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Determination of LY355703 in Dog and Mouse Plasma by Positive Ion Liquid Chromatography/Tandem Mass Spectrometry with Atmospheric Pressure Chemical Ionization

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A liquid chromatographic/mass spectrometric assay was developed for the determination of LY355703, a potent anti-tumor drug, in mouse and dog plasma. Empore (3M) C_{18} solid-phase extraction cartridges were used for sample preparation in conjunction with a positive pressure manifold. Chromatographic separation was obtained with a cyano high-performance liquid chromatographic column and detection was conducted using atmospheric pressure chemical ionization tandem mass spectrometry in the selected reaction monitoring mode. A structural analog, compound LY354504, was used as the internal standard. The assay was validated for the determination of LY355703 in mouse (ICR and NuNu) and dog (beagle) plasma. The lower and upper limits of quantitation were 2.1 and 527 ng ml^{-1}, respectively, using a 0.1 ml plasma aliquot. The signal-to-noise ratio of a typical 2.1 ng ml^{-1} standard was ~40:1. The inter-day precision (relative standard deviation) and accuracy (relative error) derived from the analysis of validation samples at five concentrations ranged from 2.7 to 7.6% and from 4.8 to 4.5%, respectively. Throughput is approximately one sample every 3 min. This assay is simple, sensitive, accurate, precise and is being used to support toxicokinetic studies in dog and mouse. © **1998 John Wiley & Sons, Ltd.**

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INTRODUCTION

The compound LY355703 (Fig. 1) is one of a series of natural products, known as the cryptophycins, from cyanobacteria (blue–green algae) of the genus *Nostoc*, that has shown potent anti-tumor activity.[1–5] A rapid, sensitive liquid chromatographic/tandem mass spectrometric (LC/MS/MS) assay was required to analyze samples to support toxicokinetic and, potentially, clinical studies. A structural analog, compound LY354504, was chosen as the internal standard (Fig. 1).

Previously, no assay for the determination of LY355703 or any similar cryptophycin compound in biological matrices has been reported. An LC/MS/MS assay was established for LY355703 prior to this work and was used to support pilot pharmacokinetic studies in mouse, rat and dog (M. Berna and P. Wood, unpublished results). This method had adequate sensitivity and was used for the simultaneous quantitation of four cryptophycin analogs. However, owing to mass spectral interference between two of the analytes, chromatographic resolution was necessary and resulted in low sample throughput.

In this paper, we present a sensitive LC/MS/MS method that is rugged and can rapidly determine LY355703 concentrations in mouse and dog plasma. LC/MS/MS with atmospheric pressure chemical ionization (APCI) is a common technique used for the rapid and sensitive quantitation of pharmaceuticals in biological matrices.[6–8] Owing to the sensitivity of LC/MS/MS, a sample size of 0.1 ml is all that is necessary to

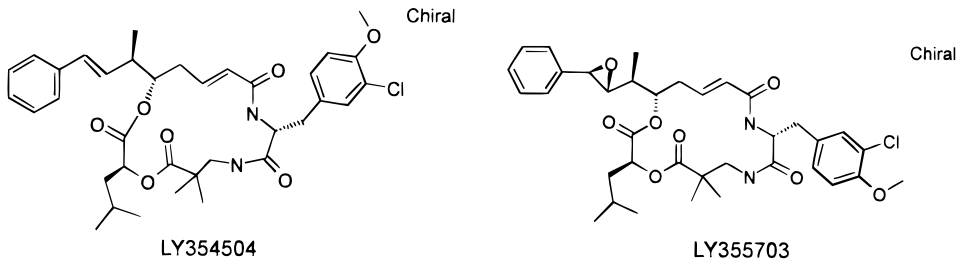

Figure 1. Structures of LY355703 and LY354504 (internal standard).

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obtain the desired lower limit of quantitation (LLOQ) (2.1 ng ml^{-1}), producing a signal-to-noise ratio of 40:1. Should the need to lower the LLOQ arise, initial studies indicate that an LLOQ of 250 pg ml^{-1} could be obtained.

