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Short communication

High-performance liquid chromatographic determination of 4,4′-dihydroxybenzophenone-2,4-dinitrophenylhydrazone in plasma

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Abstract

An analytical method has been developed for the determination of 4,4′-dihydroxybenzophenone-2,4-dinitrophenylhydrazone (I, trade name A-007) in plasma. Plasma samples are primed with the internal standard, 2,2′-dihydroxybenzophenone-2,4-dinitrophenylhydrazone (II), deproteinized with acetonitrile, centrifuged and filtered prior to assay. The components are then separated on a reversed-phase column with retention times of 4.4 and 6.0 min for I and II, respectively. Ultraviolet detection at 365 nm was employed and little interference with the analyte or the internal standard was noted from other plasma components. This method has been applied to the plasma of rats and monkeys dosed for pharmacokinetic and toxicity studies.

1. Introduction

4, 4′ - Dihydroxybenzophenone - 2, 4 - dinitrophenylhydrazone (I) (Fig. 1) is a new compound under evaluation for cancer management [1–8]. Phase 1 clinical trials of topical I are scheduled with patients suffering from metastatic malignancies including Kaposi's sarcoma spread to cutaneous areas [9]. This report details the analytical method used to assay I in rat and monkey plasma [10,11].

2. Experimental

2.1. Materials and reagents

4, 4' - Dihydroxybenzophenone - 2, 4 - dinitrophenylhydrazone (CAS: 002675-35-6) (I), and the internal standard 2,2′- dihydroxybenzophenone-2,4-dinitrophenylhydrazone (CAS: 109689-81-8) (II) were obtained from DEKK-TEC (New Orleans, LA, USA) and used as received. The

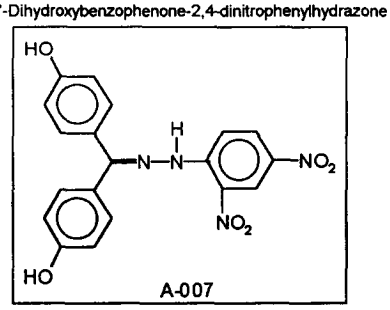

Fig. 1. Chemical structure of 4,4′-dihydroxybenzophenone-2,4-dinitrophenylhydrazone (I).

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acetonitrile used was HPLC grade purchased from J.T. Baker (Phillipsburg, NJ, USA). Acrodisc syringe filters, 0.45 μm, were obtained from Gelman Sciences (Ann Arbor, MI, USA).

2.2. Instrumentation

HPLC analysis was performed using an LKB Model 2248 pump and LKB Uvicord SII detector (Bromma, Sweden) fitted with a fixed-wavelength 365 nm filter. A Rheodyne Model 7125 injection port (Cotati, CA, USA) with a 20-μl sample loop was used to inject the samples. Chromatograms were recorded with a Hewlett-Packard 3390A integrator (Avondale, PA, USA). Samples were chromatographed on a 250 × 4.6 mm Keystone Hypersil ODS column (Bellefonte, PA, USA) packed with 5 μm spherical packing of pore diameter 120 Å and 10% carbon loading.

2.3. Mobile phase

The mobile phase consisted of 90% acetonitrile–10% water degassed and filtered through a 0.45-μm Rainin filter (Woburn, MA, USA) delivered at a flow-rate of 0.6 ml/min.

2.4. Sample collection and deproteinization

Blood samples were collected from Sprague–Dawley rats that had been administered I orally (1 g/kg, single dose), intravenously (0.1 g/kg, single dose) or dermally (0.25% gel, multiple applications). Blood was also collected from African Green monkeys that had received multiple dermal applications of I gel. Plasma was separated by centrifugation (800 g, 10 min) and frozen at −20°C until analysis. To 0.2 ml of plasma, 10 μl of 114 μ*M* internal standard (II) solution and 0.79 ml acetonitrile–water (90:10) were added. The samples were then shaken for 30 s and centrifuged for 10 min at 3200 *g*. The supernatant was decanted and filtered through a 0.45-μm Acrodisc syringe filter and then injected onto the column.

2.5. Calibration standards

Standard solutions were prepared by dissolving 2.1 mg of I or 4.5 mg of II in 100 ml of acetonitrile. These standard solutions were stable for at least 12 months at 5°C. Control blank plasma samples (obtained from the Blood Center for South East Louisiana) were spiked with I in the concentration range 10–1000 ng/ml (range narrowed or extended depending upon the expected plasma concentrations) and subjected to the treatment procedure described above. Peak-area ratios of I/II versus the concentration of I (ng/ml) were subjected to linear regression. The concentration of I in samples was then obtained from this regression equation.

