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Determination of MDL 74,405, a synthetic analogue of α-tocopherol, in dog plasma and heart tissue by high-performance liquid chromatography with electrochemical detection

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Abstract

A high-performance liquid chromatographic (HPLC) procedure with electrochemical detection was developed to analyze MDL 74,405 (I), the (−)-(*S*)-enantiomer of an α-tocopherol analogue, in dog plasma and heart tissue after infusion (0.3 mg/kg/h). The sample preparation involved the addition of an internal standard to either the dog plasma and heart tissue prior to protein precipitation. After centrifugation, the resulting supernatant was directly analyzed by HPLC with a Zorbax Rx C_8 column and a mobile phase of acetonitrile–0.04 *M* potassium phosphate, pH 7.0 (70:30, v/v). The concentration of I found was *ca.* 100 ng/ml in plasma and *ca.* 3 μg/g in heart tissue. A chiral separation with an Ultron ES-OVM column and a mobile phase of methanol–0.025 *M* potassium phosphate, pH 6.5 (17:83, v/v) was also used to investigate the enantiomeric conversion of I. Preliminary results of a probe study indicated that I is not inverted to the (+)-(*R*)-enantiomer *in vivo*.

1. Introduction

Oxygen-derived free radicals and active oxygen intermediates such as superoxide anion, hydrogen peroxide and hydroxyl radicals have been implicated as the major culprits in causing pathophysiological damages to muscle tissue in myocardial ischemia and reperfusion [1–3]. Under normal physiological conditions these highly reactive free radical species are scavenged by endogenous antioxidants such as α-tocopherol (vitamin E). In fact, α-tocopherol is the principal chain breaking, lipid soluble antioxidant in mammalian tissue [4,5], and is capable of quenching the propagation of free radical reactions within cell membranes. The efficiency of this defense mechanism is decreased during myocardial reperfusion-induced free radical injury [6]. However, due to its high lipophilicity and lack of cardioselectivity, the acute administration of α-tocopherol is unlikely to rapidly lead to increased levels in heart tissue.

MDL 74,405 (I) (Fig. 1) is the (−)-(*S*)-enantiomer of a synthetic quaternary ammonium analogue of α-tocopherol that has been shown in rats to be a hydrophillic and cardioselective free radical scavenger [7–10]. Due to its cardioselectivity and its ability to decrease infarct size, I is

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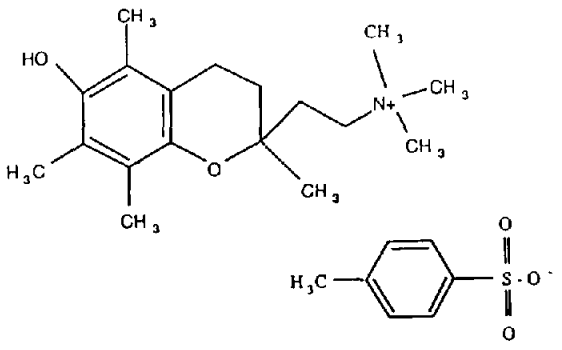
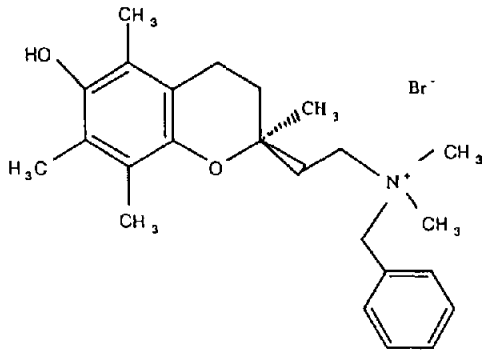


MDL 73,404 (racemate) III
MDL 74,405 [(-)-(S)-enantiomer] I
MDL 74,406 [(+)-(R)-enantiomer] II



MDL 100, 449 (internal standard IS)

Fig. 1. The chemical structures of I, II, III and I.S.

being developed as a therapeutic agent for cardiac reperfusion injury.

In order to get a better understanding of the pharmacological activities of I, a sensitive and reliable analytical procedure is needed to measure this compound in animal models. The present paper describes a reversed-phase high-performance liquid chromatographic (HPLC) procedure coupled with electrochemical detection for the determination of the levels of I in dog plasma and heart tissue after intravenous infusion. The report also describes a probe study that has been carried out to investigate if I is converted to its (+)-(*R*)-enantiomer, MDL 74,406 (II), *in vivo*.