EXPERIMENTAL

Chemicals and materials

Compounds LY355703 and LY354504 were obtained from Eli Lilly (Indianapolis, IN, USA). Control beagle dog plasma was obtained from the Toxicology Division at Eli Lilly and ICR and NuNu mouse plasma from Harlan Bioproducts for Science (Indianapolis, IN, USA). High-performance liquid chromatographic (HPLC)-grade acetonitrile, methanol and propan-2-ol were obtained from Burdick & Jackson (Muskegon, MI, USA) and ethanol (190 proof) from Quantum Chemical (Tescola, IL, USA). Water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Empore (3M, Minneapolis, MN, USA) 3 ml/7 mm C_{18} solid-phase extraction (SPE) extraction cartridges and positive pressure extraction manifolds were obtained from Varian (Sugar Land, TX, USA).

LC/MS equipment and analytical conditions

A Shimadzu HPLC system consisting of two LC-10AD pumps, an SCL-10A system controller, a GT-104 solvent degasser and a SIL 10A-XL autosampler was used for all HPLC analyses. The chromatographic system consisted of a Waters HP CN column (3.9 × 150 mm, d_p 4 μm) and mobile phase (63% acetonitrile–30% water–7% propan-2-ol) was delivered at 1 ml min^{-1}. directly into the APCI source. The autosampler was setup to make 25 μl sample injections every 2.2 min. Mass spectrometric detection was performed on a Sciex API III Plus instrument operating in the positive ion APCI mode. The heated nebulizer was set at 500 °C with the discharge ionization current at 5 μA. The orifice potential was set at 65 V and the collision gas thickness was 90:10 Ar–N_2 at $\sim 330 \times 10^{12}$ atoms cm^{-2}. Nitrogen was used as the auxiliary and nebulizer gas and was set at 1.5 l min^{-1} and 80 psi, respectively. Quantitation was performed using selected reaction monitoring (SRM) of the transitions LY355703 m/z 669 → 238 and internal standard m/z 653 → 143 with a scan time of 1.33 scans s^{-1} and a dwell time of 249.98 ms. The product ion spectra of LY355703 and its internal standard, LY354504, are presented in Fig. 2. Mass calibration, data acquisition, chromatographic and mass spectral representation and post-acquisition quantitative analyses were performed via a suite of PE Sciex software applications: Tune 2.5, RAD 2.6, MacSpec 3.3 and MacQuan 1.3.

Standard solutions

A stock standard solution of LY355703 was prepared by dissolving 2.11 mg of compound in 50 ml of ethanol to give a final concentration of 42.2 μg ml^{-1}. This solution was diluted to give a series of working solutions with concentrations of 2110, 844, 422, 211, 105, 42.0, 21.0 and 8.4 ng ml^{-1}. A stock standard solution of LY354504 (internal standard) was also prepared by dissolving 2.18 mg of compound in 50 ml of ethanol to give a final concentration of 43.6 μg ml^{-1}. This solution was further diluted to give a working solution of 545 ng ml^{-1}. The stock standard and working solutions were stored at −20 °C when they were not in use.

Sample preparation

The samples for calibration were prepared by placing 100 μl of plasma in a 1.5 ml polypropylene centrifuge tube and spiking 25 μl of the appropriate working standard solution followed by 25 μl of the internal standard working solution. Duplicate standards were prepared for each analysis during the validation and study sample analysis at concentrations of 527, 211, 106, 52.7, 26.3, 10.5, 5.3 and 2.1 ng ml^{-1}.

The validation samples, used to evaluate the accuracy and precision during the validation, and the quality control samples, used during the analysis of study samples, were prepared in the same fashion as the samples for calibration. During the validation, five replicates at each of the following concentrations were prepared and analyzed on each of three days: 527, 211, 52.7, 5.3 and 2.1 ng ml^{-1}. During the analysis of study samples, quality control samples were prepared in duplicate (or more) at the following concentrations and run with each analysis: 211, 52.7 and 5.3 ng ml^{-1}.

