have better therapeutic indexes in preclinical systems than didemnin B.

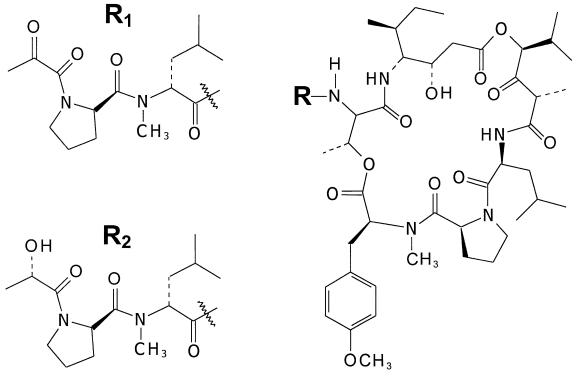

Fig. 1. Chemical structures of aplidine (monoisotopic *Mr* 1109.63) and didemnin B (monoisotopic *Mr* 1111.64). R_1 is the side chain of aplidine and R_2 is the side chain of didemnin B.

To support an imminent clinical study of aplidine pharmacokinetics, it was necessary to develop a method for the determination of plasma and urine drug concentrations.

In previous studies, didemnin B was quantified by high-performance liquid chromatography (HPLC) [24], radioimmunoassay (RIA) [25], and enzyme-linked immunosorbent assay (ELISA) [26] methods.

The HPLC method required high amounts of sample and was not sensitive enough for clinical studies. Only RIA and ELISA achieved low quantification levels, but they did not allow distinguishing aplidine from didemnin B.

In the last years LC–ESI–MS–MS (liquid chromatography–electrospray ionisation tandem mass spectrometry) has proven to be a very powerful technique for drug analysis in complex samples, such as biological fluids, giving high sensitivity and very high specificity, allowing to develop methods with simple and partial sample purification and fast chromatographic separations.

Here we describe a new method for the quantification of aplidine, allowing to perform sensitive, reproducible and a very highly selective analysis. Furthermore, the method requires low quantities of sample with a simple extraction procedure.

2. Experimental

2.1. Chemicals and materials

Acetonitrile, chloroform (both HPLC grade), formic acid and ethanol (both analytical grade) were purchased from Merck (Darmstadt, Germany). Sterile water and saline solutions were purchased from Laboratori Diaco Biomedicali (Trieste, Italy). Aplidine and didemnin B standards were kindly provided by Pharma Mar S.A. (Tres Cantos, Madrid, Spain). Cremophor EL was obtained from Sigma (St. Louis, MO, USA). Sodium pentobarbital was purchased from Siegfried CMS (Zofingen, Switzerland) and sodium heparin (Liquemin 5000 U.I./ml) was purchased from Roche (Milan, Italy). Drug-free heparinised plasma and urine were prepared in our laboratories from male Sprague-Dawley rats.

2.2. Stock solutions

Standard stock solutions of aplidine and the internal standard (I.S.) didemnin B were prepared in acetonitrile, both giving a final concentration of 1 mg/ml.

Didemnin B stock solution was diluted in rat blank plasma and urine to achieve final concentrations of 100 ng/ml and 20 ng/ml, respectively. These I.S. solutions were aliquoted and 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 Reverse Phase Hypersil-5 ODS column (100×3.0 mm; C_{18}, 5 μm, 120 Å) provided with a Chromsep guard column (10×2.0 mm; C_{18}, 5 μm, 120 Å), both purchased from Chrompack (Middelburg, The Netherlands). Samples were injected using a Perkin-Elmer 200 autosampler (thermostated at 4°C) with a 20 μl injection-loop. Separations were carried out using a linear gradient of acetonitrile (solvent B) in water (solvent A), both with 0.5% of formic acid (50% B at 0 min; 50% of B for 1 min; to 82% of B 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 was performed with a Macintosh System 7600/132 (Apple, Copertino, CA, USA) using Masschrom 1.0 software (PE Sciex, Foster City, CA, USA). The Mass Spectrometer was calibrated with polypropylene glycol (PPG) obtained from PE Sciex and the resolution was set in the range 0.6–0.9 amu.

