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Validation of a sensitive assay for thiocoraline in mouse plasma using liquid chromatography–tandem mass spectrometry

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Abstract

A sensitive high-performance liquid chromatography–tandem mass spectrometry assay for thiocoraline, an anti-tumor depsipeptide, in mouse plasma is described. Echinomycin, a quinoxaline peptide, was used as an internal standard. Thiocoraline was recovered from the mouse plasma using protein precipitation with acetonitrile and followed by solid-phase extraction of the supernatant. The mobile phase consisted of methanol (0.1% formic acid)–water (0.1% formic acid) (90:10, v/v). The analytical column was a YMC C_{18}. The standard curve was linear from 0.1 to 50 ng/ml (R^2>0.99). The lower limit of quantitation was 0.1 ng/ml. The assay was specific based on the multiple reaction monitoring transitions at m/z 1157→215 and m/z 1101→243 for thiocoraline and the internal standard, echinomycin, respectively. The mean intra- and inter-day assay accuracies remained below 5 and 12%, respectively, for all calibration standards and quality control (QC) samples. The intra- and inter-day assay precisions were less than 11.4 and 9.5% for all QC levels, respectively. The utility of the assay was demonstrated by a pharmacokinetic study of i.v. (bolus) thiocoraline on CD-1 mice. Thiocoraline was stable in mouse plasma in an ice-water bath for 6 h and for three freeze–thaw cycles. The reconstituted thiocoraline after extraction and drying sample process was stable in the autosampler for over 24 h. The assay was able to quantify thiocoraline in plasma up to 48 h following dose. Pharmacokinetic analysis showed that thiocoraline has distinct pharmacokinetic profiling when dosed in different formulation solutions. The assay is currently used to measure thiocoraline plasma concentrations in support of a project to develop a suitable formulation with a desirable pharmacokinetic profile.

Keywords: Thiocoraline

1. Introduction

Thiocoraline (Fig. 1) is a cyclic thiodepsipeptide, isolated from an actinomycete, *Micromonospora marina*, a microorganism collected off the Mozambique coast [1,2]. Thiocoraline has shown in vitro anti-tumor activity against human non-small cell lung, breast, colon, renal, and melanoma cancer cells and in vivo against human carcinoma xenografts [1–3].

There are several methods that have been published for the bioanalysis and quantification of

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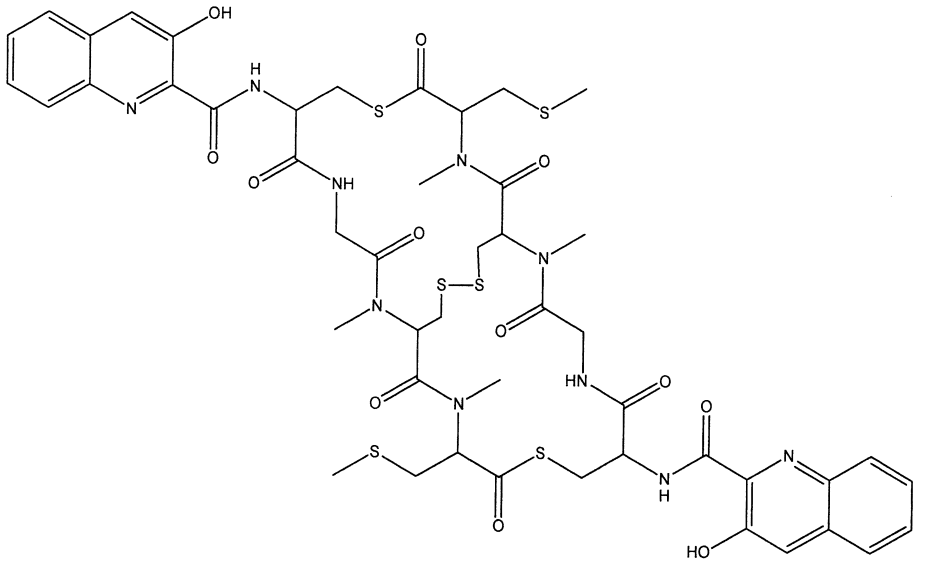
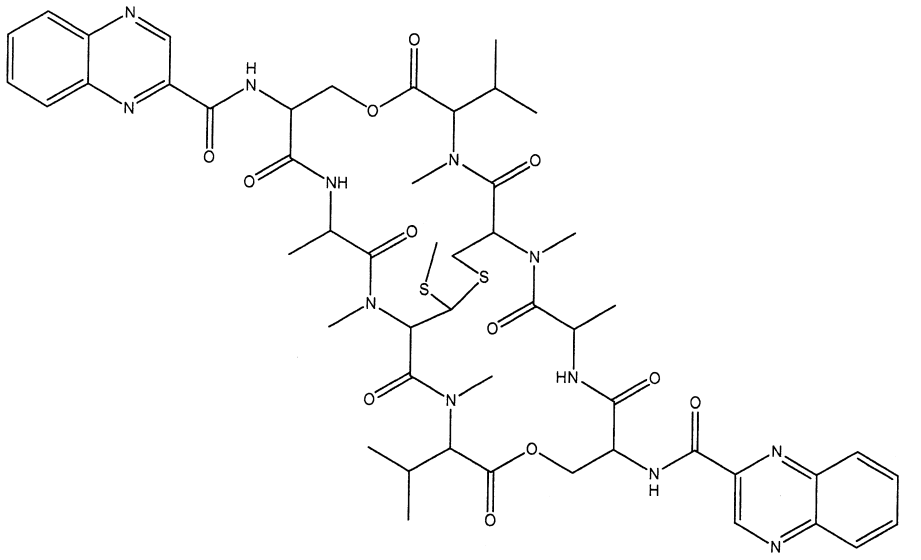