Two types of blank samples, with and without internal standard, were prepared in duplicate and run with each analysis. The blanks were included in both validation and study sample analyses. The blank with internal standard was prepared by spiking 25 μl of ethanol and 25 μl of the internal standard working solution into 100 μl of plasma. The blank without internal standard was prepared in the same fashion, but an additional 25 μl of ethanol replaced the addition of the internal standard.

The study samples were prepared by adding 100 μl of the sample, 25 μl of ethanol and 25 μl of the internal standard working solution to the polypropylene centrifuge tube. All samples were extracted by adding 200 μl of acetonitrile to each tube to precipitate the proteins followed by vortex mixing. Next, the samples were centrifuged at 3000 × g for ∼5 min followed by the addition of 1 ml of water to the supernatants. The Empore C_{18} extraction cartridges were preconditioned with methanol (3 ml) followed by water (3 ml). A Cerex (Varian) positive pressure extraction manifold was used to force the wash and elution solvents through the extraction cartridges. A 1 ml volume of water was added to the cartridges followed by the sample supernatants; the samples were forced through the cartridges. The samples were washed with 1 ml of 35% acetonitrile in water and eluted with 1 ml of acetonitrile. Using a Savant SpeedVac, the effluents were concentrated to dryness (ambient temperature) and the dry residues were reconstituted with 50 μl of reconstitution solution (50% water–45% acetonitrile–5% propan-2-ol).