Analytical conditions were optimised by direct infusion of standards using a Sciex ionspray interface (Fig. 2). The HPLC system was connected to the mass spectrometer through a Sciex turboionspray interface and the analysis was performed by multiple reaction monitoring (MRM) mode. Briefly, the first quadrupole (Q1) filters ions m/z 1111 amu for aplidine and m/z 1113 amu for didemnin B and, after collision-activated dissociation (CAD) fragmentation in the second quadrupole (Q2), specific ions m/z 295 amu for aplidine and m/z 297 amu for didemnin B were selected by the third quadrupole and then detected by the channel electron multiplier (CEM).

The nebulizing gas flow (air) and the curtain gas flow (N_2) were set at 1.9 l/min and 2.2 l/min, respectively. The turboionspray interface was heated at 400°C and the auxiliary gas flow (air) was set at 8 l/min. The ionisation voltage was set at +5000 V, and the orifice and ring potentials were set at +50 V and +340 V, respectively. The collision-activated dissociation gas pressure (N_2) was maintained at 2.84×10^{-3} torr. Dwell time was set at 600 ms.

Peak areas were calculated using extracted ion chromatograms (1111→295 for aplidine and 1113→297 for didemnin B) with MacQuan 1.5 software (PE Sciex).

2.4. Extraction procedure

2.4.1. Plasma samples

In a 2.0 ml Eppendorf microfuge tube (Hamburg, Germany), 10 μl of the I.S. and 10 μl of formic acid were added to 100 μl of rat plasma. 400 μl of acetonitrile were added to precipitate proteins, and after 1 min vortexing and 10 min of centrifugation at 16 000 *g* in an Eppendorf microfuge, the supernatant was transferred to a new 2.0 ml Eppendorf microfuge tube. 1.2 ml of chloroform was added, and after 2 min vortexing and 5 min of centrifugation at 16 000 *g* in an Eppendorf microfuge, the upper aqueous phase was discarded. The organic phase was evaporated to dryness using a Savant speed-vac system (Farmingdale, NY, USA). The dried residue was stored at 4°C and reconstituted just before analysis with 100 μl of water 49.5%, acetonitrile 49.5% and formic acid 1%.

2.4.2. Urine samples

Extractions were performed on 500 μl of urine in a 15 ml Falcon polypropylene conic tube (Becton Dickinson Labware, Franklin Lakes, NJ, USA). 50 μl of the I.S., 50 μl of formic acid and 1.5 ml of acetonitrile were added, and after 3 min vortexing and 10 min of centrifugation at 2500 *g* in a benchtop centrifuge, the supernatant was transferred to a new