3. Results

3.1. Standard calibration curves

Standard calibration curves were constructed by plotting the peak-area ratios of I/II against the concentration of I. Table 1 lists the peak-area ratios for seven concentrations of I. A linear regression analysis revealed a standard curve of [I] = 84.2 × peak area ratio + 2.4 with a correlation coefficient (r^2) = 0.996.

3.2. Recovery

Distilled water and blank plasma samples were spiked with I standards in the concentration range 42–840 ng/ml and carried through the

Table 1
Peak area ratios for standard 4,4′-dihydroxybenzophenone-2,4-dinitrophenylhydrazone in plasma

Area I/II	[I] (ng/ml)
0.157	12
0.488	48
0.835	72
1.084	96
1.430	120
1.680	144

Table 2
Recovery data from water and plasma samples

Concentration of I (ng/ml)	Water standard (peak area)	Plasma standard (peak area)	Recovery of I from serum (%)
42	12 403	12 424	100
84	14 670	16 147	110
210	29 280	29 294	100
420	61 602	59 915	97
840	118 900	109 110	92
Mean (%)			100
± S.D. (%)			7

extraction procedure. The absolute recovery of I was calculated by comparing the area of I for standards spiked in distilled water to blank plasma (Table 2). The mean recovery of I from five determinations was 100% with a standard deviation of 7%.

3.3. Chromatography of plasma samples

Fig. 2 shows typical chromatograms of normal blank rat plasma with II (internal standard) included. The retention time of the internal standard is 6.05 min (left). Also shown is the same rat's plasma 4 h after the oral administration of I equivalent to 1 g of I per kg animal body weight. The retention time of I is 4.43 min and the internal standard (II) 5.95 min (right).

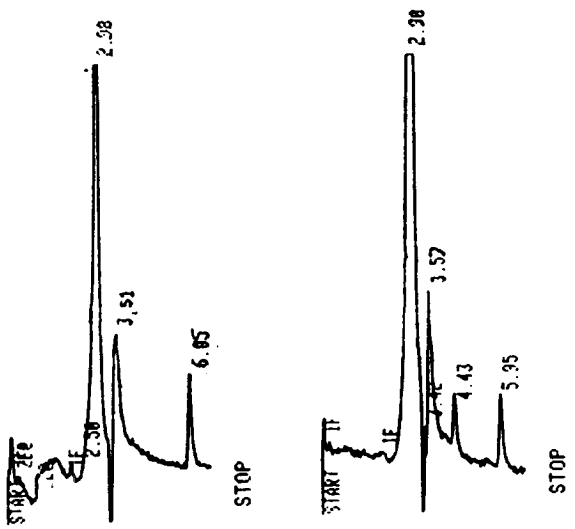

Fig. 2. Left chromatogram: blank rat plasma primed with internal standard and pretreated as in text. Retention time of internal standard 6.05 min. Right chromatogram: plasma of the same rat 4 h after the oral administration of 4,4'-dihydroxybenzophenone-2,4-dinitrophenylhydrazone (I) equivalent to 1 g of I per kg animal body weight. The retention time of I is 4.43 min and of the internal standard 5.95 min. The concentration of I is 65 ng/ml of plasma.

3.4. Reproducibility

Blank plasma samples ($n = 5$) were spiked with 100.8 ng/ml I and stored at −20°C. This batch was assayed and the mean concentration of I was determined as 96.6 ng/ml with a standard deviation of ± 9.3 ng/ml (10%).

4. Discussion

The goal of this study was to establish a fast, accurate and specific method for the quantitative analysis of I in plasma. To achieve these goals the method progressed through the following revisions.

The initial method used the diacetate of I as the internal standard (III; this compound is made by acetylating the hydroxyl groups of I). However, this internal standard was replaced as it was found to hydrolyze to I in the presence of biological fluids and hence increased the amount of I in the sample. The method also originally used 70% acetonitrile as the mobile phase. However, the 90% mobile phase was found to produce base line resolution between I and the

internal standard.

The method limit of quantitation is 12 ng/ml (limits of detection 4 ng/ml). Such low limits were achievable by selecting the optimum absorption wavelength for I at 365 nm.

The pharmacokinetic data for I is reported elsewhere [10,11]. However, I was not detected in the plasma of rats treated topically with I but only in those animals receiving oral or intravenous doses of the chemical. For animals receiving large (5 g/kg) oral doses of I, only small quantities were found in the plasma, such that I plasma levels were typically found to be less than 150 ng/ml.

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