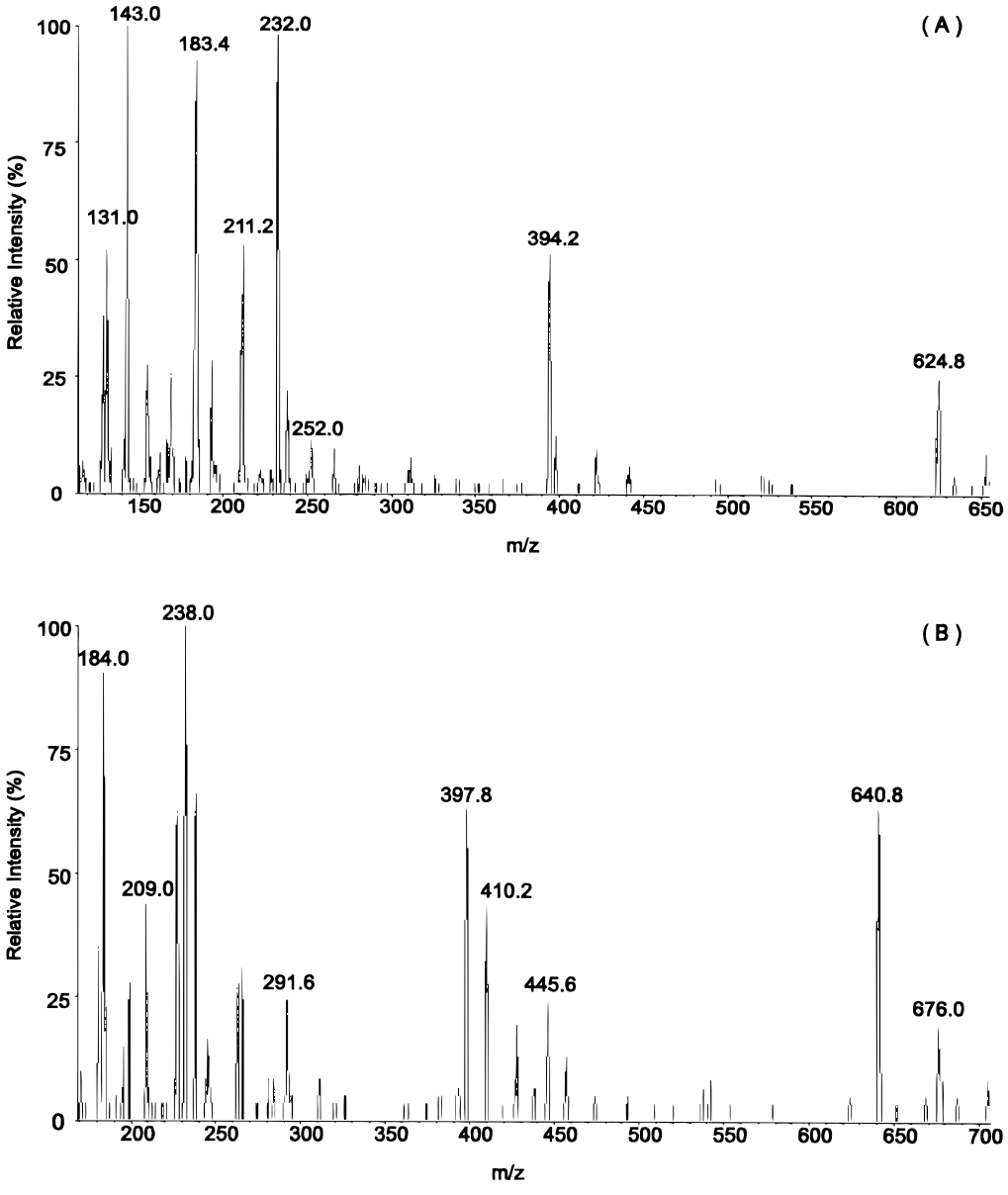

Figure 2. Product ion mass spectra obtained by collision-induced dissociation of (A) LY354504 (internal standard) and (B) LY355703.

Calibration

Two standard curves were prepared in the range 2.1–527 ng ml^{-1} at concentrations of 527, 211, 106, 52.7, 26.3, 10.5, 5.3 and 2.1 ng ml^{-1}. One of the standard curves was run at the beginning of each analysis and the second at the end of each analysis. The peak area ratio of LY355703 to the internal standard was related to concentration using a linear regression with $1/y^2$ weighting.

Validation procedures

Validation samples were prepared and analyzed to evaluate the intra-and inter-day accuracy and precision of the analytical method in each of the matrices: beagle plasma and ICR and NuNu mouse plasma. Five replicates of each of the validation concentrations (527, 211, 52.7, 5.3 and 2.1 ng ml^{-1}) were analyzed along with two standard curves on each of three days using the same instrument.

The extraction efficiency of LY355703 and the internal standard were determined by comparing the peak areas of extracted samples to those of extracted blanks spiked with standard and internal standard. This was performed by analyzing three replicates at each of two concentrations, 5.3 and 211 ng ml^{-1}. In addition, the effect of the matrix on the detection of the analytes (matrix effect) was evaluated by comparing the extracted blanks spiked with standard and internal standard with neat standards at the same concentrations. The selectivity of the assay was investigated by processing and analyzing blanks prepared from two independent lots of control plasma; the blanks were surveyed for interfering peaks. The selectivity was tested in each of the three matrices.

The stability of LY355703 in mouse (ICR and NuNu) and dog plasma was studied under a variety of storage and process conditions. The freezer stability (−70 °C)

was evaluated by preparing three validation samples at 5.3 and 211 ng ml^{-1} and analyzing them after being stored at $-70\,^{\circ}C$ for a period of 1, 2 and 4 weeks. Additional freezer stability data will be collected while conducting toxicology studies. The stability of the analytes in the injection solvent was studied to verify that the compounds would not degrade over the course of an analysis. This was accomplished in two ways: first, by running identical calibration graphs at the beginning and end of each analysis throughout the validation and during study sample analysis and, second, by extracting samples and storing them overnight at room temperature and injecting them into the LC/MS/MS system the following day with a fresh standard curve. The back-calculated standard sample concentrations were used to evaluate the stability of the analysis. The effect of three freeze–thaw cycles on the stability of the analytes was determined by analyzing triplicate validation samples at 5.3 and 211 ng ml^{-1} after 1, 2 and 3 freeze–thaw cycles. Room temperature stability was studied by analyzing triplicate validation samples at 5.3 and 211 ng ml^{-1} after being incubated in the biological matrix, prior to extraction, for 4 and 24 h. Finally, sample dilutions were tested by analyzing triplicate 106 ng ml^{-1} samples that were diluted from a 10.6 μg ml^{-1} (dilution factor 1:100) concentrated plasma sample. Stability of the spiking stock solutions was tracked by placing system suitability samples in front of the run at 5.3 and 211 ng ml^{-1}. The same stock solutions were sampled for over 1 month to ensure instrumental accuracy and sensitivity for the day, as well as stock solution stability.