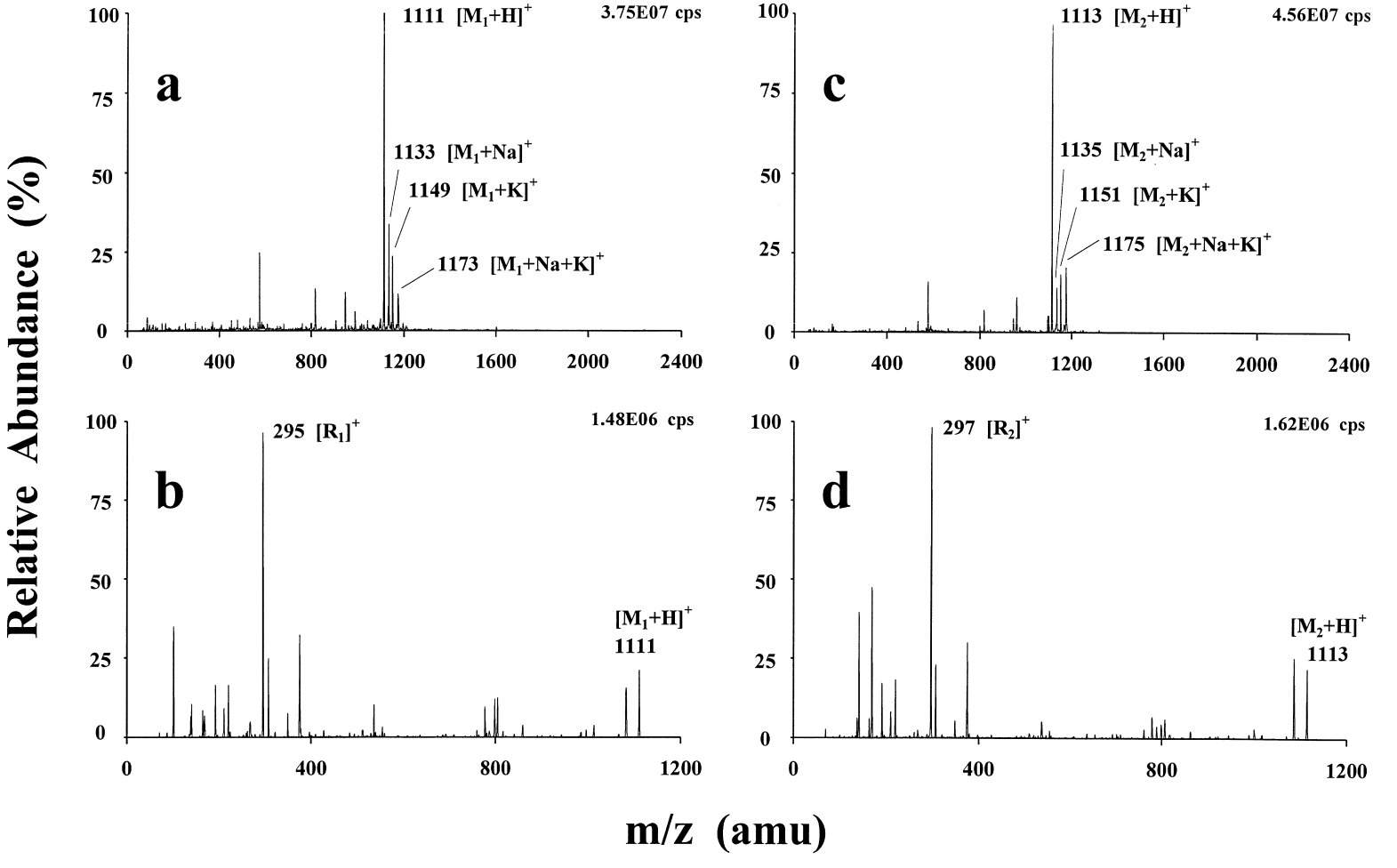

Fig. 2. Mass spectra of aplidine and didemnin B obtained by infusion of the standards: aplidine Q1 scan spectrum (a) and product ion spectrum (b); didemnin B Q1scan spectrum (c) and product ion spectrum (d). Standard solutions (1 μg/ml in water 49.5%, acetonitrile 49.5% and formic acid 1%) were delivered by a model 11 syringe pump (Harvard Apparatus, South Natick, MA, USA) at the flow-rate of 5 μl/min. The infusion pump was connected to a Sciex ionspray interface through a fused-silica capillary (1 m×75 μm I.D.; 150 μm O.D.; Polymicro Technology, Phoenix, AZ, USA). Q1 scan mass spectra were acquired with a mass range 30–2400 amu using a step size of 0.1 amu and a dwell time of 0.250 ms. Product ion spectra were acquired with a mass range 30–1200 amu using a step size of 0.1 amu and a dwell time of 0.500 ms.

15 ml Falcon polypropylene conic tube. Five milliliters of chloroform were added, and after 2 min vortexing and 5 min of centrifugation at 2500 *g* in a benchtop centrifuge, the upper aqueous phase was discarded. The organic phase was evaporated to about 1 ml under nitrogen flow, and then evaporated to dryness with a Savant speed-vac system. The dried sample was reconstituted just before analysis with 500 μl of water 49.5%, acetonitrile 49.5% and formic acid 1%.

