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Bioanalysis of thiocoraline, a new marine antitumoral depsipeptide, in plasma by high-performance liquid chromatography and fluorescence detection

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Abstract

A sensitive bioanalytical assay for thicoraline, an investigational marine anticancer agent, in plasma, based on reversed-phase liquid chromatography and fluorescence detection, is reported. The proteins in the sample are precipitated by the addition of acetonitrile. After centrifugation, the supernatant is injected directly into the chromatograph. The analyte is quantified by fluorescence detection with excitation and emission at 365 and 540 nm, respectively. The method has been validated in the 1–100 ng/ml range, 1 ng/ml being the lower limit of quantification. Precision and accuracy both meet the current requirements for a bio-analytical assay and are $<$15% at 1 ng/ml and $\leq$5% in the 5–100 ng/ml range. Plasma samples can be stored for at least 4 months at -80°C. Finally, the usefulness of this method for pharmacological research was shown in a pilot study of the pharmacokinetics of thiocoraline in rats.

Keywords: Thiocoraline; Depsipeptides

1. Introduction

Thiocoraline, a cyclic thiodepsipeptide, is one of several recently discovered depsipeptides isolated from marine organisms. This depsipeptide originates from the fermentation broth of a marine *Micromonaspora* [1]; the structure has recently been elucidated [2] (Fig. 1). The chemistry shows a symmetrical molecule with a disulfide bridge in the middle, containing two thioester links and two 3-hydroxyquinaldic acid groups. The component is soluble in several organic solvents and insoluble in water. This agent has been selected for further investigation of its anti-cancer properties; in vitro, thiocoraline showed inhibition of polymerase α [3]. Before the start of phase I clinical pharmacokinetic studies, it is essential to develop a bioanalytical

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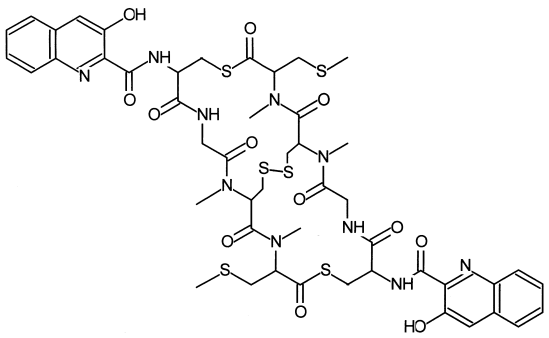

Fig. 1. Chemical structure of thicoraline.

assay; a sensitive validated bioanalytical method is therefore an important tool in drug development. Such an assay has not yet been developed for thiocoraline, although an applicable chromatographic system was reported for the analysis of raw material isolated from the fermentation broth [2].

The bioanalysis of depsipeptides and other compounds originating from marine organisms is a relatively new analytical field. It started with a chromatographic assay for didemnin B [4], using UV detection; in plasma, 5 ng/ml was obtained as the detection limit. Later, more sensitive assays were developed for didemnin B, based on immunologic techniques. With a radioimmunoassay [5] and an indirect competitive inhibition enzyme immunoassay [6] detection limits of 20 pg/ml and 1 ng/ml were obtained, respectively. Recently, chromatographic assays were published for the depsipeptide FR901228, based on LC–MS [7], and for ecteinascidin 743, based on LC–UV [8]; the lower limit of quantification (LLQ) is 1 ng/ml for both methods. For aplidine, also a depsipeptide, HPLC with fluorescence detection was used in combination with pre-column derivatization of a carbonyl function, obtaining 2 ng/ml as the LLQ [9]. For thiocoraline, a depsipeptide with a totally different, unique structure, sensitive detection may be facilitated by the native fluorescent properties of the 3-hydroxyquinaldic acid groups. The use of a MS detector, unfortunately not yet commonly available in every hospital laboratory, can then be avoided. The efficiency of a reversed-phase liquid chromatographic system in combination with fluorescence detection will probably be an ideal starting point for the development of a sensitive assay for thiocoraline in plasma. The analytical separation, though, requires a sample clean-up, since the proteins, especially, in the plasma sample may disturb the LC separation. In this article the development and validation of a bioanalytical assay for thiocoraline in plasma is presented. The suitability has been investigated in a pharmacokinetic experiment in the rat.