Thiocoraline



Echinomycin

Fig. 1. Chemical structures of thiocoraline and the internal standard, echinomycin.

depsipeptides, including didemnin B, aplidine, ecteinascidins, and thiocoraline. For didemnin B in human plasma, a chromatographic assay was developed using UV detection and a detection limit of 5 ng/ml was reported [4]. For aplidine, a dehydroxyl analog of didemnin B, two methods were reported; one used fluorescence detection a carbonyl functional group after precolumn derivatization, the lower limit of quantification (LLOQ) was 2 ng/ml [5]; the other explored the liquid chromatography–tandem mass spectrometry (LC–MS–MS) technique for a highly selective analysis, obtaining 1.25 ng/ml as the LLOQ [6]. Ecteinascidin 743 (ET 743) was analyzed based on LC–UV [7] and LC–MS [8]. The LLOQs were 1 ng/ml for the LC–UV method and 0.01 ng/ml for the LC–MS method. The previous studies on thiocoraline were described for the analysis of raw material from fermentation [9] and in rat plasma by high-performance liquid chromatography (HPLC)–fluorescence detection [10]. The assay quantitated thiocoraline concentrations ranging from 1 to 100 ng/ml using 100 μl sample volumes.

Here we describe a HPLC–MS–MS method for the quantification of thiocoraline in mouse plasma, allowing us to perform a very sensitive, reproducible, and highly selective analysis. This method is the first validated method to quantify thiocoraline in mouse plasma using MS–MS. Recently, this method has been used to support more than 10 thiocoraline pharmacokinetic (PK) studies in CD-1 mice to optimize thiocoraline formulation and PK profiles.

2. Materials and methods

2.1. Materials

Thiocoraline was provided by PharmaMar (Colmenar Viejo, Madrid, Spain). Echinomycin (Fig. 1), a quinoxaline peptide, was used as an internal standard and obtained from Sigma (St. Louis, MO, USA). The drug-free (blank) mouse plasma containing sodium EDTA as anticoagulant was obtained from Bioreclamation (Hicksville, NY, USA). HPLC-grade methanol and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA, USA). Formic acid, ACS grade, was obtained from Sigma. Deionized water was purified by an laboratory Barnstead E-pure system.

2.2. Instrumentation

The HPLC system consisted of a Shimadzu LC-10ADvp solvent delivery unit, an on-line degasser, a gradient mixer and a system controller (Shimadzu Scientific, Columbia, MD, USA). A CTC-PAL autosampler (LEAP Technologies, Carrboro, NC, USA) was used to inject samples. The autosampler allowed the use of two separate wash solvents (water with 0.1% formic acid and methanol with 0.1% formic acid) to eliminate sample carry-over. The mass spectrometer was a PE Sciex API 4000 LC–MS–MS system (Toronto, Canada). The API 4000 mass spectrometer was interfaced with Shimadzu HPLC system using a turboionspray (TIS) ion source. A Dell GX400 Optiplex computer with PE Sciex Analyst software (version 1.2) was implemented to control the API 4000 mass spectrometer, the Shimadzu LC-10ADvp solvent delivery unit (via the system controller), and the CTC PAL autosampler. The API 4000, Shimadzu LC-10ADvp, and the CTC PAL autosampler were also connected by contact closures to synchronize time events.