2.5. Validation study

2.5.1. Standard curves

Aplidine stock solution was diluted in rat blank plasma and urine to prepare standards ranging from 100 ng/ml to 0.5 ng/ml for plasma and from 125 ng/ml to 0.5 ng/ml for urine. Lower concentrations were also prepared for the determination of the limit of detection (LOD). These standard solutions were aliquoted and stored at −20°C until use.

Five samples for each concentration were extracted and analysed in five different serial calibrations for both plasma and urine. The internal ratios (aplidine peak area/I.S. didemnin B peak area) were calculated for each point and standard curves were constructed using all points by least squares linear regression analysis of internal ratios vs. concentrations. Precision was expressed as coefficient of variation (CV %) of the internal ratios, and, after back-calculation of concentrations from the standard line, accuracy was calculated as [(*mean calculated concentration-nominal concentration*)/*nominal concentration*] × 100. The limit of quantification (LOQ) was considered as the lowest concentration yielding precision and accuracy of less than 15%, and the limit of detection (LOD) was considered as the lowest concentration that can be discriminated from the baseline level, with a signal intensity which is at least three times greater than the background level.

2.5.2. Intra- and inter-day assays

Variability studies were performed by spiking drug-free rat plasma and urine with 5 ng/ml and 50 ng/ml of aplidine. Five samples at each concentration were used for three consecutive days. Precision and accuracy were calculated as described for standard curves.

2.5.3. Recovery

The recovery of the extraction procedure was separately determined for both aplidine and didemnin B, using spiked plasma and urine at 5 ng/ml and 50 ng/ml. Six samples for each concentration were used. Recovery percentages were estimated by comparing the mean peak areas of extracted plasma and urine samples to the mean peak areas of standard solutions (in water 49.5%, acetonitrile 49.5% and formic acid 1%).

2.6. Pharmacokinetic studies

2.6.1. Animal preparation

Four male Sprague-Dawley rats, from our Institute breeding facilities, weighing 300–320 g were used for this study, housed under controlled conditions and fed with a standard diet ad libitum. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national [27] and international [28,29] laws and policies.

Animals were anaesthetised by sodium pentobarbital 40 mg/kg as a bolus via intra-peritoneal administration. Silastic catheters (Silmedic, medical silicon tube; 0.51 mm I.D., 0.94 mm O.D.; Lepetit S.p.A., Linate, Milan, Italy) were placed into the jugular vein according to Sigma Tau S.p.A. (Pomezia, Italy) internal protocol (unpublished method). Briefly, two incisions, at right angles, were made in the skin at the back of the neck to apply a skin button device (membrane valve for blood withdraw; mod. 617, Umberto Danuso, Bresso, Milan, Italy). The catheter was passed under the skin to the back of the neck, excess tubing was cut off, and then it was connected to the valve of the skin button. Finally, two sutures were used to fix the skin button to the underlying muscle and skin incisions were closed by sutures. Once the device and cannulas had been placed, blood samples were simply withdrawn by inserting an insulin syringe into the membrane valve of the button device.

2.6.2. Drug administration and sample collection

Four mg of aplidine were dissolved in 4 ml of ethanol and diluted with cremophor EL 10% in saline sterile solution to a final concentration of 210 μg/ml and was administered to rats via intra-venous ($n=4$) at 700 μg/kg. After drug administration, the rats were put in metabolic cages for urine collection. Blood samples (about 300 μl) were withdrawn and collected in a 2.0 ml Eppendorf microfuge tube containing 5 μl of heparin, after 2, 5, 10, 20, 60, 120, 240 min. Blood samples were immediately centrifuged to avoid haemolysis, and 100 μl of plasma were stored in a 2.0 ml microfuge tube at −20°C until extractions were performed. Urine was collected at 8 h and then at 24 h after the treatment. Urine samples were centrifuged and the supernatants were stored at −20°C until extractions were performed. Plasma samples with concentrations higher than the chosen linearity range were diluted in rat blank plasma extract and reanalysed.

