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Bioanalysis of aplidine, a new marine antitumoral depsipeptide, in plasma by high-performance liquid chromatography after derivatization with *trans*-4′-hydrazino-2-stilbazole

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Abstract

A sensitive bio-analytical assay in plasma of the depsipeptide aplidine is reported, based on reversed-phase liquid chromatography and fluorescence detection of the *trans*-4′-hydrazino-2-stilbazole (4′H2S) derivative of the analyte. At ambient temperature, two conformations of the depsipeptide are observed in solution due to *cis–trans* isomerism at the proline–pyruvoyl peptide bond. Aplidine is isolated from the matrix by solid-phase extraction on an octadecyl modified silica stationary phase. After evaporation of the acetone eluate, a derivatization with 4′H2S is performed in a water–acetonitrile mixture at pH 4. The reaction mixture is injected directly into the chromatograph and the analyte is quantified by fluorescence detection at 410 and 560 nm for excitation and emission, respectively. The method has been validated in the 2–100 ng/ml-range, 2 ng/ml being the lower limit of quantification. Precision and accuracy both meet the current requirements for a bioanalytical assay. The identity of the 4′H2S reaction products of aplidine have been confirmed by mass spectrometric analysis. Finally, the method has been employed for a pilot pharmacokinetic study of aplidine in mice which demonstrated its usefulness for pharmacological research.

Keywords: Derivatization, LC; Aplidine; Depsipeptide; *trans*-4′-Hydrazino-2-stilbazole

1. Introduction

Aplidine (dehydrodidemnin B), a cyclic depsipeptide (Fig. 1A), is one of several recently discovered depsipeptides isolated from marine organisms. This novel member of the didemnins was isolated from the Mediterranean tunicate *Aplidium albicans* [1,2], other sources are unknown. The drug exhibits strong antitumoral activities, specifically breast, melanoma

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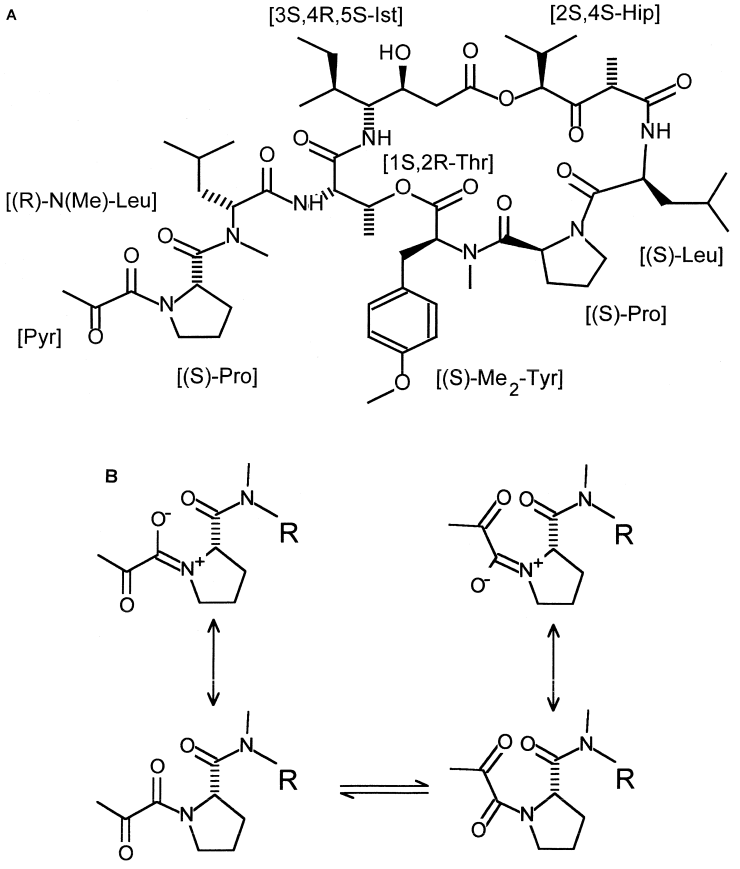

Fig. 1. Chemical structure of aplidine (A) and the *cis–trans* isomerism (B) at the peptide binding between the proline and the pyruvoyl moiety. Hip: hydroxyisovalerylpropionyl; Ist: isostatine; Leu: leucine; Pro: proline; Pyr: pyruvoyl; Thr: threonine; Tyr: tyrosine.

and non-small-cell lung cancer appeared to be sensitive to low concentrations of aplidine [3], and showed a very potent inhibition of protein synthesis [2]. Before the start of phase I clinical pharmacokinetic studies in the (very) near future it is essential that a bioanalytical assay is available. Such an assay has not been reported yet for aplidine, although an applicable chromatographic system was reported for the analysis of raw material, isolated from the tunicates [4]. Unfortunately, the molecule does not possess properties that facilitate sensitive detection by a common HPLC detector. A second complicating factor are the two chromatographic peaks observed for this compound [4], probably caused by *cis–trans* isomerism of the peptide bond between the proline and pyruvyl moieties in the side chain of the molecule (Fig. 1B).