RESULTS AND DISCUSSION

Linearity

A typical calibration graph for LY355703 is shown in Fig. 3. It was constructed by plotting the peak area ratio of LY355703 to the internal standard versus the plasma concentration of LY355703. Over an 11 day period during the validation of LY355703 in the three biological matrices, a total of 14 calibration graphs were analyzed. The correlation coefficients obtained were 0.99 or higher.

Assay precision and accuracy

The data for the intra-day precision and accuracy of the method determined by analyzing five replicates at 2.1, 5.3, 52.7, 211 and 527 ng ml^{-1} on each of three days for ICR mouse plasma, and on a single day for NuNu mouse and beagle dog plasma, are reported in Tables 1–3. The inter-day precision and accuracy data for ICR mouse plasma are reported in Table 4. The accuracy of the method was determined by calculating the relative error (RE) and the precision by calculating the relative standard deviation (RSD). The inter-day precision (RSD) ranged from 2.7 to 7.6% and the mean inter-day accuracy (RE) ranged from −4.8 to 4.5%, over the five concentrations evaluated.

Stability

Stock solutions of LY355703 and LY354504 were found to be stable for a minimum of 4 weeks when prepared in ethanol and stored in polypropylene vials at $\sim -20\,^{\circ}C$ prior to and after each use. Also, LY355703 was found to be stable at $\sim -70\,^{\circ}C$ (in dog and ICR and NuNu mouse plasma) for at least 3 months. No degradation of LY355703 or LY354504 was observed in the reconstitution solvent during the period when the samples were on the autosampler waiting to be injected (up to 24 h), during three freeze–thaw cycles in ICR mouse, NuNu mouse or beagle plasma or during incubation at room temperature (up to 24 h) in each of the three matrices.

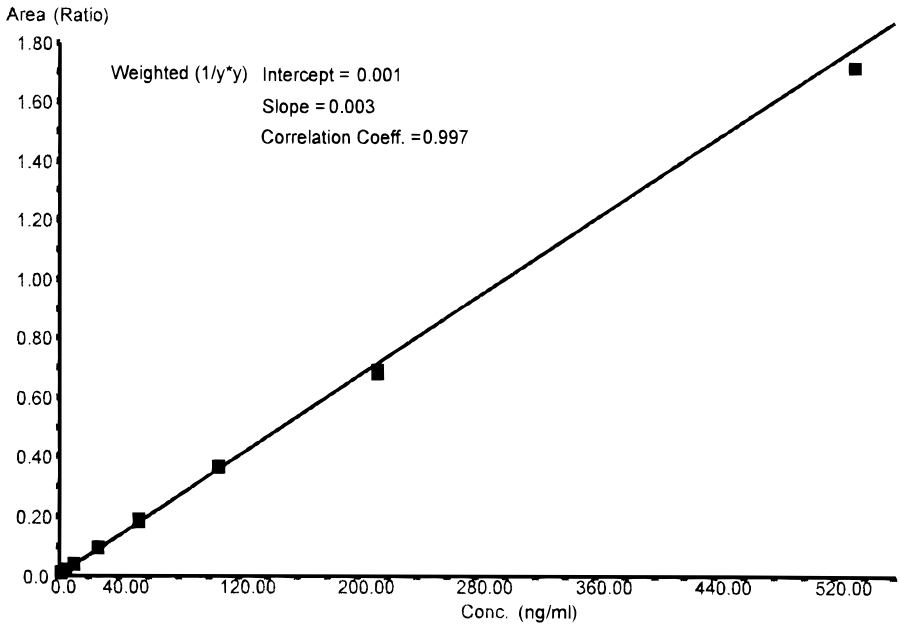

Figure 3. Representative calibration graph for LY355703 in ICR mouse plasma.