2. Experimental

2.1. Chemicals

Compounds I and II, MDL 73,404 (racemate) (III), and MDL 100,449 (internal standard) (I.S.) (Fig. 1) were obtained from Marion Merrell Dow Research Institute (Cincinnati, OH, USA). HPLC grade acetonitrile was purchased from Baxter (Burdick and Jackson division, Muskegon, MI, USA). Water was purified with a Millipore Milli-Q water system (Marlborough, MA, USA). If not specified, reagent-grade chemicals were used throughout this study.

2.2. Dog plasma and heart tissue samples

The dog experiment was performed by Dr. Roberto Bolli of the Baylor College of Medicine (Houston, TX, USA) with techniques that had been previously described [11]. Briefly, the dog was infused with I intravenously at 0.3 mg/kg/h. Blood sample 1 was taken before the infusion of I, sample 2 was taken at 5 min before occlusion (25 min after starting the infusion of the drug), sample 3 was taken at 3 min of reperfusion (48 min after start of the infusion), and sample 4 was taken at 1 h of reperfusion (105 min after start of the infusion). Heart tissue samples were taken at the end of the experiment (*ca.* 3 h after reperfusion), with TS-1 samples taken from the ischemic zone and TS-2 samples from the nonischemic zone. The samples were shipped frozen to our laboratory and stored frozen (−80°C) until analysis.

2.3. Preparation of dog plasma standards

A stock solution of I (1 mg/ml) was prepared by accurately weighing 10 mg of the compound, placing it into a 10-ml volumetric flask and diluting to volume with methanol. Appropriate dilutions of the stock were made with methanol and the spiked solutions were used to prepare spiked dog plasma standards at concentrations of 50, 100, 200 and 400 ng/ml.

2.4. Preparation of dog heart tissue standards

To prepare dog heart tissue standards, drug free dog heart tissue was obtained, and a 10% (1 g of heart + 9 ml of phosphate buffer) homogenate was prepared in 30 m*M* sodium phosphate buffer at pH 7.4 with 4 m*M* NaCl. Dog heart tissue standards containing appropriate amounts of I (800, 400, 200, 100, 50 ng/ml and blank) were prepared by adding precise aliquots (200, 100, 50, 25, 12.5, and 0 μl respectively) of a spiking solution (4 μg/ml) to 1 ml of heart tissue homogenate.

2.5. Preparation of dog plasma sample for HPLC analysis

Unknown dog plasma sample or plasma standard (0.5 ml) was added to 0.5 ml of ice-cold acetonitrile which contained 250 ng of I.S. Each sample was vortex-mixed for *ca.* 5 to 10 s to precipitate the plasma protein and then centrifuged at 900 *g* for 20 min. The supernatant was transferred and 100 μl was used for HPLC analysis.

2.6. Preparation of dog heart tissues sample for HPLC analysis

Unknown dog heart homogenate (10%) or heart tissue standard (0.2 ml) was mixed with 0.25 ml of an ice-cold solution of methanol which contained 250 ng of I.S. Each sample was vortex-mixed and centrifuged at 900 *g* for 30 min. The supernatant was decanted and 50 μl injected for HPLC analysis.

2.7. Sample preparation for the probe study of enantiomeric interconversion

For the chiral HPLC analyses of I and II in dog plasma and heart tissues, the samples were extracted by a solid-phase extraction (SPE) technique. Briefly, the method consisted of preconditioning a weak cation-exchange carboxylic acid (CBA) column (100 mg size, Analytichem International, Harbor City, CA, USA) with 1 ml of 1 *M* HCl, followed by 2 × 1 ml wash of water. Then, the sample to be extracted, either 0.5 ml of plasma or 0.2 ml of heart tissue homogenate, was diluted to a total volume of 1.0 ml with water and then applied to the CBA column. The fluid was withdrawn with small vacuum (*ca.* 34 kPa). Each CBS column was rinsed successively with 1 ml of water, 1 ml of acetonitrile, 1 ml of 2% NH_4OH in methanol and finally 1 ml of 10% methanol in water. After air-drying the column for *ca.* 5 min, the compound of interest was eluted with 2 × 250 μl of a CH_3OH–0.2 *M* K_2HPO_4 (67:33, v/v) mixture. A 5–10 μl aliquot of the extract was analyzed by chiral HPLC.