Only a few bioassays have been reported so far for related compounds, originating from marine organisms. For didemnin B, a very sensitive radioimmunoassay was reported; 20 pg/ml was obtained as the limit of detection; however, the antiserum required for this assay may no longer be available [5]. Therefore, a competitive inhibition enzyme immunoassay was also developed [5]; unfortunately, this assay was more than 10-fold less sensitive and unidentified metabolites, cross reacting with the antibody in this procedure, seemed to be formed during pharmacokinetic investigations [6]. A chromatographic assay based on LC–UV after solid-phase extraction (SPE) has also been reported [7]; the limit of detection is 5 ng/ml in plasma and 11 ng/ml in urine. Selective chromatographic methods have been reported for FR901228 [8,9], also a depsipeptide, and for ecteinascidin 743 (ET-743) [10,11] based on both LC–UV and LC–MS. The

lower limit of quantification (LLQ) is 50 [9] and 1 ng/ml [8] for the FR901228 and 1 [10] and 0.01 ng/ml [11] for the ET-743 assays using UV and MS detection, respectively. For both agents the more sensitive LC–MS method was required for a complete pharmacokinetic study; the usefulness of the LC–UV methods was limited. However, since an MS detection system is not yet available in every hospital laboratory, we started to investigate alternative methods for the development of a bio-analytical assay for aplidine. For a bio-analytical assay in the expected lower ng/ml concentration range, we selected derivatization of the analyte into a fluorescent derivative prior to LC to achieve this required sensitivity. A clean-up of the sample previous to the derivatization was considered to be essential as particularly the proteins in the plasma sample may disturb both a quantitative reaction and chromatographic separation of the analyte. In this paper, the development and validation of a bio-analytical assay for aplidine in plasma is presented.

2. Experimental

2.1. Chemicals

Aplidine originated from PharmaMar (Tres Cantos, Madrid, Spain). Acetonitrile (gradient grade) and methanol (HPLC grade) were provided by Biosolve (Valkenswaard, Netherlands) and trifluoroacetic acid (TFA) by Sigma (St. Louis, MO, USA). Deuterochloroform (C^2HCl_3) (99.8%) and hexadeuterodimethylsulfoxide (DMSO-d_6) (99.9% with 1% tetramethylsilane (TMS)) were obtained at CIL (Cambridge Isotope Laboratories, Andover, MA, USA).

Water was on a multi-laboratory scale purified by reversed osmosis and *trans*-4′-hydrazino-2-stilbazole (4′H2S) dihydrochloride originated from Fluka (Buchs, Switzerland). Dexamethasone phosphate, morphine hydrochloride and paracetamol were of pharmaceutical grade, all other chemicals were of analytical grade from Merck (Darmstadt, Germany).

2.2. Equipment

Chromatographic analyses were performed on the following configuration: A P580 isocratic pump (Gynkotek HPLC, Germering, Germany), a Basic+ Marathon autosampler (Spark Holland, Emmen, Netherlands), equipped with a 7739-005 injection valve (Rheodyne, Cotati, CA, USA) with a 100-μl sample loop, and an FP-920 fluorescence detector (Jasco, Hachioji, Japan). The column was thermostated in a water bath and the temperature was controlled by a thermomix 1420 heating device (Braun, Melsungen, Germany). Data were recorded on a Jotronics Pentium 166-32 Mb personal computer (Delfgauw, The Netherlands), equipped with a CHROMELEON chromatographic data system (Gynkotek HPLC).

For SPE, Sep-Pak cartridges (Waters Chromatography, Milford, MA, USA) containing 200 mg octadecyl-modified silica as the sorbent were processed with a 24-port Visiprep SPE vacuum manifold (Supelco, Bellefonte, PA, USA). LC–MS was performed on an LC-10 AD pump (Shimadzu, Kyoto, Japan) with the column outlet directly coupled to a VG Platform II mass spectrometer (MicroMass, Altrinchem, UK). ^{1}H-NMR spectra were recorded with a Gemini 300 BB instrument (Varian, Palo Alto, CA, USA) at 100.1 mHz.