2.3. Chromatographic conditions

The mobile phase flowing through the HPLC column consisted of methanol (0.1% formic acid)–water (0.1% formic acid) (90:10, v/v). The column was run under isocratic conditions with a flow-rate 300 μl/min. The analytical column was a YMC Pro-C_{18} minibore, 5 μm, 50×2 mm I.D. (Waters, Milford, MA, USA). The CTC-PAL Leap cooling unit was set at 4 °C. The sample injection volume was 20 μl. The syringe, injection loop, and the switching valve were postwashed (100 μl) four times by two washing solution sequentially. The first wash solution was water containing formic acid (0.1%, v/v), and the second wash solution was methanol containing formic acid (0.1%, v/v).

2.4. Mass spectrometric conditions

The ion polarity was set to the positive mode. The nebulizer, curtain, and collision gas was UHP nitro-

gen (99.999%). User controlled voltages, gas pressures, and source temperature were optimized for the detection of the parent and product ions of thiocoraline and echinomycin. The analytes were infused at 10 μl/min by an infusion pump (Harvard Apparatus, South Natick, MA, USA) teed into the mass spectrometer. The turbo temperature was set to 400 °C. The curtain gas, gas 1 (nebulizer gas), and gas 2 (turbo gas) pressures were 68.95, 275.79, and 448.14 kPa, respectively. The nitrogen gas was delivered from a nitrogen Dewar with the gas regulator maintained at 689.48 kPa. The ion spray and entrance potential were 5500 and 10 V. The declustering potential, collision energy, and collision cell exit potential were optimized at 135, 58, and 10 V, respectively, for thiocoraline; and 130, 56, and 25 V, respectively, for echinomycin. The dwell time was 100 ms with a 5 ms pause between scans.

2.5. Data acquisition and analysis

Data acquisition was performed using multiple reaction monitoring (MRM) of thiocoraline with its internal standard, echinomycin (Fig. 2). Transition was monitored at m/z 1157.3 to 215.2 for thiocoraline and m/z 1101.5 to 243.1 for echinomycin. Automated data acquisition and data analysis were performed using PE Sciex Analyst soft-