2.8. HPLC analysis

The HPLC system consisted of a Waters 600E system controller (Milford, MA, USA), a Waters WISP Model 714B autosampler, and an ESA Model 5100A Coulochem detector equipped with an ESA Model 5011 analytical cell and an ESA Model 5020 guard cell (Bedford, MA, USA). The guard cell was connected prior to the autosampler. Data analysis was performed using a Beckman computer automated laboratory system (CALS) (Allendale, NJ, USA).

For quantitation of I in plasma and heart tissue, separation was performed on a Zorbax Rx C_8 column (5 μm, 250 × 4.6 mm I.D.), with a Zorbax Rx C_8 guard column (5 μm, 12.5 × 4.6 mm I.D.; Mac-Mad Analytical, Chadds Ford, PA, USA). The mobile phase was CH_3CN–0.04 *M* K_2HPO_4, pH 7.0 (70:30, v/v) with a flow-rate of 1.0 ml/min. For chiral separation of I and II, separation was performed on an Ultron ES-OVM column (5 μm, 150 × 4.6 mm I.D.), with an Ultron ES-OVM guard column (5 μm, 24 × 4.6 mm I.D.; Mac-Mad Analytical). The mobile phase was CH_3OH–0.025 *M* KH_2PO_4, pH 6.5 (17:83, v/v) with a flow-rate of 1.0 ml/min. All HPLC separations were performed at ambient temperature. The potential of the analytical cell 1 of the electrochemical detector was set at 0.05 V, of the analytical cell 2 (monitor) at 0.25 V and of the guard cell at 0.50 V. The gain was set at × 1 × 100.

2.9. Data processing

Calibration standards were placed at the beginning and the end of each set of samples to be analyzed. The peak areas of I and I.S. were determined using the CALS described above. The peak-area ratios of I/I.S. were calculated and plotted against the standard concentrations. The parameters obtained by linear regression were used to calculate the concentrations of I in the unknown samples.

3. Results and discussion

3.1. Analysis of I in dog plasma and heart tissue

Analysis of I in dog plasma and heart tissue by HPLC coupled with electrochemical detection showed good sensitivity and specificity. Under the described HPLC conditions, no interfering peaks were observed, and the retention times for I and I.S. were *ca.* 7.4 and 10.9 min, respectively. Typical chromatograms obtained from the spiked dog plasma standards and unknown dog plasma samples are shown in Figs. 2 and 3. A typical chromatogram obtained from an unknown dog heart tissue sample is shown in Fig. 4. The detection limit for I in dog plasma is *ca.* 0.4 pmol (0.19 ng) (signal-to-noise ratio = 3). Since the experimental results of a probe study indicated that most of the dog plasma samples contained I at *ca.* 100 ng/ml, a five point calibration curve (0, 50, 100, 200 and 400 ng/ml) was routinely prepared and employed for sample analysis. For a typical calibration curve the line fit is essentially linear with correlation coefficient greater than 0.999 and a slope of 0.2758.

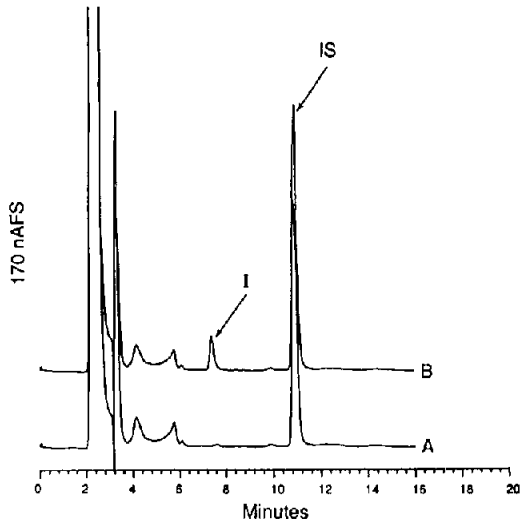


Fig. 3. HPLC of (A) pre-dosed plasma, and (B) pre-occlusion plasma.