2.3. LC–MS conditions

A Superspher 100 C_{18} (2H100 mm, Merck) column was used at ambient temperature and the eluent was acetonitrile–water–TFA (60:39.9:0.1, v/v). The injection volume was 20 μl. The eluent flow (0.2 ml/min) led directly into the electrospray interface in the positive ion mode. The total mass range, 500–2000, was scanned in 2.1 s per cycle. The mass resolution was 15 (instrumental units), the cone voltage 30 V and the source temperature 120°C.

2.4. NMR procedure and conditions

Aplidine (1 mg) was dissolved in 0.65 ml C^2HCl_3 or DMSO-d_6. In C^2HCl_3, TMS was used as internal reference, in DMSO-d_6 the central DMSO line was set at 2.50 ppm.

2.5. Chromatographic conditions

Twenty-μl injections were made on a Symmetry C_{18} column (100×4.6 mm, d_p=3.5 μm, average

pore diameter = 10 nm, Waters). The column temperature was 64±2°C. The eluent comprised acetonitrile–water–TFA (47:52.9:0.1, v/v) at a flow-rate of 1.5 ml/min. The fluorescence detection wavelengths were 410 nm for excitation and 560 nm for emission, respectively.

2.6. Analytical procedure

The SPE column was pre-treated with 2×2 ml methanol and 2×2 ml water. A 1-ml plasma sample was then loaded onto the column, followed by washing with 2 ml water, 2 ml of acetonitrile–water (50:50, v/v) and 0.3 ml acetone (the sorbent was kept wetted in between all the solvent additions). Next, the analyte was eluted with 1.5 ml acetone and collected in a polypropylene microtube. After evaporation of the eluate under a nitrogen gas stream at ambient temperature, the sample was reconstituted in 50 μl acetonitrile by vortex-mixing. Next, 50 μl of 0.5 *M* ammonium chloride (acidified to pH 4.0 with 0.1 *M* hydrochloric acid) and 50 μl of 10 mg/ml 4′H2S dihydrochloride in water were added. After vortex-mixing, the mixture was heated at 80°C for 20 min in a thermostated water bath; next, after cooling to ambient temperature, the mixture was transferred to an injection vial with a glass 250-μl insert.

2.7. Method optimization

For the optimization of the derivatization reaction a 25 μg/ml aplidine solution in acetonitrile (8 μg/ml in the reaction mixture) was used. All conformations of both aplidine and its derivative were detected in one analytical chromatographic run, using a non-endcapped C_{18} column (Microspher 100, Merck) and an eluent comprising acetonitrile–water–THF (65:34.9:0.1, v/v/v) at a 1 ml/min flow-rate. UV absorbance detection at a low wavelength (208 nm) was used in addition to fluorescence detection at 400 nm for excitation and 540 nm for emission.

2.8. Validation

Stock solutions of 116 and 157 μg/ml aplidine in acetonitrile were prepared (with separate weighing) and were stored at −20°C. For calibration a 1 μg/ml dilution in plasma, pooled from five individuals, was made from the 157 μg/ml stock solution and stored at −20°C. Dilutions of the 1 μg/ml standard of aplidine in plasma to yield 2, 5, 10, 20, 50 and 100 ng/ml aplidine calibration samples in plasma, respectively, were made daily for each analytical run. Least-squares regression, weighted by $1/X$ (reversed concentration) was employed, using the area of the first and largest of both derivative peaks of aplidine.

From a 1 μg/ml dilution in plasma made from the 116 μg/ml stock solution, validation samples in plasma were made at 2, 5, 200 and 1000 ng/ml, respectively, and stored at −20°C; plasma of different individual donors was used. Precisions and accuracies were determined by quintuplicate analysis of each validation sample in three different analytical runs. The repeatability (intra-day precision) is calculated according to

$$\text{Repeatability} = \frac{\sqrt{\text{ErrMS}}}{\text{GM}} \cdot 100\%$$

where ErrMS = error mean square and GM = grand mean, and the reproducibility (inter-day precision) according to

$$\text{Reproducibility} = \frac{\sqrt{(\text{DayMS} - \text{ErrMS})/n}}{\text{GM}} \cdot 100\%$$

where DayMS = day mean square and n = number of replicates in each run, for each individual concentration. Six individual blank plasma samples are also tested.

Samples of 5, 50 and 500 ng/ml aplidine in plasma were made for stability studies; these samples were stored at ambient temperature for 1 day or shorter, at 2°C for 1 week and at −20 and −80°C for 3 weeks and longer. In addition, plasma samples spiked at 10, 20 and 100 ng/ml aplidine, respectively, are stored at −80°C. These samples were used to test the effects of additional freeze–thaw cycles and to determine any deterioration of aplidine in plasma at ambient temperature and at 37°C.