3. Results and discussion

3.1. Liquid chromatography–tandem mass spectrometry

Analysis of the standards was performed by infusion in order to optimise the ionisation conditions. As showed in Fig. 2, both aplidine and didemnin B give predominant quasi-molecular ions $[M+H]^+$ when analysed in Q1 scan mode (m/z 1111 amu and m/z 1113 amu, respectively) and a main fragment, corresponding to the side chain of the molecules (R_1 and R_2 in Fig. 1), in product ion scan mode (m/z 295 amu and m/z 297 amu, respectively). Different modifiers were tested in order to increase sensitivity. Best results were obtained under acid conditions, which also minimise sodium and potassium adducts. Formic acid has shown to enhance sensitivity at least three times more than trifluoroacetic acid and almost two times more than acetic acid. The addition of ammonium acetate and ammonium formate did not increase the sensitivity and produced a considerable amount of ammonium adducts. Beyond 0.5% of formic acid, no further increase of sensitivity was achieved.

The fragmentation pattern was used for the LC–MS–MS analysis by MRM mode, which, combined with liquid chromatography separation, usually gives good sensitivity (also due to a very low signal/noise ratio) and very high specificity by either eliminating or minimising matrix interferences.

Under our chromatographic conditions, aplidine has shown two partially separated peaks, eluted after the single peak of I.S. didemnin B (Fig. 3).

The two peaks of aplidine (peaks 2 and 3) were collected separately and reinjected. Each peak gave rise to two peaks, having an identical chromatographic profile and the same peak 2/peak 3 ratio. This ratio is temperature-dependent, in that a shift to peak 3 was observed if temperature was increased (data not shown). This behaviour suggests that the two peaks of aplidine are rotamers, which are in a temperature-dependent equilibrium. Therefore, aplidine was quantified as the sum of both peak areas.

The extraction procedure with acetonitrile and chloroform in the presence of formic acid allowed to avoid most of the coeluting matrix interferences, which dramatically inhibited the ionisation of the analytes.

3.2. Linearity, precision, accuracy and recovery

The results of calibrations are shown in Tables 1 and 2. The method was linear over the range from 100 ng/ml to 5 ng/ml for plasma and from 125 ng/ml to 1.25 ng/ml for urine. The resulting standard lines were $y=0.330x-1.283$ ($r^2=0.990$) for plasma and $y=0.081x+0.115$ ($r^2=0.992$) for urine. The LOQ was 5 ng/ml for rat plasma and 1.25 ng/ml for rat urine, and the LOD was 1 ng/ml for rat plasma and 0.5 ng/ml for rat urine.

The intra-day and the inter-day variabilities are shown in Table 3. For all samples precision and accuracy were below 13% and 10%, respectively.

In plasma samples the mean recoveries were 69% (CV% <12%) for aplidine and 68% (CV% <10%) for didemnin B. Whereas in urine samples the recoveries were 91% (CV% <12%) for aplidine and 87% (CV% <7%) for didemnin B.

3.3. Pharmacokinetic findings

The applicability of the method was tested by measuring aplidine concentrations in plasma and in