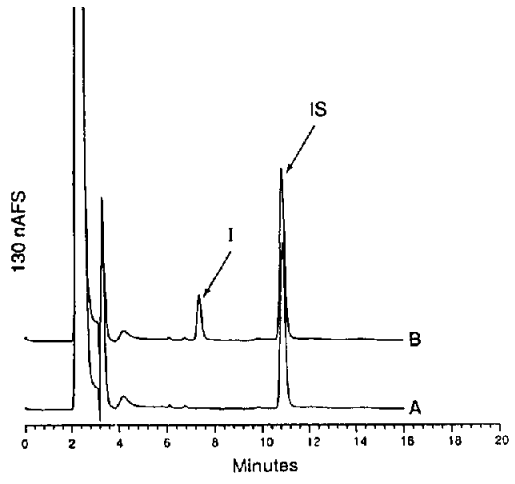


Fig. 2. HPLC of dog plasma samples. (A) Blank plasma; (B) plasma spiked with compound I (50 ng/ml).

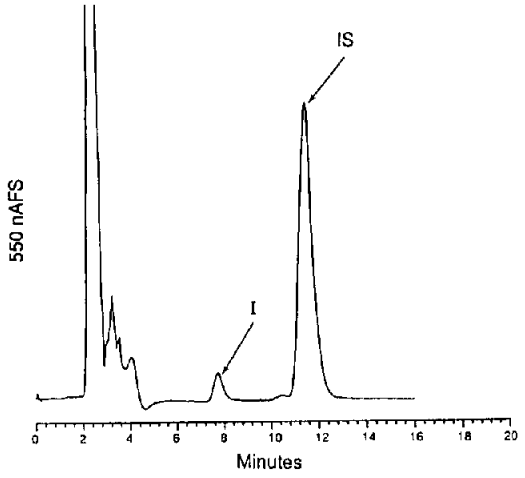


Fig. 4. HPLC of dog heart tissue at non-ischemic zone after treatment with compound I.

Table 1
Reproducibility and accuracy of measurements of I in dog plasma

Concentration added (ng/ml)	n	Concentration found (mean ± S.D.) (ng/ml)	R.S.D. (%)	Accuracy (%)
50.0	8	50.3 ± 4.2	8.3	100.6
100.0	10	103.1 ± 3.8	3.7	103.1
200.0	9	189.8 ± 11.1	5.8	94.9
400.0	8	407.7 ± 11.3	2.8	101.9
800.0	6	797.9 ± 19.6	2.5	99.7

Table 1 summarizes the results of a validation study in dog plasma carried out over a three-day period. The precision of the method, as measured by the relative standard deviation (%R.S.D.) ranged from 2.5 to 8.3%. The accuracy of the assay ranged from 94.9 to 101.9%.

As expected, the results showed that no I was detected in the pre-dose (baseline) plasma samples. The mean concentration of I found in the pre-occlusion samples (25 min post-infusion) was 100.9 ng/ml, in the plasma samples taken 3 min after re-perfusion was 109.9 ng/ml, and in the plasma samples taken one hour post-reperfusion was 121.6 ng/ml. The cardioselectivity of I in dog heart tissue was demonstrated by the much higher drug level found in both the ischemic and non-ischemic zones (means of 3.2 μg/g and 3.1 μg/g of heart tissue, respectively). These results are comparable to those found in a similar experiment performed with rats where a mean concentration of 3.2 μg/g of heart tissue was detected for rats dosed with 0.3 mg/kg/h of III [12]. Compound III is the racemic mixture of I and II.

3.2. Study of enantiomeric interconversion of I

The chiral HPLC separation was done on an ES-OVM column. An ES-OVM guard column was also used to protect the analytical column and keep the retention time and peak resolution reproducible. Without the guard column, the retention time of I in plasma samples seemed to gradually increase from injection to injection. This is most likely due to the deposition of plasma proteins on the column causing a change in the surface characteristics of the chiral stationary phase. Fig. 5 shows the chiral separation of I and II.

Fig. 6 shows the experimental results for the racemization study of I in dog plasma. Dog plasma standard (200 ng/ml) spiked with 3% of II, and one pre-dose and two post-dose dog plasma samples were processed by solid-phase extraction and analyzed by chiral HPLC. The chiral HPLC data showed that II was clearly detectable in the 3.0% spiked plasma standard, and no II was detected in the other three plasma samples, indicating that no enantiomeric interconversion had occurred. A similar experiment was also carried out with dog heart tissues. As shown in Fig. 7, only from the heart tissue sample spiked with 3% of II shows the com-