For the analysis of the validation and stability samples at 50, 200, 500 and 1000 ng/ml only 100 μl samples were used and were supplemented with 900 μl of pooled blank plasma to give 1-ml samples.

For the determination of the extraction yield, 50 μl of an aplidine standard in acetonitrile, made from the same stock as the calibration samples in plasma, in the 40–2000 ng/ml range is added to the SPE eluate resulting from blank plasma. The yield was calculated for two calibrations in two separate ana-

lytical runs by dividing the slopes of the two different calibration lines.

The stability of the derivatives of aplidine stored in the autosampler at ambient temperature overnight was verified. The recovery was calculated identically to the extraction yield.

The selectivity of the assay was also tested by investigating the influence of possible co-medications. Stock solutions of 2–5 mg/ml in water were made from dexamethasone, morphine and paracetamol. Blank pooled plasma samples were spiked with one of the individual drugs to obtain approximately 10-μg/ml samples. For each drug duplicate analysis was performed.

The pharmacokinetics of aplidine in mice (≈25 g) were investigated after an i.v. bolus injection of 1 mg/kg aplidine. Sixteen mice were treated and sacrificed to take a plasma sample after 6, 24, 60 and 120 min ($n=4$ for each interval) respectively; blank mouse plasma was also tested. The mouse plasma sample volumes ranged from 0.2 to 0.45 ml and were supplemented to 1 ml with pooled human plasma prior to extraction and further analysis.

3. Results and discussion

3.1. Method development

The necessity for a bio-analytical assay for aplidine prior to the start of phase I clinical trials with doses in the order of 1 mg, urged us to develop a sensitive method in the lower ng/ml-range. In order to enhance the detectability of the analyte in an LC assay we focused on the development of a derivatization with a fluorescent label. Useful reactive groups in the molecule may be a hydroxyl group or carbonyl groups. The carbonyl group of the pyruvyl moiety in the side chain may be relatively good sterically accessible. For the derivatization of carbonyl functions, the reaction with several hydrazino compounds has been described [12]. We chose 4′H2S because of its (recent) commercial availability, its reactivity in an aqueous solution and therefore the possible direct injection of the reaction mixture in an RPLC system [13–15]. The reaction equation is shown in Fig. 2.

The presence of two conformations of aplidine in solution complicated the development of a quantitative analytical method. Recently, Hamada et al. [16] showed with ^{13}C-NMR that the two conformers are the *cis* and *trans* isomers of the pyruvoyl–proline amide bond. In C^2HCl_3, we also found two conformations to be present, in approximately a 1:1 ratio (NH protons and methyl protons of pyruvoyl group) using ^{1}H-NMR. The spectrum in DMSO-d_6 however, shows the presence of four conformations in approximately a 35:35:15:15 ratio (methyl protons of pyruvoyl group). The additional signals probably originate from *cis–trans* isomerism of the second proline amide bond. These four signals coalesce into two if the temperature is raised; at 105°C only the

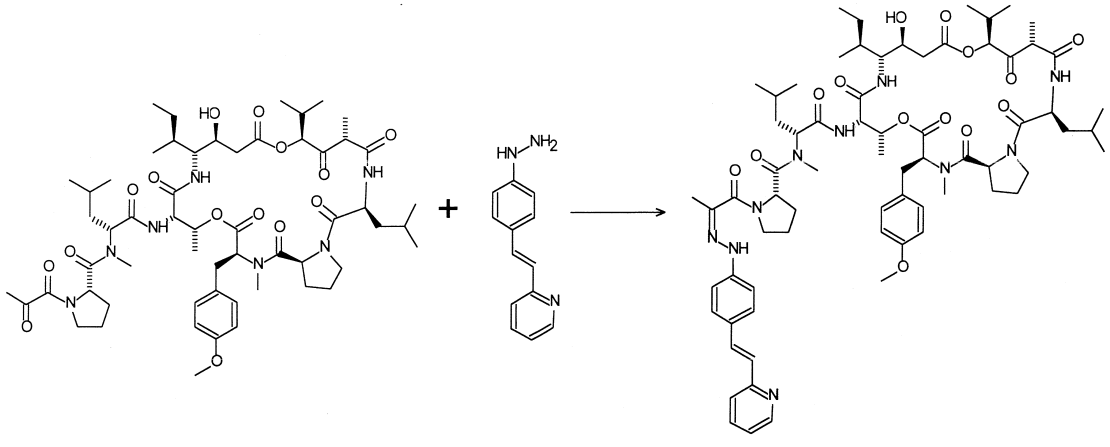

Fig. 2. Derivatization reaction of aplidine with 4′H2S.

two signals for the pyruvoyl methyl group remain. In the C^2HCl_3 spectrum, the threonine NH signal of one conformer is more shielded than that of the other conformer, shown by a lower dependency of the chemical shift on the temperature. This indicates that the two conformers arise, indeed, as a result of *cis–trans* isomerism at the pyruvoyl–proline residue because this shielding is caused by a hydrogen bond of the threonine NH proton with the carbonyl function of the pyruvoyl residue of the *cis* isomer [16].