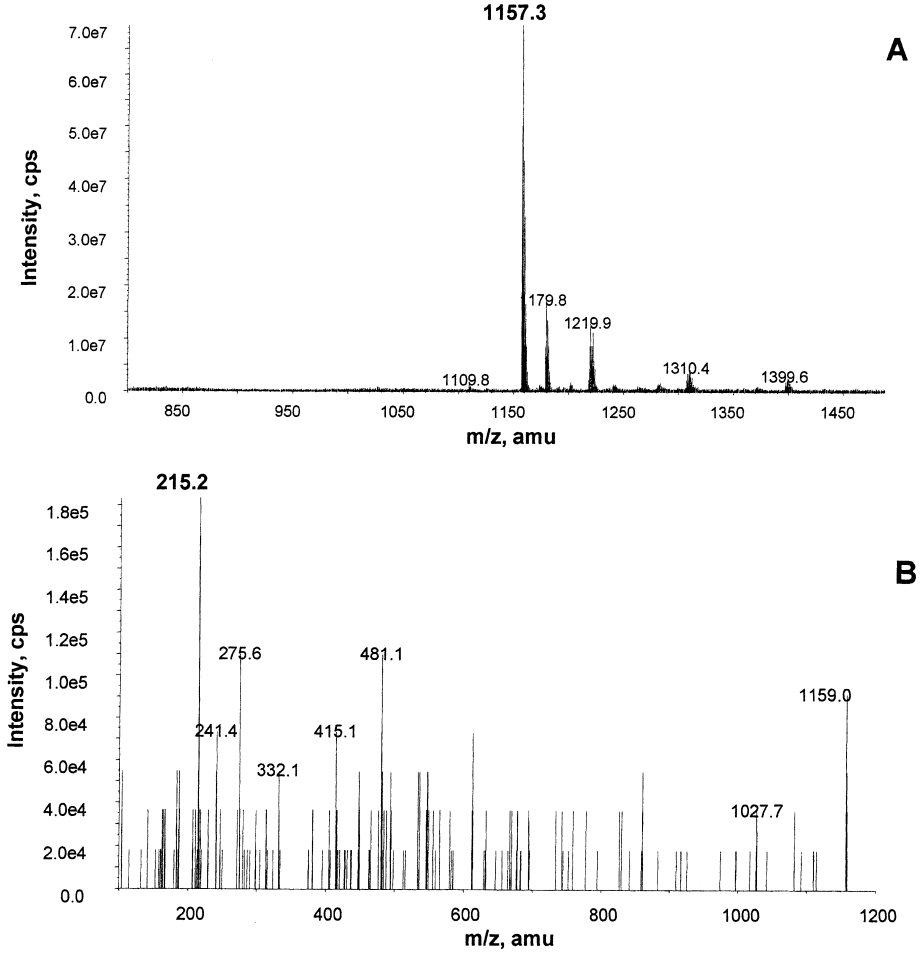


Fig. 2. Reconstructed Q1 mass spectrum and product ion scan of thiocoraline. (A) Reconstructed Q1 mass spectrum obtained by positive turbo ion spray ionization. (B) Product ion scan of the m/z 1157 at collision energy of 58 V.

ware. Unknown sample concentrations of thiocoraline were calculated from the linear regression equation $y = ax + b$ with a weighted factor of $1/x$. The peak area ratios of thiocoraline to its internal standard versus corresponding concentrations were used for the linear least-squares regression of the calibration lines.

2.6. Preparation of stock and working solutions

Thiocoraline stock solution was prepared in acetonitrile at a concentration of 0.25 mg/ml. A series dilution led to a concentration of 2.5 μg/ml in acetonitrile. The working solutions were prepared in acetonitrile–water (1:1) with concentrations of 500, 250, 100, 50, 25, 10, 5, and 1 ng/ml. All stock and working solutions were stored at −30 °C and used within 1 month of preparation.

2.7. Preparation of calibration standards and quality control samples

The calibration standards, with thiocoraline concentrations of 50, 25, 10, 5, 2.5, 1, 0.5, and 0.1 ng/ml, were prepared from 10 times dilution of the working solutions in mouse plasma. Quality control (QC) samples, containing 40, 20, 1.25, 0.5, 0.25, and 0.1 ng/ml were prepared in mouse plasma. The preparation of calibration standards and quality control samples were processed in an ice-water bath.

2.8. Sample preparation

First, 300 μl of acetonitrile was added to a microcentrifuge vial containing 400 μl of plasma samples. After vortexing, the mixture was centrifuged for 5 min at 19 000 *g* at 4 °C. The supernatant was then transferred to a 3M C_{18} extraction disk plate. Before loading the supernatants the disk plate was activated using 400 μl of acetonitrile, equilibrated using 400 μl of water, and then loaded 400 μl of water. After loading the disk plate was washed using 2×400 μl of water and then eluted using 2×400 μl of acetonitrile. The elution solutions were evaporated to dryness under a nitrogen stream at 25 °C, using a TurboVap 96 concentration workstation (Zymark, Hopkinton, MA, USA). Finally, the dry residues were reconstituted in 200 μl of 50 ng/ml echinomycin in acetonitrile–20 m*M* ammonium acetate (1:1). An aliquot containing 20 μl of mixture was injected onto the analytical column.

2.9. Method validation

The analytical method was validated to demonstrate the specificity, recovery, limit of quantitation (LOQ), accuracy, and precision of measurements. Triplicate sets of calibration standard and QC samples were analyzed on 3 different days to determine the intra- and inter-day validations.

Acceptable specificity was defined as the absence of any detectable MRM LC–MS–MS ion currents at the retention time regions of thiocoraline and echinomycin in six different double blank plasma samples.

The recovery of thiocoraline from the extraction procedure from mouse plasma was determined by a comparison of the peak area of thiocoraline in the spiked plasma samples in three replicates to the peak area of thiocoraline samples prepared in reconstitution solvent. The three different concentrations were applied to represent low-, medium-, and high-levels of the LOQ concentration range.

The calibration range for thiocoraline was established using triplicate set of standards from 0.1 to 50 ng/ml thiocoraline in blank CD-1 mouse plasma. The calibration standard concentrations were 0.100, 0.500, 1.00, 2.50, 5.00, 10.0, 25.0, and 50.0 ng/ml. The internal ratios (thiocoraline peak area versus I.S. echinomycin peak area) were calculated for each point and standard curves were constructed using least square linear regression analysis of internal ratios over concentration ratios. The linear regression is weighted by $1/x$ (the reciprocal of the concentration ratio). The correlation coefficient is used to evaluate the linearity of the calibration curve. According to the guidance set by the US Food and Drug Administration (FDA) for bioanalytical method validation, the minimum acceptance criterion for the intra- and inter-assay mean accuracies (% bias) of the calibration standards are within ±15% of the expected concentration (20% at the lower LOQ). The intra- and inter-assay precisions (RSDs) of the calibration standards should within ±15% (20% at the lower LOQ). The linearity of the calibration standards should be a correlation coefficient of

determination (R^2) of greater than 0.98, using at least six sets of calibration standard concentrations.