2. Experimental

2.1. Chemicals

Thiocoraline was isolated from the culture broth using filtration, liquid–liquid extraction and several preparative chromatographic techniques according to Romero et al. [1] and was provided by PharmaMar (Tres Cantos, Madrid, Spain). Acetonitrile (Gradient grade) was provided by Biosolve (Valkenswaard, The Netherlands) and trifluoroacetic acid (TFA) by Sigma (St. Louis, MO, USA). Water was home-purified by reversed osmosis.

2.2. Equipment

Chromatographic analyses were performed with the following equipment: a P580 isocratic pump (Gynkotek HPLC, Germering, Germany), a Basic+ Marathon autosampler (Spark Holland, Emmen, The Netherlands), equipped with a 7739-005 injection valve (Rheodyne, Cotati, CA, USA) with a 100-μl sample loop, and a FP-920 fluorescence detector (Jasco, Hachioji, Japan). The column was thermostated in a water bath with the temperature being controlled by a thermomix 1420 heating device (B. Braun, Melsungen, Germany). Data were recorded on a Jotronics Pentium 166, 32 Mb personal computer (Delfgauw, The Netherlands) equipped with a Chromelion chromatographic data system (Gynkotek HPLC). A Hettich Universal centrifuge, type 1200 (Hettich, Tuttlingen, Germany), was used for centrifugation.

2.3. Chromatographic conditions

Injections (50 μl) were made on a SYMMETRY C_{18} column (100×4.6 mm, d_p=3.5 μm, average pore diameter 10 nm, Waters Chromatography, Mil-

ford, MA, USA). The column temperature was kept at 39±2°C. The eluent comprised a mixture of 60% (v/v) acetonitrile, 39.9% (v/v) water and 0.1% (v/v) TFA; the eluent flow-rate was 1 ml/min. The fluorescence detection wavelengths were 365 nm for excitation and 540 nm for emission.

2.4. Analytical procedure

A 100-μl plasma sample is transferred into a polypropylene micro tube and 200 μl acetonitrile is added. The tube is closed and shaken vigorously by vortex-mixing and then centrifuged at $1.3 \cdot 10^3 g$ for 5 min. Finally, the supernatant is transferred to an injection vial with a 250-μl glass insert.

2.5. Validation

Stock solutions of 65.3 and 65.9 μg/ml thiocoraline in acetonitrile were prepared (with separate weighings) and stored at −20°C. For calibration a 500 ng/ml dilution in plasma, pooled from four individuals, was made from the 65.9 μg/ml stock solution and stored at −20°C. Dilutions of the 500 ng/ml standard of thiocoraline in plasma to yield 1, 2, 5, 10, 20, 50 and 100 ng/ml thiocoraline calibration samples in plasma were prepared daily and analysed in duplicate for each analytical run. Least-squares regression, weighted by $1/X^2$ (reciprocal of squared concentration), of peak height versus concentration was employed for calibration.

From a 500 ng/ml dilution in plasma originating from the 65.3 μg/ml stock, validation samples in plasma were prepared at 1, 2, 5, 20 and 100 ng/ml and stored at −20°C; plasma of different individual donors was used. Precision and accuracy were determined by six-fold analysis of each validation sample in three different analytical runs. The repeatability (intra-day precision) is calculated according to:

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

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

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

(DayMS, day mean square; n, number of replicates in each run) for each individual concentration. Six individual blank plasma samples were also tested.

The validation samples of 5, 20 and 100 ng/ml thiocoraline in plasma were also used for stability studies; these samples were stored at ambient temperature for 1 day, at 37°C for 1–32 h, at 2°C for 1 week, at −20°C for ≥1 week and at −80°C for ≥3 weeks. In addition, plasma samples were removed from the freezer to adapt to ambient temperature to test the effects of extra freeze–thaw cycles ($n=2$ or 4).