Table 1. Intra-day validation statistics for LY355703 in ICR mouse plasma

Day	Parameter	Validation sample level (ng ml^{-1}) 2.1	5.3	52.7	211	527
1	Average (ng ml^{-1})	2.04	5.42	54.47	218.52	528.25
	SD (ng ml^{-1})	0.18	0.28	2.01	8.94	26.47
	Accuracy (RE%)	−2.86	2.26	3.36	3.56	0.24
	Precision (RSD%)	8.92	5.11	3.70	4.09	5.01
	n	5	5	5	5	5
2	Average (ng ml^{-1})	1.98	5.05	55.60	214.07	513.84
	SD (ng ml^{-1})	0.15	0.14	1.34	2.41	10.57
	Accuracy (RE%)	−5.71	−4.72	5.50	1.45	−2.50
	Precision (RSD%)	7.79	2.81	2.42	1.34	2.06
	n	5	5	5	5	5
3	Average (ng ml^{-1})	1.98	5.14	55.10	221.23	512.84
	SD (ng ml^{-1})	0.14	0.26	1.06	8.65	12.78
	Accuracy (RE%)	−5.71	−3.03	4.55	4.85	−2.69
	Precision (RSD%)	7.20	5.01	1.93	3.91	2.49
	n	5	5	5	5	5

Assay specificity

The specificity of the assay is demonstrated by the absence of endogenous substances, in the drug-free matrices, that interfere with the quantitation of LY355703 at the LLOQ. Potential interferences were minimized by combining an SPE clean-up step with the separation power of HPLC and the specificity of MS/MS detection. Representative ion chromatograms of a blank sample (no drug or internal standard) and a 2.1 ng ml^{-1} standard are presented in Figs 4 and 5, respectively.

Initial *in vivo* and *in vitro* metabolism studies in dog and rat indicate that significant levels of LY355703 metabolites are not present in plasma. Metabolites of LY355703, if present, could result in mass spectral interferences with the SRM transition of the analyte.

Table 2. Intra-day validation statistics for LY355703 in NuNu mouse plasma

Parameter	Validation sample level (ng ml^{-1}) 2.1	5.3	53.7	211	527
Average (ng ml^{-1})	2.18	5.28	53.81	208.73	498.30
SD (ng ml^{-1})	0.16	0.16	1.44	8.47	16.34
Accuracy (RE%)	3.81	−0.38	2.11	−1.08	−5.44
Precision (RSD%)	7.20	3.02	2.67	4.06	3.28
n	5	5	5	5	6

Table 3. Intra-day validation statistics for LY355703 in beagle dog plasma

Parameter	Validation sample level (ng ml^{-1}) 2.1	5.3	52.7	211	527
Average (ng ml^{-1})	2.06	5.30	55.06	216.45	526.32
SD (ng ml^{-1})	0.23	0.36	2.22	5.56	50.93
Accuracy (RE%)	−1.90	0.00	4.48	2.58	−0.13
Precision (RSD%)	11.16	6.75	4.03	2.57	9.68
n	5	5	5	5	4

Table 4. Inter-day validation statistics for LY355703 in ICR mouse plasma

Parameter	Validation sample level (ng ml^{-1}) 2.1	5.3	52.7	211	527
Average (ng ml^{-1})	2.00	5.20	55.06	217.94	518.31
SD (ng ml^{-1})	0.15	0.27	1.49	7.43	18.22
Accuracy (RE%)	−4.76	−1.89	4.48	3.29	−1.65
Precision (RSD%)	7.58	5.22	2.71	3.41	3.51
n	15	15	15	15	15