Twelve replicates at 0.250, 0.500, 1.25, 20.0, and 40.0 ng/ml were used to make the low- to high-range QC concentrations. The target values for intra- and inter-assay mean accuracies and precisions of each QC sample should be within ±15% of the expected concentration.

The stability of thiocoraline in mouse plasma was investigated by analyzing triplicate QC samples at 1.25 and 40 ng/ml. Freeze–thaw stability was determined after three cycles of freezing at −80 °C and thawing at room temperature. For assessment of short-term stability, the QC samples were thawed and left to stand in an ice-water bath for 6 h. The stability of the extracted samples (reconstituted in acetonitrile–20 m*M* ammonium acetate) in the CTC-PAL autosampler (equipped with a cooled sample stack, ~4 °C) was determined over 24 h. Concentrations following storage were compared to freshly prepared samples of the same concentrations.

2.10. Pharmacokinetic studies

Male and female CD-1 mice (with three mice per sampling time point) were used to monitor samples for the thiocoraline pharmacokinetic studies. Each animal received an intravenous bolus injection of 1 mg/kg thiocoraline in differently formulated solutions. Blood samples were taken by cardiac puncture at 5, 15, 30 min, and 1, 2, 4, 6, 8, 24, and 48 h after dosing. Blood samples were heparinized and kept on ice. Plasma was harvested from the blood samples following centrifugation for 15 min at 3000 rpm. The plasma was immediately frozen and remained frozen at −80 °C until analyzed. The plasma samples taken at 5 min were diluted 20-fold with mouse plasma prior to analysis.

On the basis of the plasma levels of the test substances, the following noncompartmental analysis parameters were calculated as follows [11]: peak plasma concentrations (C_{max}) were determined as the plasma concentration found at the shortest sampling time. The area under the concentration–time curve ($AUC_{0\rightarrow Cp}$) from the time zero to the last measured time point (Cp) was determined by the log-linear trapezoidal rule. The area term was extrapolated to infinity ($AUC_{0\rightarrow\infty}$) using Cp divided by the elimination rate constant (β), which is obtained by nonlinear iterative least squares regression of the terminal log-linear portion of the concentration–time curve. The elimination half-life ($t_{1/2\beta}$) is calculating by dividing β into the natural logarithm of 2. The steady-state volume of distribution (V_{ss}) was calculated by dividing the administered dose by the combination of $AUC_{0\rightarrow\infty}$ and β. Total body clearance (Cl) was calculated by dividing the administered dose by the $AUC_{0\rightarrow\infty}$. The above parameters were calculated using the WinNonlin Professional Version 4.01 (Pharsight, Mountain View, CA, USA).

3. Results and discussion

3.1. Chromatography and mass spectrometry

The ionization and fragmentation of thiocoraline was studied using electrospray tandem mass spectrometry. The collision-induced dissociation spectra of parent ion at m/z 1157 produced an intense fragment ion at m/z 215 under optimum collision energy at 58 V. The parent mass spectrum and product ion mass spectrum of thiocoraline are shown in Fig. 2A and B.

MRM, based on m/z 1157→215 transition, was specific for thiocoraline; based on m/z 1101→243 transition, was specific for echinomycin. A typical chromatogram from blank mouse plasma is shown in Fig. 3A, showing no interfering endogenous materials with the analysis of thiocoraline and echinomycin.

The carryover problem was solved successfully by using a CTC PAL autosampler equipped with a dual washing system. With dual extensive washes (eight washes per injection) of the syringe, valve, and injection loop, as described in Materials and methods, the carryover was reduced to less than 1%, allowing us to reach the LLOQ of 0.1 ng/ml by using 40 μl of plasma samples (Fig. 3B). Clearly, the current assay based on the MRM MS–MS detection provided much better specificity and sensitivity over the previous HPLC–fluorescence assay [10].