For the determination of the extraction yield two calibrations were performed: one according to the standard procedure and a second by making dilutions of the same concentrations in acetonitrile; 100 μl of these samples was mixed with 200 μl acetonitrile–water (50:50, v/v) prior to injection into the chromatograph. The yield was calculated from these two calibrations in two separate analytical runs by dividing the slopes of the calibration lines and was corrected for the normal value of the plasma protein content (75 mg/ml).

The stability of thiocoraline in deproteinized plasma samples standing in the autosampler was tested by injecting a second portion of the calibration samples from a separate closed sample vial the next morning after a run. The recovery was calculated for two calibrations in two separate analytical runs by dividing the slopes of the two different calibration lines.

The pharmacokinetics of thiocoraline in rats (ca. 150 g) were investigated for an intravenous bolus injection of 1.56 mg/kg thiocoraline. Nine rats were treated and sacrificed to obtain a serum sample after 0, 5, 10, 15, 20, 30, 45 and 60 min and 24 h. The samples taken in the interval 5–60 min were diluted 20-fold with the pooled human plasma prior to analysis.

3. Results and discussion

The necessity for a bioanalytical assay for thiocoraline prior to the start of phase I clinical trials with doses in the 1-mg order urged us to develop a sensitive method in the lower ng/ml range. The native fluorescent properties of the analyte allowed

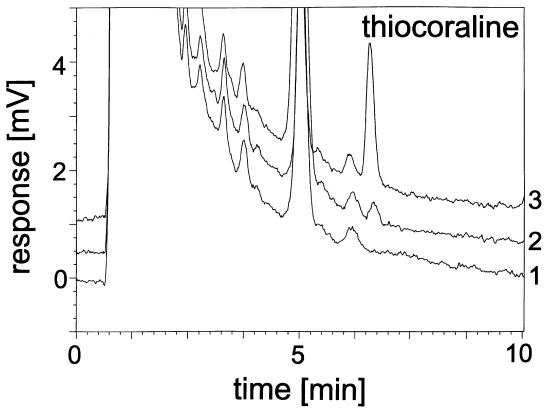

Fig. 2. Chromatograms of thicoraline in deproteinized plasma. (1) Blank; (2) 2 ng/ml spiked to blank plasma; (3) 10 ng/ml spiked to blank plasma. The retention time of thiocoraline is 6.3 min.

us to develop a sensitive assay. In combination with HPLC and a simple pre-treatment of the sample by precipitating the proteins, a method ready for validation was obtained. 99.7% of the proteins are precipitated using the current method [10] with acetonitrile. The calculated thiocoraline yield of the extraction is 101%.

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

Table 1
Results for back-calculated thiocoraline calibration plasma samples ($n=10$) in five runs

c (Nominal) (ng/ml)	Found (ng/ml)	RSD (%)	Accuracy (%)
1	1.04±0.07	6	105
2	1.86±0.07	4	93
5	4.67±0.23	4	93
10	10.5±0.3	3	105
20	19.4±1.0	5	97
50	48.9±2.8	6	98
100	109±9	8	109

Table 2
Overall results of the analysis of the validation samples ($n=18$)

c (ng/ml)	Repeatability (%)	Reproducibility (%)	Accuracy (%)
1	10	2	107
2	12	4	101
5	5	1	98
20	3	1	102
100	4	1	98

lower limit of detection, based on a signal-to-noise ratio of 3, is also 1 ng/ml. All values for the precision and accuracy clearly meet the demands for a bioanalytical assay: ≤20% for the LLQ and ≤15% at higher concentrations [11]. No internal standard was available, and the validation data show precise and accurate results after using the method without an internal standard, as expected for a chromatographic assay with only one simple sample pretreatment step. In six individual blank plasma samples, no interferences in the chromatograms are observed which could influence the quantification of thiocoraline; the calculated 'thiocoraline concentration' in these blanks is 0.20±0.16 ng/ml.