At ambient temperature, the equilibrium reaction is too slow to elute both isomers as one chromatographic peak but also too fast to elute them as two baseline-separated peaks. If the individual conformations are collected at the detector outlet, stored at ambient temperature and reinjected, the peak ratio is constant after approximately 2 h, the equilibrium then being established. After derivatization of aplidine with 4′H2S, again two chromatographic peaks (Fig. 3) are observed, corresponding to two conformations of the aplidine derivative. Both peaks are well separated, indicating a slower equilibrium reaction compared to the parent compound. If now the individual conformations are collected, the equilibrium is not yet totally established after 24 h at ambient temperature or 1 h at 80°C. Because didemnin A and didemnin B, analogues of aplidine without a carbonyl function in the side chain, do not show conversion with 4′H2S under identical reaction conditions, we presume that only the carbonyl group at the side chain of aplidine reacts with 4′H2S and not the carbonyl function in the cyclic part of the structure (Fig. 2). This assumption was supported by LC–MS analysis (Fig. 4); for both derivatives ($C_{70}H_{98}N_{10}O_{14}$) the singly charged ($m/z=1303$) and doubly charged ($m/z=652$) molecular ions are found, corresponding to aplidine with one stilbazole group.

The derivatization reaction was optimized with respect to reaction time and temperature. The yield of the derivatives and the recovery of aplidine as a function of the reaction time is shown in Fig. 5. The figure shows a maximum yield of the derivatives with complete conversion of aplidine into the derivatives when the reaction mixture is sufficiently long heated at 70 or 80°C; we choose 20 min at 80°C for further use. The quotient of the response of both derivatives of aplidine, for both fluorescence and UV signal, is constant in these experiments and remains in the range 3.4–3.9 for the fluorescence signal and 1.8–2.2 for the UV signal; quantification of aplidine using only the largest (first) peak can then be allowed.

3.2. Validation

Examples of chromatograms at different concentrations are shown in Fig. 3. In an analytical run, calibration samples in the range 2–100 ng/ml were used for quantification of the validation samples, averaged back calculated concentrations are given in Table 1 for the different concentration levels in nine analytical runs. The results of the validation samples, precision and accuracy at each level in three different analytical runs, are listed in Table 2. The lowest

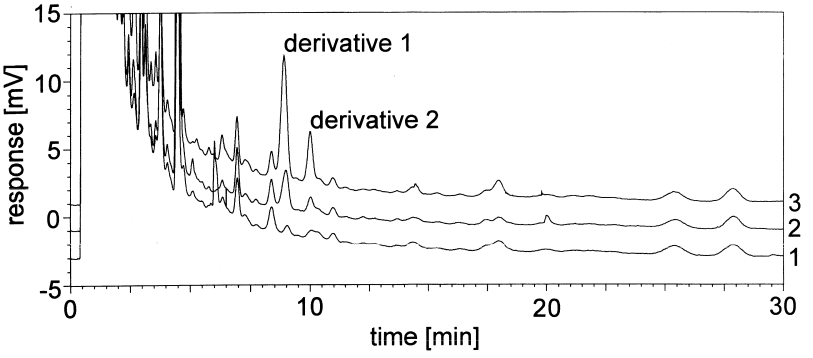

Fig. 3. Chromatograms of derivatized aplidine in plasma. (1) blank; (2) 2 ng/ml spiked to blank plasma; (3) 10 ng/ml spiked to blank plasma.