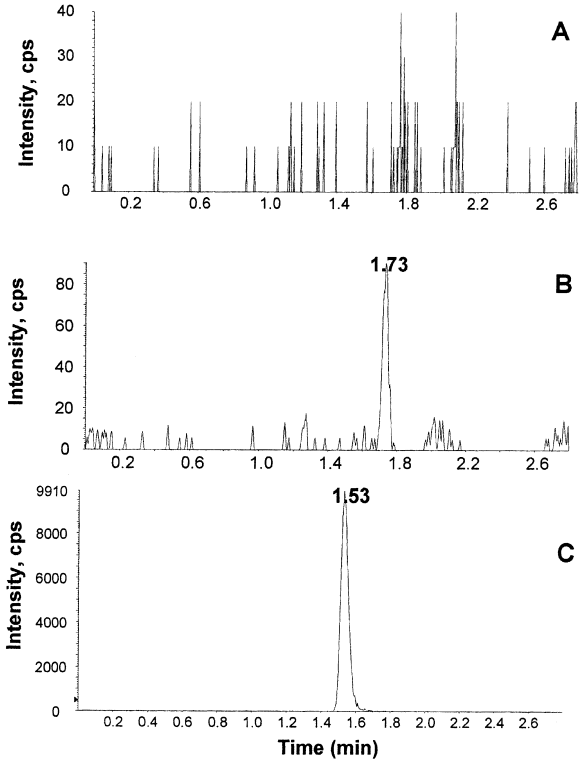


Fig. 3. Reconstructed ion chromatograms for thiocoraline and echinomycin specific MRM transition from blank and spiked mouse plasma. (A) Reconstructed ion chromatogram for the MRM transitions at m/z 1157→215 and m/z 1101→243 of double blank mouse plasma. (B) Reconstructed ion chromatogram for the MRM transition m/z 1157→215 of blank mouse plasma spiked with 0.1 ng/ml thiocoraline. (C) Reconstructed ion chromatogram for the MRM transition m/z 1101→243 of blank mouse plasma spiked with 50 ng/ml echinomycin.

3.2. Method validation

The mean overall recoveries of thiocoraline from mouse plasma following the protein precipitation and extraction procedure were 68.4±1.4 ($n=3$), 71.1±0.5 ($n=3$), and 76.4±3.1% ($n=3$) at 1.25, 20, and 40 ng/ml, respectively.

The assay was linear over the concentration range of 0.1–50 ng/ml. The correlation coefficients for the calibration curves (weighted by $1/x$, the reciprocal of the concentration ratio) ranged from 0.9866 to 0.9997. Typically, the calibration curve was defined by a slope of 1.323 and an intercept of 0.0018. The RSD of the slopes was 6.4%. The mean inter-day accuracy for all calibration standards ranged from 96 to 105% (≤5% bias), and the mean inter-day precision for all calibration standards was less than 13.3% (Table 1).

Table 1
Summary of the back-calculated thiocoraline calibration standards ($n=9$) in mouse plasma

Nominal concentration (ng/ml)	Concentration found (ng/ml)	RSD (%)	Accuracy (%)
0.100	0.097±0.013	13.3	97
0.500	0.523±0.052	9.9	105
1.00	1.009±0.103	10.2	101
2.50	2.45±0.194	7.9	98
5.00	4.82±0.366	7.6	96
10.0	9.9±0.835	8.5	99
25.0	24.5±1.744	7.1	98
50.0	51.1±1.808	3.5	102

RSD, Relative standard deviation; SD, standard deviation.

The accuracy and precision values for all QC replicates ($n=36$) are summarized in Table 2. The mean intra- and inter-day assay accuracies, determined at each QC level throughout the validated runs, remained below 12 and 6%, respectively. The mean intra- and inter-day assay precisions were less than 11.4 and 9.5% for all QC levels, respectively.

Based on the previous report [10] and our investigation, thiocoraline was unstable in plasma at room temperature. To overcome this problem we prepared calibration standards and QC samples, as well as plasma samples from PK studies, in an ice-water bath. The short-term stability in the ice-water bath was evaluated and the mean deviations from freshly prepared QC samples did not exceed 4% (Table 3). As has been reported previously [10], storage at −80 °C is suitable as a long-term storage condition for thiocoraline. We investigated freeze–thaw stability at −80 °C during the three freeze–thaw cycles. Table 3 shows that thiocoraline was stable after three freeze–thaw cycles and the extracted samples (reconstituted in acetonitrile–20 m*M* ammonium acetate) were stable in the autosampler (~4 °C) for 24 h.