Special attention was paid to the stability of thiocoraline in the plasma samples. The performance of three replicates, as in our investigation, at several levels and storage conditions in the study of the stability of new pharmaceutical agents is common practice [12–14]. The results for the storage of different concentrations of thiocoraline at different temperatures are given in Table 3, which shows that storage at −80°C is suitable as a long-term storage condition for at least 4 months. The decline of the thiocoraline concentration in plasma samples stored at 37°C, investigated at 20 and 100 ng/ml, clearly shows first-order kinetics with a half-life of 8.3 h. In our opinion, the degradation of thiocoraline in plasma at both 37°C and ambient temperature is not concentration-dependent; the observed difference at ambient temperature between 20 ng/ml and the other two concentrations is not significant in a bioanalytical assay. Two minor additional peaks were observed in the chromatograms of degraded samples at the 100 ng/ml level at $t_R=3.5$ and 8.7 min. This was confirmed by the degradation of 500 ng/ml thiocoraline in plasma at 37°C. The influence of

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

Temperature (°C)	Storage time (days)	Concentration (ng/ml)		
		5	20	100
Ambient	1	76±3	82±1	74±1
2	7	100±7	100±5	91±2
−20	8	102±4	107±2	105±2
	21	106[a]	110±8	102±9
	119	93±6	93±5	88±2
−80	21	106±9	108[a]	101±7
	119	94±6	95[a]	96±4

[a] $n=2$.

additional freeze–thaw cycles indicated that at least four cycles are permitted without loss of thiocoraline. The recovery of thiocoraline after overnight storage (average difference in time of analysis: 14.5 h) in the autosampler at ambient temperature is 88%, which is rather low; however, the good repeatability, reproducibility and accuracy for the randomly injected samples indicate that using a non-cooled autosampler is permitted if the total run time and the ambient temperature do not exceed the values during validation (13 h at ca. 22°C for 46 samples). However, cooling in the autosampler or cooling of the samples in the refrigerator until they are placed in the autosampler can be a significant addition to the present method.

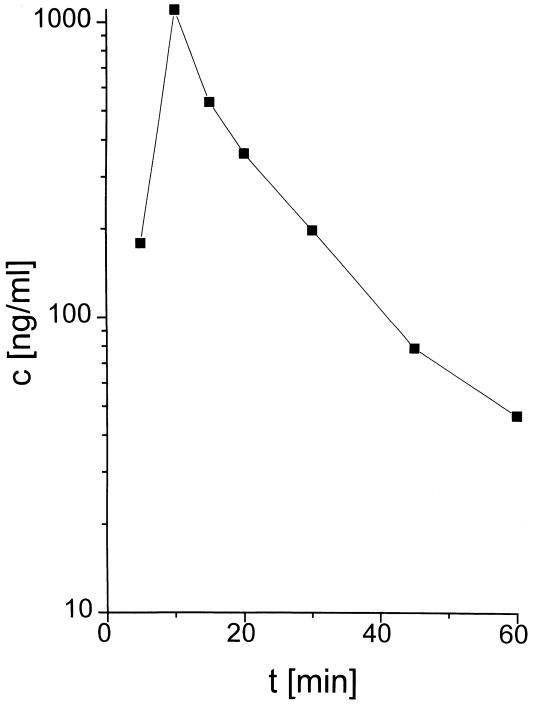

Fig. 3. Pharmacokinetic plot of thiocoraline in rat serum. Dose: 1.56 mg/kg.

The results of the pilot pharmacokinetic study of thiocoraline in rats are shown in Fig. 3. Blank human plasma was used for the dilution of these samples because of its better availability and because the presented method was actually developed for that matrix. The blank rat serum sample showed no interference and, after 24 h, 1.9 ng/ml thiocoraline was still found in the rat serum sample.

4. Conclusions

A selective, sensitive, precise and accurate assay for thiocoraline in plasma has been developed and can be used in pre-clinical pharmacokinetic investigations of thiocoraline in rats. The method can be used for future clinical studies. Thiocoraline is not stable in plasma at ambient temperature: sample cooling within ca. 5 h of sampling is necessary. The instability of thiocoraline in deproteinized plasma is similar, and cooling of the samples may be introduced. The identity of one or more degradation products of thiocoraline in plasma will be investigated in future work.

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