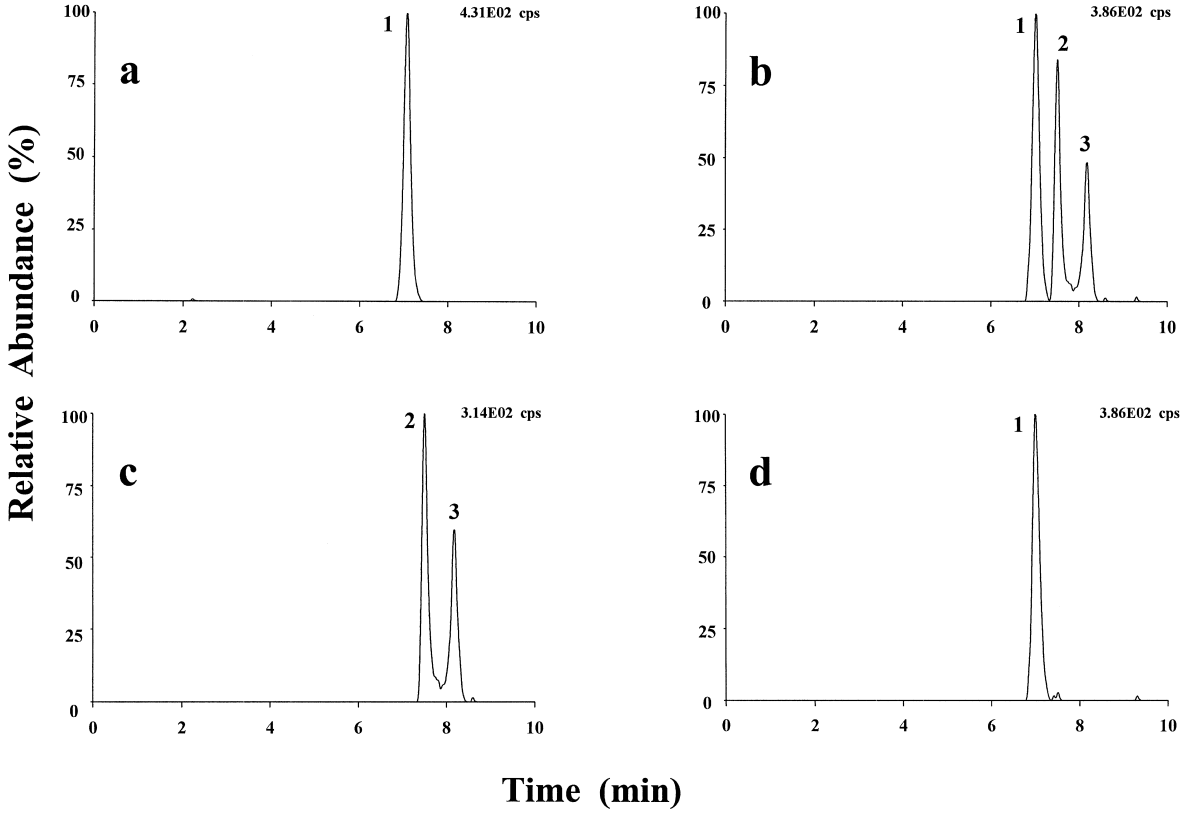

Fig. 3. Representative LC–MS–MS chromatograms of extracted plasma. Total ion chromatogram of blank (a) and of aplidine 5 ng/ml (b). Extracted ion chromatograms of m/z 1111→295 amu (c) and m/z 1113→297 amu (d). Peak 1 is didemnin B and peaks 2 and 3 are aplidine rotamers. Chromatograms of extracted urine samples are qualitatively the same as for the plasma samples.

urine samples from a pharmacokinetic study in rats. After blood withdraw, samples were immediately centrifuged to avoid haemolysis. In fact, haemolytic processes can give rise to serious errors in the evaluation of the drug kinetics, in that as previously described for didemnin B [30], about 50% of aplidine is stored in blood cells (data not shown).

At the administered dose, aplidine quickly disappeared from plasma with a half-life of 9 min. and

Table 1
Precision and accuracy data of aplidine after extraction from spiked rat plasma. ($n=5$)

Nominal conc. (ng/ml)	Mean calc. conc. (ng/ml)	Precision (CV%)	Accuracy (%)
5.00	5.72	10.72	14.42
10.00	11.38	13.64	13.81
25.00	24.86	7.50	−0.57
50.00	46.36	11.92	−7.28
100.00	101.68	4.87	1.68

Table 2
Precision and accuracy data of aplidine after extraction from spiked rat urine. ($n=5$)