3.3. Pharmacokinetic studies

Using our validated assay, the plasma concen-

Table 2
Summary of the quality control validation in mouse plasma

Nominal concentration (ng/ml)	*n*		Concentration found (ng/ml)	RSD (%)	Accuracy (%)
Intra-day assay					
0.250	3	Day 1	0.226±0.005	2.2	91
	3	Day 2	0.259±0.029	11.4	104
	3	Day 3	0.263±0.013	4.8	105
0.500	3	Day 1	0.526±0.034	6.4	105
	3	Day 2	0.531±0.024	4.4	106
	3	Day 3	0.479±0.026	5.5	96
1.25	3	Day 1	1.26±0.137	10.8	101
	3	Day 2	1.10±0.044	4.0	88
	3	Day 3	1.18±0.070	6.0	94
20.0	3	Day 1	20.8±1.193	5.7	104
	3	Day 2	19.9±1.113	5.6	100
	3	Day 3	20.2±0.351	1.7	101
40.0	3	Day 1	39.8±3.724	9.4	100
	3	Day 2	39.7±3.208	8.1	99
	3	Day 3	38.3±3.262	8.5	96
Inter-day assay					
0.250	9		0.249±0.024	9.5	100
0.500	9		0.512±0.035	6.8	102
1.25	9		1.18±0.107	9.0	94
20.0	9		20.3±0.930	4.6	102
40.0	9		39.3±3.034	7.7	98

RSD, Relative standard deviation; SD, standard deviation.

tration versus time profile of thiocoraline was determined in CD-1 mice dosed intravenously (Figs. 4 and 5). PK analysis showed that thiocoraline has distinct pharmacokinetic profiles when dosed in different formulation solutions (PM93135FE0102 and PM93135FE0102). The corresponding pharmacokinetic parameters (calculated using the WinNonlin Professional Version 4.01) are summarized in Table 4.

The sensitivity achieved with the assay described here is at least 10-times higher than with previously published method [10], enabling us to monitor thiocoraline plasma concentration up to 48 h after administration of 1 mg/kg of thiocoraline.

Table 3
Stability of thiocoraline in mouse plasma and the post-preparative stability in the autosampler

Nominal concentration (ng/ml)	Treatment	Percentage of initial value (%)
1.25	Ice-water bath for 6 h	96.4±1.3
	Three freeze–thaw cycles	98.7±13.4
	Autosampler (~4 °C)	103.6±4.5
40.0	Ice-water bath for 6 h	98.6±3.6
	Three freeze–thaw cycles	92.8±9.9
	Autosampler (~4 °C)	109.2±3.9

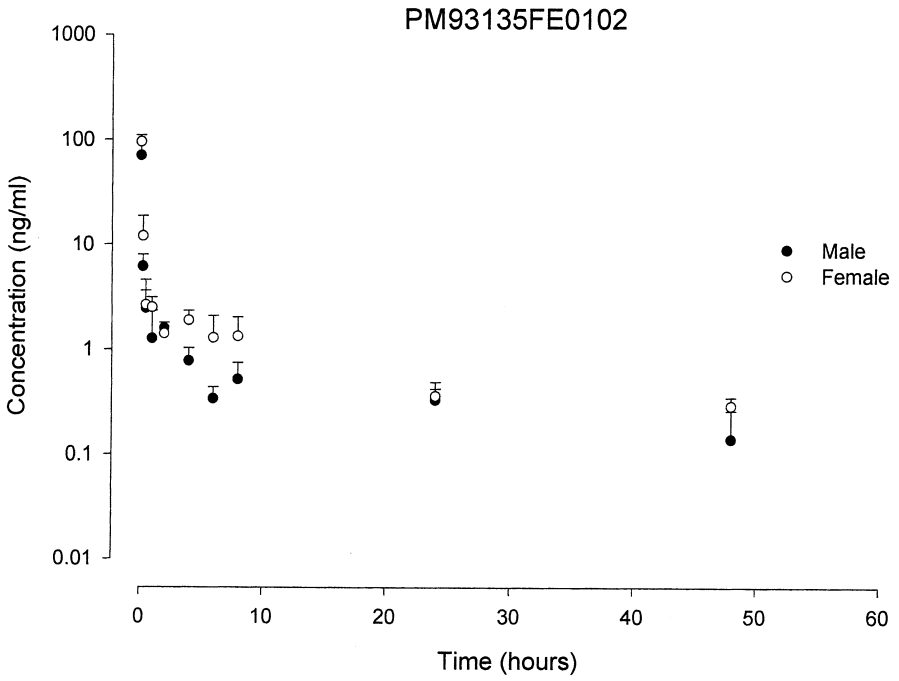


Fig. 4. Plasma concentration–time curve of thiocoraline in CD-1 mice dosed intravenously with 1 mg/ml of thiocoraline in formulation2 solution (PM93135FE0102). The plasma samples were analyzed as described in Materials and methods.