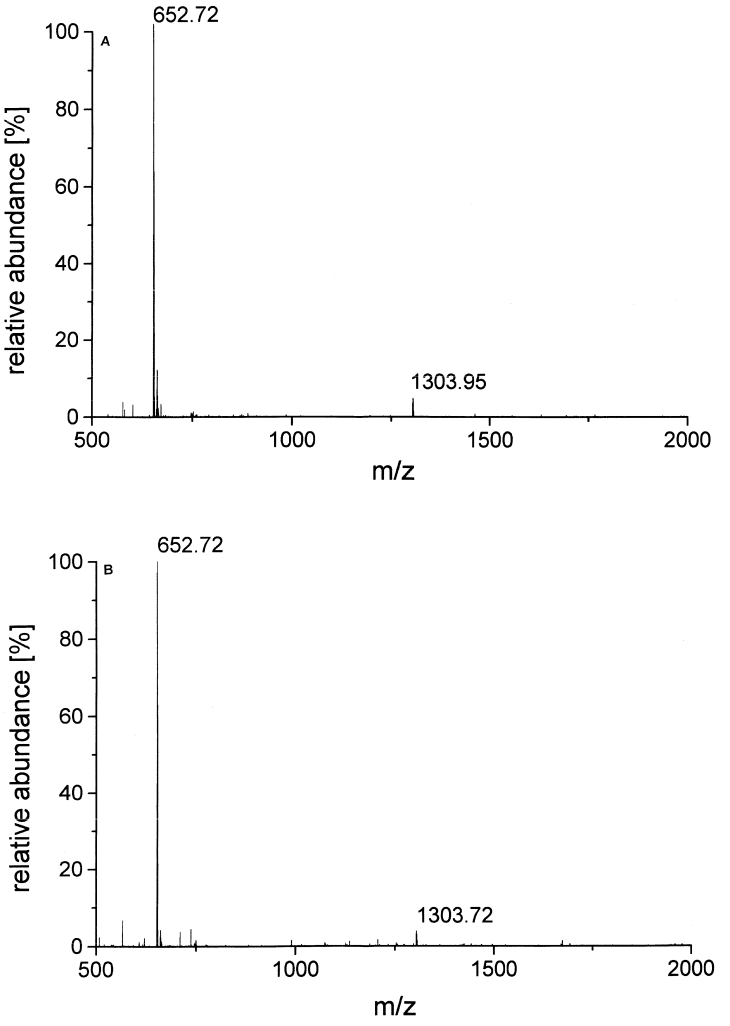

Fig. 4. (+) Electrospray mass spectra of the 4′H2S derivatives of aplidine (derivatization of 243 μg/ml aplidine in acetonitrile under standard conditions) after in-line LC separation. (A) derivative 1; (B) derivative 2.

level, 2 ng/ml, proves to be the LLQ. All values of the precision and the accuracy do meet the demands for a bioanalytical assay: ≤20% for the LLQ and ≤15% at higher concentration levels [17]. No appropriate internal standard was available, however, the validation data show precise and accurate results after using the method without internal standard. In six individual blank plasma samples, no interferences in the chromatograms are observed which could influence the quantification of aplidine; the calculated 'aplidine concentration' in these blanks is 0.1±0.6 ng/ml.

For both stock solutions of aplidine in acetonitrile, stored at −20°C, no deterioration was observed after 5.5 months; deviations <1% of the weighted value were found for both standards with the validated quality control method, based on RPLC with UV detection, at the pharmacy laboratory of the Slotervaart Hospital, Amsterdam.

Special attention was paid to the stability of aplidine in the plasma samples. Results of storage of different concentrations of aplidine at different temperatures are given in Table 3. The table shows the necessity of using −80°C as the long-term storage