Nominal conc. (ng/ml)	Mean calc. conc. (ng/ml)	Precision (CV%)	Accuracy (%)
1.25	1.08	10.00	10.42
2.50	2.29	6.12	−9.51
5.00	4.72	3.45	1.65
12.50	12.74	3.61	0.35
25.00	24.89	3.35	−1.60
50.00	51.04	1.86	−1.23
125.00	124.64	8.81	0.25

Table 3
Intra-day[a] and inter-day[b] precision and accuracy in plasma and urine samples

	Intra-day		Inter-day	
	Precision (CV%)	Accuracy (%)	Precision (CV%)	Accuracy (%)
Plasma nominal conc. (ng/ml)				
5.0	12.3	9.4	9.6	7.3
50.0	12.1	−6.9	11.9	−4.4
Urine nominal conc. (ng/ml)				
5.0	12.2	−8.3	12.3	−6.3
50.0	2.5	−7.5	3.6	−7.0

[a] Samples ($n=5$) analysed on a single day.
[b] Samples ($n=15$) analysed on three consecutive days.

Table 4
Unmodified aplidine excreted in rat urine after intravenous treatment (700 μg/kg)

Rat	0–8 h			8–24 h			0–24 h
	Conc. (ng/ml)	Urine volume (ml)	Excreted aplidine (ng)	Conc. (ng/ml)	Urine volume (ml)	Excreted aplidine (ng)	Excreted aplidine (%)
1	29.7	2.5	74.3	81.7	6.0	490.2	0.27
2[a]	7.0	6.0	42.0	–	–	–	–
3	20.1	2.0	40.2	68.6	9.5	651.7	0.31
4	42.0	1.9	79.8	110.0	5.4	594.0	0.32

[a] The animal died within 8–24 h after the treatment.

was detectable, but not quantifiable, in samples collected after 20 min (Fig. 4). The excretion of aplidine was assayed by the quantification of unmodified aplidine in the urine samples. The amount recovered in the 24 h represents the 0.3% of the administered dose. The results are shown in Table 4.

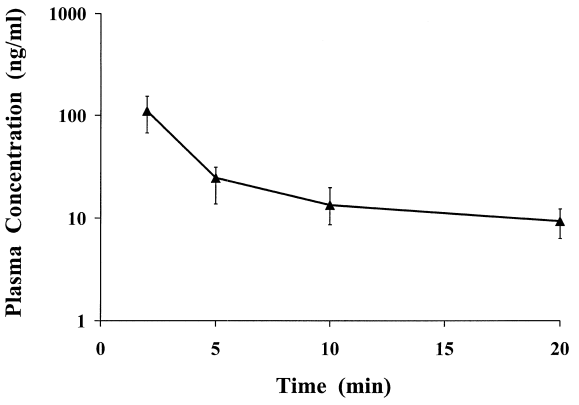

Fig. 4. Plasma concentration-time profile of aplidine (700 μg/kg) in rats after intravenous treatment (semi-logarithmic scale).

4. Conclusions

Using the LC–tandem mass spectrometry technique, we have obtained a sensitive, accurate and highly specific method able to determine aplidine (which may also be used for didemnin B) in rat plasma and urine. Moreover, the use of MRM mode increases the speed of analysis, in that partially purified samples can be used without loss of performance. This method can be suitable for supporting therapeutic monitoring of plasma and urine drug concentrations in patients, and with appropriate implementation it could become a good tool for routine clinical investigations.

Acknowledgements

The authors would like to thank Dr. Glynn T. Faircloth, Dr. José Jimeno and Dr. Ruben Henriquez of Pharma Mar S.A. for providing aplidine and

didemnin B standards and all the available information on these compounds.

The authors wish to thank also Dr. Antonio Longo, Dr. Silvia Pace, Ms. Annamaria Russo and Mr. Fabrizio Fiore of the Pharmacokinetic and Metabolism Laboratory, Sigma Tau S.p.A., for their help in setting up the animal method, and Mrs. Maria Pia De Simone and Ms. Elena Fontana for helping to prepare the manuscript. Dr. Agata Motos Gallardo is recipient of a fellowship from Zambon S.A., Spain.

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