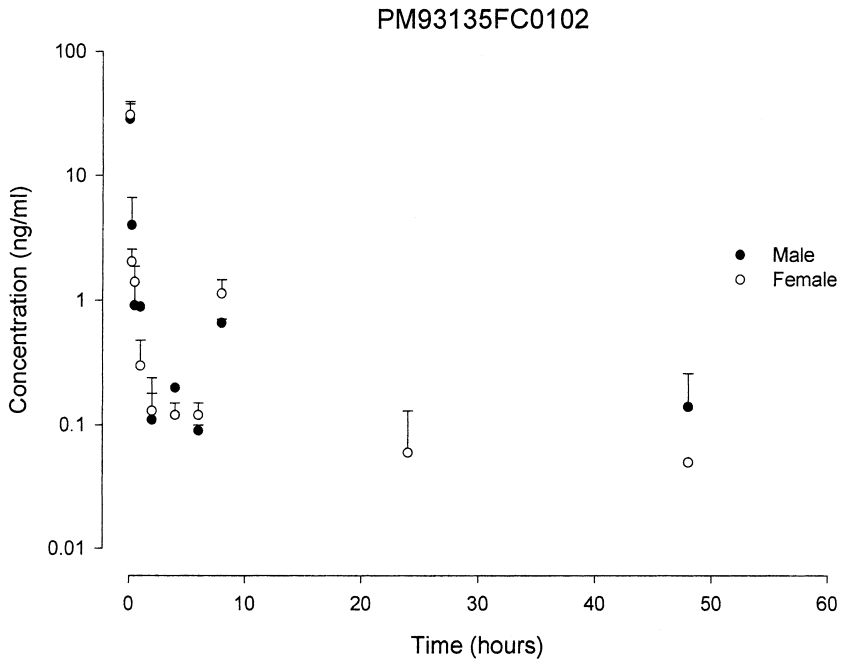


Fig. 5. Plasma concentration–time curve of thiocoraline in CD-1 mice dosed intravenously with 1 mg/kg of thiocoraline in formulation1 solution (PM93135FC0102). The plasma samples were analyzed as described in Materials and methods.

Table 4
Estimated pharmacokinetic parameters (mean±SD) after intravenous administration of thiocoraline (1 mg/kg) to CD-1 mice (three mice per sampling time point)

Study ID and gender	C_{max} (ng/ml)	$t_{1/2}$ (h)	$AUC_{0\rightarrow\infty}$ (h ng/ml)	V_d (l/kg)	CL (l/h/kg)
PM93135E0102 (female)	95.20±15.47	15.29±2.09	60.69±5.06	328.77±43.23	14.93±1.25
PM93135E0102 (male)	70.67±27.47	25.49±4.86	42.09±10.00	714.81±37.49	20.06±4.99
PM93135C0102 (female)	30.90±8.62	5.33±4.50	15.51±2.56	401.21±336.59	52.78±6.73
PM93135C0102 (male)	38.03±9.80	5.38±2.63	15.08±4.05	489.00±139.12	40.05±26.40

C_{max}, Peak plasma concentration; $t_{1/2}$, terminal elimination half live; AUC, area under the plasma concentration–time curve; V_d, apparent volume of distribution; CL, total body clearance.

4. Summary

The pharmacokinetic analysis of thiocoraline relies on a highly sensitive assay, capable of determining thiocoraline in plasma at low-pg/ml concentrations. In preclinical species, the limited volumes of plasma and interferences from the biological matrix, all add to the complexity of the trace analysis of thiocoraline. The specificity and sensitivity of the LC–MS–MS assay reported here were significantly higher than those of HPLC–fluorescence techniques. The assay also required lower volumes of plasma, which allowed for the pharmacokinetic analysis to be conducted in individual animals. The assay is currently used to measure thiocoraline plasma concentrations in support of a project to screen different formulations on thiocoraline. The information from such studies would allow us to develop a suitable formulation with a desirable pharmacokinetic profile for thiocoraline administration.

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