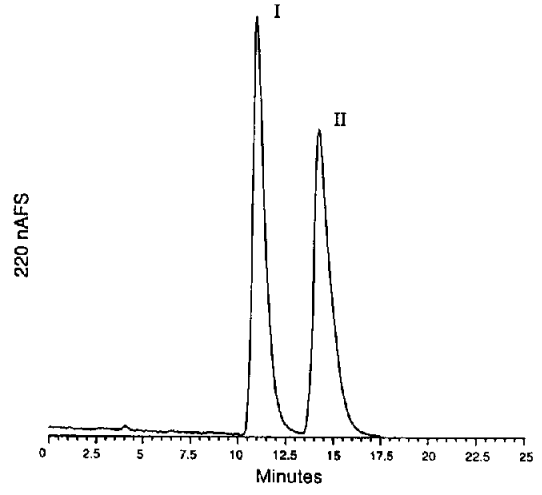


Fig. 5. Chiral separation of compounds I and II.

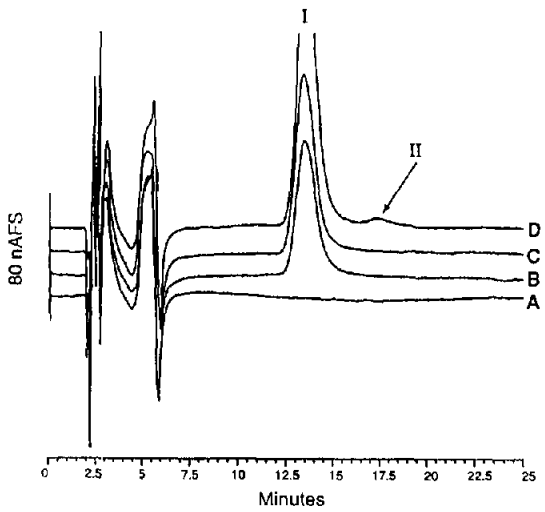


Fig. 6. Chiral HPLC screen for compound I racemization in dog plasma. (A) pre-dose plasma; (B) dosed plasma (pre-occlusion); (C) dosed plasma (re-perfusion); (D) compound I plasma standard (200 ng/ml) spiked with 3% (w/w) of compound II.

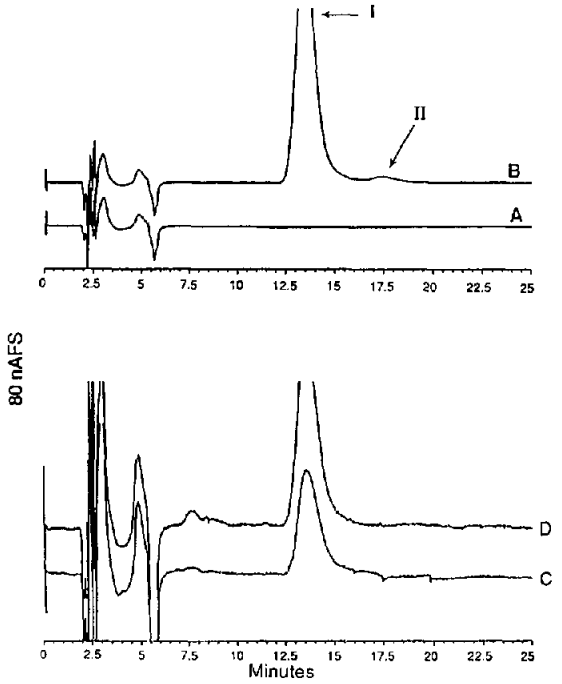


Fig. 7. Chiral HPLC screen for compound I racemization in dog heart tissue. (A) pre-dosed heart tissue; (B) compound I heart tissue standard (400 ng/ml) spiked with 3% (w/w) of compound II; (C) dosed heart tissue (ischemic zone); (D) dosed heart tissue (non-ischemic zone).

pound could be recovered. These results indicated that I was not converted to II *in vivo*.

4. Conclusions

HPLC with electrochemical detection is a very sensitive and selective method for the detection of I in dog plasma and heart tissue. This method is capable of detecting I in dog plasma down to the low picomole level. The mean value of I found in dog plasma after infusion (0.3 mg/kg/h) is *ca.* 100 ng/ml and in the heart tissue is *ca.* 3 μg/g. The cardioselectivity of the compound is demonstrated since the drug levels found in heart tissue are *ca.* 30 folds higher than those found in plasma. The compound tends to distribute evenly in the ischemic and non-ischemic zones as the analytical results show no significant differences between these two areas in the heart. Finally, the preliminary results from this dog study indicate that I does not racemize *in vivo*.

5. Acknowledgements

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