Limits of quantitation (LOQ), extraction efficiency and dilutions

The lower (LLOQ) and upper (ULOQ) limits of quantitation are defined as the lowest and highest concentrations on the calibration graph at which an acceptable accuracy of at least $100 \pm 20\%$ [(mean assay concentration/theoretical concentration) × 100] and

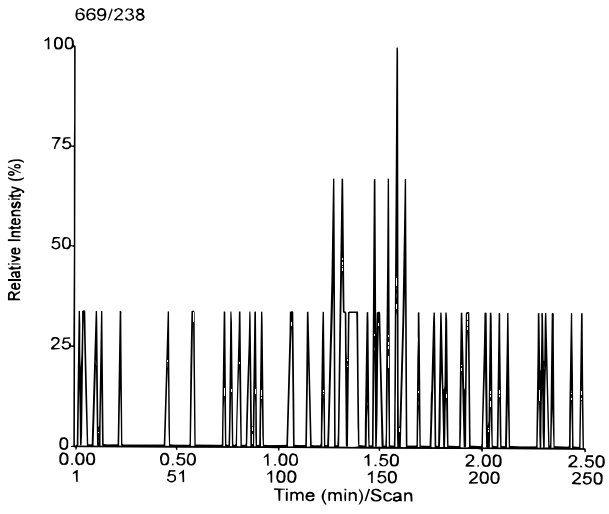

Figure 4. Representative ion chromatogram of a blank sample (no drug or internal standard).

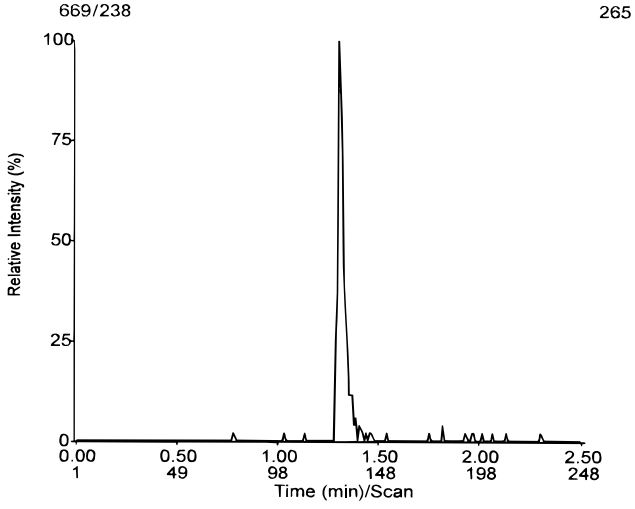

Figure 5. Representative ion chromatogram of a 2.1 ng ml^{-1} standard sample extracted from ICR mouse plasma.

precision of at least 20% (RSD) were obtained. The LLOQ and ULOQ of this assay are 2.1 and 527 ng ml^{-1}, respectively. In addition, initial experiments suggest that the LLOQ can be lowered to at least 250 pg ml^{-1} should the need arise.

The extraction efficiency for LY355703 was 84–107% and that for LY354504 was 102–106%. The biological matrices were found to have no effect on the signal of the analyte and internal standard.

Samples containing high concentrations of the analytes were diluted up to 1:100 in control plasma. It was demonstrated that the accuracy of the dilutions was acceptable (the maximum difference from the theoretical value observed was 16%).

CONCLUSION

A validated LC/MS/MS assay for the determination of LY355703 has been developed that offers several advantages over previous methodologies used to determine cryptophycin compounds in biological matrices. The extraction procedure is relatively simple and requires only 100 μl of plasma. The method offers excellent sensitivity and selectivity and it can be used to determine LY355703 concentrations in beagle dog and ICR and NuNu mouse plasma. It is likely that this assay could also be used to determine LY355703 in other species (e.g. rat and human). The freezer, freeze–thaw, analysis and room temperature stabilities of LY355703 were investigated, and no significant degradation was observed. Dilutions can be employed to represent accurately the original sample (dilution factor up to 1:100). The extraction efficiency was determined to be ~100%.

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