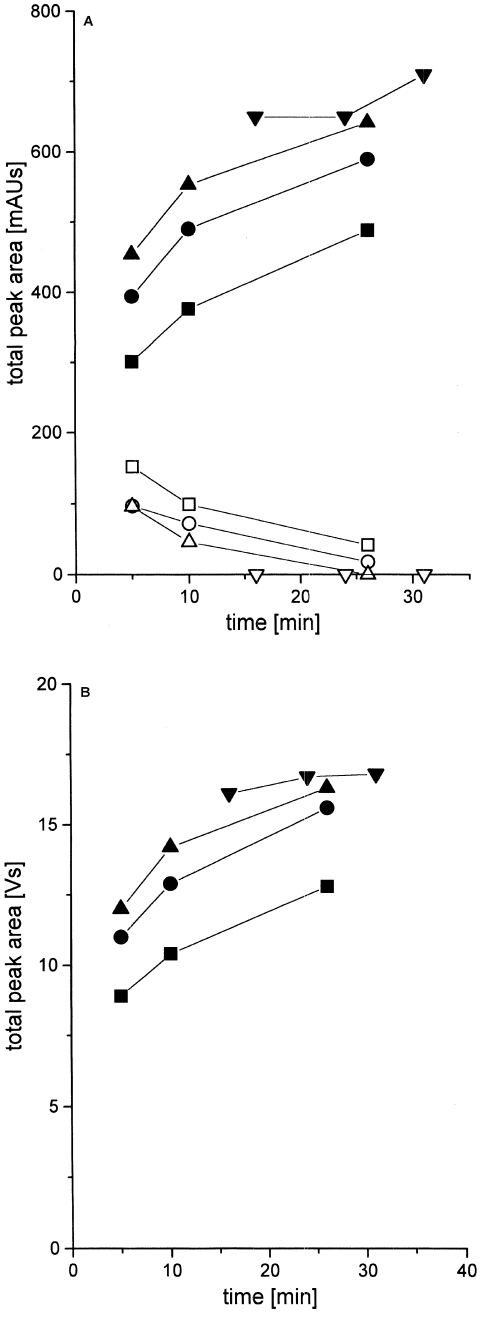

Fig. 5. Aplidine peak areas (areas of the peaks of both conformers are added) after the reaction of 25 μg/ml aplidine in acetonitrile with 4′H2S at different temperatures for different times under standard conditions. (■) 50°C; (●) 60°C; (▲) 70°C; (▼) 80°C. (A) fluorescence detection at 400 and 540 nm for excitation and emission, respectively; (B) UV absorbance detection at 208 nm. Open symbols: aplidine; closed symbols: 4′H2S derivative of aplidine.

Table 1
Results of back calculated aplidine calibration samples ($n=9$)

c (nominal) (ng/ml)	Found (ng/ml)	RSD (%)	Accuracy (%)
2	2.13±0.24	11	107
5	4.63±0.41	9	93
10	9.7±0.7	7	97
20	20.4±0.6	3	102
50	51.5±2.6	5	103
100	98.6±2.9	3	99

Table 2
Overall results of validation samples ($n=15$)

c (ng/ml)	Repeatability (%)	Reproducibility (%)	Accuracy (%)
2	14	3	107
5	10	7	97
20[a]	8	2	94
100[a]	6	1	105

[a] Diluted from a 10-fold concentration of aplidine in plasma, prior to the analysis.

condition; only a limited storage period can be tolerated. The decline of the aplidine concentration in plasma samples stored at both ambient temperature and 37°C is shown in Fig. 6. For both temperatures the figure shows rapid degradation and warrants rapid cooling down of a sample, preferably to 2°C or lower, after sampling. After being separated from the blood cells, the plasma sample should be frozen the same day when it is temporarily stored at 2°C and within 1 h when it is temporarily stored at ambient temperature. The influence of extra freeze–thaw cycles is shown in Table 4, indicating that these procedures cannot be allowed.

The yield of the SPE is 55% and reproducible. Apparently, this relatively low extraction yield, mainly caused by protein binding, did not seriously affect the precision and accuracy of the assay. The recovery of the aplidine derivative after overnight storage in the autosampler at ambient temperature is 97%, which is sufficient for chromatographic analysis during the night. No co-elution of extra peaks is observed if 10 μg/ml of respectively dexamethasone, morphine or paracetamol is present in the processed plasma sample.

The results of the pilot pharmacokinetic study of aplidine in mice are shown in Fig. 7. Blank mouse

Table 3
Recovery of aplidine in plasma ($n=3$) after storage at different temperatures

Temperature (°C)	Recovery, % (storage time)		
	5 ng/ml	50 ng/ml	500 ng/ml
Ambient	59±6 (16 h)	<40 (20 h)	65±2 (20 h)
2	43±7 (1 week)	53±3 (1 week)	49±1 (1 week)
−20	76±6 (3 week) 88±3 (9 week) 50±6 (28 week)	78±6 (4 week) 69±12 (11 week)	91±1 (4 week) 95±4 (11 week) 83±4 (29 week)
−80	90±7 (3 week) 100±4 (9 week) 73±6 (28 week)	84±4 (4 week) 86±10 (11 week)	110±4 (4 week) 98±5 (11 week) 89±16 (29 week)

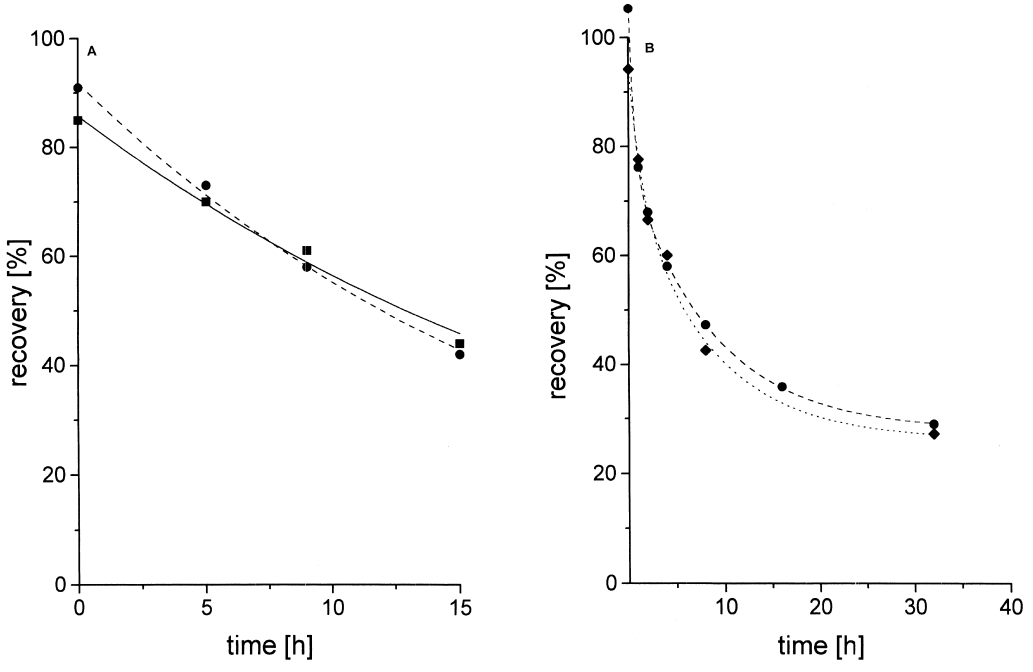

Fig. 6. Aplidine decomposition in plasma at (A) ambient temperature, fitted according to a first order exponential decline and (B) 37°C, fitted with a double exponential function (■,———) 10 ng/ml; (♦,· · · · · ·) 20 ng/ml; (●,- - - - - -) 100 ng/ml. Each point represents the average of the results of the duplo analysis.

Table 4
Recovery of aplidine ($n=3$) in plasma after additional freeze–thaw cycles in between storage at −80°C

No. of extra freeze–thaw cycles	Recovery (%)	
	10 ng/ml	100 ng/ml
2	76±15	69±2
4	71±4	65±1

plasma showed a small interference, corresponding with 3 ng/ml aplidine in mouse plasma. the pharmacokinetic data are corrected for this amount in the blank. Chromatograms of mouse samples are shown in Fig. 8. The source of the endogenous interference of mouse plasma in the present assay, larger compared to human plasma, is not known.

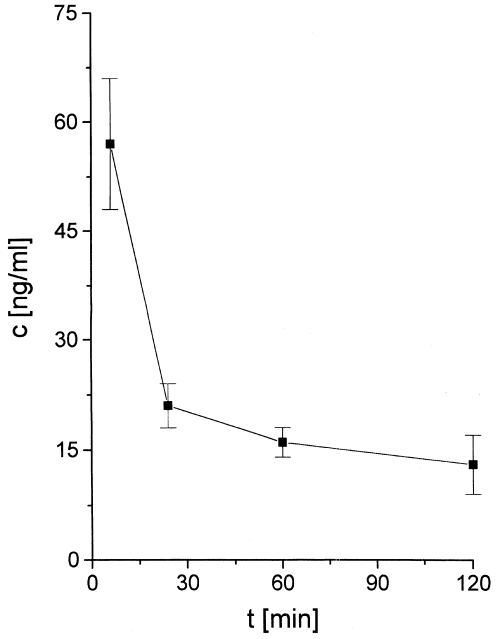

Fig. 7. Pharmacokinetic plot of aplidine in mice ($n=4$ at each time): dose: 1 mg/kg.

4. Conclusions

A selective, sensitive, precise and accurate assay for aplidine in plasma has been developed and could be used in a pre-clinical pharmacokinetic investigation of aplidine in mice. The method can be used for clinical studies in the future. Aplidine is not stable in plasma: immediate freezing to −80°C after sampling is recommended. The samples should only be thawed once, shortly before analysis. The storage time should not exceed 11 weeks.

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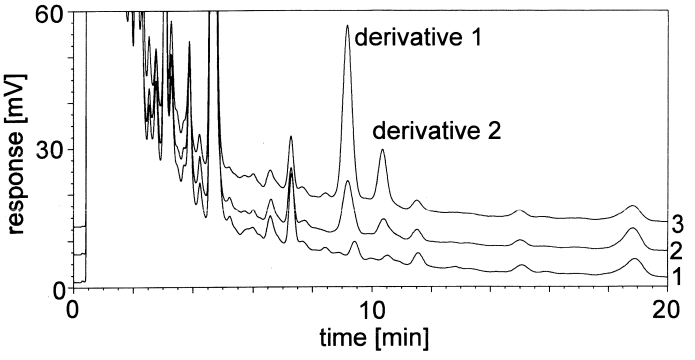

Fig. 8. Chromatograms of derivatized aplidine in mouse plasma. (1) blank (0.4 ml mouse plasma); (2) 18 ng/ml in a 0.4 ml plasma sample; (3) 55 ng/ml in a 0.3-ml plasma sample.

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